An innovative technique for the detection of begomoviruses in mucilaginous plant extracts using immunocapture PCR (IC-PCR)

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ABSTRACT

An innovative protocol for immunocapture (IC)-polymerase chain reaction (IC-PCR) is described for detecting begomoviruses in clarified plant extracts with degenerate primers without isolation of total nucleic acids. The IC-PCR procedure was successful in detecting begomoviruses that exist in low titers in plant species containing various forms of PCR-amplification inhibitors such as polysaccharides, tannins, and polyphenolic compounds. This method involves extraction with a phosphate buffer containing sodium sulfite, ethylenediaminetetracetate (EDTA), and 1.5% triton X-100 (TX-100) at pH 8.3. The DNA template was released from the captured antigen by heating the PCR tubes at 65°C/10 min in the presence of 5% TX-100. Several amplicons representing the core coat protein of begomoviruses of okra leaf curl virus (OkLCV), tomato yellow leaf curl virus (TYLCV) were successfully detected with this method.

Key words: *IC-PCR, diagnosis, begomoviruses, DNA probing, mucilaginous plant extracts, Triton X-100.*

INTRODUCTION

Diseases caused by the whitefly-transmitted geminiviruses (WTG) have recently become a major threat to the production of vegetable and field crops in Tropical and subtropical regions (Abdel-Salam, 1999b; Idris *et al*., 2002; Mansoor, *et al.* 2003). WTG belong to the genus *Begomovirus*, the family *Geminiviridae*. *Begomovirus* comprises > 100 species which infect dicot plants (Mansoor, *et al.*, 2003; Rampersad and Umaharan, 2003).

Previously, begomoviruses have been characterized by symptomatology and host range tests. A sole reliance on these measures may impede diagnosis of begomoviruses as symptoms are affected by several variables including virus isolate, time of infection, plant cultivar, and environmental conditions (Polston and Anderson, 1997). Nonetheless, more accurate, sensitive, and reproducible diagnostic assays for detection of begomoiuruses have recently been developed. The polymerase chain reaction (PCR), using degenerate primers, represents an advanced avenue for begomovirus detection (Rojas *et al*., 1993; Wyatt and Brown, 1996).

Traditional PCR methods necessitate the extraction of total nucleic acids from infected plants. Methods for nucleic acid extraction are not only cumbersome but may not also be

applicable to different plant species, especially those containing high levels of polyphenols and polysaccharides (Porebski *et al.*, 1997; Tel-Zur *et al.*, 1999; Jose and Usha, 2000; Rampersad and Umaharan, 2003). Consequently, IC-PCR, utilizing specific antibody binding, was used to alleviate inhibitors associated with plant viruses present in woody and mucilaginous plants (Wetzel *et al.*, 1992; Rowhani *et al.*, 1995; Harper *et al.*, 2002; Rampersad and Umaharan (2003).

Rampersad and Umaharan (2003) ranked the IC-PCR test as the most sensitive tool for the detection of begomoviruses present in plants belonging to *Fabaceae*, *Malvaceae*, and *Solanaceae*. Some detection problems; however, especially reproducibility have arisen upon using their described technique.

The objective of this study is to describe a modified IC-PCR technique for the detection of begomoviruses especially present in mucilaginous hosts.

MATERIALS AND METHODS

Sources of viruses and antisera

OkLCV and TYLCV were collected from infected okra (*Abelmoschus esculetus* [L.] Moench) and tomato (*Lycopersicon esculentum* Mill.) plants at the Experimental Farm, Faculty of Agriculture, Cairo University, Giza, Egypt. These viruses were isolated and then serologically identified by using antisera for OkLCV and TYLCV, produced by the author. OklCV antiserum (OkLCV-AS) was produced for the intact virus. TYLCV antiserum, on the other hand, was produced for disrupted coat protein of the virus and designated as TYLCV-cp-AS. An indirect ELISA technique utilizing the presence of TX-100 in the extraction buffer (Abdel-Salam *et al.*, 1998) was used routinely for the detection of these two viruses.

Preparation of clarified extracts

For each infected host, 0.5 g of fresh succulent tissues was pulverized in a sterilized mortar and pestle in the presence of liquid nitrogen. Tissues were then suspended (1:5, w/v) in each of two-extraction buffers. The first buffer (designated as buffer C) was described by Rampersad and Umaharan (2003). It composed of 20 mM Tris-HCl, 138 mM NaCl, 1 mM polyvinyl pyrrolidone, PVP, 0.05 % Tween 20, 3 mM KCl, and 3 mM NaN_3, pH 7.4. The second buffer in the present study, designated as innovative buffer, composed of 100 mM Na_2HPO_4-NaH_2PO_4, 20 mM Na_2SO_3, 20 mM EDTA, 1.5 % TX-100, pH 8.3. The extracts were subsequently clarified with low-speed centrifugation (8000 rpm/10 min/5°C) and each was finally diluted to 2^{-4} in each of the same buffer used for suspension.

IC-PCR

Sterile 0.2 ml polypropylene micro centrifuge tubes were pre-coated with the tested antisera (25 µl /tube) diluted with a coating buffer composed of 15 mM Na_2CO_3; 35 mM $NaHCO_3$, and 3 mM NaN_3, pH 9.6. The tubes were incubated overnight at 4°C. The antiserum was removed and the tubes were washed twice (50 µl /tube) with phosphate buffer saline (PBS, 1.5 mM KH_2PO_4, 138 mM NaCl, pH 7.4), then left to dry at 37°C for 15 min. Plant clarified extracts were then added to the antiserum-coated tubes (25 µl /tube). Tubes were then incubated for 2.5 hr at 37°C. The Plant extracts were removed and tubes were washed (50 µl /tube) three times with PBS. Tubes were then left to dry at 37°C for 15 min.

Synthesis of cDNA was performed in the coated tubes as follows: For a single-25 µl reaction, the PCR mixture included 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 0.1 % T-X100 (Promega M166B, WI, USA), 2.5 mM

$MgCl_2$, 0.2 mM dNTPs, 25 pmole of each primer, and sterile double distilled H_2O containing 5 % TX-100,. The reaction mixture was heated (65°C/10 min). 2.5 U *Taq* DNA polymerase (Promerga, M166A, WI, USA) were then added per tube. Tubes were subjected to amplification in the Techne 312 thermocycler programmed for 3 min at 94°C, followed by 35 cycles of 1 min at 92°C, 20 sec at 60°C, and 30 sec at 72°C, with a final extension of 5 min at 72°C.

The degenerate primers prV324 5` gcc(ct)at(ga)ta(tc)ag(ag)aagcc(ac)ag3` and prC889 `gg(ag)tt(atg)ga(ga)gcatg(tca)gtacat-3`, designed by Wyatt and Brown (1996) to amplify the core coat protein fragment (~575 – 579 bp) of begomoviruses were used for cDNA Synthesis.

Analysis of PCR products

The PCR amplified products were analyzed in 1.5 % agarose gel (SIGMA, A-9918, Mo., USA) prepared in 45 mM Tris-borate, 1 mM EDTA, pH 8.4. The gel was stained with ethidium bromide (1µg/ml), scanned with UV illuminator, and photographed with 35 mm camera equipped with an orange filter (85 A).

RESULTS AND DISCUSSION

Two IC-PCR techniques with two different extraction buffers were compared for their efficacy to amplify OkLCV and TYLCV. The use of buffer C (Rampersad and Umaharan, 2003) in virus extraction produced irreproducible results. For instance TYLCV-cp-AS failed to recognize its own antigen (Fig. 1, lane 2); whereas, it recognized OkLCV antigen (Fig. 1, lane 3). Similarly OkLCV-AS failed to recognize OkLCV upon using the extraction buffer C (Fig. 2, lane 2).

The innovative buffer was reproducible upon its usage with both antisera. Results showed that the innovative buffer enhanced the recognition of OkLCV (Fig. 2, lane 3) and TYLCV (Fig. 2, lane 4).

The major idea of the IC-PCR technique depends primarily on the entrapment of virus antigen on a solid surface by specific antibodies to this antigen. Such a step followed by other washing steps eliminate any other plant constituents especially polysaccharides and polyphenolics which interfere with the nucleic acid isolation, restriction enzyme digestion, electrophoresis, PCR, and sequencing (Porebski *et al.*, 1997; Rampersad and Umaharan, 2003; Rogstad, 2003). The nucleic acid is then released from the captured-virus antigen, for PCR amplification, by different methods including heat exposure (Potter and Nakhla, 2003) or by neutral detergents such as 0.05 % Tween 20 (Rampersad and Umaharan, 2003) or 0.3 % Triton X-100 (Moury *et al.*, 2000).

The extraction buffer in IC-PCR is of great importance. It has to maintain the integrity of plant virus antigen(s), should not reduce antigen binding to the solid surface, and contains additives that hamper the bad actions of polysaccharides and polyphenolics. Several investigators have used polyvinyl pyrrolidone (PVP) in extraction buffers of IC-PCR and DNA extraction because of the nature of PVP, in reducing the action of polyphenols as well as in reducing aggregation of plant extract constituents (Maliyakal, 1992; Porebski, 1997; Rampersad and Umaharan, 2003).

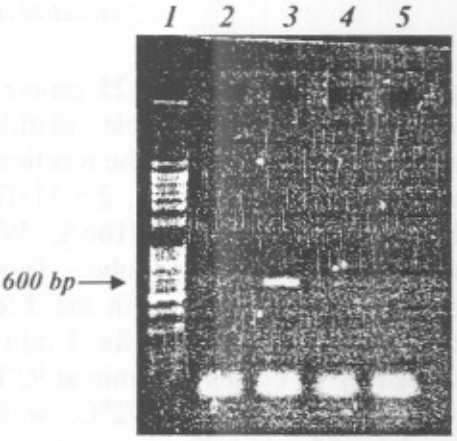

Fig. (1): IC-PCR showing the effect of extraction with buffer C on the entrapment of TYLCV (lane 2) and OkLCV (lane 3) with TYLCV-CP (AS) diluted at 1/500. Lane 4 and 5 represent healthy tomato and okra sap respectively. Lane 1 is a 1 *kb Plus DNA ladder.*

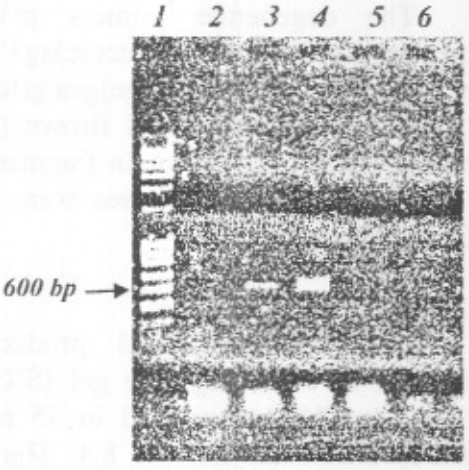

Fig.(2): IC-PCR showing the effect of the extraction buffers on the entrapment of OkLCV(AS) (1/100 dilution) to OkLCV(lanes 2,3) and TYLCV(lane 4). In lanes 2 and 3, infected tissues were extracted with buffer C and the innovated buffer respectively. TYLCV-infected tissues were also extracted with the innovative buffer (lane 4). Lanes 5 and 6 are healthy plant sap of tomato and okra, respectively. Lane 1 is a 1 *kb Plus DNA ladder.*

Application of buffer C, containing PVP, although was favored by Rampersad and Umaharan (2003), did not however worked regularly in detection of TYLCV and OkLCV in the present study. On the other hand, the innovative buffer produced repeatable results. This might be due to the involvement of TX-100 in this buffer. The present described technique has utilized TX-100 in both the extraction and the disruption steps. TX-100 was used in the extraction step of IC-PCR at a concentration of 1.5%. Such a concentration was used regularly in plant virus purification (Abdel-Salam, 1999b; Harper *et al.*, 2002) without affecting the integrity and hence the antigenecity of virions. TX-100 is known for its expedient action in releasing virus from attached plant constituents and in reducing non-specific binding in immuno-dot blotting techniques (Abdel-Salam, 1999a).

On the other hand, TX-100 was used at 5% concentration coupled with heat treatment to assure the release of nucleic acid from the disrupted virions. The high concentration of TX-100 probably has another benefit in reducing the secondary structure of the extracted nucleic acid and hence increases the fidelity of PCR.

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الملخص العربي

تقنية جديدة للكشف عن الفيروسات التوأمية بأستخدام الارتباط المناعى المتلازم مع تفاعل البلمرة المتسلسل

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تصف هذه الدراسة المجراة طريقةجديدة للارتباط المناعى المتلازم مع تفاعل تسلسل البلمرة immunocapture (IC)-polymerase chain reaction (IC-PCR) وذلك للكشف عن الفيروسات التوأمية المنقولة بالذبابة البيضاء Begomoviruses فى العصير النباتى دون اللجوء للحصول على الحمض النووى المنقى. وتتميز هذة الطريقة بتفوقها على الطرق المماثلة فى الدقة والتجانس للنتائج المتحصل عليها خاصة فى تقصى ال Begomoviruses التى تتواجد بتركيزات قليلة فى عوائل نباتية تحتوى عصاراتها المخاطية على مواد مثبطة لـ PCR كمركبا ت عديدة التسكر، التانينات، والفينولات. وتشتمل الطريقة على استخلاص العصارة فى محلول فوسفاتى منظم يحتوى على اثيلين داى امين تترااسيتات وسلفيت الصوديوم و 1.5% ترايتون اكس 100 فى وسط قلوى (pH 8.0) . ويتبع ذلك ادمصاص جزيئات الفيروس على جدران انابيب الـ PCR المشبعة بالاجسام المضادة Antibodies الخاصة بالفيروس. ويتم بعد ذلك تحرير الحمض النووى من جزيئا ت الفيروس واجراء الـ PCR من خلال تعريض الفيروس لدرجة حرارة 65Co لمدة عشرة دقائق فى وسط يحتوى على 5% ترايتون اكس 100. وقد امكن بهذة الطريقة وبأستعمال بوادىء معينة degenerate primers من الحصول على نواتج بلمرة للجزء الداخلى من جين الغلاف البروتينى core coat protein gene لفيروسى تجعد اوراق البامية okra leaf curl virus (OkLCV) و tomato yellow leaf curl virus (TYLCV) .