Polyvalent Detection of Members of the Genus *Potyvirus* by Molecular Hybridization Using a Genus-Probe

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ABSTRACT

The use of a unique riboprobe named polyprobe, carrying partial sequences of different plant viruses or viroids fused in tandem, has permitted the polyvalent detection of up to 10 different pathogens by using a nonradioactive molecular hybridization procedure. In the present analysis, we have developed a unique polyprobe with the capacity to detect all members of the genus *Potyvirus*, which we have named genus-probe. To do this, we have exploited the capacity of the molecular hybridization assay to cross-hybridize with related sequences by reducing the hybridization temperature. We observed that sequences showing a percentage similarity of 68% or higher could be detected with the same probe by hybridizing at

50 to 55°C, with a detection limit of picograms of viral RNA comparable to the specific individual probes. According to this, we developed several polyvalent polyprobes, containing three, five, or seven different 500-nucleotide fragments of a conserved region of the NIb gene. The polyprobe carrying seven different conserved regions was able to detect all the 32 potyviruses assayed in the present work with no signal in the healthy tissue, indicating the potential capacity of the polyprobe to detect all described, and probably uncharacterized, potyviruses being then considered as a genus-probe. The use of this technology in routine diagnosis not only for *Potyvirus* but also to other viral genera is discussed.

Plants are affected by a large number of viruses, which in many cases cause significant economic losses, compromising the viability of agricultural industries. Effective management of viral diseases requires an integrated approach addressed to prevent or delay the progress of the infection. The use of healthy plants represents one of the main measures to manage viral diseases by reducing the initial source of inoculum. Accordingly, rapid and reliable routine virus testing procedures is a critical step in their control (Jones et al. 2006). Traditional detection methods include bioassays with indicator plants and serological methods such as the enzyme-linked immunosorbent assay (ELISA), although the molecular detection techniques (RT-PCR, microarrays, molecular hybridization, etc.) have been incorporated in the routine diagnosis of plant viruses (Jeong et al. 2014).

During recent years, plant virus detection procedures have been addressed to get the simultaneous detection of the main pathogens affecting a crop, in order to save time, labor, and overall cost. Several strategies have been used for simultaneous detection of plant viruses. For PCR-derived assays, two main approaches have been used according to the use of a cocktail of primers targeting different pathogens (multiplex RT-PCR) or a polyvalent primer pair (polyvalent PCR) that is able to drive the amplification of a conserved region (James et al. 2006). However, the detection limit of the multiplex RT-PCR technique appears to be affected when more than six different pathogens are detected (Sánchez-Navarro et al. 2005) and the polyvalent primers are often compromised by the lack of conserved regions. In the case of the genus *Potyvirus*, the presence of a very conserved region in the NIb gene has permitted the detection of virus isolates from all major groups of the genus, including uncharacterized species (Zheng et al. 2010). The incorporation of the DNA-microarray technology has

incremented significantly the simultaneous detection and identification of multiple plant viruses and (or) virus subgroups (Barba and Hadidi 2011; Bystricka et al. 2005). However, the difficulty of adapting the array technology for routine screening of large numbers of samples and the high costs associated with equipment required have compromised its incorporation in routine plant virus analysis. The serological assay has been also adjusted to polyvalent detection of 33 (Jordan and Hammond 1991) or 14 (Liu et al. 2015) potyvirus species by using broad-spectrum monoclonal antibody (MAb) that recognize a conserved core region of the potyvirus coat proteins. In the latter case, the authors indicate that the MAb could detect many potyviruses in infected plants, but also that different binding affinities were observed in some infected samples.

Multiple detection by using the nonisotopic molecular hybridization technique was addressed first by a cocktail of the specific single probes in the hybridization solution, allowing the polyvalent detection of the different plant viruses affecting ornamental (Sánchez-Navarro et al. 1999), horticultural (Minutillo et al. 2012; Saldarelli et al. 1996), and stone fruits crops (Saade et al. 2000); and second, by using a unique riboprobe, called “polyprobe”, that contains partial nucleic acid sequences of different viruses (Aparicio et al. 2009; Herranz et al. 2005; Peiró et al. 2012) or viroids (Cohen et al. 2006; Lin et al. 2011; Zhang et al. 2012) cloned in tandem. The polyprobe has proved the simultaneous detection of six different viruses (Herranz et al. 2005) or up to eight viroids (Cohen et al. 2006; Torchetti et al. 2012), eight viruses plus two viroids (Peiró et al. 2012) or three different pathogens comprised of virus, viroid, and bacteria (Zamora-Macorra et al. 2015), with the same detection limit as the single assay. Recently, an octamer of 32-nucleotide sequence derived from the central conserved region of viroids in the genus *Coleoviroid* was used to develop a universal probe allowing the detection at least of four coleus viroids (Jiang et al. 2013).

In the present work, we have explored the potential capacity of the polyprobe technology to develop a universal probe with the property to detect all members of a specific viral genus, in this case the genus *Potyvirus*. The cross hybridization observed at 50 to 55°C

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures are published online.

between sequences sharing an identity of 68% or higher was used to design several polyprobes, carrying in tandem different 500-nucleotide fragments of the conserved Nlb gene, with the capacity to cross hybridize with all potyvirus species available in the database. A polyprobe carrying seven different conserved regions was able to detect all the 32 potyviruses assayed in the present work, revealing the potential capacity of this technology to be applied at the genus-probe (GP) level.

MATERIALS AND METHODS

Computing analysis. The phylogenetic analysis was performed with all potyviruses for which the complete nucleotide sequence is available in the database. The phylogenetic analyses were inferred in a multistep process: in the first step, the sequences were aligned using the CLUSTAL W program (Higgins et al. 1994) to generate, in a second step, the neighbor-joining phylogenetic trees, using the JTT model, implemented in the MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumar et al. 2016). Third, the statistical reliability of the constructed trees was assessed by the bootstrap method based on 10,000 pseudoreplicates.

The more conserved region observed in the potyvirus alignment, covering 500 nucleotides of the Nlb gene (Supplementary Fig. S1) was used to estimate the identity value between all the analyzed potyvirus sequences. To do this, we used the MatGAT (Matrix Global Alignment Tool) application that generates similarity/identity matrices for DNA sequences (Campanella et al. 2003).

To identify the equivalent conserved region in the rest of viruses assigned to the family *Potyviridae*, the seven selected conserved regions from *Lettuce mosaic virus* (LMV), *Watermelon mosaic virus* (WMV), *Potato virus Y* (PVY), *Pepper veinal mottle virus* (PVMV), *Plum pox virus* (PPV), *Sweet potato feathery mottle virus* (SPFMV), and *Tobacco etch virus* (TEV) were aligned with all species of the corresponding family *Potyviridae*, for which the complete nucleotide sequence is available in the database, using the CLUSTAL W program. The selected regions were also used to estimate the identity value with the MatGAT application.

Plant materials. 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 Agroforestry Institute at the Polytechnic University of Valencia (<http://www.upv.es/iam/ingles/bienvenida.htm>). Total nucleic acid extraction was performed using 0.1 g of lyophilized or fresh leaf tissue using the silica capture extraction protocol (MacKenzie et al. 1997) that renders total nucleic acids. The extracted nucleic acids were stored at -80°C until use. Alternatively, healthy

and infected tissue were homogenized with 5 volumes of cold extraction buffer (50 mM sodium citrate, 5 mM EDTA, pH 8.5) and directly applied (1 μl) onto nylon membranes (Sanchez-Navarro et al. 1998).

Synthesis of cDNA clones. Reverse transcription and PCR reactions were carried out using specific primers (Table 1), containing the 5' and 3' *XhoI* and *SalI* restriction sites, respectively. PCR products were digested with both restriction enzymes and extracted from the agarose gel. The purified PCR fragments were inserted in the pBluescript SK(+) plasmid, previously digested with the *XhoI* enzyme and dephosphorylated. The incorporation of the purified PCR fragment into the pSK+ plasmid in the right orientation allowed the inactivation of the original *XhoI* site, present in the pSK+ plasmid, by the compatible *SalI* site. This permits the use of the new 5' proximal *XhoI* for the synthesis of the riboprobe or the incorporation of a new PCR fragment (Peiró et al. 2012). Using this strategy, we introduced seven cDNA fragments in the pSK+ corresponding to the partial sequences of the following viruses: LMV, WMV, PVY, PVMV, PPV, SPFMV, and TEV. We generated three polyprobes that differed in the number of viral sequences incorporated. Thus, GP3, GP5, and GP7 contained three (PPV-SPFMV-TEV), five (PVY-PVMV-PPV-SPFMV-TEV), and seven (LMV-WMV-PVY-PVMV-PPV-SPFMV-TEV) viral sequences, respectively.

Synthesis of the digoxigenin-labeled riboprobes and hybridization procedure. For the synthesis of the riboprobes, 1 μg of the corresponding plasmid was linearized with *XhoI* restriction enzyme, purified by phenol-chloroform extraction and precipitated with ethanol. The linearized plasmid was used to synthesize the riboprobe as described previously (Mas et al. 1993; Pallás et al. 1998). 1 μl of the total nucleic acids preparations (undiluted or serially diluted in extraction buffer), were directly applied onto positively charged nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany), air dried and cross-linked by UV crosslinker ($700 \times 100 \mu\text{J}/\text{cm}^2$). Prehybridizations and hybridizations with the riboprobes were conducted as described previously, with the only difference of the temperature selected for the hybridization (Pallás et al. 1998; Sanchez-Navarro et al. 1999). All riboprobes were used at the same concentration in the hybridization solution (20 ng/ml). Chemiluminescent detection using CDP-start reagent as substrate was performed as recommended by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany). Films were exposed for 30 min.

Estimation of the detection limit of the hybridization assays. For the estimation of the detection limit of the single or the three polyprobes, the seven DNA fragments cloned in the pSK+ were PCR amplified using the corresponding antisense primer and the reverse primer. The resultant PCR fragment contains the

TABLE 1. Primer pairs used in the amplification of the genus-probes

Primer name	Nucleotide sequence (5'-3') ^a	Target viral species	Expected fragment (bp)	Accession number and location in the genome ^b
2847-s	CACACTCGCGTAAGTTTGGAGTGTGGAA	<i>Lettuce mosaic virus</i> , LMV	496	X97704
2848-As	CACAGTCGACGTCATAGCTAGCACAAACCAT			7961–8457
2869-s	CACACTCGAGAAGGGAATTTGGAAYGGTTC	<i>Watermelon mosaic virus</i> , WMV	466	EU660589
2870-As	CACAGTCGACTTGTCAACGACTGTAGATGG			7882–8348
2845-s	CACACTCGAGATTGGAACGATCATTGAA	<i>Potato virus Y</i> , PVY	472	AJ890346
2846-As	CACAGTCGACACCATGAGAGAATTATCCAC			7510–7982
2865-s	CACACTCGAGTAAACTTGGGATTG	<i>Pepper veinal mottle virus</i> , PVMV	479	DQ645484
2866-As	CACAGTCGACCATGAGAGTGTATC			7530–8009
2871-s	CACACTCGAGGAAAGAAAGGAGTGTGGAATGG	<i>Plum pox virus</i> , PPV	498	KU508427
2872-As	CACAGTCGCGTCATTGCCAAAATAACCAT			7495–7993
2873-s	CACACTCGAGATGGGATACAAAGGTCTYTGGA	<i>Sweet potato feathery mottle virus</i> , SPFMV	493	KU511268
2874-As	CACAGTCGACGCTAACACAACCATAAGTGT			8563–9056
2861-s	CACACTCGAGGAAAGCTGGGAATTTGG	<i>Tobacco etch virus</i> , TEV	479	DQ986288
2862-As	CACAGTCGACCATGAGTGTGTTGTC			7468–7947

^a Restriction sites of the *XhoI* and *SalI* are underlined.

^b Numbers refer to the corresponding nucleotide of the sequence available in the indicated accession number of the GenBank database.

Fig. 1. Phylogenetic trees of the 94 potyviruses for which the complete sequence is known. Trees were developed using nucleotide sequences of the complete genome and inferred by the minimum-evolution method. Values at the nodes are bootstrap values based on 10,000 pseudoreplicates. Nodes with bootstrap support <50% are not indicated. Sequences in gray boxes were used for the synthesis of the genus-probes. Sequences underlined correspond to the potyvirus used for the hybridization detection using the genus-probes.

corresponding potyvirus clone plus the T3 promoter. The amplicons were purified from agarose gel and then used directly for the synthesis of unlabeled transcripts complementary to the corresponding dig-RNA probes. Known amounts of the free transcripts were serially diluted (fivefold) in sterile water since previous results showed similar detection limit (pg/μl of viral RNA) for the no-radioactive molecular hybridization procedure when the dilutions were performed in sterile water (Peiró et al. 2012) or heathy tissue (Sanchez-Navarro et al. 1996, 1998). The dilutions were applied directly onto nylon membranes and the dot-blot hybridization was conducted as described previously.

RESULTS

Design of the polyprobes and analysis of the cross-hybridization. In order to get representative common sequences that would allow us to design a potential polyprobe at the genus level we performed a phylogenetic analysis using all potyvirus sequences from 94 species. The resultant phylogenetic tree grouped all viruses into different clusters supported with significant bootstrap values (Fig. 1). We selected the following seven potyviruses that cover the full spectrum of disparate sequences (viral names in gray boxes, Fig. 1): LMV, WMV, PVY, PVMV, PPV, SPFMV, and TEV. Sequence alignment of all the 94 potyvirus species revealed a highly conserved region of 500 nt in the NIb gene. The corresponding regions of the seven selected potyvirus were cloned separately in a bacterial plasmid to generate the individual probes but also in tandem to obtain three different polyprobes carrying three (PPV, SPFMV, and TEV), five (PVY, PVMV, PPV, SPFMV, and TEV), or seven (LMV, WMV, PVY, PVMV, PPV, SPFMV, and TEV) viral sequences (Fig. 2A). MatGAT analysis of the seven selected sequences revealed a percent identity that ranged between 64.8 and 72.5% (Fig. 2B).

In the next step, we analyzed the capacity of each individual riboprobe to cross-hybridize to the rest of the selected sequences. To do this, we synthesized complementary transcripts of the seven selected sequences that were serially diluted (1:5) and applied on

Fig. 2. Schematic representation of the different genus-probes (GP) 3, 5, and 7 clones introduced in the pSK+ plasmid. **A**, The corresponding virus fragments are indicated by a box in which the numbers represent the corresponding nucleotide of the database sequences: X97704 (*Lettuce mosaic virus*, LMV), EU660589 (*Watermelon mosaic virus*, WMV), AJ890346 (*Potato virus Y*, PVY), DQ645484 (*Pepper vein mottle virus*, PVMV), KU508427 (*Plum pox virus*, PPV), KU511268 (*Sweet potato feathery mottle virus*, SPFMV), and DQ986288 (*Tobacco etch virus*, TEV). The *XhoI* restriction site used either to synthesize the riboprobe with the T7 RNA polymerase or to insert additional cDNA probes is indicated. **B**, Schematic representation of the identity percentage estimated by MatGAT between the sequences used for the genus-probes LMV, PPV, PVMV, PVY, SPFMV, TEV, and WMV.

Fig. 3. Evaluation of the cross hybridization between the seven potyvirus sequences indicated in Figure 2. **A**, Influence of the temperature in the cross hybridization between the *Plum pox virus* (PPV) probe and the rest of the indicated potyvirus sequences. Known amounts of the positive transcripts corresponding to the sequences indicated in Figure 2 were applied on nylon membranes. For the healthy control, 100 ng of total RNA extracted from melon (A), cucumber (B), *Nicotiana benthamiana* (C), tomato (D), chrysanthemum (E), and gynura (F) were applied. Replicas of the same membrane were hybridized with the PPV riboprobe, complementary to the sequence indicated in Figure 2 at 68, 60, 55, and 50°C, respectively. Numbers on the top of the left panel indicate the picograms of transcripts applied on the membrane, while numbers on the right side indicate the identity percentage estimated by the MatGAT program (Campanella et al. 2003) between the PPV sequence and the rest of the potyvirus sequences indicated in Figure 1. **B**, Summary of the results obtained in A. Numbers represent the identity percentage between the PPV sequence and the rest of the potyvirus sequences, while the colors indicate the lowest amount for which transcripts showed hybridization with the PPV probe. The colors shown online are blue (200 pg), dark blue (40 pg), green (8 pg), yellow (1.6 pg), orange (0.32 pg), and red (0.06 pg). White indicates no hybridization signal. **C**, Schematic representation of the hybridization observed with the seven individual potyvirus probes. Replicas of the membrane described in A were hybridized at 55°C with the indicated probes. Numbers represent the corresponding identity percentage estimated by MatGAT program, while the colors represent the lowest amount of transcripts showing hybridization with the corresponding probe, as indicated in B. Films were exposed for 15 min.

nylon membranes, together with total RNA extracted from several healthy tissues (Fig. 3). Replicas of the same membrane were hybridized first with the PPV riboprobe at different hybridization temperatures, to evaluate the effect of such parameter in the cross-hybridization (Fig. 3A). We observed no cross-reaction when the hybridization was performed at 68°C. However, the reduction of 8 degrees during the hybridization assay (60°C) was sufficient to start to see cross-hybridization with the TEV sequence at high concentration (200 pg/μl), meanwhile the reduction of another 5 (55°C) or 10 (50°C) degrees was sufficient to detect two (TEV, SPFMV) or all sequences at different concentrations. In the case of the hybridization performed at 50°C, we also observed a weak signal in the negative controls (100 ng of total RNA extracted from healthy tissue). Accordingly, we evaluated the cross-hybridization of the remaining riboprobes at 55°C (Fig. 3C). In general, we observed that sequences with an identity percentage below 65% with the corresponding probe were not detected at 55°C but also that sequences with such percentage higher than 67% were all detected, except the PPV probe that rendered negative results with LMV, which shared 69.7% identity. Between 65 and 67% identity, we observed different behaviors suggesting that other factors influence the cross-hybridization (e.g., the size of the identical regions, the percentage of cytosine or guanine nucleotides, etc.).

Next, we evaluated the cross-reactivity of the three polyprobes (GP3, GP5, and GP7) (Fig. 4). For this purpose, we used replicas of the same membrane described in Figure 3 that were hybridized at 50°C, since no cross-hybridization was observed with the negative controls at this temperature. First, we observed that the shorter polyprobe carrying only three sequences (GP3: PPV, SPFMV, and TEV) was able to detect the seven potyvirus sequences in which the lower detection limit corresponded to the PVMV transcripts at 8 pg/μl (Fig. 4). The inclusion of another two viral sequences in the GP5 polyprobe (PVY, PVMV, PPV, SPFMV, and TEV) allowed the detection of all seven viral sequences with

lower detection limits, at least for the sequences present in the polyprobe. Thus, we observed positive hybridization signal for all sequences included in the GP5 until a concentration of 0.06 pg/μl, while the other two sequences not present in the polyprobe were detected until a concentration of 1.6 pg/μl, five times less sensitive than the GP3. Apparently, the enlargement of the polyprobe has a slight negative effect in the detection limit of the heterologous sequences. Finally, we hybridized the membrane with the GP7 polyprobe and we obtained the same detection limit for all analyzed viruses, corresponding to 0.32 pg/μl. As observed for the GP5, the increment of the size of the polyprobe affected the detection limit, reducing five times the best signals obtained with GP3 or GP5.

In silico analysis of the capacity of genus-probe to detect all potyviruses available in the database and other members of the family *Potyviridae*. According to the results obtained, sequences showing an identity percentage of 68% or higher with the selected probes, could be detected by cross-hybridization. To identify how many potyvirus sequences could be potentially hybridized with the cloned potyvirus sequences, we performed a MatGAT analysis (Campanella et al. 2003) using the equivalent region of the 94 potyvirus species used for the phylogenetic analysis (Supplementary Fig. S2). The results showed that all potyvirus species presented an identity percentage of 68% or higher with two or more of the seven selected sequences. Only *Onion yellow dwarf virus*, with a percentage of 69.3%, showed such identity with only PPV. In addition, we observed that the majority of the sequences presented identity percentages of 70% or higher with any of the selected sequences. Only *Daphne mosaic virus*, *Habenaria mosaic virus*, *Hordeum mosaic virus*, *Onion yellow dwarf virus*, *Ornithogalum mosaic virus*, and *Vallota speciosa virus* presented identity percentages below 70%. Also, we observed that the 94 potyvirus species could be theoretically detected with the smaller genus-probe of GP3, since all sequences presented an identity percentage higher than 68% with any of the PPV, TEV, and SPFMV cloned fragments.

To identify if other members of the family *Potyviridae* could be potentially detected with the seven selected potyvirus sequences, the equivalent regions from 27 species representatives of the seven genera of the family *Potyviridae*, except potyvirus, were subjected to a MatGAT analysis (Campanella et al. 2003) to determine the identity percentage. The results obtained revealed that all viruses, except rymovirus, presented an identity percentage below 65%, suggesting that they should be not detected with any of the different probes assayed herein. In the case of rymovirus, the three species analyzed showed identity percentage of 68% or higher with one or more of the seven selected sequences and, thus, susceptible of being detected by the genus-probes.

Analysis of field samples by nonradioactive nucleic acids spot hybridization (NASH). Finally, we evaluated the capacity of the three genus-probes to detect heterologous potyviruses or other viral species of the family *Potyviridae* by analyzing 49 different field samples, including different hosts, for the presence of 32 potyvirus species or 7 viral species assigned to the genus *Bymovirus*, *Ipomovirus*, *Rymovirus*, and *Tritimovirus* (Table 2). First, it should be mentioned that the infected starting material was lyophilized or fresh tissue proportionated by the German Collection of Microorganisms and Cell Cultures (DSMZ) or the Mediterranean Agroforestry Institute of the Polytechnic University of Valencia. The samples were extracted using the silica protocol (MacKenzie et al. 1997) but also with a fast protocol in which the tissue is homogenized with citrate buffer and directly applied onto the membrane (Sanchez-Navarro et al. 1998, 1999). First, we observed no hybridization signal with any of the eight healthy hosts analyzed, in spite of the hybridization being performed at 50°C. The 32 potyviruses and the 2 rymoviruses were detected by NASH with the three genus-probes except for PVMV that rendered negative signal with GP3. Interestingly, GP3 presents the most unfavorable situation for PVMV detection since its identity percentage with

Fig. 4. Evaluation of the cross hybridization between the seven potyvirus sequences and genus-probes (GP) 3, 5, and 7. **A**, Replicas of the membrane described in Figure 3A were hybridized with the indicated genus-probe at 50°C. For the healthy control, 100 ng of total RNA extracted from melon (A), cucumber (B), *Nicotiana benthamiana* (C), tomato (D), chrysanthemum (E), and gynura (F) were applied. Numbers on the top of the left panel indicate the picograms of transcripts applied on the membrane. Films were exposed for 15 min. **B**, Summary of the results obtained in A. The colors indicate the lowest amount of transcripts showing hybridization with the corresponding genus-probe. The colors shown online are blue (200 pg), dark blue (40 pg), green (8 pg), yellow (1.6 pg), orange (0.32 pg), and red (0.06 pg). White indicates no hybridization signal.

the three cloned sequences present in GP3 is around 66 to 68% (Fig. 3). On the other hand, we observed that *Onion yellow dwarf virus*, which theoretically could be detected only with the PPV clone (identity percentage of 69.3%), was detected with all three genus-probes. In addition, *Ryegrass mosaic virus* was clearly detected with GP3 and GP5, in spite of the fact that the higher identity percentage in both probes corresponded to the PPV cloned sequence at 67.2%. When the plants were analyzed using the fast citrate buffer protocol, we observed that 90.2% of silica positives (83 out 92) were correctly detected. We also observed two samples that were positive by citrate buffer extraction and negative by silica extraction, using the GP3 and GP5 probes (samples 30 and 34, Table 2). These results could indicate differences in the viral titer associated to the extraction protocol used but we cannot discard other effects derived of the starting material (lyophilized)

or the host (*Nicotiana benthamiana* or *Cucurbita pepo*) that could interfere with the detection procedure.

DISCUSSION

In the last few years, a significant effort has been made to develop strategies for simultaneous detection of multiple plant viruses. Although PCR-based approaches have received special attention, molecular hybridization assay represents an attractive methodology for the detection of plant viruses since it is accurate, sensitive (pg/μl of viral RNA), robust, and very powerful for large screening with a reduced cost (James et al. 2006; Pallás et al. 2011, 2017 for review). Previous works showed that this technology allows multiple detection of plant viruses by a cocktail of specific probes or by a unique probe carrying the different viral fragments fused in

TABLE 2. Analysis of the capacity of genus-probes (GP) 3, 5, and 7 to detect different potyvirus, bymovirus, ipomovirus, rymovirus, and tritovirus in field samples by nucleic acids spot hybridization (NASH)^a

Virus	Acronym	Genus	Source ^b	Host	NASH (silica/citrate buffer)		
					GP3	GP5	GP7
<i>Carrot virus Y</i>	CarVY	<i>Potyvirus</i>	UPV	<i>Daucus carota</i>	+/+	+/+	+/+
<i>Onion yellow dwarf virus</i>	OYDV	<i>Potyvirus</i>	UPV	<i>Allium cepa</i>	+/-	+/-	+/+
<i>Turnip mosaic virus</i>	TuMV	<i>Potyvirus</i>	UPV	<i>Lactuca sativa</i>	+/+	+/+	+/+
<i>Bean common mosaic necrosis virus</i>	BCMN	<i>Potyvirus</i>	DSMZ	<i>Phaseolus vulgaris</i>	+/+	+/+	+/+
<i>Bean common mosaic virus</i>	BCMV	<i>Potyvirus</i>	DSMZ	<i>Phaseolus vulgaris</i>	+/+	+/+	+/+
<i>Beet mosaic virus</i>	BtMV	<i>Potyvirus</i>	DSMZ	<i>Nicotiana benthamiana</i>	+/+	+/+	+/+
<i>Johnsongrass mosaic virus</i>	JGMV	<i>Potyvirus</i>	DSMZ	<i>Zea mays</i>	+/+	+/+	+/+
<i>Leek yellow stripe virus</i>	LYSV	<i>Potyvirus</i>	DSMZ	<i>Chenopodium quinoa</i>	+/+	+/+	+/+
<i>Pepper mottle virus</i>	PepMoV	<i>Potyvirus</i>	DSMZ	<i>Nicotiana tabacum</i>	+/*	+/+	+/+
<i>Peru tomato mosaic virus</i>	PTV	<i>Potyvirus</i>	DSMZ	<i>Nicotiana tabacum</i>	+/*	+/+	+/+
<i>Potato virus A</i>	PVA	<i>Potyvirus</i>	DSMZ	<i>Nicotiana occidentalis</i>	+/+	+/+	+/+
<i>Potato virus V</i>	PVV	<i>Potyvirus</i>	DSMZ	<i>Nicotiana benthamiana</i>	+/+	+/+	+/+
<i>Potato virus Y</i>	PVY	<i>Potyvirus</i>	DSMZ	<i>Nicotiana tabacum</i>	+/*	+/+	+/-
<i>Soybean mosaic virus</i>	SMV	<i>Potyvirus</i>	DSMZ	<i>Nicotiana benthamiana</i>	+/+	+/+	+/*
<i>Tobacco vein banding mosaic virus</i>	TVBMV	<i>Potyvirus</i>	DSMZ	<i>Nicotiana benthamiana</i>	+/+	+/+	+/+
<i>Maize dwarf mosaic virus</i>	MDMV	<i>Potyvirus</i>	DSMZ	<i>Zea mays</i>	+/+	+/+	+/+
<i>Dasheen mosaic virus</i>	DsMV	<i>Potyvirus</i>	DSMZ	<i>Nicotiana benthamiana</i>	+/+	+/+	+/+
<i>Apium virus Y</i>	ApVY	<i>Potyvirus</i>	DSMZ	<i>Ami majus</i>	+/+	+/+	+/+
<i>Pepper yellow mosaic virus</i>	PepYMV	<i>Potyvirus</i>	DSMZ	<i>Nicotiana tabacum</i>	+/*	+/+	+/+
<i>Sweet potato virus 2</i>	SPV-2	<i>Potyvirus</i>	DSMZ	<i>Nicotiana benthamiana</i>	+/+	+/+	+/+
<i>Bean yellow mosaic virus</i>	BYMV	<i>Potyvirus</i>	DSMZ	<i>Nicotiana benthamiana</i>	+/+	+/+	+/+
<i>Carrot thin leaf virus</i>	CTLV	<i>Potyvirus</i>	UPV	<i>Daucus carota</i>	+/+	+/+	+/+
<i>Celery mosaic virus</i>	CeMV	<i>Potyvirus</i>	UPV	<i>Apium graveolens</i>	+/nt	+/nt	+/nt
<i>Clover yellow vein virus</i>	CIYVV	<i>Potyvirus</i>	UPV	<i>Phaseolus vulgaris</i>	+/nt	+/nt	+/nt
<i>Freesia mosaic virus</i>	FreMV	<i>Potyvirus</i>	UPV	<i>Freesia</i>	+/+	+/+	+/-
<i>Papaya ringspot virus</i>	PRSV	<i>Potyvirus</i>	UPV	<i>Citrullus lanatus</i>	+/nt	+/nt	+/nt
<i>Tobacco etch virus</i>	TEV	<i>Potyvirus</i>	UPV	<i>Nicotiana tabacum</i>	+/+	+/+	+/+
<i>Tobacco etch virus</i>	TEV	<i>Potyvirus</i>	UPV	<i>Physalis floridana</i>	+/+	+/+	+/+
<i>Lettuce mosaic virus</i>	LMV	<i>Potyvirus</i>	DSMZ	<i>Chenopodium quinoa</i>	-/+	-/+	+/+
<i>Pepper vein mottle virus</i>	PVMV	<i>Potyvirus</i>	DSMZ	<i>Nicotiana benthamiana</i>	-/-	+/+	+/+
<i>Sweet potato feathery mottle virus</i>	SPFMV	<i>Potyvirus</i>	DSMZ	<i>Nicotiana benthamiana</i>	+/+	+/+	+/+
<i>Tobacco etch virus</i>	TEV	<i>Potyvirus</i>	UPV	<i>Nicotiana tabacum</i>	+/+	+/+	+/+
<i>Watermelon mosaic virus</i>	WMV	<i>Potyvirus</i>	DSMZ	<i>Cucurbita pepo</i>	-/+	-/+	+/+
<i>Plum pox virus</i>	PPV	<i>Potyvirus</i>	Lab	<i>Prunus domestica</i>	+/nt	+/nt	+/nt
<i>Barley yellow mosaic virus</i>	BaYMV	<i>Bymovirus</i>	DSMZ	<i>Hordeum vulgare</i>	-/-	-/-	-/-
<i>Ugandan cassava brown streak virus</i>	UCBSV	<i>Ipomovirus</i>	DSMZ	<i>Manihot esculenta</i>	-/-	-/-	-/-
<i>Cucumber vein yellowing virus</i>	CVYV	<i>Ipomovirus</i>	UPV	<i>Cucumis sativus</i>	-/-	-/-	-/-
<i>Sweet potato mild mottle virus</i>	SPMMV	<i>Ipomovirus</i>	DSMZ	<i>Nicotiana tabacum</i>	-/-	-/-	-/-
<i>Agropyron mosaic virus</i>	AgMV	<i>Rymovirus</i>	DSMZ	<i>Triticum aestivum</i>	+/+	+/+	+/+
<i>Ryegrass mosaic virus</i>	RGMV	<i>Rymovirus</i>	DSMZ	<i>Lolium multiflorum</i>	+/+	+/+	+/+
<i>Brome streak mosaic virus</i>	BrSMV	<i>Tritovirus</i>	DSMZ	<i>Hordeum vulgare</i>	*/-	*/-	*/-
Healthy control			Lab	<i>Cucumis melo</i>	-/nt	-/nt	-/nt
Healthy control			Lab	<i>Cucumis sativus</i>	-/nt	-/nt	-/nt
Healthy control			Lab	<i>Nicotiana benthamiana</i>	-/-	-/-	-/-
Healthy control			Lab	<i>Solanum lycopersicum</i>	-/nt	-/nt	-/nt
Healthy control			Lab	<i>Chrysanthemum</i>	-/nt	-/nt	-/nt
Healthy control			Lab	<i>Gynura aurantiaca</i>	-/nt	-/nt	-/nt
Healthy control			Lab	<i>Nicotiana tabacum</i>	-/-	-/-	-/-
Healthy control			UPV	<i>Arabidopsis thaliana</i>	-/-	-/-	-/-

^a All samples were extracted with the silica and the citrate buffer protocols. The hybridization was performed at 50°C. +, -, and * correspond to positive, negative, and uncertain results, respectively. nt, no tissue available.

^b Samples were obtained from Isabel Font's group at the Universidad Politécnica de Valencia (UPV), the German Collection of Microorganisms and Cell Cultures (DSMZ), or our laboratory (Lab).

tandem called a polyprobe. The polyprobes permit the detection of several pathogens with comparable detection limit to the individual probes (Herranz et al. 2005), although with long polyprobes (e.g., 10 probes in tandem or more) a reduction of the hybridization temperature is required (Peiró et al. 2012). A challenge of this strategy has been its adaptation by generating a polyvalent polyprobe able to detect all species within a genus, named genus-probe. To answer this question we have selected the genus *Potyvirus* for three reasons: (i) it is one of the largest groups of plant viruses including 146 species (Revers and Garcia 2015), (ii) potyviruses affect many species which are economically important (Scholthof et al. 2011), and (iii) some other diagnosis assays have been adjusted for the detection of a broad spectrum of potyviruses with varied success (Chen et al. 2001; Hsu et al. 2005; Jordan and Hammond 1991; Liu et al. 2015; Wei et al. 2009). To obtain the genus-probe we have used the versatility of the molecular hybridization that permits the detection of closely related sequences by reducing the hybridization temperature. Thus, we selected the more conserved region, representing 500 nt of the NIb gene, of seven potyviruses distributed along the genus phylogenetic tree and we observed that cross-hybridization occurs at 55°C (single probes) or 50°C (polyprobes) when the target sequences share 68% or higher identity percentage with the probe, meanwhile negative hybridization signal was obtained when the percentage was below 65%. We observed some exceptions to these rules (e.g., LMV is not detected by PPV probe sharing 69%) suggesting that factors other than the identity percentage are important in the cross-hybridization. In this sense, the presence of large identical regions, the percentage of cytosine and guanines or the probe mismatches, among others, are critical factors to take into account (Kessler 2000). In these hybridization conditions, there were no positive signals in the different healthy controls used, opening the applicability of this assay for routine diagnosis. According with the identity percentage required for cross-hybridization, the three genus-probes described herein have the capacity to detect the 94 potyvirus species analyzed. However, we observed some negative results (samples 29, 30, or 33) using the GP3 that were positive by GP5 or GP7. Apparently, the presence of more conserved fragments with the capacity to hybridize with a target sequence increments the detection limit of the GP. In our hands, we were able to detect 100% of potyviruses analyzed (32 species in 34 samples), representing a comparable (33 species in 55 samples) (Jordan and Hammond 1991) or a significant increment from previous approaches using broad-spectrum MAb (14 species) (Liu et al. 2015) or polyvalent primer pairs that ranged between 100% (23 species in 40 samples) (Zheng et al. 2010), 80% (32 out 40 samples corresponding to 21 species out 23) (Pappu et al. 1993), or 50% (20 out 40 samples corresponding to 15 species out 23) (Gibbs and Mackenzie 1997). We also observed a positive hybridization signal with the two rymovirus analyzed, while no hybridization was observed with the rest of the viral species assigned to bymovirus, ipomovirus, or tritrovirus. Interesting, the two rymovirus AgMV and RGMV (samples 39, 40) plus BrSMV (tritrovirus, sample 41) and BaYMV (bymovirus, sample 25) were also detected by RT-PCR using the universal potyviruses NIb primers (Zheng et al. 2010) (data not shown), indicating high conserved sequences in this region in the family *Potyviridae*. Positive hybridization signal was also observed in some species that share only an identity percentage close to the estimated limit of 68% for cross-hybridization, with one of the conserved fragments of the genus-probe (e.g., *Onion yellow dwarf virus* or *Ryegrass mosaic virus*). In addition, the possibility to use a fast protocol, which detected 90% of positive samples analyzed by NASH, together with the robustness of the methodology that permits the detection of species with significant nucleotide sequences variability or even uncharacterized potyviruses and the reduced time and cost make the nonradioactive hybridization of using a genus-probe a very suitable approach for the routine diagnosis of potyvirus in large surveys. Currently, the use of a broad-spectrum MAb PTY1 (Jordan and Hammond 1991),

combined with a direct tissue extract procedure, represents a valuable tool for diagnostic and screening applications for the detection of aphid-transmissible potyviruses, although it is not known how large the spectrum of viruses that could be detected by the MAb PTY1. The genus-probe developed in this work anticipates that all potyviruses can be broadly detected (identity percentage of 68/69% or higher). Furthermore, this technology presents the advantage to be easily updated by introducing new sequences of interest (e.g., new potyviruses, etc.). In addition, the use of this new genus-probe, in conjunction to other molecular techniques that permit the identification of the corresponding potyvirus species (e.g., degenerate primer-based RT-PCR or HTS methods, etc.), could be a very good approach to detect any potyvirus isolate, previously described or uncharacterized. Finally, the observation that the genus-probe could detect also members of the genus *Rymovirus*, opens the possibility to design a genus-probe against the family *Potyviridae*. An open question related to the genus-probe technology is the size of the conserved regions to be introduced in the polyprobe. In the present work, we have selected fragments of around 500 nt but further analysis will be addressed to delimitate the minimal size of the conserved regions that still are able to cross-hybridize.

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