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Protocols

Tissue blot immunoassay and direct RT-PCR of cucumoviruses and potyviruses from the same NitroPure nitrocellulose membrane

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A B S T R A C T

A method is described for using Nitropure nitrocellulose (NPN) membranes as an effective solid matrix for retrieval of template RNA of three potyviruses, *Tobacco etch virus*, *Soybean mosaic virus* and *Turnip mosaic virus*, and two cucumoviruses, *Cucumber mosaic virus* and *Peanut stunt virus*. These NPN membranes were also used for tissue blot immunosorbent assays (TBIAs) to identify and detect plant viruses. For RNA detection, discs from dried membranes blotted with infected tissue were minimally cleaned with Triton X-100 and placed directly into reverse transcription (RT) reactions to initiate cDNA synthesis. Aliquots of cDNA plus primers specific for coat protein produced PCR amplicons of expected sizes for each of the viruses. Intensity of PCR-amplified bands from cDNA transcribed from both non-processed and TBIA-processed NPN membranes was comparable to those using FTA Card protocols. Direct sequencing of PCR products yielded high quality runs enabling identification to species. NPN membranes retained immunologically detectable virus particles, as well as intact template viral RNA, for more than a year at room temperature. The quantity of amplification product decreased after several months of storage, but could be increased by increasing the number of PCR cycles.

1. Introduction

Serological techniques, including enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977), lateral flow devices (Tsuda et al., 1992), and tissue blot immunosorbent assay (TBIA) (Lin et al., 1990) are powerful tools for the detection and diagnosis of plant viruses. Of the several ELISA variations used for plant viruses, all are performed in microtiter plates, require extraction of virus from fresh tissue, and take several hours to complete. Lateral flow devices available as immunostrips (Agdia Inc.) are quick and simple to use, but costly when testing large numbers of samples. In contrast, plant tissues are squashed or blotted directly onto nitrocellulose membranes in TBIA, which can be processed at a later date (Makkouk and Kumari, 2009). The method was first described for the detection of plant viruses by Lin et al. (1990) and has since become used widely as a sensitive and reliable method for plant virus detection (Cambra et al., 2000; Chen et al., 2004; Comstock and Miller, 2004; Hsu and Lawson, 1991; Jonson et al., 2007; Makkouk and Comeau, 1994; Martin et al., 2002; McDonald et al., 2004). TBIA is also less costly than immunostrips for high throughput and can be completed in less time than ELISA.

Nucleic acid-based approaches are also used extensively for plant virus detection and identification. Until recently, the source of viral RNA for cDNA synthesis and PCR was from purified virus particles or total RNA. Burgoyne (1996) patented the use of Flinders Technology Associates (FTA) Cards for the collection and storage of DNA to be used either directly or indirectly in PCR. The FTA technology was then licensed to Whatman Inc. (2006) and distributed in several formats, including the FTA PlantSaver Card. The FTA Card matrix is a cotton-based cellulose fiber membrane on a supported backing, to which infected tissue is blotted. According to US Patent No. 6645717 (Smith et al., 2003), "the fibers are conditioned with chaotrophic and other agents which lyse cells, releases and immobilizes the genetic material while inhibiting their degradation". These cards were soon adapted for plant virus detection and plant gene expression (Roy and Nassuth, 2005). Early reports of FTA technology focused on detection of geminivirus DNA, including *Cassava mosaic virus*, *Maize streak virus*, and *Tomato yellow leaf curl virus* (Ndunguru et al., 2005). Effective retrieval of viral RNA sequences from FTA Cards was first reported by Ndunguru et al. (2005) by RT-PCR of eluted RNA of *Tobacco etch virus* (TEV), *Tobacco mosaic virus*, and *Potato virus* Y. Rogers and Burgoyne (2000), using *Coxsackievirus* B4 (CVB-4), added cleaned discs from FTA Cards directly into RT reaction mixes (or one-step RT-PCR mixes) instead of using RNA eluted from several discs.

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As both FTA Cards and NitroPure nitrocellulose (NPN) membranes are solid matrices, and TBIA is used extensively in our laboratory (Chen et al., 2004), it was hypothesized that NPN membranes could be used as a source of plant viral RNA for RT-PCR. Singh et al. (2004) described a method for using nitrocellulose membranes to immobilize RNA from tissues macerated in a buffered detergent and RT-PCR from water-eluted spots. Three potyviruses, TEV, *Turnip mosaic virus* (TuMV), and *Soybean mosaic virus* (SMV) (Genus *Potyvirus*, Family *Potyviridae*) and two cucumoviruses *Cucumber mosaic virus* (CMV) and *Peanut stunt virus* (PSV) (Genus *Cucumovirus*, Family *Bromoviridae*) were used to test this hypothesis. Both genera are economically important and widespread and are often found in mixed infections in cucurbits (Pinto et al., 2008; Wang et al., 2002), ornamentals (Arneodo et al., 2005), and other crops. The objective of the present study was to establish the versatility of NPN membranes for binding proteins of plant viruses for immunoassay, and as a direct source of viral RNA for RT-PCR. The protocol required no mechanical tissue maceration or elution of viral RNA, and yielded amplicons of expected sizes from RT-PCR. Sequences obtained directly from PCR products were high quality reads and enabled accurate separation of the viruses into the expected species and confirmation of immunological assays. NPN membranes were comparable to FTA Cards in efficiency and archival storage properties.

2. Materials and methods

2.1. Virus cultures and maintenance

All viruses used, with the exception of TEV, were isolated in Montgomery County, VA. TEV was isolated in Scott County, VA from burley tobacco and was maintained in tobacco (*Nicotiana tabacum* L. cv. Burley 21) or hot pepper (*Capsicum chinense* Jacq. cv. Scotch bonnet), seeds of which were obtained from Bodles Research Station of the Ministry of Agriculture and Fisheries, St. Catherine, Jamaica, West Indies (McDonald et al., 2004). SMV, strain G1, was from soybean (*Glycine max* (L.) Merrill) cv. Lee and was maintained in either cultivar Essex or Lee 68 soybean (Hunst and Tolin, 1982). TuMV was isolated from Dame's Rocket (*Hesperis matronalis* L.) and maintained in turnip (*Brassica rapa* L. subsp. *rapa*) cv. Green Seven Top (Ferry-Morse Seed Co., Fulton, KY) or *Nicotiana benthamiana* Domin. CMV was isolated from flowering tobacco (*Nicotiana* sp.) from the Historic Smithfield Plantation and maintained on *Nicotiana tabacum* L. cv. Xanthi. PSV was isolated from bean (*Phaseolus vulgaris* L.) and maintained on cv. Bronco. All viruses were propagated on greenhouse-grown plants and periodically transferred to new susceptible hosts by preparing sap in 0.01 M phosphate buffer, pH 7.0, and rubbing onto young plants dusted with silicon carbide powder, grit 600 (Buehler, Lake Bluff, IL). For long term storage, leaf pieces from infected plants were stored frozen at −70 °C or desiccated at 4 °C.

2.2. Solid matrices and virus deposition

Two solid matrices were tested. The first was a NPN, a supported nitrocellulose transfer membrane with 0.45 μ pore size, type WP4HY, now available from GE Osmonics Labstore (Minnetonka, MN). Strips of desired sizes were cut and taped by the edge to index cards for support. A template with an array of 6 mm drilled holes made from the membrane's protective paper was taped over the NPN membrane for positioning tissue blots and protecting non-blotted areas of the membrane. A leaf from plants non-infected or infected with each of the viruses was rolled into a tight coil, torn in half, and the freshly torn edges were pressed gently and repeatedly onto the membrane surface until the spot was uniformly green (Fig. 1, panel A). The second matrix was the FTA Card (Karle et al., 2004). Leaf tissue was blotted to the FTA Card in the same manner except that template arrays were not used. After sample application, cards and membranes were allowed to air dry a minimum of 2 h at room temperature (26 ± 2 °C) before continuing.

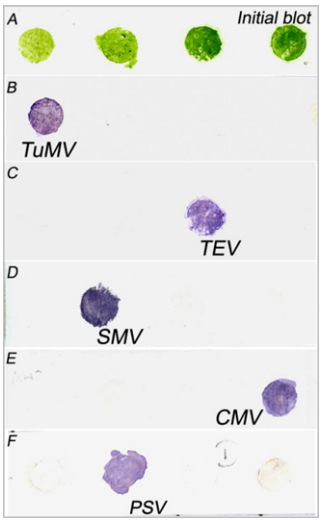

Fig. 1. NitroPure nitrocellulose (NPN) membranes with five viruses (*Cucumber mosaic virus* (CMV), *Peanut stunt virus* (PSV), *Soybean mosaic virus* (SMV), *Tobacco etch virus* (TEV), and *Turnip mosaic virus* (TuMV). Panels A to D are NPN membranes blotted with leaves infected with TuMV, TEV, or SMV (lanes 1–3, respectively) or healthy tissue (lane 4). Panel A is the initial untreated membrane showing uniform green spots. Panels B–D are NPN membranes after tissue blot immunoassay processing with the antibody to TuMV, TEV, or SMV, respectively, showing positive reactions as a purple spot. Panels E and F are NPN membranes blotted with healthy tissue (lane 1, 3), PSV (lane 2) or CMV (lane 4) processed with antibodies to CMV (panel E) or PSV (panel F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.3. Tissue blot immunoassay

Virus identity and presence were confirmed by TBIA using a protocol modified from Lin et al. (1990) and Chen et al. (2004). The dried membranes were placed in 5% Triton X-100 (octylphenolpoly(ethyleneglycolether)$_x$) for 10 min to remove plant debris and green color, rinsed for 3 min in potassium phosphate saline (KPS) (0.02 M K_2HPO_4, 0.15 M NaCl, pH 7.4) containing 0.05% Tween-20 (Lin et al., 1990), then blocked for 20 min with 5% non-fat dry milk (Nestle Carnation, Nestle USA Inc., Solon, OH) and 0.5% bovine serum albumin (BSA) (Sigma–Aldrich, St. Louis, MO) in KPS. Without rinsing, the membranes were then placed for 90 min into a combination of primary antibody specific for each virus and a secondary enzyme-labeled anti-animal antibody. Membranes were then rinsed for 10 min in Tris buffered saline (TBS) (0.05 M Tris base, 0.15 M NaCl, pH 7.6) containing 0.05% Tween-20, followed by two additional rinses in TBS–Tween, each for 5 min. Finally, membranes were immersed in substrate, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Zymed Laboratories Inc., Invitrogen Corporation, Carlsbad, CA), for 5–10 min until a purple precipitate was visible on positive samples, then rinsed thoroughly in de-ionized water and left to air dry. All procedures were conducted at room temperature with constant, gentle agitation on a platform shaker set at 200 rpm. All chemicals were purchased from Fisher Scientific Inc., USA unless otherwise stated.

Antibodies for PSV, SMV, TEV and TuMV were polyclonal rabbit antiserum used at 1:10,000 in KPS. Antisera to SMV and PSV were prepared in-house (Hunst and Tolin, 1982; Tolin and Miller, 1975). Antisera to TEV and TuMV were from G.V. Gooding (Raleigh, NC) and T.P. Pirone (Lexington, KY), respectively. The secondary antibody