Validation of a microarrays protocol for detection and genotyping isolates of *Plum pox virus*

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Abstract

A genomic strategy for PPV identification has been recently developed (Pasquini et al., 2008). The method is based on using a 70-mer oligonucleotide DNA microarray chip capable of simultaneously detecting and genotyping PPV strains. Universal and specific probes have been identified and used with a sensitive protocol of hybridization using an indirect fluorescent labelling of cDNA product with cyanine able to enhance the sensitivity of the virus detection avoiding the use of the PCR amplification step. In order to evaluate the protocol fitness for diagnostic use, about 30 samples belonging to a PPV isolates collection, including M, D, EA and C strains, have been used for its validation, that was determined, estimating the performance criteria that include the following parameters: diagnostic sensitivity (D-SN), diagnostic specificity (D-SP) and diagnostic accuracy (D-AC).

Keywords: oligonucleotides chip, PPV, sensitivity, specificity, accuracy, performance criteria.

Introduction

The developing of innovative viral diagnostic methods is one of the major objectives of plant virologists to increase the sensitivity and rapidity of detection of low titer viruses. In recent years, great emphasis has been made towards the development of technologies to identify simultaneously a wide range of different pathogens, including plant viruses in a single sample, thus avoiding the use of parallel tests. The availability of systems that identify a large number of plant virus targets in a single event is the prerequisite for the control of viruses in quarantine to prevent their introduction in a country through the international movement of germplasm and in national and local certification programs to reduce their spread within a country. Furthermore, a single simultaneous diagnostic protocol could be useful in the certification programs which include testing of a large number of viruses in evaluated germplasm.

Several techniques were developed for the simultaneous detection of viruses. They consist essentially of multiplex-PCR or multiplex-real time PCR, however, the number of virus targets that can be identified is limited by possible primers interactions (multiplex-PCR) or by the number of fluorochromes that can be added to the reaction (multiplex-real time PCR). Currently, DNA microarrays represent the major known technology able to identify in a single event a large number of viruses and other pathogens (Boonham et al., 2007). They were first described in 1995 (Schena et al., 1995) for simultaneous analysis of large-scale gene expression patterns. Since then, this technology was developed to extend its use to other fields including detection of human and plant viruses (Barba and Hadidi, 2007, 2010; Hadidi and Barba, 2008; Hadidi et al., 2004)

An oligonucleotide microarrays chip for simultaneously detection and genotyping strains of *Plum pox virus* (PPV) has been recently developed using PPV universal and strain-specific 70-mer oligonucleotides probes (Pasquini et al., 2008). The method has a sensitive hybridization protocol, based on the indirect fluorescent labeling of cDNA incorporating cyanines. The protocol, which avoids the use of a PCR amplification step, was sensitive and specific and may represent a model for further technology developments.

In this paper the above mentioned system was evaluated to validate its performance. The validation of a protocol is the evaluation of its fitness for diagnostic use. It is determined by estimating performance criteria that include the following parameters: diagnostic sensitivity (D-SN), diagnostic specificity (D-SP) and diagnostic accuracy (D-AC). D-SN is the proportion of known infected reference samples that test positive in the assay. D-SP is the proportion of uninfected

reference samples that test negative in the assay. D-AC is the average of D-SN and D-SP which gives a complete evaluation of the protocol.

Materials and methods

Reference samples: The number and source of reference samples used to validate a protocol are significant. The reference samples ideally should represent known infected and non-infected plants from the pathogen population. A series of reference samples, target (representing all genomic and geographical PPV variability) and non-target (representing other viruses and healthy controls), have been selected to perform all experiments (Table 1).

Tab. 1 List of PPV target and other virus and healthy non target samples used to obtain the validation parameters.

N°	Name	Specie	Variety	Origin	Pathogen	Strain
1	Ispave 2	peach	Bela c1	Ex-Yugoslavia	PPV	M
2	Ispave 8	plum	P2774	Hungary	PPV	D
3	Ispave 11	peach	Marcus	Greece	PPV	M
4	Ispave 12	apricot	Canino	Spain	PPV	M
5	Ispave 13	plum	Delickia	Austria	PPV	M
6	Ispave 17	apricot	Tolda di Castigliole	Italy	PPV	D
7	Ispave 21	plum	Centenar	Italy	PPV	D
8	Ispave 29	apricot	Canino Ancian	France	PPV	M
9	Ispave 32	plum	-	Italy	PPV	D
10	Ispave 38	apricot	Priana	Spain	PPV	D
11	Ispave 39	apricot	El Amar	Egypt	PPV	EA
12	Ispave 40	plum	Plodiuv	Bulgaria	PPV	M
13	Ispave 42	peach	Big Top	Italy	PPV	M
14	Ispave 46	plum	Goccia d'oro	Italy	PPV	D
15	Ispave 51	peach	Calipso	Italy	PPV	D
16	Ispave 53	peach	-	Greece	PPV	M
17	Ispave 148.2	N. benthamiana	-	Italy	PPV	C
18	-	apricot	-	Egypt	PPV	EA
19	-	Brassica rapa	-	Italy	TuMV	-
20	-	artichoke	C3	Italy	ArLV	-
21	-	peach	GF 305	Italy	ApMV	-
22	-	peach	GF 305	Italy	ACLSV	-
23	-	peach	GF 305	Italy	PNRSV	-
24	-	peach	GF 305	Italy	PDV	-
25	-	peach	GF 305	Italy	PLMVd	-
26	-	peach	GF 305	Italy	HSVd	-
27	-	peach	GF 305	Italy	healthy	
28	-	plum	??	Italy	healthy	
29	-	apricot	??	Italy	healthy	

Target samples were as follow:

- PPV-infected isolates originated from different Mediterranean countries and maintained as the PPV collection at the C.R.A.–Plant Pathology Research Centre of Rome, Italy (PPV-ISPaVe collection). The isolates represented four PPV strains (PPV-D, PPV-M, PPV-EA and PPV-C) and different host species (peach, plum, apricot, *Nicotiana benthamiana*).

Non-target samples were as follow:

- species of Potyviruses (*Turnip mosaic virus* - TuMV and *Artichoke latent virus* - ArLV) to verify possible cross-reaction with homologous viruses of the same genera;
- viruses commonly infect stone fruits to verify possible cross-reactions with pathogens potentially present in the assayed vegetative hosts:
- c.1. Ilarvirus species (*Prunus necrotic ringspot virus* – PNRSV, *Prune dwarf virus* – PDV, *Apple mosaic virus* – ApMV);
- c.2. Trichovirus species (*Apple chlorotic leafspot virus* – ACLSV) and
- c.3. viroid species (*Hop stunt viroid* – HSVd and *Peach latent mosaic viroid* – PLMVd);
- healthy samples from different species (peach, plum and apricot) to verify the interferences due to the assayed matrices.

DNA Microarrays protocol: Experiments were performed using microarray chips prepared with UltraGAPS Microarray Slides (Corning, NY, USA) printed at the Istituto Superiore di Sanità, Dipartimento di Biologia Cellulare e Neuroscienze, Rome, Italy with twenty-one 70-mer oligonucleotides (Pasquini et al., 2008).

The microarray design was as follows:

- The chip contained eight rows with four sub arrays (total of 32 subarrays), each sub array contained five rows of twelve spots. Each oligonucleotide was printed randomly in 12 replicated spots on sub arrays.
- Total RNA was extracted from 100 mg of each virus infected and uninfected leaf tissue using a RNeasy Plant mini kit (Qiagen Inc., Valencia, CA). A cDNA was obtained from the above plant.
- Total RNAs by using oligodT and random primers as described in Pasquini et al., 2008.
- The indirect chemical labelling of cDNA was done by adding 8 µl of cDNA to 1 µl of $Na(HCO_3)_2$ buffer 0.1M pH 9.0 and 1 µl of Cyanine 3 (Cy3) or Cyanine 5 (Cy5) dye (Amersham Bioscience, Buckinghamshire, UK) suspended in DMSO buffer and incubated for 18 h at room temperature. The labelled cDNA was purified using a QiaQuick PCR cleaning kit (Qiagen).
- Slides were pre-hybridized for 45 min at 55 ºC with a pre-heated (55 °C) blocking buffer (1 % BSA, 0.1 % SDS, 5X SSC).
- The slides were then washed and dried by centrifugation.
- The hybridization buffer, contained 1 µg of each purified labelled cDNA (typically 30 pmoles of incorporated dye), 50 % formamide, 0.1 % SDS, 5X SSC, was applied to the slide after denaturation and covered with a cover slip (HybriSlip, Schleicher and Schuell Bioscience, Keene, NH). Hybridization was performed for 20 h at 55 ºC in the dark.
- The slides were then washed with pre-heated 2X SSC, 0.1 % SDS at 55 °C (5 min, twice), followed by 0.5X SSC, 0.1 % SDS (10 min at room temperature) and finally with 0.05X SSC (5 min at room temperature, four times).
- The hybridized slides were scanned using a GenePix 4200 A array scanner (Axon Instruments Ltd., Aberdeen, Scotland, UK).
- The parameters 'mean signal–mean local background' (mean Cy3 minus B or mean Cy5 minus B) and the 'mean local background' (B) were used in further calculations. Local background was calculated using the adaptive circle method. To estimate D-SN, D-SP and D-AC the cut-off point of reaction (positive/negative threshold) was established to be at least five fold fluorescent signal above the local background.

Establishment of performance criteria: The 29 target and non-target samples were assayed in 36 experiments performed by scientists in two laboratories (CRA-PAV and Istituto Superiore di Sanità) to verify the reproducibility of the protocol. The same array scanner read the slides.

In each experiment two samples, previously labeled with the two different fluorochromes Cy3 and Cy5, were combined in the hybridization mixture. Eighteen experiments were performed by mixing target samples and eighteen experiments by mixing target and non-target samples.

The D-SE, D-SP and D-AC parameters were calculated, on the averages values of the 36 experiments, using a two-way (2x2) table (Table 2). Results of the test were classified as TP (true positive) or TN (true negative) if they were in agreement with the oligonucleotide specificity determined in the set up of the chip (Pasquini *et al.*, 2008). Alternatively, they were classified as false positive (FP) or false negative (FN) if they disagreed with the previously determined oligonucleotides strain-specificity.

Tab. 2 Two-way table for calculating performance criteria

+ obtained/+ expected (TP*)	+ obtained/- expected (FP)
- obtained/+ expected (FN)	- obtained/- expected (TN)
D-SE = TP / (TP + FN)	D-SP = TN/(FP + TN)
D-AC = TP + TN / (TP + FP + FN + TN)	

*TP = true positive; FP = false positive; FN = false negative; TN = true negative
D-SE = diagnostic sensitivity; D-SP 0 diagnostic specifity; D-AC = diagnostic accuracy

The performance criteria were estimated for each single probe and for the entire chip. The probes designed on the basis of the alignment of the RNA genome of a member of PPV-W strain were not included in the experiments because of the unavailability of isolates of this strain.

Results

In all experiments good hybridizations were always obtained, without any background problems or weak signals. No significant differences were obtained in experimental results performed in the two laboratories (data not shown). Eleven

probes showed D-AC percentages from 97 to 100 %. The lower percentages of D-AC were always determined by the lower values of D-SE, as oligonucleotide probes did not hybridize occasionally with some infected reference samples. Four probes showed very low D-AC percentages (83-87 %) due to lower percentages both of D-SE or D-SP. Two of these probes are specific for PPV-D strain (V3 and P2) and were both designed on P3 gene of the PPV genome, whereas the other two probes (F3 and D3) recognized more strains and were designed on two different genes (HCPro and in the region between CP and 3'UTR, respectively) (Table 3).

Tab. 3 Percentages of validation parameters of each probe

Probe name	PPV genes	% D-SE*	% D-SP	% D-AC
D-CP (D)	CP	96	99	97
F-2 (D)	CP	97	98	97
V-3 (D)	P3	90	85	87
P-2 (D)	P3	83	84	83
F-3 (D + M)	HCPro	82	87	85
D-3 (D + M + C)	CP-3'UTR	84	83	83
V-2 (D + M + C + EA)	3'UTR	96	100	98
M-1 (M)	NIb	99	99	99
M-CP (M)	CP	98	99	98
SoC-1 (C)	P1	100	100	100
Soc-2 (C)	HCPro	99	100	99
SwC-1 (C)	NIb	99	100	99
SwC-2 (C)	P3	100	100	100
EA-1 (EA + D)	CI	96	99	97
EA-CP (EA)	CP	98	99	98

* = D-SE: diagnostic sensitivity; D-SP: diagnostic specificity; D-AC: diagnostic accuracy

Oligonucleotide probes specific for PPV-C isolates showed the highest D-AC percentages, but only one homologous reference sample was used from *N. benthamiana.* Also the two PPV-M specific probes showed high percentages of D-AC. The V2 universal oligonucleotide probes, designed on the conserved 3'UTR region of the PPV genome, showed a D-SE of 96 %, a D-SP of 100 % and a D-AC of 98 %. When the percentages of D-AC of each probe were correlated with the percentage of sequence homologies of each oligonucleotide probe it was evident that a cut off of 80% can be established for the genotyping of the virus in this set up of DNA microarrays system (Table 4).

Tab. 4 Comparison among percentages of probe sequence homology to PPV strain sequence and percentage of D-AC

	% Homology				
Probe name	**PPV-D**	**PPV-M**	**PPV-C**	**PPV-EA**	**D-AC**
D-CP (D)	100	77	64	65	97
F-2 (D)	98	77	62	61	97
V-3 (D)	100	82	74	74	87
P-2 (D)	100	82	74	74	83
F-3 (D + M)	100	100	74	84	85
D-3 (D + M + C)	97	90	90	88	83
V-2 (D + M + C + EA)	95	91	91	90	98
M-1 (M)	80	100	72	80	99
M-CP (M)	77	100	35	67	98
SoC-1 (C)	71	65	100	65	100
Soc-2 (C)	70	42	98	31	99
SwC-1 (C)	62	71	98	65	99
SwC-2 (C)	72	72	98	77	100
EA-1 (EA + D)	88	78	77	100	97
EA-CP (EA)	65	67	24	100	98

The D-SE, D-SP and D-AC of the complete chip were established on the averages of each probe values. On the whole the protocol showed: D-SE of 94 %, a D-SP of 96 % and a D-AC of 95 %. These parameters were obtained including also the lower values of the V3, P2, F3 and D3 oligonucleotide probes.

Discussion

The 36 experiments performed in two different laboratories confirmed the versatility of the protocol as good results were always obtained, which indicated the reproducibility of the system, using different scientists, equipment and environmental conditions.

The parameters obtained by assaying the designed oligonucleotide probes against a wide range of PPV isolates substantially confirmed the probe strain-specificity reported previously (Pasquini et al., 2008). The universality of the V2 probe, designed on the conserved 3' UTR region of PPV genome, was confirmed, as it reacted with all strains and, practically, with all tested isolates. Also the specificity of the probes directed against a single PPV strain (M1 and M-CP specific for PPV-M strain; EA1 and EA-CP specific for PPV-EA strain; Soc1, Soc2, Sw1, Sw2 specific for PPV-C strain and D-CP specific for PPV-D strain) was confirmed in this investigation. The values obtained with the probes V3, P2, F3 and D3, respectively, were lower than expected which may be explained by the percentage of sequence homology of the probe. A threshold of 80% of sequence homology is need for PPV genotyping in these protocol conditions.

The validation parameters obtained for the PPV microarrays chip were very good, as the values of D-SE, D-SP and D-AC were high. These data confirmed the usefulness of this innovative technique in the diagnosis and typing of PPV and it has a great potential to be applied to other plant viruses as well as viroids and phytoplasmas. The possibility to test an infected plant sample against an high number of probes is a new research field in plant pathology without frontiers. The DNA microarrays for plant pathogen detection are a technology in a developing stage. The set up protocol, without the use of commercial kits and the amplification steps, is not expensive and very easy to hande. It means that it can be performed also in phytosanitary laboratories without the necessity of specialized personal. Moreover, the technology cost is decreasing because have been developed chips re-usable more than one time. For all these reasons DNA microarrays technology represents a diagnostic tool with great potential, as it could be used to test simultaneously in a single event all possible pathogens of a crop as previously suggested (Hadidi and Candresse, 2001, 2003).

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