Print-capture PCR: a simple and highly sensitive method for the detection of Plum pox virus (PPV) in plant tissues

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Plum pox virus (PPV) is the causal agent of sharka disease of stone fruit trees which is considered to be one of the most serious plant diseases. Sensitive techniques are needed for its diagnosis. Immunosorbent assays based on the use of monoclonal antibodies (1) and different PCR techniques have been described with or without previous immunocapture (2,3). In all instances plant extracts are needed, even though the sample grinding operation is time consuming and entails risks of contamination and of release of PCR inhibitors. Several reports have demonstrated the potential of using immobilized targets in PCR (4,5) or tissue of printing techniques for the detection of viral agents by immunological (6) or nucleic acid-based techniques (7). In this article we describe a simple direct tissue blotting PCR assay called print-capture PCR (PC-PCR) that allows the rapid and sensitive detection of PPV from infected plants without the need for grinding the samples. We demonstrate that a number of proteins may be used for the capture phase of PC-PCR, thus obviating the need for virus-specific immunoglobulins. For tissue printing, the fresh sections of leaves or stems from infected or healthy control plants (GF305 peach seedlings, apricots, *Nicotiana benthamiana*) were pressed onto Whatman 3MM paper. The prints can be processed directly or stored at room temperature for up to 1 month without any detrimental effects on amplification. Plant extracts used for immunocapture-PCR (IC-PCR) (3) or for spot-capture PCR (SC-PCR) were prepared by grinding (1/30, w/v) the samples used for tissue printing in PBS (3) supplemented with 2% polyvinylpyrrolidone and 0.2% sodium diethyl dithiocarbamate. For SC-PCR, 4 µl of the plant extracts were spotted on small squares of Whatman 3MM paper; the paper dried and processed as the tissue prints. Similar 0.5 ml microtubes (Sarstedt) were used for all capture techniques. For IC-PCR, the plant extracts were submitted to an immunocapture performed directly in the tubes used for the reverse transcriptase–PCR (RT–PCR) as described previously (3). The coating of the immunocapture tubes was performed using either a specific monoclonal antibody against PPV, immunoglobulins purified from an antiserum against PPV or skimmed milk as described below for SC-PCR and PC-PCR tubes. For PC-PCR and SC-PCR, the squares of paper harboring either the tissue prints or the spotted extracts were introduced in an Eppendorf tube and 120 µl 0.5% Triton

X-100 was added, vortexed and incubated for 2 min at room temperature. Triton extracts (100 µl) were then recovered and transferred to tubes previously coated in carbonate buffer (3) with one of the following proteins: anti-PPV monoclonal antibodies 5B (1) (1 µg/ml), antiserum against PPV (2 µg/ml), anti-citrus tristeza virus (CTV) monoclonal antibodies [3DF1+ 3CA5 (8), 1 µg/ml], skimmed milk (Sveltesse, Nestlé, 5%), bovine serum albumin (BSA fraction V, Boehringer, 5%) or Triptone (Oxoid, 5%). The Triton extracts were incubated in the coated tubes for 2 h at 37°C and washed twice with PBS-Tween (3). The RT–PCR one step protocol (3) was used for amplification purposes. Ten microliters of PCR products were analyzed by electrophoresis in 3% agarose gels and stained by ethidium bromide. Comparison of IC-PCR (3), SC-PCR and PC-PCR indicates that all three techniques allow the successful amplification and detection of PPV from infected herbaceous and woody plant hosts (Fig. 1). Evaluation of various substrates for the preparation of tissue prints or the spotting of extracts in SC-PCR indicated that both paper such as Whatman 3MM and nylon membranes (Immobilon-N, Millipore) are suitable but that no amplification products could be obtained from printed or spotted nitrocellulose membranes (HA45, Millipore) (results not shown). For obvious cost reasons, Whatman paper was therefore selected for the rest of this study. Attempts at PCR amplification by directly including the pieces of paper in the RT–PCR mix resulted in non-specific amplifications and were therefore not pursued. Attempts to amplify material released from the prints or spotted extracts in the absence of Triton X-100 proved unsuccessful, indicating the requirement for Triton in order to release amplifiable targets, possibly by disruption of the viral particles (9). Similarly, attempts to directly amplify the Triton extracts prepared from the printed or spotted papers failed, demonstrating the need for the capture step. Direct comparison, using the same plant extracts of IC-PCR and SC-PCR indicated that both techniques have sensitivities in the same range, with positive amplification still observed with crude infected *Nicotiana* extracts diluted 10^6-fold in healthy control extract (results not shown). One surprising observation made during the course of this work is that while specific anti-PPV immunoglobulins are necessary for a successful capture in IC-PCR (Fig. 1, compare tracks 1 and 3), the capture
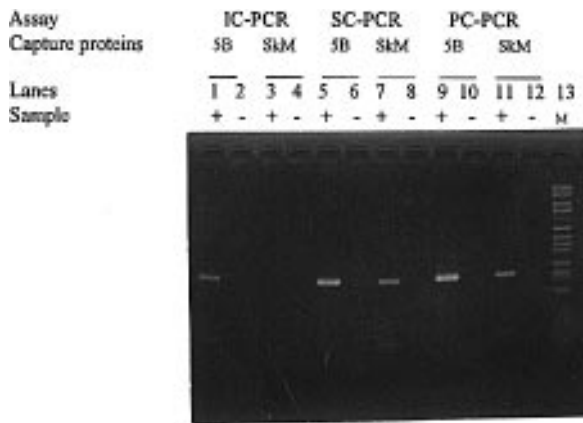
Figure 1. PPV detection using various capture-PCR techniques. The techniques used were IC-PCR (tracks 1–4), SC-PCR (tracks 5–8) and PC-PCR (tracks 9–12). In each case, the capture was performed using tubes coated with PPV-specific monoclonal antibodies 5B (5B, tracks 1, 2, 5, 6, 9 and 10) or skimmed milk (SkM, tracks 3, 4, 7, 8, 11 and 12). Samples from healthy *Nicotiana benthamiana* (–, tracks 2, 4, 6, 8, 10 and 12) were analyzed in parallel to the PPV infected samples (+, tracks 1, 3, 5, 7, 9 and 11). Track 13, DNA molecular marker VI from Boehringer Mannheim.

Figure 2. PC-PCR amplification products obtained from PPV-infected GF305 peach seedlings using capture tubes coated with various proteins: PPV-specific monoclonal antibodies 5B (track 2), PPV-specific rabbit polyclonal antibodies (track 3), skimmed milk (track 4), tryptone (track 5), citrus tristeza virus-specific monoclonal antibodies 3DF1+3CA5 (track 6), BSA (track 7) or tubes coated with carbonated buffer alone (track 8). Track 1, DNA molecular marker VI from Boehringer Mannheim.

phase of PC-PCR or SC-PCR can also be performed, although usually with a slightly lower efficiency, using non-PPV-specific capture agents such as skimmed milk (Fig. 1, compare tracks 5 and 9 with 7 and 11). Therefore, it appears that the amplifiable material captured during IC-PCR and PC-PCR or SC-PCR could be, at least partially, of a different nature. In order to understand this phenomenon better, a number of other non-specific proteins were evaluated for their ability to be used as capture agents in PC-PCR. The results presented in Figure 2 indicate that, although the efficiency of the capture may vary, all proteins tested allowed the capture of amplifiable material. At the same time, the requirement for a protein is demonstrated by the failure to amplify captured material using a tube coated with carbonate buffer alone (Fig. 2, track 8). To date, there is no information on the nature of the amplifiable material captured in our assay. It could either be intact or partially decapsidated PPV virions captured through non-specific protein–protein interactions or naked PPV RNA retained through protein–nucleic acid interactions. The fact that the signal obtained using PPV-specific immunoglobulins is usually somewhat stronger (Fig. 2, compare tracks 2 and 3 with 4–7) indicate that, in addition to non-specific interactions, specific immunocapture can also participate in the trapping of amplifiable material. The possibility to use cheap and nonspecific proteins for the capture phase of PC-PCR has both the advantage of reducing the cost of the assay and of offering the possibility to use PC-PCR for the sensitive detection of viruses for which no specific immunoglobulins arc available. The PC-PCR method avoids the preparation of extracts and consequently the release of inhibitors of plant origin as well as potential contamination problems. The prints preparation is simpler and much faster than extractions or squash preparations and can be used with quarantine viruses without risks. Another advantage is that, contrary to plant extracts, the printed or spotted membranes can be stored for a long time before being used or mailed, thus allowing their preparation directly in the field if needed. The PC-PCR method and its SC-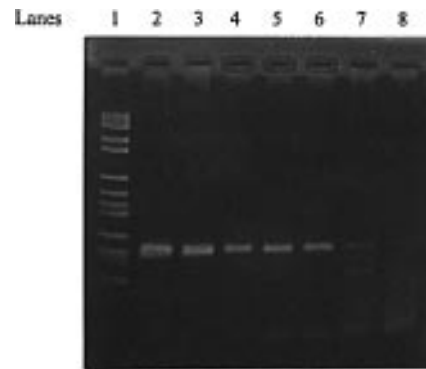PCR variation are simple, fast, cheap and very sensitive, and are thus very well adapted for use in routine indexing programs. In addition, these techniques should easily be adapted to the detection of other plant viruses and pathogenic agents. The detection of Apple chlorotic leaf spot virus (ACLSV) and the bacterium *Erwinia amylovora* has been assayed by PC-PCR and SC-PCR respectively, by using primers and conditions of amplification previously described (10,11). The capture phase was done with skimmed milk. Amplified fragments of the expected size were obtained, thus confirming the suitability of these methods.

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