DNA MICROARRAY: PARALLEL DETECTION OF POTATO VIRUSES

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Summary. – DNA microarray assay has become a useful tool for gene expression studies. Less frequent is its application to detection of viruses or diagnostics of virus diseases. Here we show design of a microscope slide-based microarray assay for simultaneous identification of several potato viruses. Different primer pairs were designed or adopted to obtain specific amplicons from six potato viruses: Potato virus A (PVA), Potato virus S (PVS), Potato virus X (PVX), Potato virus Y (PVY), Potato mop-top virus (PMTV) and Potato leaf-roll virus (PLRV). Purified viral DNA probes were spotted on a microscope slide coated with poly-L-lysine. The same primers were used for preparation of fluorochrome-labeled targets. The latter were denatured and hybridized on the microarray slide (chip). An example of simultaneous assay of two pathogens is given and possibilities of practical application of this type of assay are discussed.

Key words: DNA microarray assay; potato viruses; diagnostics

Introduction

Potatoes can be attacked by about 50 different viruses and viroids (Brunt *et al*., 1996), but only some of the resulting diseases have an economic impact. Among the most worldwide distributed potato pathogens are PVA and PVY (the family *Potyviridae*), PVM and PVS (the genus *Carlavirus*), PVX (the genus *Potexvirus*), PLRV (the family *Luteoviridae*), and PMTV (the genus *Pomovirus*). All of them are ssRNA viruses. Five of these viruses, PLRV, PVY, PVA, PVM and PVS, are transmitted by aphids, while PVX is transmitted mechanically and PMTV spreads via the fungus *Spongospora subterranea*. These pests are most frequently detected by enzyme-linked immunosorbent assay (ELISA).

The microarray technique including DNA microarray (chip) technology (Schena *et al.*, 1995; Lockhart *et al*., 1996; Desprez *et al.*, 1998) represents a new tool for molecular biology. It belongs to the group of hybridization-based methods combining miniaturization and fluorescent dye labeling. In comparison to other molecular methods, it has some important advantages: high capacity, flexibility and sensitivity. Up to 10,000 probes could be deposited onto solid surface all at once (Schena *et al.*, 1995).

The principle of the DNA microarray analysis resides in hybridization of fluorescein-labeled DNA fragments prepared from unknown samples to specific DNA sequences prepared from known viruses, robotically spotted onto solid support. The spotted DNA is designated a probe and the labeled DNA fragment a target. Cy3-dUTP and Cy5-dUTP are frequently used as fluorescent dyes, because they incorporate with a relatively high efficiency into DNA. Furthermore, they have a good photo stability and yield, and their emission spectra are different enough. Several types of solid support as nylon, nitrocellulose, silicon or glass are used. Glass is characterized by a low inherent fluorescence; glass-based arrays are most often done on microscopic slides. Their surface is coated with poly-L-lysine, aminosilane or epoxysilane, which enable immobilization of spotted DNA.

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Abbreviations: ELISA = enzyme-linked immunosorbent assay; PLRV = Potato leaf-roll virus; PMTV = Potato mop-top virus; PVA = Potato A virus; PVM = Potato virus M; PVS = Potato virus S; PVX = Potato virus X; PVY = Potato virus Y; RT-PCR = reverse transcription – polymerase chain reaction; SDS = sodium dodecyl sulfate; SSC = saline-sodium citrate; TBE = Tris-borate-EDTA

Table 1. Characteristics of primers used in RT-PCR and PCR

Virus	Primer fragment	Sequence	Origin	Sense in genome	No. of nucleotides	Accession No. (bp)
PVA	PVA1	CCAACACAGACGCCCAAAAC	a	+	249–268	
	PVA1r	TCCTCGCCAGAAAACATCCC	a	–	1063–1082	AJ131400 (834)
PVA	PVA2	TGCCAACCAAGAACCATC	this work	+	1978–1955	
	PVA2r	GAAGTGCCTCCAACCCTGTAGT	this work	–	2413–2434	AJ131400 (457)
PVS	PVS1	TGGCGAACACCGAGCAAATG	a	+	2619–2638	
	PVS1r	ATGATCGAGTCCAAGGGCACTG	a	–	2784–2805	D00416 (187)
PVX	PVX3	GCTGTGGCTTCGATTTTGAGACC	this work	+	4878–4900	
	PVX3r	TTGGTGCCGTCCCTGTAAGC	this work	–	5307–5326	M38480 (449)
PVX	PVX4	AGGGCGAGGCACTGTCTG	this work	+	5500–5519	
	PVX4r	GGGGCTCTAGGCTAAACTCTGG	this work	–	6155–6176	M38480 (677)
PVY	PVY1	ACGTCCAAAATGAGAATGCC	b	+	8723–8742	
	PVY1r	TGGTGTTCGTGATGTGACCT	b	–	9183–9202	D00441 (480)
PLRV	PLRV1	CGCGCTAACAGAGTTCAGCC	c	+	3669–3688	
	PLRV1r	GCAATGGGGGTCCAACTCAT	c	–	3985–4004	X74789 (336)
PMTV	PMTV1	GTATTTTTAAGTCTAAACAGTTTGTGAC	d	+	1–29	
	PMTV1r	AATGAGTTCTACCTAAATTAGACAC	d	–	500–524	AJ243719 (524)

(a) = Nie and Singh, 2001; (b) = Singh *et al*., 1996; (c) = Singh *et al*., 1995; (d) = Petrzik, unpublished data.

This approach is suitable for detection of many pathogens in a single hybridization assay. The aim of this study was to design a model DNA-microarray assay for detection of some important potato virus pathogens and to test its applicability to diagnostics.

Materials and Methods

Total nucleic acids were isolated from leaves and/or tubers of infected plants (supplied by the Potato Research Institute, Havlíčkův Brod, Czech Republic) derived from tissue cultures. All plants were previously tested by ELISA to identify particular viruses. Approximately 200 mg of a tissue was homogenized in 3 ml of an extraction buffer (500 mmol/l Tris-HCl pH 8.3, 150 mmol/l NaCl, 0.05% Tween; 2% polyvinylpyrrolidone; 1% polyetyhyleneglycol 6000, 3 mmol/l NaN_3 and 1 mmol/l EDTA) and extracted by phenol-chloroform-isopropanol (Sambrook *et al.,* 1989). The nucleic acid pellet was resuspended in 500 µl of sterile water.

Reverse transcription–polymerase chain reaction (*RT-PCR*) *and PCR.* Viral RNAs were amplified by RT-PCR and reamplified by PCR. For RT-PCR the Access RT-PCR System Kit (Promega) was used according to the manufacturer's protocol. Eight primers were designed using the PrimerSelect (DNASTAR, Inc.) or Vector NTI Suite 5.5 (InforMax, Inc.). Another eight primers were adopted from other studies (Nie and Singh, 2001; Singh *et al.*, 1995, 1996; Petrzik, personal communication). Multiple sequence alignment was performed using the ClustalW program (Thompson *et al.,* 1994). Sequence comparison was done using the Internet service BLAST at URL http://www.ncbi.nih.gov/BLAST/. Sequences, length of amplicons and origin of all used primers are listed in Table 1. The reaction proceeded as follows. A 1 µl aliquote of extracted nucleic acids was added to 25 µl of a RT-PCR mixture. The reaction was carried out in one tube. RT proceeded at 48°C for 45 mins, PCR consisted of 94°C/3 mins (heat-inactivation), 35 cycles of 93°C/45 secs (denaturation), 55–60°C/1 min (according to the primer pair used) (primer annealing) and 68°C/1 min (primer extension). Final extension was done at 68°C for 10 mins. For reamplification RED-Taq PCR DNA Polymerase (Sigma) was used and 1 µl of the RT-PCR mixture after reaction (diluted 1:100) was added to 25 µl of a PCR reaction-mixture. The PCR conditions were: 93°C/2 mins, 30 cycles of 93°C/30 secs, 55–60°C/30 secs (according to the primer pair used) and 72°C/45 secs, followed by 72°C/10 mins. Both RT-PCR and PCR were performed on Mini Cycler (MJ Research, Watertown, USA). The obtained amplicons were purified with Qiaex II Gel Extraction Kit (Qiagen) or phenol-chloroform-isopropanol method and controlled on 1% agarose gel electrophoresis in 1x TBE buffer, stained with SyberGreen and visualized under UV light.

Preparation of microarray chip. Pre-synthesized DNA probes and a negative control (buffer without DNA) were spotted ("printed") onto a poly-L-lysine-coated microscopic slide using a robotic arrayer GeneSurfer (GeneAge Technologies). In addition, a landmark control (Cy-5-labeled DNA) was spotted to check the efficiency of probe immobilization. The slide was incubated at 80°C for 2 hrs to immobilize the DNA probes and cooled at room temperature in dark. Then it was incubated in isopropanol at room temperature for 10 mins and placed in boiling water to denature the DNA probes. The printed probes were stained with SyberGreen to control quality of printing and the whole chip was washed.

Fluorescein labeling of target DNAs amplified by RT-PCR and PCR was carried out using the KlenTaq DNA polymerase (GeneAge Technologies) and 2 mmol/l dNTPs labeled with Cy-3 or Cy-5 (NEN) tin the presence of non-labeled dNTPs in a ratio of 1:3. The reaction conditions consisted of 95°C/2 mins, 33 cycles of 95°C/30 secs, 60°C/30 secs and 72°C/45 secs, and 72°C/10 mins. The labeled DNAs were purified using Microcon 30 minicolumns (Millipore), denatured at 95°C for 4 mins and then kept on ice to retain their single-stranded structure. Cy-3- and Cy-5-labeled viral ssDNA fragments were simultaneously hybridized with the DNA probes on the chip at 42°C for 4 hrs in humid conditions. Then the chip was washed in three different washing buffers (I: 2 x SSC with 0.1% SDS, II: 1 x SSC, and III: 0.1 x SSC). The results were analyzed using the GeneTAC LS IV scanner.

Results and Discussion

Design of probes is crucial for the microarray technology. To avoid cross-hybridization the spotted sequences (probes) should be unique or should have only a low homology with other sequences. In order to detect all possible strains of a particular virus successfully, the probes should be highly conservative across the virus strain or group of strains. Considering these presumptions, eight specific primers were designed. Another eight primers, which have been optimized for a multiplex reaction by Nie and Singh (2001), were adopted.

The suitability of all the probes was tested. Their sequences were compared by the BLAST program with those of other viruses. A high homology to related viruses was observed for probes of PLRV and PVY. The PLRV probe showed a similarity with Barley yellow dwarf luteovirus (75.6%) and Soybean dwarf luteovirus (70.5%). The PVY probe shared similarity with Tamarillo mosaic potyvirus (64.0%) and Pepper mottle potyvirus (92.3%). However, none of these viruses attacks potatoes. Homologies, which were found for the rest of the probes, did not exceed 13% of their length. To ensure that none of the probes has similarity with others, alignment of their sequences – each versus each – was performed, and no identical continuous region, longer than 12 bp, was found. Moreover, the probe similarity did not exceed 53% and all homologies were distributed equally. From this viewpoint, all the probes used seemed convenient for detection of all the viruses investigated.

The amount of spotted DNA probe represents another important factor. According to Cheung and coworkers (2001) the concentration of DNA in the probe before application (printing) may range from 100 to 500 μg/ml.

In our case, the probes were checked by gel electrophoresis (Fig. 1) and solutions of approximately 30 μg/ml of purified DNA were prepared and spotted onto a slide in specific pattern. The microarray, prepared in this way, contained probes of five common potato viruses: PVS, PVA, PVX, PMTV and PLRV.

Some difficulties with the DNA chip hybridization may be caused by a low abundance of target DNAs. In order to prepare a suitable sample of target DNA, reamplification of RT-PCR products with a labeled dNTP was performed. Obviously, if virus concentration in a plant is sufficiently high, the labeled RT-PCR product itself provides enough viral DNA for detection. Another improvement of DNA microrray technique can be achieved by use of a multiplex RT-PCR in DNA target preparation. In this case, amplicons need not have different size, because they are not discriminated by size but by sequence. At present we are testing this possibility for all primers used in this work.

Initially, to avoid eventual cross-hybridization, a target DNA derived fom only one virus was hybridized with DNA probes on the microarray chip. Later, to mimic the situation in detecting mixed infections, two target DNAs were used. Fig. 2 shows a good detection selectivity achieved for two different viruses, PLRV and PVS. Various more complex mixtures of target DNA are being tested currently.

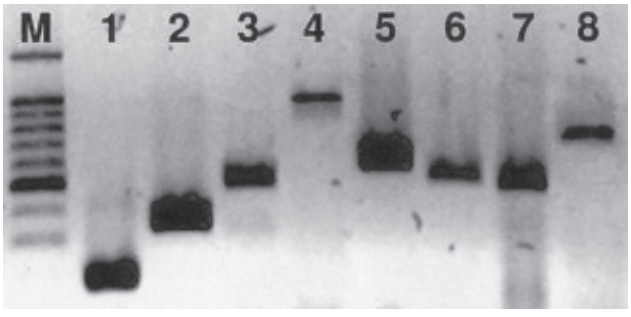

Fig. 1
Agarose gel electrophoresis of amplicons used as DNA probes

100 bp DNA ladder, Biotools (lane M); PVS, 187 bp (lane 1); PLRV, 336 bp (lane 2); PVY, 480 bp (lane 3); PVA (primers PVA1, PVAr), 834 bp (lane 4); PMTV, 524 bp (lane 5); PVA (primers PVA2, PVA2r), 457 bp (lane 6); PVX (primers PVX3, PVX3r), 449 bp (lane 7); PVX (primers PVX4, PVX4r), 677 bp (lane 8).

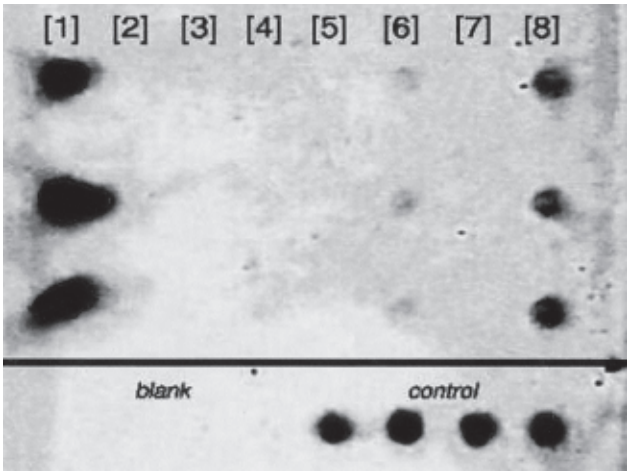

Fig. 2
DNA array (chip) analysis – simultaneous detection of PVS and PLRV

DNA probes were spotted in triplicate. Upper part: PLRV, 336 bp (lane 1); PMTV, 524 bp (lane 2); PVA (primersPVA2, PVA2r), 457 bp (lane 3); PVX (primer PVX4, PVX4r), 677 bp (lane 4); PVY, 480 bp (lane 5); PVA (primers PVA1, PVA1r), 834 bp (lane 6); PVX (primers PVX3, PVX3r), 449 bp (lane 7); PVS, 187 bp (lane 8). Lower part: negative control (left part) and positive controls (lanes 5–8).

The DNA microarray technique promises to become a routine diagnostic tool of the future, as it can be already seen for example in gene expression studies in medicine. Anyhow, its reliability for diagnostics of plant pathogens should be further investigated.

In conclusion, the results obtained with the DNA microarray technique are stimulating. Although, at present, the equipment for ”printing” and ”reading” microarrays is very expensive, the cost of production of a simple DNA chip for simultaneous detection of several pathogens is competitive provided a middle scale production is considered. Potato pathogens are a good object for testing this possibility, as the diseases they cause are economically important and the potential market for a ”potato microarray (chip)” is promising.

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