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Protocols

Oligonucleotide microarray-based detection and identification of 10 major tomato viruses

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A DNA microarray chip was developed for screening 10 major economically important tomato viruses from infected plants using "Combimatrix" platform 40-mer oligonucleotide probes. A total of 279 oligonucleotide virus probes were specific for simultaneous multiple detection, identification, differentiation and/or genotyping of each of the following tomato RNA viruses and/or strains and a virus satellite: *Cucumber mosaic virus*, *Cucumber mosaic virus* satellite RNA, *Tomato infectious chlorosis virus*, *Tomato chlorosis virus*, *Tomato spotted wilt virus*, *Pepino mosaic virus*, *Potato virus Y*, *Tobacco mosaic virus* and *Tomato mosaic virus*. This selection included both positive and negative single-stranded RNA viruses. The single-stranded DNA viruses, *Tomato yellow leaf curl virus* and *Tomato yellow leaf curl Sardinia virus* were detected but were not differentiated using probes designed from their coat protein genes.

A sectored oligonucleotide microarray chip containing four sets of 2000 features (4 × 2K) was designed. In this way, four samples were tested simultaneously in a hybridization event and 16 samples were analyzed by re-using the chip four times. The hybrids had low background signals. Many of the 40-mer oligonucleotide probes were specific for the detection and identification of each RNA viral species, RNA viral satellite and genotyping strains of *Cucumber mosaic virus*, *Pepino mosaic virus* and *Potato virus Y*. Universal probes were developed for strains of the last three viruses and also for the genus *Tobamovirus* which includes both *Tobacco mosaic virus* and *Tomato mosaic virus*.

1. Introduction

Tomato (*Lycopersicon esculentum* Mill) is a vegetable crop cultivated world-wide which is affected by many viruses that induce significant losses economically. Therefore, the detection and identification of viruses and their strains as well as RNA satellites affecting tomato crops is of critical importance to plant virologists in general and to plant quarantine and certification programs world-wide. Tomato viruses belong to diverse groups and do not share nucleotide sequences. Therefore, it is very important to use multiplexed methods for their detection and differentiation, as the demand of trade globalization requires pathogen-free seeds and fruit products.

The first reports on detecting viruses by the microarray technology were published in 2002 (Chizhikov et al., 2002; Lapa et al., 2002; Wang et al., 2002; Wilson et al., 2002). As of 2010, over 230 reports have been published.

Different microarray platforms have been developed depending on the different solid support (surfaces or beads) and probes synthesis: oligonucleotide probes are first synthesized and then spotted onto a glass; alternatively, probes are synthesized directly onto the array surface. The Combimatrix (Mukilteo, WA, USA) platform provides a chip where each oligonucleotide probe, designed by the costumer, is synthesized by an electrochemical synthesis process on a platinum electrode that is controlled independently by the synthesizer's computer. By this process, a developed CustomArray is a particular chip that is divided into four sectors or chambers, each of which may contain up to 2240 different oligonucleotide probes. The advantages of this system are that the sectors may be hybridized individually with different targets using a sectored hybridization cap and this new generation chip may be re-used up to 8 times by stripping the target from a hybridized recently CustomArray thus enabling subsequent re-hybridization.

Microarray-based technology has been used for plant virus detection since 2002 (Esteban et al., 2010; Zhang et al., in press; Abdullahi and Rott, 2009; Wei et al., 2009; Pasquini et al., 2008; Lenz et al., 2008; Abdullahi et al., 2005; Deyong et al., 2005; Bystrická et al., 2003, 2005; Boonham et al., 2003; Lee et al., 2003). DNA microarray provides the highest capability for parallel yet

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specific testing which can be used to detect individual plant virus or combinations of many plant viruses and/or virus-like pathogens (Hadidi et al., 2004; Hadidi and Barba, 2008; Barba and Hadidi, 2007, in press).

In this paper, the fluorescent-based microarray system developed by Combimatrix was used to create a "tomato viral chip" that detects and identifies 10 tomato viruses, which are important economically. The suitability of the Combimatrix system to detect and identify simultaneously single and multiple tomato virus infection is demonstrated. Virus-specific probes, strain specific probes and universal probes have also been designed and synthesized successfully onto glass slides for detection, identification and genotype characterization of tomato viruses.

2. Materials and methods

2.1. Tomato viruses and their sources

Tomato viruses included in this study were positive and negative-stranded RNA as well as single-stranded DNA. The virus species were: *Cucumber mosaic virus* (CMV), *Tomato infectious chlorosis virus* (TICV), *Tomato chlorosis virus* (ToCV), *Tomato spotted wilt virus* (TSWV), *Pepino mosaic virus* (PepMV), *Tomato yellow leaf curl virus* (TYLCV), *Tomato yellow leaf curl Sardinia virus* (TYLCSV), *Potato virus Y* (PVY), *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV). Tomato virus isolates used in this investigation are of Italian origin. They were characterized and maintained in the CRAPAV Collection as lyophilized leaves of plants (*Nicotiana benthamiana*, *L. esculentum*) infected naturally or experimentally. With the exception of isolate CRAPAV-44 [mixed infection of PVY strain N (PVY-N) and CMV strain II (CMV II)], isolate CRAPAV-263 [CMV II + satRNA] and isolate CRAPAV-155 [mixed infection of TYLCV and TYLCSV], each isolate of the other viruses was obtained from a single infection (Table 1). An isolate of *Impatiens necrotic spot virus* (INSV) (Family: Bunyaviridae, Genus: *Tospovirus*), was added as a control to determine the specificity of TSWV designed probes. Healthy tomato seedlings were used as internal negative controls in each test.

2.2. Total RNA extraction and RT-PCR assays for confirming the identity of tomato viruses

Total RNA was extracted from 100 mg of virus-infected leaf tissue using a Rneasy Plant Mini Kit (Qiagen Inc., Hilden, Germany). Total RNA concentration was determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (NanoDrop Techologies Inc., Wilmington, DE, USA). This procedure was applied also to the DNA single-stranded TYLCV or TYLCSV as total RNA extraction from leaves infected with these viruses which also contains their mRNAs was used successfully in a reverse transcription-polymerase chain reaction (RT-PCR) diagnostic assay (A. Tiberini, unpublished).

RT-PCR assays were performed using specific primers (Table 2) to test and verify the presence of the expected virus in each sample. Two μl of total RNA were submitted to one-step RT-PCR in a 25 μl volume. The reaction mixture contained 5 μl 5× buffer (Promega, Madison, WI, USA), 2.5 mM of each dNTP, 0.5 mM of each primer, 20 U of Rnase-OUT (Invitrogen, Carlsbad, CA, USA), 1.25 U of AMV RT (Promega), 0.75 U of Go-Taq polymerase (Promega). All viruses tested were amplified according to the following conditions: reverse transcription at 42 °C for 60 min, followed by denaturation at 95 °C for 5 min and by amplification consisting of 35 cycles with the following steps: 30 min at 94 °C, 30 min at 56 °C and 60 s at 72 °C, with a final extension for 10 min at 72 °C. Amplified products were electrophoresed in 1.5% agarose gels and visualized by staining with ethidium bromide.

2.3. 40-mer oligonucleotides design and slide construction

The nucleotide sequences of 28 virus isolates and strains of the 10 tomato viruses and one virus satellite investigated were retrieved from NCBI GenBank: CMV-Tfn: Y16926, Y16925,

Table 1
Major tomato viruses and their strains or isolates used.

Virus	Genus	Family	Isolate-ID	Strain
RNA viruses				
Cucumber mosaic virus (CMV)	*Cucumovirus*	*Bromoviridae*		
			CRAPAV-78	CMV IA
			CRAPAV-291	CMV IB
			CRAPAV-263	CMV II
Pepino mosaic virus (PepMV)	*Potexvirus*	*Flexiviridae*		
			IVV-Roggero	Ch2
			DSMZ	EU/TOM
Potato virus Y (PVY)	*Potyivrus*	*Potyviridae*		
			CRAPAV-71	PVY - C
			CRAPAV-117	PVY-N
Tomato infectious chlorosis virus (TICV)	*Crinivirus*	*Closteroviridae*		
			CRAPAV-259	
Tomato chlorosis virus (ToCV)	*Crinivirus*	*Closteroviridae*		
			CRAPAV-267	
Tobacco mosaic virus (TMV)	*Tobamovirus*	*Virgaviridae*		
			CRAPAV-385	
Tomato mosaic virus (ToMV)	*Tobamovirus*	*Virgaviridae*		
			CRAPAV-384	
Tomato spotted wilt virus (TSWV) (a negative strand RNAvirus)	*Tospovirus*	*Bunyaviridae*		
			CRAPAV-61	
Mixed infections				
CMV II + PVY-N			CRAPAV-44	
DNA viruses				
Tomato yellow leaf curl virus (TYLCV)	*Begomovirus*	*Geminiviridae*		
Tomato yellow leaf curl virus Sardinia (TYLCVS)	*Begomovirus*	*Geminiviridae*		
Mixed infections				
TYLCV + TYLCSV			CRAPAV-155	