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SHORT COMMUNICATION

Polyvalent detection of twelve viruses and four viroids affecting tomato by using a unique polyprobe

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Abstract Non-radioactive molecular hybridization represents an attractive approach for the detection of multiple plant virus and/or viroids and a good alternative to the more extended serological and PCR-based detection methods. The use of polyprobes or riboprobes carrying partial sequences of different plant viruses or viroids fused in tandem, has permitted the detection of up to 10 different pathogens or the development of genus-specific probes. In the present article, the polyprobe technology has been adapted for the detection of the main viruses and viroids affecting tomato crops. To do this, three polyprobes have been developed covering four viroids (Poly4), twelve viruses (Poly12) or the four viroids plus the twelve viruses (poly16). The detection limit of the three polyprobes was comparable to the individual probes allowing the detection of up to 0,2 pg/μl of viral or viroidal RNA. A survey of 50 field samples revealed that all positive samples detected with the individual probes were also detected with the corresponding poly12 (98%) or poly16 (100%) probes. The analysis of tomato seeds revealed that both, single and polyprobes, were able to detect an infected seed in a pool of 250 healthy seeds. Finally, a ring-test analysis among six laboratories revealed a high reproducibility of the non-radioactive molecular hybridization procedure using the three polyprobes. The use of this technology in the routine analysis of tomato samples is discussed.

Keywords Multiplex · Non-radioactive molecular hybridization · Tomato viruses and viroids · Dig-RNA probe · Seed

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Tomato (*Solanum lycopersicum*) is among most important vegetable crops, representing the 72% of the value of fresh vegetables produced worldwide (source: Food and Agricultural Organization, United Nations). The number of viral species that infect tomato crops is up to 136, representing one of most susceptible host of plant virus, with the exception of cucumber (*Cucumis sativus*) with 153 viral pathogens (Brunt et al. 1996). The most important RNA or DNA viruses infecting tomato include among others: alfalfa mosaic virus (AMV), cucumber mosaic virus (CMV), parietaria mottle virus (PMoV), pepino masaic virus (PepMV), potato virus Y (PVY), tobacco etch virus (TEV), tomato chlorosis virus (ToCV), tomato infectious chlorosis virus (TICV), tomato mosaic virus (ToMV), tomato spotted wilt virus (TSWV), tomato torrado virus (ToTV) and tomato yellow leaf curl virus (TYLCV). Tomato crops

infected with these viral species, alone or in combination, can display a variety of 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. Seed and insect transmission of some viruses makes control difficult. Tomato crops are also affected by viroids including citrus exocortis viroid (CEVd), potato spindle tuber viroid (PSTVd), tomato apical stunt viroid (TASVd) and tomato planta macho viroid (TPMVd). Tomato plants affected by these viroid species display symptoms such as chlorosis, bronzing, leaf distortion and general stunting and reduction on size fruit (Ling and Zhang 2009). One of the main measures to control viruses/viroids disorders represents the exclusion of the infected seeds or planting material together with hygiene practices addressed to prevent subsequent crops infection. Except for viroids, the most extended detection method of pathogens is the enzyme-linked immunosorbent assay (ELISA) due to its easy use, sensitivity and automation (Clark and Adams 1977; Lopez et al. 2003). However, during the last 10 years, the detection methods based on the genome component of pathogens (e.g. molecular hybridization, RT-PCR, etc.) have become very attractive due to their sensitivity, specificity, economy and the capacity to multiple or polyvalent detection of several pathogens in a unique assay (James et al. 2006; Olmos et al. 2007; Pallás et al. 2018). Virus/viroid detection via non-isotopic molecular hybridization (MH) technique offers several advantages over the ELISA approach. First, both virus and viroid can be detected with the same procedure (see James et al. 2006 for review). Second, the detection limit of MH is, in most of the cases, greater than that of the ELISA test (Sakamoto et al. 1989; Sánchez-Navarro et al. 1996, 1998), allowing the detection of picograms of nucleic acids of virus and/or viroid (Guo and Bowden 1991; Herranz et al. 2005; Peiró et al. 2012; Sánchez-Navarro et al. 1999). And third, MH permits the polyvalent detection of several pathogens in a unique test (see Pallás et al. 2018 for review). Polyvalent detection of virus/viroid by using the non-isotopic MH technique has been performed by two different forms: first, by a cocktail of the specific single probes in the hybridization solution (Saldarelli et al. 1996); and second, by using a unique riboprobe, called 'polyprobe', that contains partial nucleic acid sequences of different viruses (Herranz et al. 2005) or viroids (Cohen et al. 2006) cloned in tandem. Both approaches have been successfully applied to the detection of up to six different plant viruses affecting tomato (Aparicio et al. 2009; Saldarelli et al. 1996). Also, the polyprobe technology has allowed the detection of pathogens with very different life cycle styles (virus, viroid and bacteria) in a single assay (Peiró et al. 2012; Zamora-Macorra et al. 2015). The largest polyprobe described so far, allowed the polyvalent detection of eight viruses and two viroids (Peiró et al. 2012) or potentially all members of the genus Potyvirus by using genus-specific probe (Sánchez-Navarro et al. 2018), although in the case of the tomato crops, the largest polyprobe assayed until now, permitted the polyvalent detection of six viruses, without compromising the specificity and/or the detection limit of the assay (Aparicio et al. 2009). In the present study, three aspects of the polyprobe technology have been analyzed: first, the capacity to detect up to 16 different pathogens (12 viruses and four viroids); second, the applicability of such technology to tomato seeds and third, the reproducibility of such technology in different laboratories of plant pathology. To do this, three different polyprobes were elaborated with the capacity to either detect 12 viruses, four viroids or all the 16 pathogens and both the polyprobes and the single probes were assayed in tomato leaves or seed tissues. Finally, an inter-laboratory assay was carried out to evaluate the reproducibility of the non-radioactive MH technique using the three polyrpobes.

To generate the different polypobes, the corresponding DNA fragments were amplified using total RNA (MacKenzie et al. 1997) extracted from infected tissue as template and the specific primers, containing the *Xho*I/*Sal*I restriction sites (Online Resource 1). The new fragments, ranging between 243 nt–360 nt, except the 757 nt of the TYLCV amplicon, were introduced in the pSK+ plasmid (single probes) or in the previously described polyprobe with the capacity to detect six tomato viruses (Aparicio et al. 2009), using the unique *Xho*I restriction site. The introduction of the corresponding amplicon in the right orientation allows the inactivation of the original *Xho*I site located in the plasmid by the compatible *Sal*I site, permitting the use of the new 5′ proximal *Xho*I for the synthesis of the riboprobe or the incorporation of a new PCR fragments (Peiró et al. 2012). To discriminate between virus and viroid, two additional polyprobes were generated with the capacity to detect 12 viruses (poly12) and four viroids (poly4) (Online Resource 2).

First, the detection limit of the single probes and the three polyprobes were evaluated. Thus, the digoxigenin-labeled probes and the corresponding unlabeled complementary transcripts were synthesized by using the pSK+ plasmid and the T7 or T3 RNA polymerase, respectively (Más et al. 1993; Peiró et al. 2012). Known amounts of the free transcripts were serially diluted (five-fold) in sterile water since previous results showed similar detection limit (pg/μl of viral RNA) for the no-radioactive MH procedure when the dilutions were performed either in sterile water (Peiró et al. 2012) or in heathy tissue extracts (Sánchez-Navarro et al. 1996; Sánchez-Navarro et al. 1998). Replicates of the same membrane carrying known amount of unlabeled transcripts from the 16 viruses and viroids, were hybridized with digoxigenin-labelled single probes or the three polyprobes (Online Resource 3). Pre-hybridizations and hybridizations with the single probes or the polyprobes were conducted as described previously (Pallás et al. 1998; Sánchez-Navarro et al. 1999). In the case of the polyprobes, the hybridization was performed at 50 °C instead of the 68 °C used for the single probes. The detection limit observed for the individual probes ranged between 5 and 0.2 pg/μl of complementary transcript, being in the range of previously described probes (Sánchez-Navarro et al. 1996, 2007). No cross-hybridization with the unrelated complementary transcripts was observed, except for the viroid probes in spite that the hybridization was performed at 68 °C. Previous results showed that cross hybridization occurs between two sequences when they shared identity percentages higher than 68% (Sánchez-Navarro et al. 2018). The four viroid sequences used in the present analysis showed identity percentages that ranged between 78 and 89%, covering the full or part of the sequence. The same detection limit was observed when the membranes were hybridized with the three polyprobes, indicating that the presence of 4, 12 or 16 sequences fused in tandem do not affect the sensitivity of the procedure.

In the next step, the three polyprobes were used to analyze 50 field samples. The plant tissue was obtained from the Leibniz Institute DSMZ-German Collection of Microorganism and Cell Cultures (https://www.dsmz.de/home.html) or from the Mediterranean Agroforestal Institute at the Polytechnic University of Valencia (http://www.upv.es/iam/). Total nucleic acids were obtained from 0.1 g of leaf tissue using the Silica capture extraction protocol (MacKenzie et al. 1997) and resuspended in 100 μl of water. 1 μl of the corresponding extracts were directly applied onto nylon membranes (positively charged, ROCHE. Basel, Switzerland), air dried and cross-linked by UV crosslinker (700×100 μJ/cm^2). Replicas of the same membrane were hybridized with either the single probes or the three polyprobes at 68 °C or 50 °C, respectively (Fig. 1). All virus positive samples detected with the individual probes were also detected with the poly12 and poly16 polyprobes, except the sample c2 that rendered negative result with poly12 (Table 1). Apparently, the low ToTV signal observed in sample c2 with the single probe is in the detection limit of the poly12. For the viroid positive samples, the same situation was observed in which all positive samples detected with the single probes were also detected with the Poly4 and Poly16, although a cross hybridization signal was observed with the single probes. Thus, the majority of the TPMVd (c8), TASVd (b7, c1, c2), PSTVd (b4, b9) and CEVd (b8) infected samples were detected with the single probes (Fig. 1). The use of polyprobes with the capacity to detect six viruses (Aparicio et al. 2009; Herranz et al. 2005) or eight viruses plus two viroids (Peiró et al. 2012) has proved to detect all positives obtained using the single probes. In the present analysis, we show that such observation could be applied to polyprobes detecting at least 16 different pathogens. An obvious question emerges from these results. What is the maximum number of pathogens that could be detected using a polyprobe without affecting the detection limit? In this sense, it is interesting to note that the reduction of the hybridization temperature below 50 °C, to compensate the less hybridization observed for the polyprobes, renders unspecific signals in the healthy controls. This observation indicates that it is possible to design polyprobes with the capacity to detect more than 16 pathogens but with a significant cost to the detection limit. An alternative to that limitation could be the design of polyprobes with the capacity to detect 10–15 pathogens and mixed them in the same hybridization solution. Previous results showed that the mix of up to three probes do not increment the undesirable background associated to the high probe concentration (Saade et al. 2000; Sánchez-Navarro et al. 1999).

To further characterize the new polyprobes, their capacity to detect infected tomato seeds was evaluated. To do this, seeds from PepMV-infected tomato plants were mixed with different proportions of healthy tomato seeds. The pools of seeds were

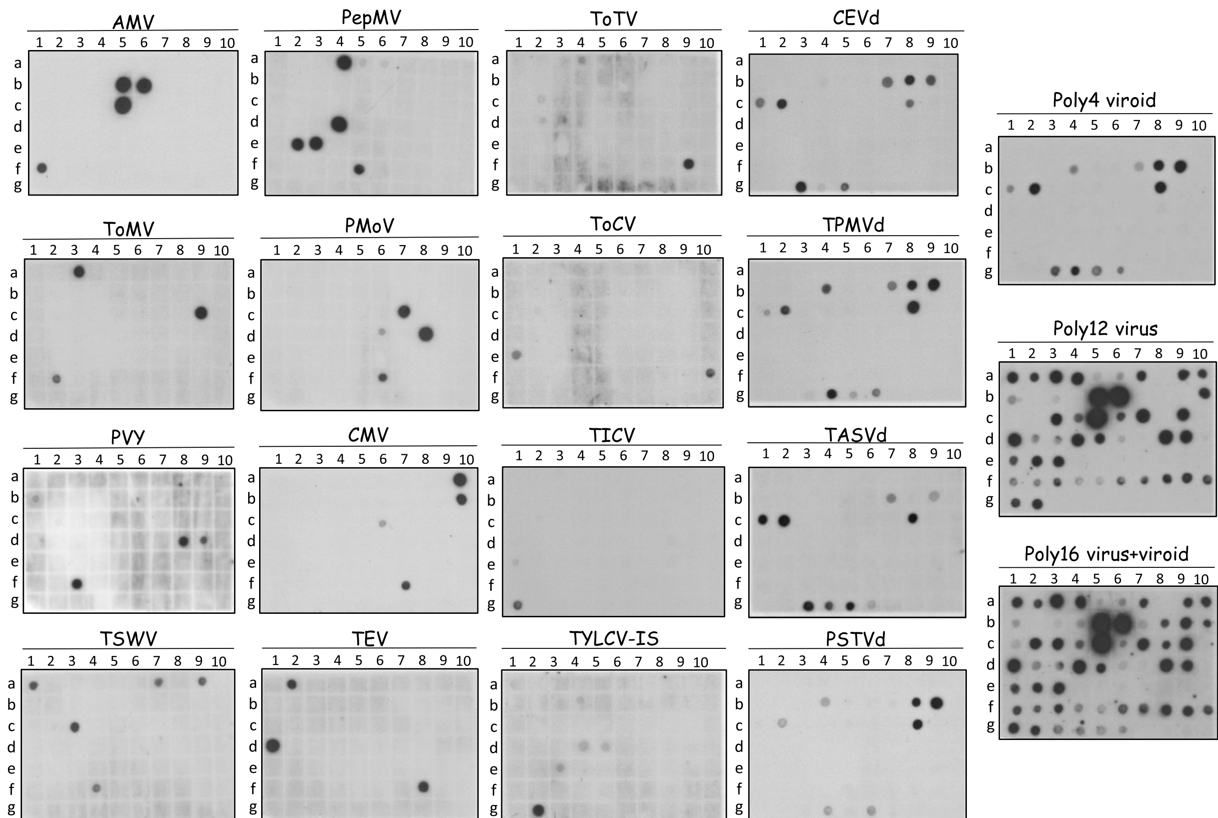

Fig. 1 Routine analysis of 50 tomato samples by non-isotopic molecular hybridization using single probes of the three polyprobes. Replicas of the same membrane were analyzed using the single riboprobe (virus name-probe) or the corresponding polyprobe (poly4, poly12 or poly16). Healthy tissue extracts were applied in boxes e4 to e10. Controls (5 pg) for AMV, ToMV, PVY, TSWV, PepMV, PMoV, CMV, TEV, ToTV, ToCV, TICV, TYLCV-IS, CEVd, TPMVd, TASVd, PSTVd were applied in the boxes: d1, f2, f3, f4, f5, f6, f7, f8, f9, f10, g1, g2, g3, g4, g5 and g6, respectively. In all cases, films were developed after 30-min exposure

subjected to total nucleic acids extraction using the silica capture extraction protocol (MacKenzie et al. 1997). To facilitate the extraction, the seeds were triturated in a mortar using liquid nitrogen. A volume of 1 μl of the different extracts was applied onto nylon membranes and hybridized with the PepMV single probe or the Poly16 polyprobe at 68 °C or 50 °C, respectively (Fig. 2). Both probes were able to detect an infected seed in a pool of 250 healthy seeds, meanwhile no signal was observed in healthy seeds of with higher proportions of uninfected seeds (1/300 or 1/350). Similar results were obtained using ToTV infected seeds (data not shown). Previous results showed that serological and Real-time TaqMan RT-PCR assays were able to detect an infected seed in a background of 1000 healthy seeds (Ling et al. 2007). In spite of such differences, the EPPO indicates that for seed testing, the maximum subsample size recommended is 250 since the sensitivity of both ELISA and real-time RT-PCR, is sufficient to detect one infected seed in a sub sample of 250 (https://doi.org/10.1111/epp.12023).

In the last step, the reproducibility of the use of these polyprobes was evaluated by an inter-laboratory screening. To do this, a ring-test analysis was performed between 6 laboratories located in different regions of Spain. Each laboratory received three replicas of the same membrane carrying different dilutions of the positive controls plus a set of 22 positive (five samples with viroids and 17 samples with virus) and 14 negative samples, together with the three polyrprobes. The hybridization of the membranes with the corresponding polyprobe,

Table 1 Summary of the positive results obtained in Fig. 1

Membrane code	AMV	ToMV	PVY	TSWV	PepMV	PMoV	CMV	TEV	ToTV	ToCV	TICV	TYLCV-IS	CEVd	TPMVd	TASVd	PSTVd	Poly4	Poly12	Poly16
a1				+														+	+
a2								+										+	+
a3		+																+	+
a4					+				+									+	+
a5					+													+	+
a6					+													+	+
a7				+														+	+
a8																			
a9				+														+	+
a10							+											+	+
b1			+															+	+
b2																			
b3																		+	+
b4												+		+		+	+		+
b5	+																	+	+
b6	+																	+	+
b7													+	+	+		+		+
b8													+	+		+	+		+
b9													+	+	+	+	+		+
b10							+											+	+
c1													+	+	+		+		+
c2									+				+	+	+	+	+		+
c3				+														+	+
c4																		+	+
c5	+																	+	+
c6							+											+	+
c7						+												+	+
c8													+	+	+	+	+		+
c9		+																+	+
c10																			
d1								+										+	+
d2									+									+	+
d3									+									+	+

Table 1 (continued)

Membrane code	AMV	ToMV	PVY	TSWV	PepMV	PMoV	CMV	TEV	ToTV	ToCV	TICV	TYLCV-IS	CEVd	TPMVd	TASVd	PSTVd	Poly4	Poly12	Poly16
d4					+							+						+	+
d5												+						+	+
d6						+												+	+
d7																			
d8			+			+												+	+
d9			+															+	+
d10																			
e1										+	+							+	+
e2					+													+	+
e3					+							+						+	+
e4																			
e5																			
e6																			
e7																			
e8																			
e9																			
e10																			
f1	+																	+	+
f2		+																+	+
f3			+															+	+
f4				+														+	+
f5					+													+	+
f6						+												+	+
f7							+											+	+
f8								+										+	+
f9									+									+	+
f10										+								+	+
g1											+							+	+
g2												+						+	+
g3													+		+		+		+
g4													+	+	+	+	+		+
g5													+	+	+		+		+
g6														+	+	+	+		+

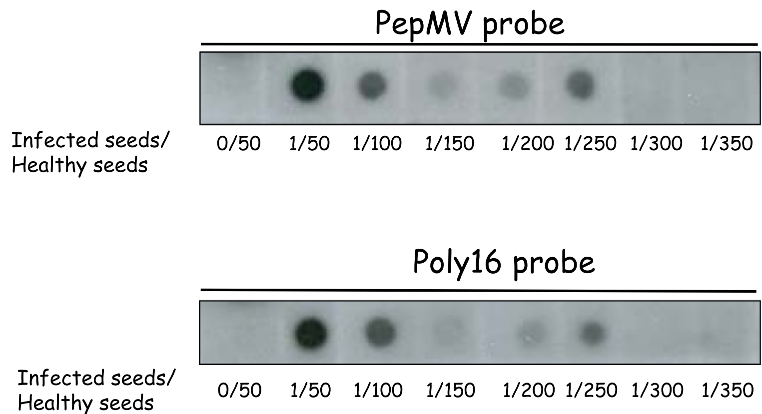

Fig. 2 Analysis of PepMV-infected tomato seeds by non-isotopic molecular hybridization using a PepMV single probe or the poly16. The fractions below the membrane indicate the number of infected seeds mixed in a pool of the indicated healthy seeds. Replicas of the same membrane were hybridized at 68 °C (single probe) or at 50 °C (polyprobe). The films were developed after 30-min exposure

revealed that all laboratories were able to detect picograms of all positive controls with the three polyprobes (between 5 to 1 picograms). In addition, the identification of the right positive and negative samples rendered a Kappa index (Cohen 1960) that ranged between 0.81 and 1 for poly12 and poly16 or between 0,53 and 1 for Poly4 in which a value between 0.61–0.8 represents a substantial agreement meanwhile a value between 0.81–1 correspond to an almost perfect agreement (Online Resource 4). The low Kappa index observed in some laboratories using the Poly4 was due to the presence of false positives meanwhile the same laboratories rendered a good Kappa index using both the poly12 and poly16 probes. The increment of the size of the polyprobes implies a less hybridization that was compensated by reducing the hybridization temperature. In this sense, the cross hybridization observed only with the Poly4 in some laboratories could reflect an inadequate low hybridization temperature, an aspect that was not observed with the bigger poly12 and poly16 probes. Interesting, 3 out 6 laboratories obtained a Kappa index of 1 with the three polyprobes indicating the high reproducibility of this technology.

In summary, our results revealed that MH technique using polyprobes allowed the polyvalent detection of up to 16 different pathogens, including viruses and viroids, both in tomato leaves and seeds and with a detection limit at the picogram level. The high reproducibility of this technology together with the high analysis capacity and the reduced cost of the analysis, make this approach very attractive for the routine diagnosis of the main viruses and viroids affecting tomato crops. The polyprobes described herein represent a very good option as the first step in a virus/viroid screening to evaluate incidence and certification of mother plants or in sanitation or quarantine programs where broad-spectrum tests are required. When the precise knowledge of the pathogen and/or disease are required, the use of single probes or other molecular detections techniques, would be necessary.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. The manuscript was prepared under compliance with ethical standards.

Animal studies and human participants This article does not contain any studies with human participants or animal performed by any of the authors.

Informed consent All authors consent to this submission.

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