Eur J Plant Pathol (2009) 123:117–123
DOI 10.1007/s10658-008-9347-5

Simultaneous detection of six RNA plant viruses affecting tomato crops using a single digoxigenin-labelled polyprobe

Frederic Aparicio · Salvador Soler · José Aramburu · Luis Galipienso · Fernando Nuez · Vicente Pallás · Carmelo López

Received: 9 April 2008 / Accepted: 9 June 2008 / Published online: 8 July 2008

Abstract A polyprobe for the simultaneous detection by non-isotopic molecular hybridisation has been developed to detect any of the following six viruses causing important economic losses in tomato crops: *Tomato spotted wilt virus*, *Tomato mosaic virus*, *Pepino mosaic virus*, *Cucumber mosaic virus*, *Potato Y virus* and *Parietaria mottle virus*. The polyprobe detected all six viruses with similar sensitivity to that obtained using individual riboprobes. In addition, we evaluated the possible use of the tissue-printing as a sample preparation technique applied to routine diagnosis of tomato plants with the polyprobe.

Electronic supplementary material The online version of this article (doi:10.1007/s10658-008-9347-5) contains supplementary material, which is available to authorized users.

F. Aparicio · V. Pallás
Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia (UPV-CSIC), Avenida de los Naranjos s/n, 46022 Valencia, Spain

S. Soler · F. Nuez · C. López (✉)
Instituto de Conservación y Mejora de la Agrodiversidad Valenciana, Universidad Politécnica de Valencia (COMAV-UPV), Avenida de los Naranjos, s/n, 46022 Valencia, Spain
e-mail: clopez@upvnet.upv.es

J. Aramburu · L. Galipienso
Institut de Recerca i Tecnología Agroalimentaries (IRTA), Ctra. de Cabrils s/n Cabrils, 08348 Barcelona, Spain

Keywords Diagnosis · Dig-RNA polyprobe · Tissue-printing · Tomato

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops in the Mediterranean area including Spain. Tomato cultivars are susceptible host for a wide range of DNA and RNA viruses which cause significant economic losses. Relevant RNA viruses infecting tomato include among others: *Tomato spotted wilt virus* (TSWV), *Tomato mosaic virus* (ToMV), *Pepino mosaic virus* (PepMV), *Cucumber mosaic virus* (CMV), *Potato Y virus* (PVY) and *Parietaria mottle virus* (PMoV). Moreover, these viruses can frequently occur in mixed infections (Gallitelli et al. 1995: Soler et al. unpublished results). Tomato crops infected with these viruses, alone or in combination, can display a variety of strong and severe disorder patterns such as necrotic symptoms in leaves and fruits, reduction in fruit yield, irregular fruit maturation and in some cases plant growth collapse. In addition, whereas ToMV and PepMV (Córdoba-Sellés et al. 2007) are seed-borne, CMV, TSWV and PVY are insect-transmitted, which contributes to their rapid spread. The natural spread of PMoV in tomato is still unknown; it appears not to be seed-transmitted in tomato and its presence seems to be related to the proximity of infected *Parietaria officinalis* plants (Ramasso et. al. 1997).

Commercially available enzyme-linked immunosorbent assay (ELISA) kits are used for the routine diagnosis of PepMV (DSMZ, Germany) and CMV,

PVY, ToMV and TSWV (Loewe, Germany). However, there are some limitations. For example, the protocols can differ for different viruses, so multiple extractions must be completed, increasing the time and expense of testing. Currently, there is not a commercial antibody against PMoV, although recently detection of PMoV in tomato by non-isotopic molecular hybridisation and reverse-transcriptase polymerase chain reaction (RT-PCR) methods have been reported (Galipienso et al. 2005). In the last years, simultaneous detection of multiple plant viruses by non-isotopic molecular hybridisation has become an attractive method for routine diagnosis (reviewed in James et al. 2006). Dot-blot hybridisation system using a mixture of specific single digoxigenin-labelled probes (riboprobes) has been developed for the simultaneous detection of six different viruses in tomato seedlings (Saldarelli et al. 1996). This approach based on cocktails of several riboprobes has also been successfully applied to the detection of different plant viruses affecting ornamentals (Sanchez-Navarro et al. 1999), stone fruits (Saade et al. 2000) and geranium crops (Ivars et al. 2004). The ultimate alternative to the mixture of single probes is the use of polyprobes. This strategy consists in the cloning in tandem of partial nucleic acid sequences of several viruses to allow the synthesis of a single probe called polyprobe (Herranz et al. 2005). Thus, digoxigenin-labelled polyprobes designed to detect two, four or six stone fruit viruses show comparable detection limits and specificity with respect to the same individual riboprobes. The validity of this polyprobe strategy was confirmed by analysis of field samples (Herranz et al. 2005). Recently, this strategy has been applied for the simultaneous detection of up to four viroids infecting citrus trees (Cohen et al. 2006). Besides improving sensitivity, non-isotopic molecular hybridisation diagnostic methods have been concerned with reducing the processing time and the overall cost. In this way, in addition to the dot-blot, the tissue-printing technique has proved to be a simple and reliable method for sample preparation to detect plant viruses (Más and Pallas 1995; Cañizares et al. 2001) and viroids (Romero-Durbán et al. 1995; Mandic et al. 2007).

The main goal of this study was to evaluate the use of a polyprobe in tissue-printing analysis, for the simultaneous detection of the above mentioned six RNA viruses in tomato plants by non-isotopic molecular hybridisation using a rapid, sensitive and reliable methodology of sample manipulation with a low cost. Thus, we first constructed a plasmid containing in tandem partial nucleic acid sequences of the coat protein of these six RNA viruses affecting tomato: CMV, PepMV, PMoV, PVY, ToMV and TSWV. Second, we confirmed the capability of the corresponding digoxigenin-labelled polyprobe (ribopoly-6) synthesised from this plasmid, to detect any of the six viruses in a single hybridisation. Finally, we evaluated the possible use of the tissue-printing as a sample preparation technique applied to routine diagnosis of tomato plants with the ribopoly-6.

Infected tomato plants with each virus, and kept in a greenhouse as part of the Centro de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV-UPV, Valencia, Spain) and the Institut de Recerca i Tecnología Agroalimentaries (Barcelona, Spain) collections, were used to extract viral RNAs: (1) to construct the polyprobe, (2) to use them as infected samples for testing polyprobe capability by dot-blot hybridisation. To construct the polyprobe, total nucleic acids were extracted from 0.1 g of fresh leaf tissue using TRI-reagent (SIGMA) following the manufacturer's protocol. The design of the primers was based on conserved sequences to detect the different strains from each virus. These primers amplified a fragment around 200 bp length corresponding to a region of the coat protein genes (CP; see Supplementary Table). Reverse transcription and PCR reactions were carried out using the SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen) following the manufacturer's instructions. Amplified products corresponding to the six viral CP fragments were cloned individually into a pTZ57R/T vector (MBI Fermentas). For each virus, CP clones in both orientations were selected (pTZ/CP:minus-strand and pTZ/CP:plus-strand). In a second step, the cloned PCR fragments were subcloned in tandem into a pSK+ vector using the corresponding restriction enzymes. This strategy rendered the pSK/poly6 plasmid which contained a fusion of all six viral sequences in the order from the T7 promoter: CMV-ToMV-PVY-TSWV-PepMV-PMoV (Fig. 1a).

The limit of the detection for each individual probe was determined by using the corresponding complementary unlabelled plus-strand viral RNA. Plasmids containing individual CP sequences in plus-strand polarity (pTZ/CP:plus-strand) were linearised with the

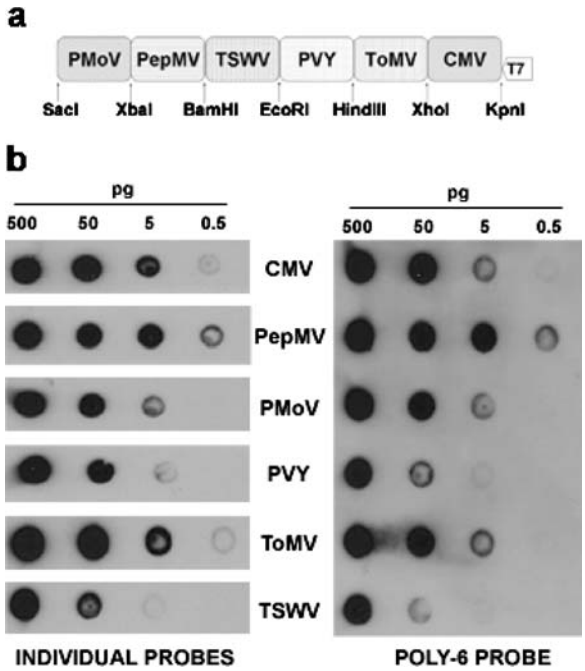

Fig. 1 **a** Schematic representation of the pSK/poly 6 plasmid. The corresponding virus fragment and restriction sites used for the cloning procedure are shown. After linearisation with *Sac*I restriction enzyme, a polyprobe of the negative polarity corresponding to around 200 nt-lengths from the CP region of each virus is synthesised using T7 RNA polymerase. **b** Comparison of the sensibility between single and the ribopoly-6 riboprobes. The limit of the detection level for each probe was determined by using the corresponding complementary unlabelled plus-strand of viral RNA. For each virus, RNA transcripts were spectrophotometrically quantified and ten-fold dilutions were either individually applied to six nylon membranes (*left*) or all of them in the same membrane (*right*). Numbers above panels represent picograms of unlabelled transcribed RNA applied onto the membranes (pg). Individual membranes (*left*) were hybridised with its specific digoxigenin-labelled riboprobe. Virus acronyms indicate both the unlabelled RNA virus applied onto the membrane as its corresponding riboprobe used in the hybridisation. The membrane containing all six RNA transcripts (*right*) was hybridised with the ribopoly-6

appropriate restriction enzyme and RNA transcribed with T7 RNA polymerase (ROCHE) following the manufacturer's instructions. To test the capability of the polyprobe to detect infected samples, total nucleic acids from 0.1 g of fresh leaf tissue were extracted using a silica extraction procedure (Thompson et al. 2003). Total nucleic acids were serially diluted in extraction buffer and dotted onto positively charged nylon membranes (ROCHE). For tissue-printing analysis, three different petioles were detached from each plant with a razor blade and the cross-sections were directly blotted onto nylon membranes. Membranes were air-dried and the nucleic acids were covalently fused by UV cross-linking (700×100 J/cm^2). The pTZ plasmid containing individual viral CP sequences in minus-strand polarity and pSK/poly6 plasmid were linearised with the appropriate restriction enzyme and used to synthesise digoxigenin-labelled single or poly-riboprobes as described previously (Más et al. 1993; Pallás et al. 1998). Pre-hybridisations and hybridisations with the single probes or the polyprobe were conducted as described previously (Pallás et al. 1998). Chemiluminescent detection with CSPD reagent (ROCHE) as the substrate was performed as recommended by the manufacturer.

In this study we first evaluated the sensitivity of the ribopoly-6 compared to the individual riboprobes. Thus, unlabelled RNA transcribed from pTZ/CP: plus-strand plasmid of each the six viruses were quantified by spectrophotometry and ten-fold dilutions in sterile water of the transcripts were applied to nylon membranes (Fig. 1b). One membrane containing plus-strand unlabelled RNA transcripts of the six viruses was hybridised with the ribopoly-6 (Fig. 1b right), whereas the remaining membranes containing only a transcript of each single virus were hybridised with its corresponding riboprobe (Fig. 1b left). Altogether no significant differences in sensitivity were observed between single and the poly-6 hybridisations for CMV, PepMV, PMoV, and PVY. The endpoint detection limit ranged between 5 and 0.5 pg of RNA transcript for these viruses (Fig. 1b, compare left and right panels). In the case of ToMV and TSWV, the detection limit of the ribopoly-6 was slightly lower than its corresponding individual riboprobe (5–0.5 and 50–5 pg, respectively, Fig. 1b). This result indicates that the ribopoly-6 can detect RNA transcripts corresponding to the CP of any of the six tested viruses without losing sensitivity with respect to individual riboprobes until at least 50 pg of viral RNA.

We also tested the specificity of the individual probes and the capability of the ribopoly-6 to detect simultaneously these six viruses in infected plants. Total nucleic acids were extracted with a silica extraction procedure from healthy and single infected tomato plants with the six viruses. Total nucleic acids corresponding to 2, 1 and 0.5 mg of tissue were blotted onto seven nylon membranes (Fig. 2). Six membranes were hybridised separately with the

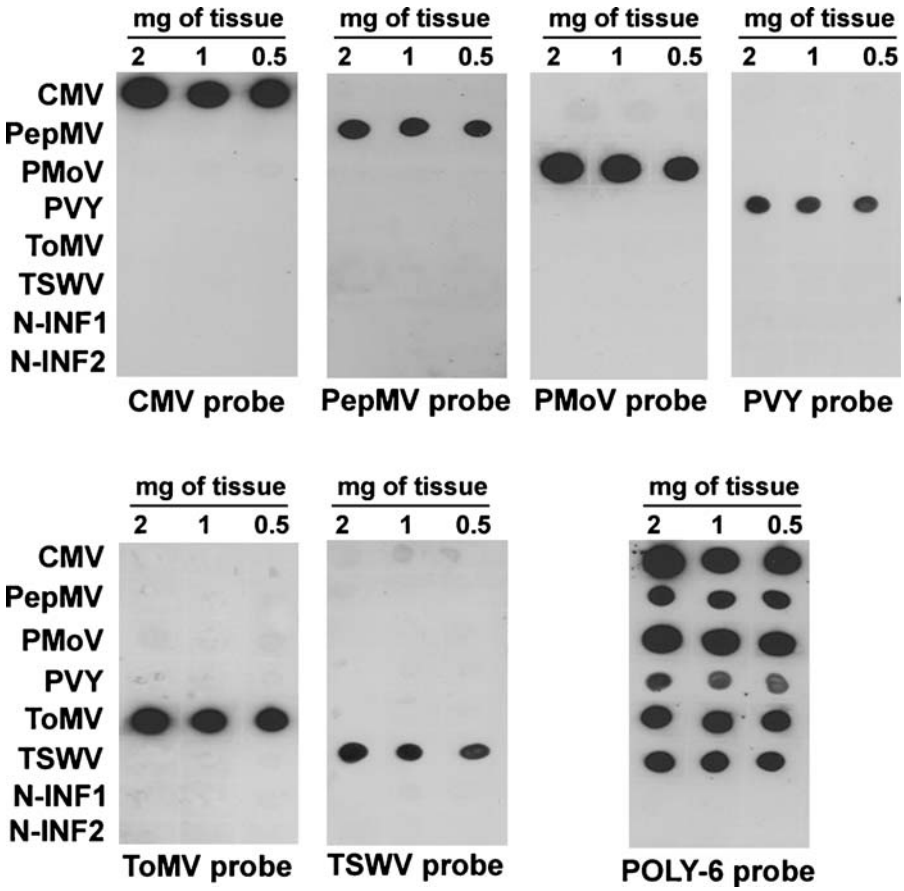

Fig. 2 Detection of the six RNA viruses in infected tomato plants by non-isotopic molecular hybridisation using individual riboprobes or the ribopoly-6. Extracts of infected or healthy tomato plants were diluted in extraction buffer and dotted onto seven nylon membrane replicas. Note that each membrane contains infected extract from the six viruses and from two healthy plants. Each replica was analysed using either the corresponding individual or the polyprobe as indicated at the bottom of each membrane. Virus acronyms and the healthy plants denoted as N-INF1 and N-INF2 are on the left. The amount of infected tissue (mg) dotted onto the membranes are above them

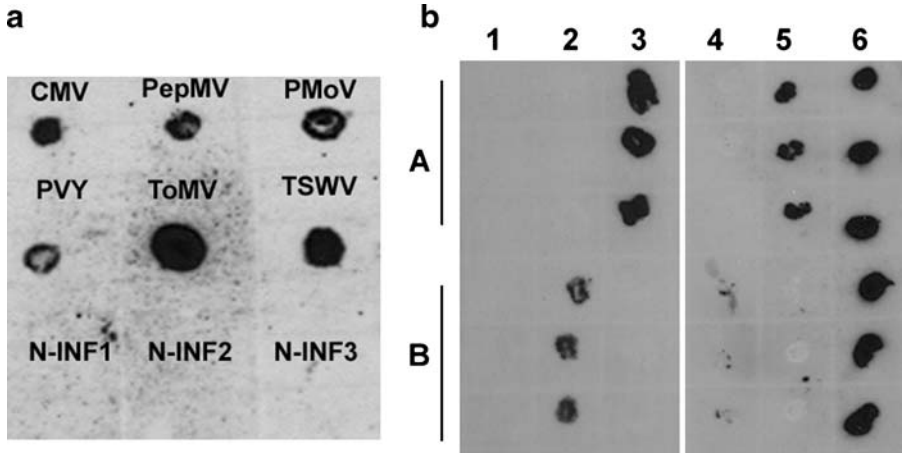

Fig. 3 Tissue-printing approach for the detection of the six viruses with the polyprobe by non-isotopic molecular hybridisation. Freshly cut leaf petioles from healthy and single infected tomato plants were directly pressed onto nylon membranes and hybridised with the polyprobe. **a** tissue-printings from single infected tomato plants with CMV, PepMV, PMoV, PVY, ToMV and TSWV and three healthy plants (denoted as N-INF1 to N-INF3). The ribopoly-6 detects all infected samples and discriminates the non-infected plants. **b** a representative example of a tissue-printing membrane from a large-scale tomato crop survey analysed in this study. In this case, freshly cut leaf petioles were applied in triplicate onto nylon membranes. Thus, in this example only samples A3, A5, A6, B2, B4 and B6 are infected

Table 1 Comparative analysis for the presence of five tomato viruses made by ELISA and non-isotopic molecular hybridisation (NIMH) with the ribopoly-6

Sample	ELISA					NIMH
	CMV	PepMV	PVY	ToMV	TSWV	Poly-6
80.1						
80.2						
80.3						
80.4						
80.5						
80.6						
80.7						
80.8	+					+
80.9						
80.10						
80.11						+
80.12						+
80.13						
80.14					+	+
80.15						
81.1						
81.2						
81.3						
81.4						
81.5						
82.1						
82.2						
82.3						
82.4						
82.5	+					+
82.6						
82.7						
204.4			+	+		+
204.5			+			+
204.6			+			+
204.7		+	+	+		+
204.8			+	+		+
205.1			+	+		+
205.2			+	+		+
205.3		+	+	+		+
205.4			+	+		+
205.5			+	+		+
206.1	+			+		+
206.2	+		+			+
206.3	+		+			+
206.4	+		+	+		+
206.5	+		+	+		+
207.1	+		+			+
207.2			+			+
207.3		+	+	+		+
207.4			+	+		+
207.5			+	+		+
207.6			+	+		+
207.7			+	+		+
208.1			+	+		+
208.2			+	+		+
208.3				+		+
208.4				+		+
208.5			+	+		+
208.6			+	+		+
208.7				+		+
208.8				+		+
209.1				+		+
209.2				+		+
209.3			+			+
209.4			+			+
209.5						+
209.6			+			+
209.7	+					+
210.1			+	+		+
210.2			+			+
210.3						+
210.4			+			+
210.5			+			+
211.1			+			+
211.2						+
211.3						+
211.4			+			+
211.5			+	+		+
212.1						
212.2						
212.3				+		+
212.4						
213.1			+	+		+
213.2			+	+		+
213.3			+	+		+
213.4			+	+		+
213.5			+	+		+
214.1				+		+

72 samples from a large-scale survey for tomato crops were analysed in this study.

specific riboprobes, while the remaining one was with the ribopoly-6. The single riboprobes were specific for its corresponding virus since no hybridisation signal was detected in the non-related infected extracts (Fig. 2). Moreover, the ribopoly-6 hybridisation rendered specific signals for all six viruses without losing sensitivity (as shown in Fig. 2, both single and ribopoly-6 hybridisations showed specific signals at the equivalent of 0.5 mg of infected leaf

tissues), whereas no signal was detected in extracts from non-infected tomato plants (Fig. 2, rows N-INF1 and N-INF2). These results validate the simultaneous detection of these six viruses with the polyprobe approach as an alternative and reliable method to single hybridisations.

In order to simplify the sample handling procedure, several healthy and single infected tomato plants (including the same ones used in the previous described blot analysis) were analysed by the tissue-printing method (Más and Pallás 1995; Mandic et al. 2007). Freshly cut leaf petioles from tomato leaves were directly pressed onto nylon membranes and hybridised with the ribopoly-6. As shown in Fig. 3a, the ribopoly-6 showed a high specific detection of tomato plants infected with any of the six viruses discriminating between healthy and infected samples. This result validates the tissue-printing procedure to detect the six viruses by non-radioactive molecular hybridisation with the polyprobe. In order to emulate a routine field assay, a tissue-printing set of 72 tomato plants, collected from a large-scale survey carried out over several commercial crop growing in different areas of the Comunidad Valenciana (Spain), was analysed with the ribopoly-6. All the samples of this survey had been previously tested by ELISA for CMV, PepMV, PVY, ToMV and TSVW at the COMAV (unpublished data). Figure 3b shows a representative tissue-printing membrane in which freshly cut leaf petioles were applied in triplicate onto the membranes. Thus, only samples A3, A5, A6, B2, B4 and B6 were infected. Table 1 summarises the results obtained by ELISA and hybridisation for the 72 samples. All samples recorded as positive by ELISA for any of the five viruses were also positive by the ribopoly-6. However, six samples detected as positives by the polyprobe were negative by the ELISA test (Table 1, samples 80.11, 80.12, 209.5, 210.3, 211.2 and 211.3). In order to clarify these differences between both methods, RNA extracted with TRI-reagent from these six samples was hybridised with the six single specific riboprobes. Thus, samples 80.11 and 80.12 tested positive for PMoV, which could not be analysed by ELISA since no antiserum was available, whereas samples 209.5, 210.3, 211.2 and 211.3 rendered positive for PVY. Much genetic and serological diversity of PVY isolates infecting tomato in Spain has been reported (Aramburu et al. 2006). The results of this work showed that some PVY isolates from tomato only provided a weak positive reaction when the ELISA test was achieved using polyclonal antiserum, and some of them were not detected when specific monoclonal antibodies were used. The presence in these samples of some PVY strains clearly detected by the ribopoly-6, but not recognised with the specific monoclonal antibody used in our ELISA analysis, could be the simplest explanation of the discrepancies observed with 209.5, 210.3, 211.2 and 211.3 samples.

In summary, the polyprobe/tissue-printing strategy presented here to detect any of the six viruses analysed in this work was shown to be a reliable alternative detection method to the ELISA test. In addition, it reduced labour and overall cost, since only one synthesis reaction and one hybridisation assay is needed to detect all six viruses, whereas the handling and manipulation of the samples is reduced to a minimum. Overall the results obtained in this work indicate that this approach could be a very useful tool as a first detection-step to determine the phytosanitary status of tomato plants in routine diagnosis.

Acknowledgements F.A. and C.L. were recipients of contracts Juan de la Cierva and Ramón y Cajal, respectively, from the Ministerio de Educación y Ciencia of Spain. We thank C. Estellés for her excellent technical assistance. This research was supported by grant RTA2006-00024-C02-02 from the Instituto Nacional de Investigaciones Agrarias (INIA) and by grant ACOMP06/067 from the Generalitat Valenciana.

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