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Development of a polyprobe for the simultaneous detection of four grapevine viroids in grapevine plants

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Abstract This study aimed to develop a polyprobe for the simultaneous detection of four viroids that infect grapevine: *Hop stunt viroid* (HSVd), *Australian grapevine viroid* (AGVd), *Grapevine yellow speckle viroid*-1 and 2 (GYSVd-1, 2), using a non-isotopic dot blot hybridization technique. A polyprobe was constructed by cloning tandem full-length sequences of HSVd, AGVd and GYSVd-1 into a single vector. The cRNA polyprobe detected all four viroids with similar sensitivity to that obtained using individual probes. In addition, samples of 78 varieties from Beijing and Xinjiang were analyzed using the polyprobe to survey the incidence of grapevine viroids in China. The result demonstrated that grapevine viroids were detected in 56 (71.8%) varieties. In this study, a rapid, reliable and cost-effective approach to the simultaneous detection of four grapevine viroids has been developed which has the potential for routine use in quarantine and certification programs.

Keywords Detection · Dot blot hybridization · Grapevine viroid · Polyprobe

Abbreviations

AGVd	*Australian grapevine viroid*
CCR	Central conserved region
CEVd	*Citrus exotic viroid*
CTAB	Cetyltrimethylammonium bromide
EDTA	Ethylenediaminetetraacetic acid
EGME	Ethylene glycol monomethyl ether
GYSVd-1, 2	*Grapevine yellow speckle viroid* 1 and 2
HSVd	*Hop stunt viroid*
LAMP	Loop-mediated isothermal PCR
nt	Nucleotide
PAGE	Polyacrylamide gel electrophoresis
PVP-40	Polyvinylpyrrolidone-40
RT-PCR	Reverse transcriptase polymerase chain reaction

Viroids are the smallest known plant pathogens and have been identified as agents of several economically significant crop diseases. They comprise small (246–401 nt), uncapsidated, covalently closed circular, non-

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coding RNA molecules (Flores et al. 2005; Ding 2009) which rely entirely on host factors for their replication. All of the known viroid species are classified into two families (Flores et al. 1998), *Pospiviroidae* and *Avsunviroidae*, based on the presence or absence, respectively, of a central conserved region (CCR). Five viroids, HSVd, AGVd, GYSVd-1, 2 and CEVd have been reported to infect grapevines (Flores et al. 1985; Sano et al. 1986; Garcia-Arenal et al. 1987; Koltunow and Rezaian 1988; Rezaian et al. 1988; Rezaian 1990) although only GYSVd-1 and 2 have been shown to induce yellow speckled symptom expression (Koltunow et al. 1989; Szychowski et al. 1998). HSVd, AGVd and CEVd produce no obvious disease symptoms and replicate in the grapevine unnoticed, acting as a symptomless reservoir, which represents a potential threat to other crops.

Viroids are commonly detected by bioassay, polyacrylamide gel electrophoresis (PAGE), molecular hybridization and RT-PCR (Owens and Diener 1981; Hadidi and Yang 1990; Astruc et al. 1996; Ragozzino et al. 2004). However, these methods have limited practicality. Bioassays are associated with constraints of time and space, while PAGE is restricted by the number of samples for analysis. Molecular hybridization and routine RT-PCR have become important tools for viroid detection (Owens and Diener 1981; Hadidi and Yang 1990). The sensitivity of RT-PCR is higher than that of molecular hybridization, although this approach is associated with a high rate of false positive identification due to sample contamination. More recently, LAMP and real-time PCR procedures have been used to reduce the effects of contamination (Boonham et al. 2004; Boubourakas et al. 2009; Monger et al. 2010; Luigi and Faggioli 2011). However, the cost of these methods is substantially higher than molecular hybridization techniques. In recent years, non-isotopic molecular hybridization techniques have been used for the simultaneous detection of multiple plant viroids and viruses. This approach has become an attractive method for routine diagnosis due to advantages of low-cost and practicality (Herranz et al. 2005; Cohen et al. 2006; Aparicio et al. 2008; Lin et al. 2011).

This study aimed to develop a rapid, sensitive, reliable and low-cost method for simultaneous detection of the four grapevine viroids; namely, a polyprobe containing tandem full-length sequences of HSVd, AGVd and GYSVd-1, was constructed and the efficiency of viroid detection by dot blot hybridization was evaluated. Furthermore, this approach was applied to survey the incidence of viroid infection of grapevines in China.

Total RNAs of infected grapevine plants with each viroid, kept in Grapevine Germplasm Resources Garden in Beijing, were extracted using a modified CTAB method (Lin et al. 2011). In brief, fresh leaf tissue (0.1 g) was ground in the presence of liquid nitrogen in a 1.5-ml tube and mixed with pre-heated (65°C) CTAB buffer (2% CTAB, 2% PVP-40, 100 mM Tris–HCl pH 8.0, 2.0 M NaCl, 20 mM EDTA and 2% β-mercaptoethanol). The homogenate was incubated at 65°C for 15 min and centrifuged at 12,000× *g* for 5 min. The upper phase was transferred to a new tube and extracted with an equal volume of phenol/chloroform (1:1) twice before centrifugation at 12,000× *g* for 10 min. The supernatant was mixed with an equal volume of potassium phosphate dibasic (2.5 M) and EGME to remove polysaccharides. After centrifugation at 12,000× *g* for 10 min, the supernatant was homogenized with an equal volume of isopropanol, and the mixture was centrifuged at 15,000× *g* for 10 min. The pellet was washed twice with 70% ethanol, air-dried and dissolved in 20 μl sterile water. The quality of the extracted RNA was measured using a NanoDrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop, USA) and visualized by agarose gel (1%) electrophoresis.

The full-length cDNAs of HSVd, AGVd, GYSVd-1 and 2 were amplified from infected plants by RT-PCR using specific primers (Table 1). The resulting products were cloned into the pGEM-T vector (Promega, Madison, WI, USA) according to the instructions provided by the manufacturer. The recombinant plasmids containing individual full-length cDNAs of the viroids were used: (1) to produce single probes and plus-strand unlabelled RNAs of each viroid, (2) to construct recombinant plasmids, containing tandem individual full-length cDNAs of the viroids, which were then used to generate polyprobe. The cloned fragment of AGVd was subcloned in tandem into a recombinant plasmid containing the HSVd fragment (pGEM/HS) to generate a recombinant plasmid of the two viroids (pGEM/HS+AG). The pGEM/HS+AG+GY plasmid which contained a fusion of the full-length sequences of the three viroids was constructed (Fig. 1a) using the same strategy.

Table 1 Primers used for the construction of polyprobe cDNA

Viroid	Primer name	Primer sequences 5′-3′[a]	Position	Restriction enzymes
HSVd	HSF	AA*CCCGGG*GCAACTCTTCTC	78–96	*Sma* I
	HSR	AA*CCCGGG*GCTCCTTTCTCA	84–66	
GYSVd-1	GV1F	*CCATGG*GTCGACGAAGGGGTGCA	98–114	*Nco* I
	GV1R	*CCATGG*GTCGACGACGAGGCTCACT	103–84	
GYSVd-2	GV2F	*ACTAGT*ACTTTCTTCTATCTCCGAAGC	188–208	*Spe* I
	GV2R	*ACTAGT*CCGAGGACCTTTTCTAGCGCTC	187–166	
AGVd	AGF	*CTGCAG*GGAAGCTAGCTGGGTC	240–261	*Pst* I
	AGR	*CTGCAG*GTTTCGCCAGCAACAG	245–218	

[a] Italics—restriction sites

Three single probes and a polyprobe were prepared from the linearized recombinant plasmids containing individual full-length cDNA of each viroid and a polyprobe by transcription using T7 RNA polymerase and DIG RNA Labeling Kit (Roche, USA) according to the manufacturer's instruction. The specificity and sensitivity of each single probe and a polyprobe were evaluated using the corresponding plus-strand viroid RNAs transcribed from the same recombinant plasmids by SP6 RNA polymerase. Serial five-fold dilutions of 1 ng of transcripts were denatured using formaldehyde and applied onto positively charged nylon membranes. To survey the incidence of grapevine viroids in China, total RNAs extracted from grapevines using modified CTAB method as above were applied similarly. The membranes were air-dried and UV cross-linked (1,200×100 J/cm^2). Pre-hybridization and hybridization was performed at

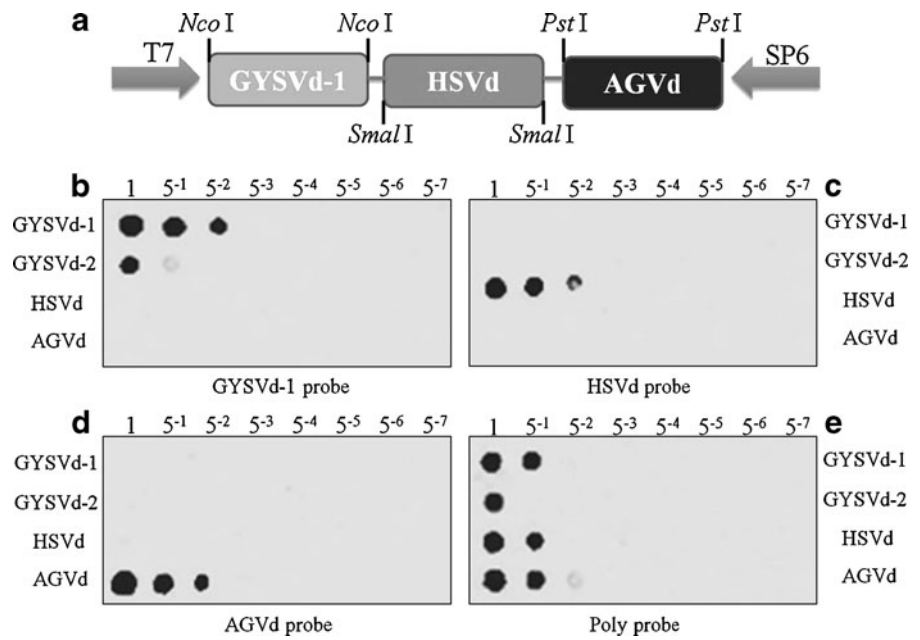

Fig. 1 **a** Schematic representation of the pGEM-t/HSVd+AGVd+GYSVd-1 plasmid. The marked restriction sites were used to subclone the full-length cDNA sequence of the corresponding viroids into a single plasmid. The polyprobe of negative polarity corresponding to viroid RNA was transcribed by T7 RNA polymerase. **b**–**e** Comparison of specificity and the detection limit between individual probes and the polyprobe. The corresponding complementary unlabelled plus-strand of viroid RNAs were used to test the sensitivity of the probes. The RNA transcripts were quantified by spectrophotometry and serial five-fold dilutions of 1 ng transcripts in RNA dilution buffer were spotted separately onto four nylon membranes. Three membranes (**b**–**d**) were hybridized with the corresponding individual probes as indicated and the remaining membrane (**e**) was hybridized with the polyprobe

68°C according to the instructions by the manufacturer (Roche Diagnostics GmbH, Germany). Each membrane, containing RNA transcripts of the four viroids, was hybridized separately with the specific single probe (Fig. 1b–d) or with the polyprobe (Fig. 1e). As shown in Fig. 1b–d, two viroids, HSVd and AGVd, were detected specifically by the corresponding probes, however, cross-hybridization was observed between GYSVd-2 transcript and GYSVd-1 probe. GYSVd-2 was detected by GYSVd-1 probe at an end point of 5^{-1} (200 pg), whereas GYSVd-1 was detected down to 5^{-2} dilutions (40 pg). This reconfirmed the former observation that GYSVd-2 cross-hybridized with GYSVd-1 (Koltunow and Rezaian 1988; Semancik and Szychowski 1992). Moreover, all four viroids were detected by the polyprobe (Fig. 1e). These results demonstrated successful detection of all four viroids simultaneously using the polyprobe. Following above experiments, the sensitivity of the polyprobe was compared to that of the individual probes by estimation of the lower limit of detection in each case. The sensitivity of the polyprobe was approximately equivalent to that of the individual probes (Fig. 1b–e). The end points for the detection of GYSVd-2 (5^{-1}) and other three viroids (5^{-2}), corresponding to 200 pg and 40 pg respectively, were identical using both the polyprobe and the individual probes. These data demonstrated reliable detection of the viroids by dot blot hybridization assay using the polyprobe.

To validate viroid detection in field samples using the polyprobe and to survey the incidence of viroid infection of grapevines in China, three samples each from 78 varieties, a total of 234 samples, were collected and were mixed as one detection sample. The majority of the grapevine samples of different varieties investigated in this study was obtained from the Grapevine Germplasm Resources Garden in Beijing, which holds a collection of different varieties including commercial and wild varieties from the major grapevine growing regions in China. The remaining samples were collected from Xinjiang, the major grape producing region in China. Therefore, these samples represent host diversity and a wide geographical distribution of grapevine viroids. Figure 2 showed the hybridization results of different samples using the three single riboprobes and the polyprobe. In all the cases, plants that were maintained as positive controls gave intense hybridization signals (Fig. 2, H10), whereas no signals were obtained from healthy controls (Fig. 2, H9). All samples that tested positive for HSVd, AGVd and GYSVd-1 using the individual probes also tested positive by hybridization using the polyprobe. Furthermore, no signal was obtained using individual probes of HSVd, AGVd and GYSVd-1 in samples that tested negative using polyprobe. These data further confirmed the reliability of the polyprobe.

Table 2 summarized the results of hybridization analyses of 78 varieties using individual probes and the polyprobe. The numbers of the 78 varieties in Table 2 correspond to A1-H8 in Fig. 2 orderly. Of the 78 samples analyzed, 47 (60.7%) samples were positive for HSVd, 5 (6.4%) were positive for AGVd, and 46 (59.0%) samples were positive for GYSVd-1 when using single riboprobes respectively. Following sample analysis using the polyprobe displayed that 56 (71.8%) samples tested positive, of which 37 samples were co-infected with HSVd and GYSVd (GYSVd-1 or 2). It is worth noticing that the samples positive for GYSVd-1 may be infected by GYSVd-1 and/or GYSVd-2, and possible infection of GYSVd-3, a tentative species, also cannot be excluded, because GYSVd-3 shared 87.77 to 88.59% sequence similarity with GYSVd-1(Jiang et al. 2009). Of the five samples that tested positive for the case of AGVd, four samples contained mixtures of HSVd and GYSVd. The results obtained in this study showed that occurrence of HSVd and GYSVd was widespread both in field and Germplasm Resources Garden samples, although the distribution of AGVd in China was sporadic. More recently, when we were underway in examining the polyprobe, CEVd infection in grapevines has been reported in China (Shu et al. 2010). We have also examined 64 grapevine samples collected from Xinjiang and Beijing, but CEVd infection was not detected in any of the samples by RT-PCR (data not shown). CEVd-grapevine has been reported only in a few countries and the infection rate is much lower than the other grapevine viroids, so CEVd detection was tentatively excluded in the present analysis.

It is known that some viroids induced no obvious symptoms in their corresponding natural hosts and act as a symptomless reservoir that may represents a threat to other sensitive crops. Globalization of agriculture has promoted the exchange of plant

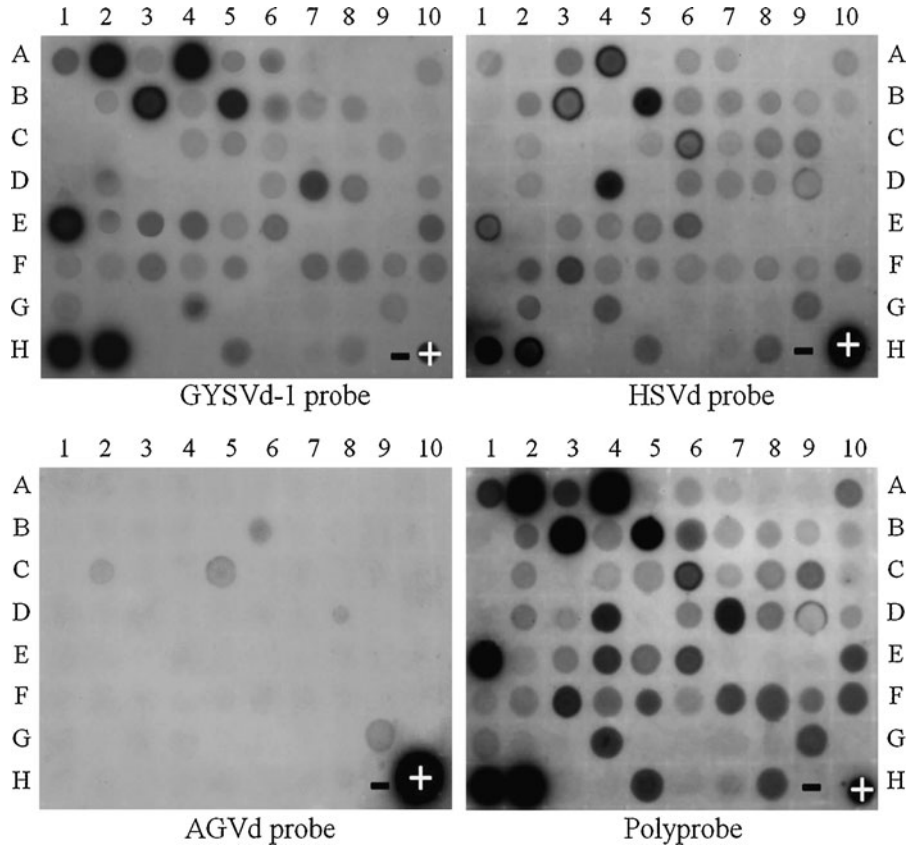

Fig. 2 Survey of the presence of grapevine viroids detected by individual probes and the polyprobe. Total nucleic acids were extracted from the collected grapevine samples using the modified CTAB method. Three microliters of samples (1 μg/μl) were first denatured using formaldehyde and then applied to four separate nylon membranes. One membrane was hybridized with the polyprobe indicated on the bottom of the membrane, while the remaining membranes were hybridized separately with the individual probes (HSVd, AGVd, and GYSVd-1). The negative controls (−) were located at positions H9 respectively, and the positive controls (+) located at positions H10 for GYSVd-1, HSVd and AGVd probes were samples infected with GYSVd-1, HSVd and AGVd individually. Plus-strand RNA transcripts of pGEM/HS+AG+GY was located at H10 as positive control for polyprobe. All other positions represented field samples

germplasm among different countries and also increased the potential for epidemics of viroid diseases. It had been reported that latent HSVd infection of cultivated grapevines can evolve and be transmitted to hop crops causing epidemics of hop stunt disease (Kawaguchi-Ito et al. 2009). Therefore, the availability of sensitive and reliable detection techniques is critical for the diagnosis of viroids in both germplasm collections and mother propagating materials in order to facilitate the protection of viroid-free crops. In this study, a non-isotopic dot blot hybridization technique was developed using a polyprobe for the simultaneous detection of four grapevine viroids. Such strategies involving the construction of polyprobes for the simultaneous detection of several viroids have become important for routine diagnosis of viroids due to the reduction of cost, time and labour requirements compared with methods involving detection of individual viroids (Cohen et al. 2006; Lin et al. 2011). However, the individual viroids resulting in a positive signal using the polyprobe were not distinguished. This should not be regarded as a limitation since infection with any one of the viroids would result in rejection of infected budwood source trees. Furthermore, any samples that tested positive using the polyprobe can be further investigated using multiplex RT-PCR (Matsushita et al. 2010). Future research will focus on the development of a multiplex RT-PCR detection and identification system for HSVd, AGVd, CEVd, GYSVd-1 and 2.

Table 2 Detection results of grapevine viroids from 78 grapevine varieties by hybridization using individual probes and the polyprobe

Variety[a]	Single probe			Polyprobe
	HSVd	AGVd	GYSVd-1	
23(A1[b])	+		+	+
71(A2)			+	+
50(A3)	+		+	+
72(A4)	+		+	+
40(A5)			+	+
37(A6)	+		+	+
54(A7)	+			+
73(A8)				
74(A9)				
53(A10)	+		+	+
52(B1)				
45(B2)	+		+	+
42(B3)	+		+	+
60(B4)			+	+
61(B5)	+		+	+
59(B6)	+	+	+	+
64(B7)	+		+	+
66(B8)	+		+	+
24(B9)	+			+
67(B10)	+			+
63(C1)				
13(C2)	+	+		+
7(C3)				
35(C4)			+	+
47(C5)	+	+	+	+
44(C6)	+		+	+
9(C7)	+			+
22(C8)	+			+
21(C9)	+		+	+
29(C10)				
56(D1)				
48(D2)	+		+	+
28(D3)				
57(D4)	+			+
65(D5)				
55(D6)	+		+	+
41(D7)	+		+	+
27(D8)	+	+	+	+
31(D9)	+			+
8(D10)			+	+
4(E1)	+		+	+
2(E2)			+	+
26(E3)	+		+	+
39(E4)	+		+	+
49(E5)	+		+	+
19(E6)	+		+	+
1(E7)				
3(E8)				
5(E9)				
6(E10)			+	+
10(F1)			+	+
11(F2)	+		+	+
12(F3)	+		+	+
14(F4)	+		+	+
15(F5)	+		+	+
16(F6)	+			+
17(F7)	+		+	+
18(F8)	+		+	+
20(F9)	+		+	+
30(F10)	+		+	+
32(G1)			+	+
33(G2)	+			+
34(G3)				
36(G4)	+		+	+
38(G5)				
43(G6)				
46(G7)				
51(G8)				
58(G9)	+	+	+	+
62(G10)				
68(H1)	+		+	+
70(H2)	+		+	+
75(H3)				
76(H4)				
77(H5)	+		+	+
78(H6)				
79(H7)				
25(H8)	+		+	+
Infection rate	47/78 (60.3%)	5/78 (6.4%)	46/78 (59.0%)	56/78 (71.8%)

[a] Names of cultivars are available only to authorized users

[b] The notation corresponded with the position in Fig. 2

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