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Improved detection of prunus necrotic ringspot virus by the polymerase chain reaction

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Abstract

The reverse transcription – polymerase chain reaction (RT-PCR) technique was used for detection of prunus necrotic ringspot virus in dormant peach trees which tested negative by ELISA. Total RNA extracted from bark tissue, using a lithium chloride based method, were used for reverse transcription and subsequent amplification of viral sequences. The PCR product, about 300 base pairs in size, was analyzed by gel electrophoresis and visualized by ethidium bromide staining. In some cases, PCR products were not clearly visible in the stained gel and became distinct only following hybridisation with a ^{32}P-labelled virus specific probe. The RT-PCR assay described in this paper is sensitive enough for detection of PNRSV in dormant woody bark tissue and could be incorporated into testing protocols during post-entry quarantines for rapid initial screening of imported budwood and in virus elimination programs.

Introduction

Prunus necrotic ringspot virus (PNRSV) is an ilarvirus which occurs world-wide in commercially cultivated stone fruit (*Prunus* species). Like other members of the ilarvirus group, the genome of PNRSV is distributed among three single stranded RNA species (Fulton, 1983). Virus strains range in virulence from symptomless to causing severe disease symptoms in a wide range of rosaceous hosts (Nemeth, 1986). Reliable detection of PNRSV is required in certification schemes aimed to provide virus-tested propagation material to the fruit tree growing industry and to prevent entry of imported PNRSV-infected plant material. A bioassay based on grafting buds and/or chips onto the indicator plant *Prunus serrulata* Lindl. cv Shirofugen, has been used extensively for reliable detection of PNRSV and other viruses. This assay is season dependent, time consuming and cannot be completed in areas where *P. serrulata* will not grow. Testing for PNRSV by ELISA has, to some extent, superceeded this bioassay. However, its routine use is hampered by several limitations – not all isolates of PNRSV react to a specific antiserum, seasonal non-specific background problems often prevent reliable discrimination of positive from negative samples (Stein et al., 1987). In addition, PNRSV may be unevenly distributed throughout the buds and limbs of infected trees. Virus concentration fluctuates during individual growing seasons and between seasons and may become undetected by ELISA (Torrance and Dolby, 1984; Scott et al., 1989).

The highly sensitive polymerase chain reaction (PCR), developed for *in vitro* amplification of nucleic acid sequences (Saiki et al., 1986), has been used successfully for detection of various plant viral RNAs in 'problematic' plant material e.g. ligneous tissue of fruit trees (Korschineck et al., 1991; Rowhani et al., 1995), gladiolus corms (Vunsh et al., 1991), potato tubers and microtubers (Spiegel and Martin, 1993).

In this report we demonstrate that a combination of PCR and hybridisation allows detection of low

concentrations of PNRSV, undetected by ELISA, in leaf and bark samples collected from dormant peach trees during winter.

Materials and methods

Plant material

PNRSV-infected peach trees (*P. persicae* L. Batsch) cv. Hermosa grown for 5 years in a screen-house provided material for this study. Virus-free peach trees derived from *in vitro*, heat-treated shoot cultures, which had been tested periodically by ELISA during the preceding 4 years to ensure freedom from PNRSV (Stein et al., 1991) were included as healthy controls. For the comparison of PNRSV detection by RT-PCR and ELISA, duplicate bark samples (about 250 mg each) were collected during winter 1995 from young branches of four PNRSV-infected dormant trees (four to five samples per tree). Two bark samples from a healthy tree were included as controls. This sampling strategy was repeated three times at monthly intervals between mid-December to mid-February. Each bark sample was divided in two, one part was tested for PNRSV by ELISA and the other was processed for RT-PCR.

RNA extraction

Two extraction procedures were initially compared for efficient RNA extraction from leaves and bark samples scraped off from branches of peach trees: (i) a phenol extraction procedure (Hooft van Huijsduijnen et al., 1985), (ii) a lithium chloride-based protocol (Hughes and Galau, 1988) with minor modifications, ('lithium method'). Total RNA was recovered from peach tissue by both methods. In this study, the lithium method was adopted for RNA extraction. Each sample (ca. 100 mg of leaf or bark) was homogenised in a roller press (Elektrowerk, Behncke & Co., Hannover, Germany) with 5 volumes of buffer (200 mM Tris-HCl, pH 8.5, containing 1.5% sodium dodecylsulphate, 300 mM lithium chloride, 10 mM EDTA, 1% sodium deoxycholate, 1% NP-40) and 0.5% 2-mercaptoethanol. The extract was collected into a 1.5 ml microfuge tube, half a volume of 6M potassium acetate, pH 6.5 was added and the mixture centrifuged at 14000 g for 10 min. Nucleic acids were precipitated from the supernatant with isopropanol and centrifuged as described above. The pellet was resuspended after drying in 25 μl of sterile water.

PNRSV clone and primers

The PNRSV clone is a PCR fragment of 785 nucleotides and corresponds to the region between nucleotides 1034 and 1818 of the RNA 3 sequence of isolate 30/4 (GeneBank Accession U57046). The nucleotide sequence of this clone shows almost 95% similarity with sequence of the corresponding region of a German isolate of PNRSV (Guo et al., 1995) and 87% similarity with the sequence of the corresponding region of the PNRSV isolate of Hammond and Crosslin (1995).

The clone was sequenced at the 5′ and 3′ ends using the dideoxy chain termination method (Sanger et al., 1977). Two oligonucleotide primers were used in this study: primer #1- 5′-[TATTTCT CTAGTA-ACATCCC]-3′, primer #2- 5′-[ATACTCGGCCTACA CATCTG]-3′.

cDNA synthesis, PCR amplification and hybridisation

The reverse transcriptase (RT) and PCR reactions were performed following a single non-interrupted thermal cycling program (Sellner et al., 1992). Each reaction contained the RNA template (about 1 μg), virus specific primers (0.4 μM each), 100 μM dNTPs, 3 mM $MgCl_2$, 2.5 μl of 10× reaction buffer giving a final concentration of 67 mM Tris-HCl pH 8.8, 17 mM $(NH_4)_2SO_4$, 1 mM Mercaptoethanol, 6 μM EDTA and 0.002% Gelatin. Two units of Taq DNA polymerase (Advanced Biotechnologies, UK) and 0.1 unit M-MLV RT (Promega) were added to each reaction mixture. The total volume of 25 μl was overlayed with 50 μl of mineral oil and subjected in a thermocycler (Hybaid, UK) to the following program: 30 min at 42 °C, 3 min at 95 °C, then 40 cycles of 92 °C for 30 sec, 54 °C for 30 sec and 72 °C for 1 min, and finally 5 min at 72 °C. PCR products were electrophoresed in a 1.2% agarose gel and stained with ethidium bromide (EtBr). A ^{32}P-labelled PNRSV-riboprobe was used for dot and blot hybridisation. Reaction conditions were as previously described by Vunsh et al. (1990).

ELISA

Triple antibody-sandwich ELISA (TAS-ELISA) employing a PNRSV-specific polyclonal antiserum AS-0027 (DSM, Braunschweig, Germany) and the monoclonal PVAS-605 (American Type Culture Collection, Rockville, MD, U.S.A) were used to detect PNRSV in peach tissue and to provide a standard against which the sensitivity of RT-PCR could be assessed. TAS-ELISA was performed follow-

ing D'Arcy et al. (1989) with minor modifications: Polyvinyl Pyrrolidone (MW 40.000, 2% W/V) being included in the extraction buffer. Absorbance at 405nm (A_{405}) was read in an ELISA microplate reader. A_{405} values of healthy controls ranged from 0.00 to 0.05 and values above 0.30 were regarded as positive for PNRSV.

Results

RT-PCR amplification of PNRSV

RT-PCR amplification of RNA extracted from leaf samples of known infected peach trees, using PNRSV-specific primers, yielded a specific DNA band of about 300 base-pairs, visible in an EtBr-stained agarose gel (Figure 1 Gel-I1,I3,I4). A fragment of a similar size was obtained with RNA isolated from purified PNRSV particles (V) and from the plasmid containing the viral clone (PL). RT-PCR from samples I1 and I4 yielded very high amounts of the anticipated product, whereas RT-PCR from I3 resulted in a diffused double band of a similar size; a heterogenous background was observed for I2 and the healthy control (H). Conclusive identification of the amplified fragment was obtained by blot-hybridisation with a ^{32}P-labelled PNRSV-specific riboprobe (Figure 1 Blot) – a specific band was observed in three samples, a weaker positive band appeared in sample I2, which was not visible in the gel analysis of the PCR products. The healthy (H) control did not hybridise to the probe.

RT-PCR detection of PNRSV in dormant peach trees

The suitability of RT-PCR for PNRSV detection during seasons when ELISA is not sufficiently reliable was investigated by testing dormant peach trees. Bark samples were collected three times during the winter of 1995 from four dormant PNRSV-infected trees and one healthy control tree (as described in Materials and methods) and tested simultaneously by TAS-ELISA and RT-PCR. Results of a typical experiment are presented in Figure 2. Two of the seventeen samples (tree no. 1, branches a,b) tested positive by ELISA, whereas the other fifteen and the healthy control were negative (Figure 2, ELISA). RT-PCR of total RNA isolated from bark samples yielded a specific band in 4/4 samples of tree no. 1, 3/4 samples of trees no. 2 and no. 3 and in 2/5 samples of tree no. 4 (Figure 2, Gel). A blot hybridisation of this gel with a ^{32}P-labelled viral riboprobe (Figure 2, Blot) showed that all samples but one (tree no. 4, branch a) produced a virus-specific band.

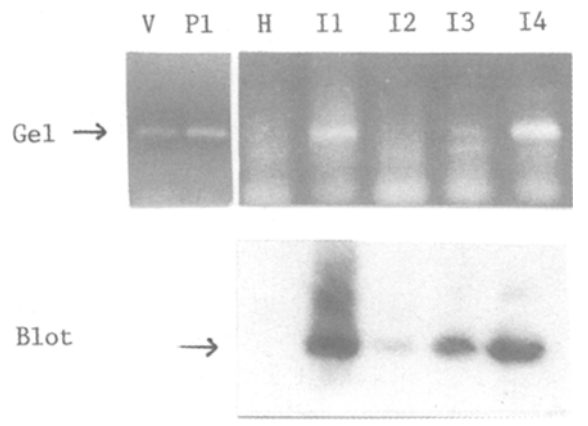

Figure 1. Ethidium-bromide-stained agarose gel of the PNRSV-specific PCR products and blot hybridisation. Total RNA extracted from: purified PNRSV (V), leaf samples of virus-infected (I1–4) peach trees and a healthy control (H) were reverse-transcribed into cDNA followed by PCR amplification using virus-specific primers. Bluescript plasmid vector carrying the PNRSV clone (Pl) was included for control. PCR products were fractionated on a 1.2% agarose gel and stained (Gel). PCR products from leaf samples were blot-hybridised with a ^{32}P-labelled cRNA probe (Blot). Arrow indicates amplified fragment (about 300 base pairs).

The same results were obtained by dot-hybridising the PCR products with the viral riboprobe (Figure 2, Dot). The healthy control (H) was negative in all tests.

Discussion

Sensitive PCR assays, increasingly used for plant virus diagnosis, are still more complex than ELISA and other serological methods. The reliability of PNRSV detection by ELISA is limited to the periods of active growth and becomes unreliable when stem elongation ceases (Scott et al., 1992). During hot summers and cold winters virus may decline, in infected trees, and become undetected by ELISA (Stein et al., 1987 and unpubl.). As a result, testing of imported dormant budwood, commonly used for international movement and other woody plant materials in certification schemes is delayed for up to months.

In this study we demonstrated that RT-PCR can be applied for detection of PNRSV in peach trees when ELISA is not reliable. A PNRSV-specific PCR fragment, visualized as distinct EtBr-stained band in agarose gel, was amplified from infected peach tissue (Figure 1). The amplified fragment corresponds to part of the 5′ untranslated region and the movement protein region (Scott and Xin, unpubl.). The authenticity of

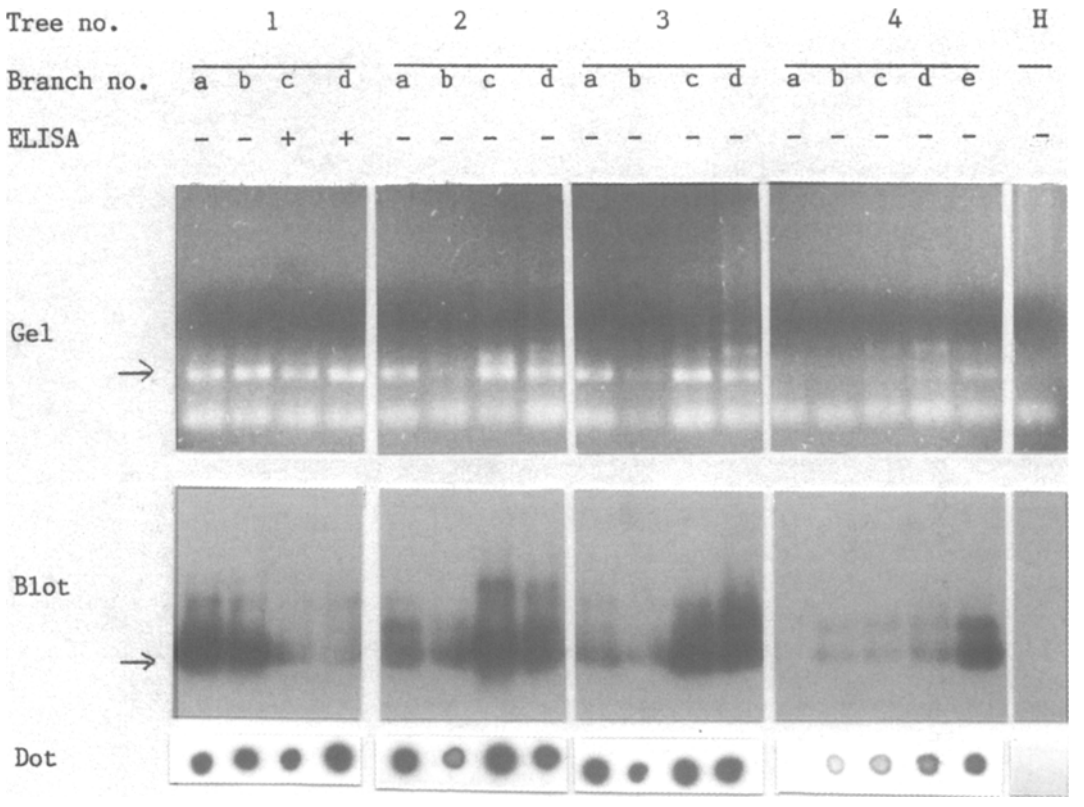

Figure 2. Comparison between ELISA, RT-PCR for PNRSV detection in bark samples of dormant peach trees and hybridisation of PCR products to a cRNA probe. ELISA analysis of bark samples from branches of dormant PNRSV-infected peach trees, + indicates infected, – samples tested negative (ELISA). Gel analysis of RT-PCR products of the same samples (Gel). The PCR products were analyzed and blot hybridised (Blot) as in Figure 1. For dot hybridisation, aliquots of the PCR products were spotted onto a nylon membrane and hybridised with the probe (Dot).

the amplified PCR fragment was confirmed by comparing the PCR products obtained from RNA isolated from infected tissue with RNA isolated from purified virus and the cloned PNRSV as well as by hybridisation with a ^{32}P-labelled PNRSV riboprobe. Figure 2 shows that PNRSV can be detected by RT-PCR in ELISA-negative bark samples from dormant infected trees. Low amounts of products, yielded an undefined diffuse band or no visible stained product leading to inconclusive results. The blot and/or dot hybridisation of PCR products with a virus-specific riboprobe confirmed the specificity of the test and increased its reliability (Figures 1, 2). Similar results were shown earlier for PCR detection of bean yellow mosaic virus in gladiolus (Vunsh et al., 1990) and for potato leafroll virus in potato tubers (Spiegel and Martin, 1993). Data presented in Figure 2 support the observations mentioned earlier regarding the variation in PNRSV concentrations among trees and branches on a single tree and emphasize the need for multiple samples from various branches even for the sensitive RT-PCR assay. Gel analysis and blot hybridisation were initially used to confirm the specificity of the PCR products. A simpler dot hybridisation procedure, which clearly distinguished between healthy and infected samples, was later applied for PNRSV detection, whereas blot hybridisation was still required when amplified products were low (not visible in the EtBr gel).

This sensitive assay can be applied to determine virus status of regenerated plant material following a virus elimination treatment; certification schemes and for imported, dormant budwood during postentry quarantine. PCR results should allow quick decisions to be made regarding virus infected material.

In this study, several steps involved in the RT-PCR reaction were simplified: efficient extraction of RNA was obtained with a lithium chloride based buffer (Hughes and Galau, 1988) conveniently homogenising

the sample with the roller press and obviating phenol; the use of single-tube RT-PCR (Sellner et al., 1992) and dot hybridisation which proved to be technically simpler than a blot assay. This procedure allowed the application of PCR for detection of PNRSV in problematic woody material.

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