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Protocol

Development of a polyprobe to detect six viroids of pome and stone fruit trees

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A simple and sensitive dot blot hybridization assay using a digoxigenin-labeled cRNA polyprobe was developed for the simultaneous detection of six viroids that infect pome and stone fruit trees. The polyprobe was constructed by cloning sequentially partial sequences of each viroid into a single vector, with run-off transcription driven by the T7 promoter. All six viroids were detectable within a dilution range of 5^{-3} to 5^{-4} in total nucleic acid extracts from infected trees. Individual trees were co-inoculated to create mixed infections and all four pome fruit viroids and both stone fruit viroids could be detected in pear and peach trees, respectively, using the polyprobe. The results of the assays using the polyprobe were comparable to those using single probes. The methods were validated by testing geographically diverse isolates of viroids, as well as field samples from several collections in the US. The assay offers a rapid, reliable and cost-effective approach to the simultaneous detection of six fruit trees viroids and has the potential for routine use in quarantine, certification, and plant genebank programs where many samples are tested and distributed worldwide. Published by Elsevier B.V.

1. Introduction

Viroids are obligate parasite plant pathogens comprised of small (246–401 nt) unencapsidated, covalently closed, circular and non-coding RNA molecules (Diener, 1971; Flores et al., 2005a). Six viroids, *apple scar skin viroid* (ASSVd), *apple dimple fruit viroid* (ADFVd), *apple fruit crinkle viroid* (AFCVd), *pear blister canker viroid* (PBCVd), *hop stunt viroid* (HSVd) and *peach latent mosaic viroid* (PLMVd), have been reported to infect naturally pome (apple, pear and quince) and stone (peach, plum, almond, etc.) fruit trees (Hadidi et al., 2003). These viroids are distributed worldwide and can cause economic damage and yield losses in fruit production. ASSVd and ADFVd infect both apple (*Malus pumila* L.) and pear (*Pyrus communis* L.) (Di Serio et al., 2001; Hadidi et al., 2003; Lolic et al., 2007); AFCVd infects apples, hops (*Humulus lupulus* L.) and Japanese persimmon (Diospyrus kaki Thumb.) (Nakaune and Nakano, 2008; Sano et al., 2008); PBCVd infects pear, quince (*Cydonia oblonga* Mill.), nashi (*Pyrus serotina* Rehd.) (Joyce et al., 2006) and wild pear (*P. amygdaliformis* Vill.) (Kyriakopoulou et al., 2001). HSVd has been found in a wide range of herbaceous and woody hosts including hops, almond [*Prunus dulcis* (Mill.) Webb], apricot (*P. armeniaca* L.), peach [*P. persica* (L.) Batsch], pear and plum (*Prunus* spp.), grapevine (*Vitis vinifera* L.), pomegranate (*Punica granatum* L.), citrus (*Citrus* spp. L.) and cucumber (*Cucumis sativus* L.) (Astruc et al., 1996; Cañizares et al., 1999; Kofalvi et al., 1997). PLMVd infects *Prunus* species (Boubourakas et al., 2009; Desvignes, 1986; Giunchedi et al., 1998; Hadidi et al., 1997) and has also been reported in *Pyrus* species in Greece and Italy (Kyriakopoulou et al., 2001). Mixed infections of HSVd and PBCVd or HSVd and PLMVd have been reported in pear trees (Fekih Hassen et al., 2006).

These six viroids belong to two different families—PLMVd is a member of the *Avsunviroidae*, and the other five are in the *Pospiviroidae* (Flores et al., 2005b). The genomes of viroids in the *Pospiviroidae* have a central conserved region (CCR), but lack hammerhead ribozymes. Three species, including PLMVd, in the *Avsunviroidae* lack CCR, but are able to self-cleave through hammerhead ribozymes. All six viroids are pathogens of quarantine significance for the international movement of plant germplasm.

Commonly used assays for the detection of viroids in fruit trees include graft inoculation of susceptible indicator plants (bioassays) and nucleic acid-based techniques such as hybridization and RT-PCR. The bioassays are both time and space consuming, and results are affected greatly by environmental conditions. This is especially true for detecting viroids in fruit trees, which may require up to five years for symptom expression in some hosts (Desvignes et al., 1999a,b; Lolic et al., 2007). Nucleic acid hybridization, RT-PCR and Loop-Mediated Isothermal PCR (LAMP) procedures have also been used to detect viroids in pome and stone fruit trees (Ambrós et al., 1995; Boubourakas et al., 2008, 2009; Cañizares et al., 1999; Di Serio et al., 2002; Faggioli et al., 2001; Faggioli and Ragozzino, 2002; Hadidi and Yang, 1990; Podleckis et al., 1993; Ragozzino et al., 2004; Torres et al., 2004). More recently, multiplex and simultaneous RT-PCR detections of plant viruses and viroids have become a widely

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reported option for viroid detection (Cohen et al., 2006; Hassan et al., 2006; Herranz et al., 2005; Ito et al., 2002; Shamloul et al., 2002; Wang et al., 2009). However, simultaneous detection of six viroids of pome and stone fruit trees has not been reported. Such a system would benefit quarantine, certification and plant genebank programs that test and distribute germplasm routinely because it would save time and other valuable resources. This paper reports the development of a digoxigenin (DIG)-labeled polyprobe that can simultaneously detect six fruit tree viroids using dot blot hybridizations.

2. Materials and methods

2.1. Viroid and plant tissue sources

The viroid isolates used to construct the polyprobe were as follows: ADFVd provided by R. Flores (Spain); ASSVd (apple isolate) and HSVd (plum isolate) provided by W. Howell (Washington, USA); AFCVd provided by T. Ito (Japan); and PBCVd provided by J. Postman (Oregon, USA). The PLMVd isolate was intercepted in a peach importation from the People's Republic of China by the USDA quarantine program. All woody hosts (*M. domestica*, *P. communis*, *P. persica* GF 305 and *P. salicina*) infected with viroids were maintained in an insect-proof screenhouse. Additional viroid sources used as positive controls consisted of a geographically diverse collection of isolates that were either intercepted in imported germplasm by the USDA quarantine program, or were provided by the National Clean Plant Network—Fruit Trees (NCPN-F, Prosser, WA). The test samples were collected from pome and stone trees in the US National Plant Germplasm System (NPGS) genebanks in Corvallis, OR, Davis, CA, Geneva, NY, and Washington, DC; the ornamental cherry trees from the National Park Service (NPS) collection in Washington, DC, and from trees in commercial orchards in Mesa County, Colorado.

2.2. Nucleic acid extractions

Leaves or bark were collected from different positions of each tree, pooled, and used for sample preparations. Total nucleic acids were extracted by a CTAB method reported by Li et al. (2008) and used for both the RT-PCR and dot blot hybridization assays. Attempts were made to simplify this protocol as described in Section 3.4.

2.3. Viroid RT-PCR

Fragments containing partial genomic sequences of each viroid were obtained by RT-PCR using viroid-specific primers (Table 1). Each primer contained a unique restriction site at its 5′-end, and each primer pair was designed so that the reverse primer of the upstream construct had the same restriction site as the forward primer of the downstream construct. The RT-PCR was conducted using the OneStep RT-PCR Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The RT-PCR products were digested with two corresponding restriction enzymes to create inserts with 5′-overhangs at both ends. These fragments were cloned into the pKS+ vector treated with the same enzymes, and calf intestinal alkaline phosphatase, to generate single-viroid constructs by directional cloning.

2.4. Construction and generation of polyprobes

Three 2-viroid fragments consisting of ASSVd + ADFVd, PBCVd + AFCVd and HSVd + PLMVd were generated by RT-PCR, restriction enzyme digestion of the products (Table 1), and directional cloning into the pKS+ vector to produce the poly2-A, poly2-B, and poly2-C constructs. Two poly4 constructs, poly4-AB and poly4-AC, were generated by two subclonings of poly2-A + poly2-B and poly2-A + poly2-C into pGEM-T Easy vector. The poly6 construct was assembled by subcloning the poly2-B into the poly4-AC, allowing the fusion of all six viroid sequences in the order: ASSVd + ADFVd + HSVd + PLMVd + PBCVd + AFCVd (see Fig. 2). The identities of all constructs were confirmed by sequencing (Macrogen USA, Rockville, MD, USA). DIG-labeled cRNA probes were generated by transcription of linearized constructs with T7 RNA polymerase using the DIG RNA Labeling Kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instruction.

2.5. Estimating the yield of the labeled probe

One microliter aliquot of the digoxigenin-labeled poly6 probe was diluted to 10^{-6} in a 10-fold series with RNA dilution buffer (5 parts DEPC water: 3 parts 20× SSC: 2 parts formaldehyde) and spotted onto a nylon membrane along with a similarly diluted aliquot of labeled control RNA of known concentration. After air drying and UV cross-linking, the membrane was processed for chemiluminescent detection as described in Section 2.6. The concentration of labeled probes was estimated by comparing the intensity of their spots to those of the labeled control.

2.6. Dot blot analysis

Two microliters of total nucleic acid extract were heated for 20 min at 65 °C, and spotted onto a nylon membrane. The membrane was then air-dried, UV-crosslinked and used for hybridizations. Prehybridization of the membrane was performed

Table 1
Properties of the OneStep RT-PCR primers.[a]

Viroid	Primer	Primer sequences 5′–3′	Restriction enzymes	Product size (bp)
ASSVd	ASSF	ATCGT**CTCGAG**GAAAGGAGCTGCCAGC	Xho I	186
	ASSR	AGACA**ATCGAT**GCTGCGTCAAAGAAAAAG	Cla I	
ADFVd	ADFF	GTTCC**ATCGAT**GAAAACTCCGTGTGGTTC	Cla I	270
	ADFR	AGCGT**GAATTC**CTCCACTCCCTGCCAG	EcoR I	
PBCVd	PBCF	TCTTC**GAATTC**GGCTTCTCGGCTCGTC	EcoR I	215
	PBCR	TCATG**ACTAGT**GGTAAACTTCCACCCTCG	Spe I	
	PBCF1	TCTTC**CCATGG**GGCTTCTCGGCTCGTC	NcoI	
AFCVd	AFCF	TACAG**ACTAGT**GGTTCCTATGGTTACAC	Spe I	207
	AFCR	TTCA**GCGGCCGC**ACAGCGCGTTCCAGTTAC	NotI	
	AFCR1	CA**GGGCCC**GGATCCACAGCGCGTTCCAGTTAC	ApaI	
HSVd	HSF	AGAAG**GAATTC**GAGGCGTGGAGAGAGG	EcoR I	166
	HSR	AGACT**ACTAGT**GCAGAGGCGCAGATAG	Spe I	
PLMVd	PLMF	TGTGT**ACTAGT**TCCACCTTGGGGTGCC	Spe I	233
	PLMR	TCTTC**GCGGCCGC**AGACTCATCAGTGCGCTC	NotI	

[a] Bold and underlined text indicates restriction sites used for cloning.