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Microarray immunoassay for the detection of grapevine and tree fruit viruses

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Development and application of DNA microarrays for plant disease diagnosis has to date been limited, and for antibody arrays even more so. In this work, an antibody microarray procedure was developed and its usefulness for the detection of plant viruses demonstrated. Using the conventional monoplex immunoassay ELISA technique as a benchmark, the procedure was used to detect several grapevine and tree fruit viruses. In a direct labelling approach, *Arabis mosaic virus* (ArMV), and *Grapevine fanleaf virus* (GFLV) were detected after incubating the antibody array with alkaline phosphatase-conjugated viral extract. Indirect detection using a double or triple antibody sandwich format also resulted in good reaction signals, using either a chromogenic or fluorescence dye. In a multiplex system, four grapevine viruses were detected without compromising sensitivity and specificity. Compared to ELISA, the antibody microarray system is similar with respect to sensitivity and specificity, and a high correlation (R^2, 0.759) was observed in regression analysis of virus concentration measurements. Receiver operating characteristic (ROC) curve analysis provided evidence of the good performance of the microarray system (AUC > 0.8).

1. Introduction

Many standard plant disease diagnostic tools such as PCR, RT-PCR and real time PCR are amenable to multiplex analysis (Gambino and Gribaudo, 2006; Osmana et al., 2008; Wall et al., 2004), but time, sample and reagent use can be cost prohibitive especially when a large number of pathogens and test samples are involved. In addition the number of targets that can be multiplexed is limited. Microarrays are being considered increasingly as a suitable alternative for detection of multiple targets (Perlee et al., 2004; Sengupta et al., 2003; Wang et al., 2002), because of their potential to overcome the shortfalls of other techniques. While some successes have been achieved in the detection of plant pathogens using DNA macro- or microarrays (Abdullahi et al., 2005; Agindotan and Perry, 2008; Boonham et al., 2003; Pasquini et al., 2008), little effort has been made in the application of antibody array to plant disease diagnostics. Nucleic acid arrays tend to be more robust than protein arrays, partly due to intrinsic DNA stability. By contrast, proteins have remarkable structural variability that dictate and complicate their interactions with other biomolecules and microarray surfaces (Haynes and Norde, 1994; Hlady and Buijs, 1996). Many proteins are also known to lose activity when bound to a solid surface, and most will adsorb non-specifically to commonly used substrate materials (Butler et al., 1992). Despite these inherent difficulties, research efforts have produced protocols that make protein microarray an attractive technology (Fredriksson et al., 2007; Mendoza et al., 1999; Peluso et al., 2003). Some of these systems typically use covalent attachment or affinity binding of capture ligands followed by a blocking step to limit non-specific binding.

In antibody array, immobilized capture antibodies are exposed to samples containing the target antigens, which can then be detected by fluorescence (Haab et al., 2001; MacBeath and Schreiber, 2000), chemiluminescence (Arenkov et al., 2000; Bronstein et al., 1990; Huang et al., 2001) or chromogenic substrates (McGadey, 1980). Arrays can also consist of immobilized antigen that can be probed with a single antibody (Joos et al., 2000). However, the greater challenge of virus purification makes this option inappropriate for routine use in plant virus diagnostic work. Microarray immunoassays are of general interest for all diagnostic applications where plant samples need to be tested for multiple pathogens in parallel. Compared with single antibody-based tests, fewer false results are observed in a microarray (Haab et al., 2001). Improvements in computer technology and bioinformatics, which have been integrated into the development of microarray-based assay systems, have made the technique even more robust.

A variety of materials have been evaluated as substrates for spotting antibodies, including membranes (Ge, 2000), derivatized glass (Joos et al., 2000; MacBeath and Schreiber, 2000) and hydrogels (Arenkov et al., 2000; Guschin et al., 1997). From a cost perspective and to achieve the required sample throughput, antibodies are sometimes spotted on the bottom of 96-well microtitre plates (Mendoza et al., 1999; Moody et al., 2001; Wiese et al., 2001). The production of microspots on these surfaces is performed using contact or non-contact printing arrayers. The

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immobilization of proteins and antibodies is accomplished through non-covalent adsorption or covalent coupling to amine reactive surfaces or through a variety of other chemistries (Arenkov et al., 2000; MacBeath and Schreiber, 2000; Mendoza et al., 1999; Suter and Butler, 1986). Numerous studies have shown that covalent attachment producing a specific orientation as opposed to random coupling of antibodies increases their antigen-binding capacity, sensitivity, and stability (Butler et al., 1993; Lu et al., 1996; Peluso et al., 2003; Shriver-Lake et al., 1997). These benefits are usually countered by the fact that the procedures for specific orientation of proteins usually result in a lower surface density of immobilized antibody (Nakanishi et al., 1996; Vijayendran and Leckband, 2001).

The limit of detection is arguably lower in miniaturized assays than in macroscopic ones (Ekins et al., 1989; Templin et al., 2002). In a microarray, the fractional occupancy of the sensor antibody and signal per unit area is higher, and there are also less diffusion constraints (Ekins, 1998). However, sandwich microarray assays are not significantly more sensitive than state-of-the-art plate assays (Silzel et al., 1998). To increase sensitivity, various signal enhancement strategies have been employed in antibody arrays, including quantum dots, resonance light scattering, tyramide signal amplification and rolling circle amplification (Chan and Nie, 1998; Redkar et al., 2006; Saviranta et al., 2004; Zhou et al., 2004). Using microarray techniques, target molecules at a concentration of 1 pg/ml have been detected from different biological samples (Moody et al., 2001).

DNA microarrays have been used to demonstrate the effectiveness of miniaturized assays in the detection of many plant viruses (Abdullahi et al., 2005; Boonham et al., 2003; Pasquini et al., 2008), but even then, there are no reports that demonstrate the full potential of the technology. To date, we are unaware of any microarray assays that are being used for routine diagnostics of plant viruses. Considering the relatively time consuming and costly task of extracting nucleic acid from plant material for DNA/RNA analysis, this work was undertaken to explore antibody arrays as a more cost effective and simpler alternative. It represents an attempt to develop and evaluate antibody microarray procedures for plant virus detection. The number of viruses used in this work is modest and is a "proof of concept". Further development of the method will enhance work throughput by the multiplexing of many target viruses and the processing multiple test samples in a single experiment. A major drawback of microarray immunoassays lies in the

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A	1	1	1	1	2a	2a	2a	2a	2b	2b	2b	2b	1	1	1	1
B	4	4	4	4	5	5	5	5	6	6	6	6	7	7	7	7
C	4	4	4	4	5	5	5	5	6	6	6	6	7	7	7	7
D	8	8	8	8	9	9	9	9	10	10	10	10	11	11	11	11
E	8	8	8	8	9	9	9	9	10	10	10	10	11	11	11	11
F	12	12	12	12	13	13	13	13	14	14	14	14	15a	15a	15a	15a
G	12	12	12	12	13	13	13	13	14	14	14	14	15a	15a	15a	15a
H	16	16	16	16	15b	15b	15b	15b	17a	17a	17a	17a	17b	17b	17b	17b
I	16	16	16	16	15b	15b	15b	15b	17a	17a	17a	17a	17b	17b	17b	17b
J	18a	18a	18a	18a	3a	3a	3a	3a	20	20	20	20	2a	2a	2a	2a
K	18b	18b	18b	18b	3b	3b	3b	3b	19	19	19	19	2a	2a	2a	2a
L	1	1	1	1	2a	2a	2a	2a	2b	2b	2b	2b	1	1	1	1

Code	IgG (mg/ml)	Source	Code	IgG (mg/ml)	Source
1	GVB-AP	CPH	13	ToRSV (1)	PDCL
2	a=PPB, b=water	-	14	TRSV (1)	BRb
3	GFLV (a=0.5, b=1)	PDCL	15a	SLRSV ac (0.53)	ACD
4	GLRaV-1 (1)	BRb	15b	SLRSV b (1)	BRb
5	GLRaV-3 (1)	BRb	16	LChV-2 (1)	AAFC
6	PNRSV (4.9)	ATCC	17a	RpRSV-c (1)	PDCL
7	PPV (0.5)	Durviz	17b	RpRSV-g (0.7)	BRb
8	ToRSV (1)	PDCL	18	ArMV (a=0.5, b=1)	PDCL
9	ApMV (0.42)	agdia	19	GFLV (1)	BRb
10	BLShV (0.41)	ACD	20	GFLV ac (0.35)	ACD
11	SMYEaV (0.62)	ACD	21	GFKV (1)	BRb
12	RBDV (0.87)	ACD	22	TBRV (a=0.5, b=1)	PDCL

CPH Centre for Plant Health, ATCC American Type Culture Collection, PDCL Phyto Diagnostics Company Limited, AAFC Agriculture and Agri-Food Canada, BRb BIOREBA, ACD AC Diagnostics

Fig. 1. Print layout (CPH-IA-ab12) of 19 different polyclonal antibodies (Table below) spotted on glass slides used in microarray immunoassay, spot sizes = ~135 μm, spot spacing = 270 μm. Antibodies from different sources were loaded into wells of 384-well plate and used as source for printing (SpotBot 2, Telechem). Antibodies with concentrations lower than 1 mg/ml were used as received otherwise were diluted in microarray protein printing buffer PPB (Telechem, Anaheim, CA) as shown below. IgG against GVB was conjugated with alkaline phosphatase and used as positive control (GVB-AP). PPB and water were also included in the array to serve as negative control in all hybridization experiments. Other print layout (CPH-IA-ab15) was also used and differs from CPH-IA-ab12 as follows: 11, 15a, and 2a replaced with 22a, 22b, and 21, respectively.