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'Phytochip': On-chip detection of phytopathogenic RNA viruses by a new surface plasmon resonance platform

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A B S T R A C T

The surface plasmon resonance (SPR) based 'Phytochip' was developed to distinguish virus-infected plants from non-infected plants. The system detects DNA–RNA hybridization to show the presence of phytopathogenic viruses such as the RNA virus barley stripe mosaic virus (BSMV) in wheat leaves. To achieve this BSMV and wheat specific oligonucleotides, and a negative control yeast oligonucleotide, were immobilized on a SPR gold surface chip. After optimization of the hybridization parameters with purified wheat samples, wheat infected with BSMV resulted in detectable signals with both the BSMV and the wheat probes. In contrast, a hybridization reaction was not be detected with the negative probe. The method is fast and sensitive with a detection time of 3000 s (50 min), a detection limit of 14.7 pg μl^{-1} BSMV RNA and a measuring range of 14.7–84 pg μl^{-1} BSMV RNA (1.323–7.56 ng BSMV RNA per 90 μl sample). These characteristics, combined with the high throughput design, make it suitable for application in plant breeding and virus control.

1. Introduction

In recent years numerous molecular biological methods and platforms have been established for analyses in biotechnology, agriculture and medical diagnostic methods. Using DNA hybridization, antibodies or aptamers, rapid, sensitive and specific biosensors are now available to detect complex targets (whole cells, viruses, virus-infected cells), small molecules (metal ions, organic dyes, amino acids, or short peptides) or proteins such as thrombin and membrane proteins of the bacteria *Proteus mirabilis* and *Salmonella enterica* (Bockisch et al., 2005; Kang and Lee, 2012; Ye et al., 2012).

Unfortunately most of these methods are not designed for high throughput analysis and also need labels to generate and amplify the target signal. Labelling strategies allow quantification of signals from miniscule amounts of sample due to amplification, for example, thousands of dye molecules are activated for each target DNA strand. However, labels can affect interactions and for detailed studies of biomolecules in their natural state, label-free techniques are preferred (Mayer and Hafner, 2011).

Surface plasmon resonance (SPR) is an established label-free system for use as the detection element in biosensors. It is widely used in the study of biomolecular interactions, as well as antibody screening for diagnostic and therapeutic applications (Fangerstam et al., 1990; Safsten et al., 2006; Mayer and Hafner, 2011). It uses the sensitivity of surface plasmon polaritons propagating along a thin metal film to detect changes in refractive index near the metal surface, either changes in angularly or spectrally resolved reflectivity (Karlsson, 2004). SPR is powerful surface analytical technique that detects sub-monolayer quantities of analyte on a gold film surface and provides kinetic data through continuous optical measurements. However, despite its analytical capacities, SPR has to date, not been widely used in medicine and other non-research applications. This is partly due to its lower sensitivity compared with ELISA and other conventional techniques, and also because of the complexity of the optical instrumentation and the need for precise temperature control.

Despite these limitations, however, SPR is established as a standard method in label-free optical detection systems (Homola et al., 1999; Green et al., 2000). Increasing the sensor integration (Hoa et al., 2007) and increasing the number of probes bound to the chip surface and analyzing in parallel can increase sample throughput, increase the number of analytes within one sample that can be detected, or allow replication of an analysis to enhance reliability. In addition, on-chip referencing can be used to

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correct for non-specific effects (Danz et al., 2011). Applications of SPR include detection of phytopathogenic bacteria (e.g. *Acidovorax avenae* subsp. *citrulli*), enterotoxin B from *Staphylococcus aureus*, variation among different DNA samples and thrombins (Naimushin et al., 2002; Puttharugsa et al., 2011; Kick et al., 2010a, b; Danz et al., 2011; Henseleit et al., 2011).

Economic cultivation of plant food and feed crop plants such as wheat, barley, potato and maize is required to ensure sufficient food supply for the world population. However, about 30% of all cultivated crop plants are lost each year due to plant diseases such as viral and bacterial infections (Strange and Scott, 2005). Crop plant damage, as consequence of virus infection, is an enormous problem worldwide and is more significant than the damage caused by phytophagous insects, inadequate nutrient supply and weeds.

Techniques such as insertion of virus resistance genes, viral envelope protein genes, or genes encoding RNA dependent RNases in the plant genome has resulted in plants that have greater resistance to virus attacks. However, modification of the plant phenotype is not detectable until seven days after infection, which allows the virus to replicate and spread in the plant in that period and highlights the need for an early detection method.

Diagnosis by RT-PCR is very sensitive, but it is a time consuming and expensive method e.g. the RNA must be free of tanning agents, essential oils etc., because they can inhibit the PCR reaction. ELISA based on antibody-protein reactions is also a time consuming and expensive method. However DNA macro and micro arrays and multiplex PCR and qPCR are rapid but they also have limitations in that they are expensive and are not amenable to high throughput applications (Ward et al., 2004; Berendzen et al., 2005; Boonham et al., 2007, 2008; Lievens and Thomma, 2007; Gambino and Gribaudo, 2006; van Doorn et al., 2007; Vincelli and Tisserat, 2008).

In the present work a 'Phytochip' based on a SPR platform was developed for rapid, high throughput detection of phytopathogenic RNA viruses. The SPR sensor chip was modified for the specific detection of plant virus RNA through DNA–RNA hybridization. The sensor chip was first optimized for the hybridization reaction and measurements were made of real samples to confirm the functionality of the 'Phytochip' as a method to detect virus infections in crop plants.

2. Materials and methods

2.1. Instrumentation

All experiments described in this paper were performed using the Fraunhofer SPR platform as described by Danz et al. (2011). It comprises the measurement device, a lab-on-a-chip system and the SPR-software. The lab-on-a-chip system includes the SPR-chip and an on-chip-microfluidic system (Fig. 1). The sensor chips for the SPR-system were manufactured by KDS Radeberger Präzisions-Formen und Werkzeugbau GmbH, Germany, and were sputtered with gold by Fraunhofer IOF. The temperature of the lab-on-a-chip system was controlled using a heating circulator bath from GFL, Großburgwedel, Germany.

The microfluidic system is composed of an aluminium channel plate and a flow cell made of poly(dimethylsiloxane) (PDMS). The channel plate includes ports for entry and exit of fluids, heat exchanger ports and vacuum ports to restrain the sensor chip. The channel system is made of PDMS and cast from a laser micro-patterned master. Each channel has a height of approx. 80 μm and a width of 1.3 mm, which increases to 3.5 mm over the gold layer. The chips consist of a 76 mm × 26 mm × 4 mm slide, made from the cyclic olefin copolymer, TOPAS®, by injection moulding. The centre of the slide is covered with a 50 nm thick 12 mm × 3 mm layer of gold. Optical elements are integrated directly into the lower

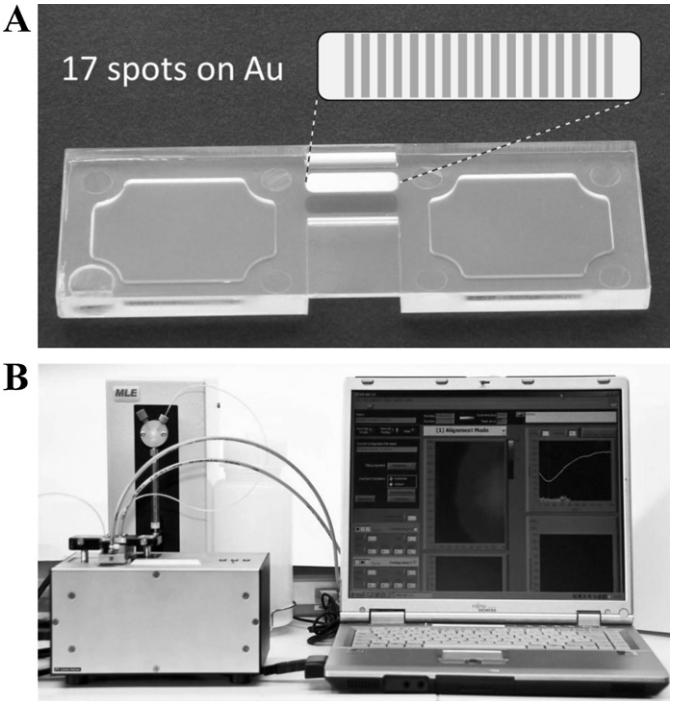

Fig. 1. (A) SPR-chip and (B), complete SPR platform with the analytical instrument, syringe pump and computer.

surface of the polymer slide, making the design relatively compact. These low-cost sensor chips with blank gold surfaces are designed for single use.

5 μl of the DNA probe solution is added to each channel of the immobilization flow cell and immobilized on the gold surface of the sensor chip by inverse micro-contact printing. The complete flow cell is formed by attaching a 17-channel connection plate to the sensor chip and the device completed by adding a reagent-handling unit with a software-controlled syringe pump and switching valve (Probensampler PS 61, Medizin- und Labortechnik Engineering GmbH Dresden, Germany).

The Fraunhofer SPR platform detects angular shifts at a constant wavelength of 810 nm. The collimated light from three near-infrared light emitting diodes (LED) irradiates the sensor chip and the integrated optical elements of the chip focus this light onto the lower surface of the gold layer where surface plasmons become excited. The reflected collimated light is detected by CCD camera. The use of three LEDs makes it possible to simultaneously monitor three different areas of the gold surface of the chip, each 0.8 mm × 9 mm which allows the simultaneous examination of three samples on one SPR-chip or alternatively, the examination of a single sample in triplicate. Note: the performance of the optical system can be further enhanced, if required, by replacing the gold layer with one-dimensional photonic crystals (Sinibaldi et al., 2012a, 2012b).

2.2. DNA probes

DNA probes for detection of the tripartite ssRNA(+) barley stripe mosaic virus (BSMV) are based on its RNA-α (Jackson et al., 1991). A thiol-modified BSMV oligonucleotide (BSMV – 5′-thioCAG CAC GAA ACT TCA TAT GAG AA-3′; T_m 64.0 °C) with complementary sequences to the virus-RNA was immobilized on the gold surface of the chip. Also immobilized was a thiol-modified oligonucleotide "UBC" for detection of the wheat rRNA transcript encoding the constitutively expressed ubiquitin conjugating protein (5′-thioGGT ACA GAC CAG CAA AGC CAG AAA TG-3′; T_m 68.0 °C) as a positive