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Direct RNA polymerase chain reaction for TMV detection in crude cell extracts

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A part of the 30,000 bp transport protein gene of tobacco mosaic virus (TMV) RNA was amplified via direct RNA PCR and via traditional reverse transcription followed by cDNA PCR. Both amplified cDNA products were restricted with *Nco*I or *Hae*III endonucleases and identical restriction fragments were produced. Two efficient methods of viral RNA concentration from an infected tobacco leaf extract were used: both 3–3.5 M sodium acetate alone and 3 M LiCl with 4 M urea quantitatively precipitated TMV RNA from the extracts. TMV RNA thus obtained could be readily amplified by direct RNA PCR. These results demonstrate that direct RNA PCR can be applied for the detection or/and analysis of high molecular weight RNA and for diagnosis of viral infections.

RNA PCR; High molecular weight RNA concentration; TMV; Virus infection diagnosis

1. INTRODUCTION

At the present time PCR appears to be the most powerful technique for identification and analysis of a gene, and for the molecular diagnosis of numerous pathogens. A number of eukaryotic viruses consist of a RNA genome. Thus in order to amplify RNA by means of the traditional PCR technique, the reverse transcription (RT) reaction must precede the polymerase chain reaction. Recently, a high RT activity of *Thermus thermophilus* polymerase was demonstrated with isolated cRNA transcribed by T7 RNA polymerase in vitro [1]. Thus it was of special interest to apply this finding to the direct detection of a viral RNA in crude extracts from infected tissues.

2. MATERIALS AND METHODS

The materials were *Taq*, *Tth* polymerase and Random Primer (RP) kit (Biopol, Moscow); reverse transcriptase (RTase) and RNAse inhibitor (NPO Vostok, Omutninsk). RT buffer was: 50 mM Tris-HCl (pH 8.3), 20 mM KCl, 10 mM $MgCl_2$. (−)primer d(5'-CCC-TTTGCGGACATCACTCTT) complementary to the tobacco mosaic virus (TMV) RNA region 5,551–5,571 and (+)primer d(5'-GCCGGTTTGGTCGTCACGGGC) corresponding to nucleotide positions 5,110–5,130 of the TMV genome [2] were a gift from Dr. V.A. Efimov.

TMV RNA isolation and electrophoretic analysis were carried out as previously described [3]. PCR products and restriction fragments were analyzed by 1.5–2% agarose gel electrophoresis. The amplifying activity of *Tth pol* was tested with lambda phage DNA according to the distributor's protocol. The RP method was used for ^{32}P labelling of the amplified cDNA according to the distributor's recommendations, except that denaturation of double stranded (ds) DNA (5 min, 100°C) was performed in the presence of the RP. For restriction the amplified TMV cDNA was eluted from the agarose gel, labelled with ^{32}P and treated with *Hae*III or *Nco*I restrictases.

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Abbreviations: RT, reverse transcription; RTase, reverse transcriptase from avian myoblastosis virus; RP, random primer; ds, double stranded.

3. RESULTS AND DISCUSSION

3.1. *RT following PCR vs. direct PCR of TMV RNA and comparison of PCR products*

The RT reaction of TMV RNA was carried out in 50 μl: viral RNA (0.5–1 μg) and (−)primer (15 pM in 10 μl) were denatured at 100°C for 2 min and chilled to 0°C. Then 5 × RT-buffer, dNTPs (200 μM each), RNasin and RTase (20 U) were added in sequence and the mixture was incubated at 37°C for 30 min. The following PCR was performed in 100 μl reaction mixture (67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 6.7 μM Na-EDTA, 0.0001% gelatin, 1.25 mM dNTPs, 15 pM of (+)- and (−)primers, 10 U *Taq pol*) at 94°C for 1 min for denaturation, 58°C for 1 min for annealing and 72°C for 3 min for extension for 30 cycles in total.

Direct RNA PCR with *Tth pol* was performed in two steps. Firstly, the RT stage of the reaction was carried out in 20 μl of RT/*Tth* buffer (67 mM Tris-HCl, pH 8.5, 17 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1 mM $MnCl_2$) containing (final concentrations indicated): 5–50 mg/μl of TMV RNA (≈25 nM), 200 nM of each dNTP, 750 nM of (−)primer, 1 U/μl of RNAsin and 0.25 U/μl of

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Tth pol. The mixture was incubated at 70°C for 15 min and then chilled to 4°C. At the second step 80 μl of PCR buffer (67 mM Tris-HCl, pH 8.5, 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1.5 mM $MgCl_2$, 0.75 mM EGTA), containing 15 pM (+)-primer, were added. At the end 75 μl of mineral oil was overlayed and the PCR was carried out according to the following program: 1st cycle, 94°C/3 min, 58°C/2min, 70°C/5 min; 2–30 cycles, 94°C/35 s, 58°C/35 s, 70°C/45 s; 31st cycle, 94°C/1 min, 58°C/2 min, 70°C/4 min; delay, 70°C/7 min.

Analysis of the products synthesized is shown in Fig. 1A. The efficiency of both methods is quite similar. In both cases the major product is a fragment with the expected mobility for the amplified TMV ds DNA (463 bp) and similar low molecular weight by-products are also synthesized. The percentage yield of these low molecular weight by-products dropped considerably, whereas the yield of the major product grew, when direct RNA PCR was carried out in the presence of 1.5 mM manganese ions (manuscript in preparation).

To prove the similarity of the major products, (1) and (2) in Fig. 1A, they were eluted from the gel, labelled with ^{32}P by the RP procedure and treated with restriction enzymes, *Nco*I or *Hae*III. From the TMV DNA fragment sequence data [2] one would predict that this treatment would generate restriction fragments with a length of 100, 116 and 247 bp for *Hae*III or 114 and 349 bp for the *Nco*I enzyme. Fig. 1B illustrates the restriction fragment pattern produced from (1) and (2) by direct or non-direct PCR, and their matching with the predicted sizes.

3.2. *Direct PCR of TMV RNA crude preparation*

Serial detection and analysis of RNA need simple and efficient methods for primary treatment of a cell sap or an extract prior to PCR which would diminish the effect of some *Tth pol* inhibitors and concentrate high molecular weight (hmw) RNAs, for example a viral RNA. We have developed two methods, of which the first part is common to both methods: 40 mg of infected or non-infected tobacco leaves frozen with liquid nitrogen, were homogenized with a pestle at 0°C in 200 μl of RNA extracting buffer (0.15 M NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM Na-EDTA). The homogenates were cleared by centrifugation at 14,000 rpm for 5 min. Then two efficient approaches for hmw RNA concentration were used:

(i) the 'Acetate' procedure: 48 mg of sodium acetate were added to 100 μl of the leaf extracts (C_{final} = 3–3.5 M) and stirred until completely dissolved. The samples were quickly frozen and exposed for 30 min at −20°C. The precipitates formed were collected by centrifugation at 14,000 rpm for 5 min, washed twice with 70% ethanol and dissolved in 20 μl of water (or 0.14 N NaCl). 5 μl aliquots were examined by agarose–EtBr electrophoresis. The procedure seems to be very efficient (see Fig. 1C). Even at this primary step of the

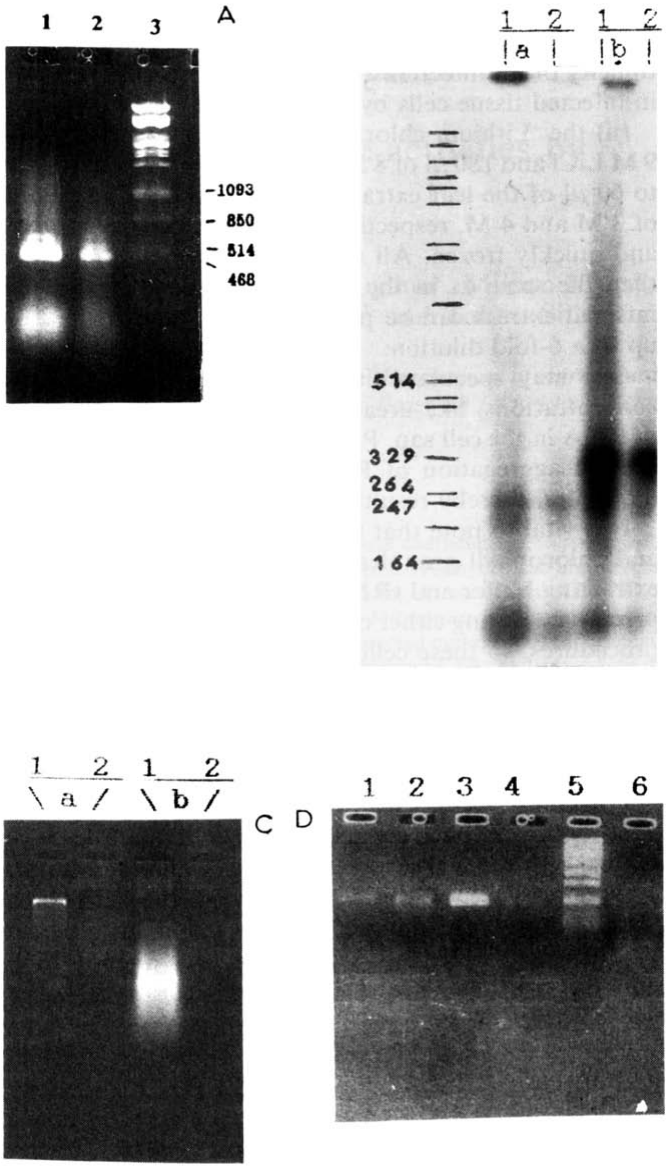

Fig. 1. Direct RNA polymerase chain reaction for TMV infection diagnosis and TMV RNA concentration. (A) Comparison of TMV cDNA amplified in the regular manner, i.e. RT followed by PCR (lane 1), and by direct virion RNA PCR (lane 2). Note that the PCR product (2) was applied at a concentration 4-times less than (1). (Lane 3) *Pst*I-restricted lambda DNA (2 μg). Numbers to the right indicate the size of the restriction fragments in base pairs. (B) Analysis of restriction fragments produced from TMV [^{32}P]cDNA amplified by direct RNA PCR (lanes 1) and RT followed by PCR (lanes 2) after *Hae*III (a) and *Nco*I (b) endonuclease treatment. Numbers to the left indicate the size of some *Pst*I-restricted lambda DNA fragments. (C) Precipitation of TMV RNA from the leaf tissue extract with 3 M sodium acetate (lanes 1) or 3 M LiCl+4 M urea (lanes 2), (a) infected with TMV and (b) not-infected. (D) Amplification of TMV RNA isolated from the infected (lanes 2–5) and non-infected (lane 6) leaf tissue (1 mg): lane 1, (control) virion TMV RNA amplified by the traditional method of RT+PCR; lanes 2 and 3, LiCl+urea and Acetate preparations, respectively (direct RNA PCR of crude RNA preparation); lane 4, direct RNA PCR of tissue nucleic acids net fraction obtained with phenol SDS; lane 5, *Pst*I-restricted lambda DNA markers; lane 6, direct RNA PCR of the Acetate crude RNA preparation obtained from non-infected tissue.

diagnosis, the TMV-infected extract is easily distinguishable from the non-infected one. Thus, at high multiplicity of an infection the viral RNA can be detected in infected tissue cells by a very simple procedure.

(ii) the 'Lithium chloride-urea' procedure: 120 μl of 9 M LiCl and 180 μl of 8 M urea were added in sequence to 60 μl of the leaf extract to give final concentrations of 3 M and 4 M, respectively. The samples were mixed and quickly frozen. All the following operations were then the same as in the previous method. Again, the infected extract can be positively recognized (Fig. 1C) up to a 6-fold dilution.

One may speculate that the acetate anion at high concentrations, like urea, induces partial denaturation of RNA in the cell sap. Presumably, this provokes non-specific aggregation of RNA and some other macromolecules, thereby resulting in their co-precipitation.

It is vital to note that the bulk of cellular chromatin and chlorophyll granules are not soluble in the RNA extracting buffer and tRNA for the most part does not precipitate during either of the viral RNA concentrating procedures, so these cellular components do not interfere with the hmwRNA detection by PCR. We believe that both procedures can find an application for mRNA detection in crude cellular extracts, its concentration and isolation.

For comparison, three TMV RNA isolation procedures followed by the direct RNA PCR method were carried out. Fig. 1D illustrates the data obtained. From our point of view both the acetate and the LiCl+urea viral RNA concentrating procedures are highly efficient and have the advantage over the tedious phenol+SDS one. In turn the one tube RNA amplification by direct RNA PCR has the advantage over the traditional RT then PCR method. It appears that the above methods could be used for serial detection of hmw viral or messenger RNAs for RNA screening or diagnosis.

3.3. *Concluding remarks*

We have described the non-radioactive diagnosis of RNA-containing potato virus X and M [4], TMV and poliovirus (Mahoney) [5] in crude cellular extracts with cDNA-peroxidase probes. Infected cell lysates or tissue sap displayed specific signals with the corresponding probes up to a 10,000-fold dilution or with 30–50 pg of the viral RNA in the fraction of total cellular nucleic acids isolated from infected cells. We predict that a combination of direct RNA PCR with non-radioactive DNA probing will be effective for the detection of a few molecules of viral RNA in crude tissue extracts at a very early stage in viral infection.

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