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Development of a portable, high throughput biosensor system for rapid plant virus detection

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A B S T R A C T

Biosensors based on living cells are characterized by high sensitivity, selectivity and rapid response times. A novel portable cell biosensor system for the detection of plant viruses, based on immobilized 'Vero' cells carrying on their membrane virus specific antibodies was developed and was designated as High Throughput Bioelectric Recognition Assay (BERA-HTP). BERA-HTP was tested for the detection of purified *Potato virus Y* (PVY), *Cucumber mosaic virus* (CMV) and *Tobacco rattle virus* (TRV) and of CMV and TRV in single, as well as in mixed infections in two different plant host species. The sensor was based on live, mammalian cells, the membrane of which has been artificially saturated with antibodies specific to different plant viruses. The attachment of PVY, CMV or TRV viral particles to the homologous electroinserted antibodies caused a virus-specific change of the cell membrane electric potential that was not observed with virus-free samples or with heterologous viruses. Fluorescence microscopy observations showed that attachment of virus particles to the cell membrane bearing the homologous antibody, was associated with a decrease of $[Ca^{2+}]$cyt. The perspective for the development of BERA-HTP as a portable, reliable and rapid (duration of assay for 96 samples: ~70 min) detection method of plant viruses in the field is discussed.

1. Introduction

Biosensors based on living cells are known to have high sensitivity, selectivity, and rapid response times. These sensing systems have been employed in a wide range of environmental, chemistry and medical applications (Kintzios, 2007). Cell biosensors used for the detection of plant viruses are based on the change of the membrane electric potential of a host cell during its interaction with the corresponding viral particles. By applying this principle on immobilized plant protoplasts, a method called Bioelectric Recognition Assay (BERA) was presented, which allowed the detection of plant viral particles (Kintzios et al., 2001, 2004). Subsequently, a technique called Molecular Identification through "membrane-engineering" was developed, which involved the electroinsertion of virus-specific antibodies on the cell membrane. The attachment of a homologous virus triggered specific changes to the cell membrane electric potential. Based on this technique, Moschopoulou et al. (2008) and Perdikaris et al. (2009) developed a Vero-cell biosensor assay for the detection of *Cucumber mosaic virus* (CMV) and *Hepatitis B Virus* (HBV), respectively. Furthermore, the combination of the assay with specialized Artificial Neural Networks that were trained to recognize plant viruses according to biosensors' responses has increased the performance of the BERA sensors (Frossyniotis et al., 2006).

In this work, a method optimized for high throughput detection of plant viruses and based on the BERA is presented. The new method, called BERA-HTP, uses "membrane-engineering" Vero cells which incorporate on their surface virus-specific antibodies at high density, rendering in this way each cell a very specific sensor of the interaction between a plant virus and the membrane-bound homologous antibodies. The method was tested for the identification of *Cucumber mosaic virus* (CMV), *Tobacco rattle virus* (TRV) and *Potato virus Y* (PVY) as purified particles as well as in sap-extracts derived from virus-infected plants. Most importantly, CMV and TRV-specific biosensors were tested for the detection of CMV and TRV in both single and mixed infections, in two different plant host species. These viruses possess a positive sense, single stranded RNA genome, belong to different virus genera and use different genome expression strategies. PVY (*Potyvirus*) infects species from the family *Solanaceae*, including potato, tobacco, pep-

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per, tomato and solanaceous weeds. Found worldwide, PVY is the most damaging viral pathogen affecting potato (Valkonen, 2007) and is widespread in tomato crops causing serious yield losses (Aramburu et al., 2006; Rosner et al., 2000). CMV (*Cucumovirus*) has the widest host range among plant viruses causing substantial economic losses mainly in *Solanaceae* and *Cucurbitaceae* crops (Haase et al., 1989; Varveri and Boutsika, 1999). TRV (*Tobravirus*) infects experimentally a very wide range of plants and is an important pathogen of potato, tobacco and plant ornamentals. TRV isolates are characterized by a great variability in the nucleotide sequence of their coat protein gene resulting in inability of serological methods to detect in a single test all TRV serotypes (Robinson, 1989). *Cucumber green mottle mosaic virus* (CGMMV) (*Tobamovirus*) which has a narrow host range confined in *Cucurbitaceae* was used as an additional heterologous virus in CMV detection experiments. CGMMV is transmitted mechanically and through seed causing severe diseases primarily in cucumber and watermelon (Varveri et al., 2002).

The novel sensor is a portable multi-channel system, suitable for a high throughput assay (eight parallel tests in 5 min), which offers an optimal combination of high speed, sensitivity and low cost.

2. Materials and methods

2.1. Sensor production

In order to construct the consumable part of the biosensor, membrane-engineered mammalian cells (see below) were prepared according to the procedure previously described by Moschopoulou et al. (2008). Briefly, Vero fibroblast cells were centrifuged at $100 \times g$ for 6 min and then resuspended in Dulbecco's medium provided with 20% (v/v) fetal calf serum (FCS). Subsequently, cells were incubated together with the antibodies ($0.5\ \mu g\ ml^{-1}$) for 20 min on ice. Then, the cells–antibodies mixture was transferred to appropriate electroporator (Thermo EC100, Waltham, MA) cuvettes. Electroinsertion was performed by applying two square electric pulses at 1800 V/cm. After electroinsertion, cells were incubated at 37 °C for 1 h. Finally, cells were centrifuged at $100 \times g$ for 6 min and resuspended in Dulbecco's medium provided with 20% (v/v) FCS.

Three different membrane-engineered cell sensors were created by electroinserting separately, CMV, TRV or PVY polyclonal antibodies, prepared in the Laboratory of Virology, Benaki Phytopathological Institute, Athens, into the membrane of Vero cells. According to this procedure, approximately 8×10^3 antibodies were incorporated on the surface of each membrane-engineered cell. Engineered Vero cells were mixed with 1% (w/v) Bactoagar® solution at 37 °C and then the mixture was transferred to a standard 96-well ELISA plate, where it was left to solidify at room temperature. Each well was loaded with 150 μl of the mixture and represented a single consumable biosensor (i.e. the biorecognition element), containing 15×10^3 engineered Vero cells. Repeating this procedure, a batch of 96 consumable sensors (ordered in 12 eight-well arrays) which corresponded to all the wells of the ELISA plate, was prepared in 3 h.

2.2. Recording and data processing

Each BERA-HTP cell-sensor row was connected to a system of titanium-made electrodes (Fig. 1). Both measurement and reference electrodes were connected to the PMD 1608-FS A/D card (Measurement Computing, Middleboro, MA). Signal and data processing were recorded with InstaCal software (Measurement Computing). The volume of each sample solution was 200 μl. The response of each sensor to the sample was estimated by recording the maximum change of the electric potential of the sensor for a period of 300 s. Since eight tests could be conducted simultaneously, a complete processing of an ELISA plate (96 tests) could be achieved in approximately 70 min (including sample handling).

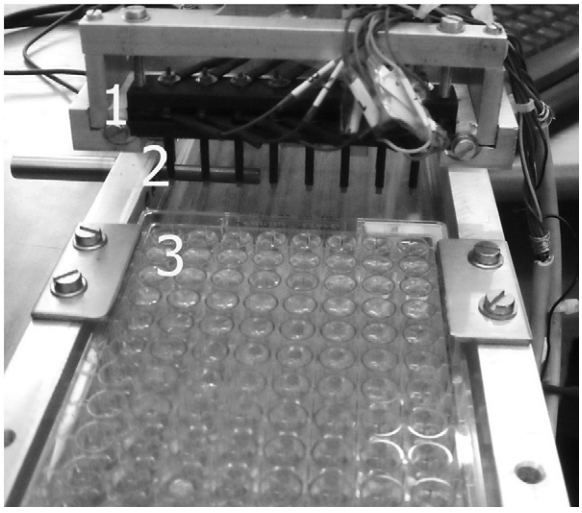

Fig. 1. The BERA-HTP biosensor. Consumable cell sensors, composed of the membrane-engineered cells immobilized in Bactoagar gel, are located into the wells of an ELISA plate. Changes of the sensor potential are measured with the aid of an adjustable moving guide (1) bearing eight pairs of electrodes (2), each corresponding to one of the eight wells of the array (3) (each plate has twelve arrays). Each pair of electrodes comprises two titanium electrodes with different length. The longer measuring electrode, insulated from most of its length, is inserted into the cell-containing gel in the well. The shorter reference electrode is immersed into the sample solution, above the gel. Measurement and reference electrodes are wired to the PMD 1608-FS data converter.

2.3. Virus detection assay

Three different sets of experiments were carried out. In the first experiment, each of CMV, TRV and PVY-specific biosensors (i.e. bearing the homologous to the respective virus antibodies) was tested on purified CMV, TRV and PVY viral particles in three tenfold serial dilutions in PBS (100, 10 and $1\ ng\ ml^{-1}$). In the second experiment, CMV, PVY and TRV-specific sensors were tested individually in extracts derived from *Nicotiana tabacum* plants infected solely with CMV, PVY or TRV. Plant extracts were prepared from infected plants by homogenizing plant tissue in 1:10 (w/v) PBS-Tween containing 2% (w/v) polyvinylpyrrolidone 40, followed by centrifugation at 5000 rpm for 5 min. Similarly to purified-virus testing, plant extracts were assayed in three tenfold serial dilutions into sap from healthy plants. Finally, in the third set of experiments each of CMV and TRV-specific sensors was tested on extracts derived from plants infected separately with CMV, TRV or PVY and in extracts from plants infected with mixtures of (i) CMV and TRV, (ii) CMV and PVY and (iii) TRV and PVY. In addition, a CMV-specific biosensor was tested using extracts derived from *Cucumis sativus* plants, infected separately and in mixture with CMV and CGMMV, in order to study the possible influence of different plant species on biosensor response. In the later set of experiments only the two higher dilutions of plant extracts were tested.

2.4. Experimental design

Experiments were set-up in a completely randomized design and each one was repeated three times. For each experiment of purified plant viruses, 80 individual assays were performed in total, consisting of 20 replications for the negative control and 20 for each of the three virus dilutions. The same process was repeated on extracts derived from virus-infected plants with the difference that

15 replications were applied for each extract dilution. The purified virus preparations and plant extract dilutions were also assayed for the presence of each virus by semi-quantified DAS-ELISA which was used as a reference method, using PVY, CMV or TRV specific antibodies according to Clark and Adams (1977).

2.5. Fluorescence Microscopy Assay

To elucidate the mode of action of the new sensor, changes in cytoplasmic Ca^{2+} concentration in cells membrane-engineered with CMV antibodies, before and after the addition of 4.0 ng ml^{-1} of homologous virus (CMV) and non-homologous viruses (TRV or PVY), were monitored by the uptake of the acetomethylester of Fluo3 as described by Moschopoulou et al. (2008). The same procedure was repeated with TRV and PVY specific biosensors. After application of 5 μl of the dye, the fluorescence of the specimens was recorded for 5 min at 10 s intervals. Slides with stained cells were mounted on a Zeiss Axiolab fluorescent microscope equipped with a BP-546 excitation filter and an FT-580 chromatic beam splitter. In order to control photobleaching, specimen exposure times were kept at a minimum level. No significant alteration of the intensity of the fluorescence took place. The number of fluorescent and not fluorescent cells was counted both manually and by using Doc-I® LS Image Acquisition and Analysis Software. For each microscopic observation, the average cell number and fluorescence assays were calculated from 10 different 7×10^3 μm^2 optical fields.

3. Results

3.1. DAS-ELISA

DAS-ELISA analysis with the appropriate virus antibodies confirmed that all plants were infected with the respective virus and virus combinations (data not shown). The detection sensitivity of DAS-ELISA, was determined using purified-virus, tenfold-diluted serially in PBS. DAS-ELISA detected purified CMV, PVY and TRV at concentrations as little as 100, 10 and 1 ng ml^{-1} PBS, respectively. In addition, PVY was detected in extracts from infected plants up to two times tenfold-diluted serially in sap of healthy plants, whereas CMV in extracts three times tenfold-diluted serially, and TRV in extracts up to four times tenfold-diluted serially (data not shown).

3.2. BERA-HTP response

BERA-HTP biosensor based on membrane-engineered cells with CMV-specific antibodies responded to all dilutions of the corresponding purified homologous virus (CMV), by considerable membrane hyperpolarization, as indicated by the *negative values* of the sensor's electric potential (Fig. 2). When no virus or a non-homologous virus was present in the sample, distinct *positive values* of the sensor's potential were observed, i.e. opposite to the response observed with the homologous viruses. A similar biosensor response pattern was observed for both TRV and PVY-specific biosensors (data not shown). In addition, and in contrast with the response of the TRV biosensor to TRV or PVY biosensor to PVY, the response of CMV specific biosensors to CMV was concentration-depended in a linear pattern ($r^2 = 0.99$). In any case the biosensors' response was quite reproducible, with 5.5–9.0% variation.

3.3. Plant samples

In the experiments with single-virus infected tobacco plants a negative potential change of CMV-, TRV- and PVY-specific biosensors was observed with extracts derived from CMV, TRV and PVY infected plants, respectively (Fig. 3A–C). Nevertheless, in the case of the TRV-specific sensor this pattern was also observed with PVY infected plants (Fig. 3B), albeit with sensor potential values close to zero.

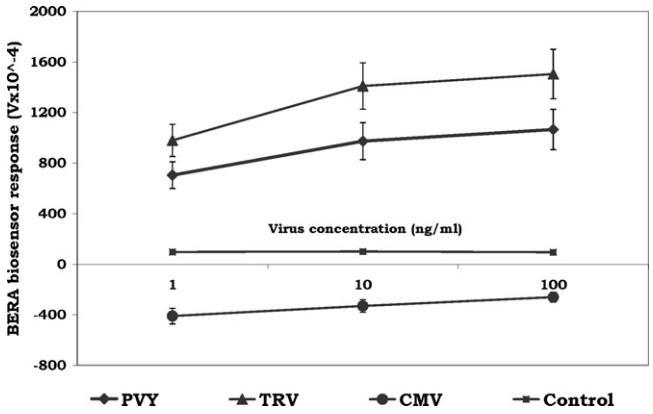

Fig. 2. Differential responses of the CMV-specific biosensor to purified CMV, TRV and PVY preparations. Sensor response is expressed as a change in the membrane electric potential of immobilized cells ($n = 20$ replications for each concentration and error bars represent standard errors of the average value of all replications with each range of concentration). The virus-specific biosensors responded to the concentrations of the homologous virus (1, 10, 100 ng ml^{-1}) with considerable cell membrane hyperpolarization as indicated by the negative values of the sensor's electric potential. Distinct positive values of the sensor's potential were observed when no virus (control) or a non-homologous virus (TRV or PVY) was tested in the sample.

In the experiments where plants were infected with mixtures of two viruses, a specific reaction, expressed as a *negative potential change* of the CMV biosensor was observed in all extract dilutions from tobacco plants either solely infected by CMV or together with TRV or PVY. An opposite, positive change was recorded in plants infected with TRV or PVY and in plants infected with both TRV and PVY, but *not* CMV (Fig. 4A). A similar specific response of the CMV biosensor was generated against the two extract dilutions from cucurbit plants infected with CMV alone or together with CGMMV, but not in extract dilutions from CGMMV-solely infected plants (Fig. 4B). The TRV biosensor also reacted specifically (i.e. with a negative potential change) only to extracts from plants infected with TRV alone or in combination with CMV or PVY but not to extracts from plants infected with the heterologous viruses (Fig. 4C). An exception to this was the slight negative response of the biosensor to extracts from plants infected with PVY, indicating that two sample dilutions are needed, at least for certain virus–host–antibody combinations, in order to achieve an unambiguous detection to evaluate the presence of the virus.

3.4. Fluorescence Microscopy Assay—engineered cell–virus interaction

Vero cells, membrane-engineered with TRV antibodies responded to the presence of the homologous TRV by a considerable reduction of their cytoplasmic Ca^{2+} concentration, as indicated by the reduced intensity of Ca^{2+}-associated fluorescence (Fig. 5A). No changes were observed in fluorescence density when Vero-TRV cells were treated with the heterologous viruses CMV and PVY (Fig. 5B) or PBS (Fig. 5C). Similar responses were monitored for both Vero-CMV and Vero-PVY cells (data not shown).

4. Discussion

Conventional methods in virus detection, such as RT-PCR and immunological assays, are used widely, despite their relatively long turnover times, as they offer high specificity, reproducibility and sensitivity. As an additional technological advance, the combination with robotic technology has enabled the analysis of an

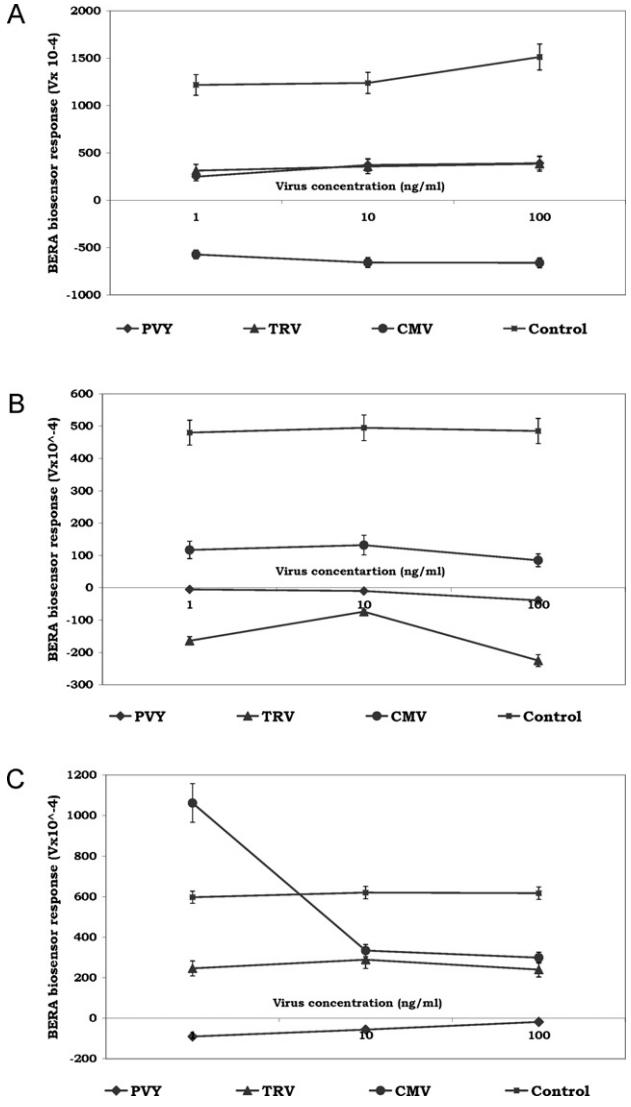

Fig. 3. Differential responses of the CMV- (A), TRV- (B) and PVY-specific biosensors (C) to plant extracts derived from tobacco plants infected with each of CMV, TRV or PVY. The virus-specific biosensors responded to the dilutions of extracts derived from plants infected with the homologous virus with considerable cell membrane hyperpolarization expressed as negative sensor potential. Healthy plant extracts were used as a negative control ($n = 15$ replications for each dilution and error bars represent standard errors of the average value of all replications with each range of concentration). On the contrary distinct positive values expressed when no virus or a non-homologous virus was present in the plant extract.

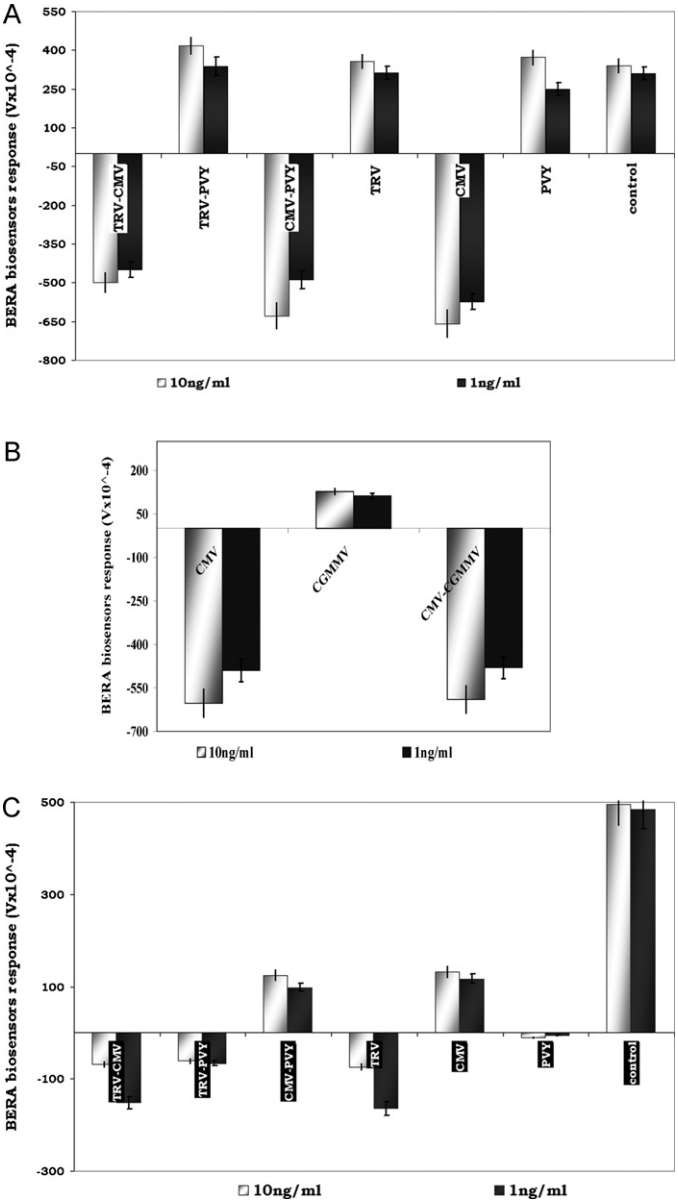

Fig. 4. Response of the CMV-biosensor, to plant extracts derived from tobacco plants infected with CMV, TRV and PVY solely or in combinations (A) and to plant extracts derived from curcubit plants infected with CMV or CGMMV, solely or in combination (B). (C) Refers to TRV-biosensors' response to plant extracts derived from tobacco plants infected with CMV, TRV and PVY solely or in combinations. Healthy plants were used as negative controls. Sensor response is expressed as a change in the membrane electric potential of immobilized cells ($n = 15$ replications for each sample). The biosensor response to the homologous virus presence solely or mixed with a different virus was expressed as negative sensor potential.

immense number of samples, nonetheless in expense of simplicity and of running cost (Lazcka et al., 2007). In certain applications such as phytosanitary control and quarantine-pathogen surveys, specificity, reliability and rapid processing of a large number of samples are essential issues, together with ease of use and low running cost.

Moschopoulou et al. (2008) have already shown that cells with electroinserted antibodies serve as recognition platforms for homologous viruses. They also demonstrated that an ultra-rapid and sensitive virus-detection assay could be developed by measuring membrane electric potential changes, which resulted from the attachment of the viral particles on the homologous membrane-bound antibodies. Using this approach, in a preliminary experiment, they were able to detect purified CMV particles at concentrations as low as 1 ng ml^{-1}, as well as to detect the virus in CMV-infected tobacco plants.

In this work, the initial BERA method was developed in a form suitable for rapid processing of a large number of samples. In an effort to simulate conditions that are common in the field, BERA-HTP was tested in a series of experiments, which involved for the first time plants infected with a mixture of viruses and different host plant species. Using BERA-HTP it was possible to successfully identify all three plant viruses tested, CMV, PVY and TRV in infected plants. Most significantly, TRV was detected in infected tobacco plants in single or in mixed infections whereas CMV was detected in single and mixed infections in tobacco and cucumber plants. The sensitivity of BERA-HTP was comparable to that of DAS-ELISA (1 ng ml^{-1}) depending on the antibodies used. The method in the present form is suitable for qualitative rather than quantitative plant virus detection and requires the use of at least two sample dilutions. Despite these limitations, BERA-HTP was able to process

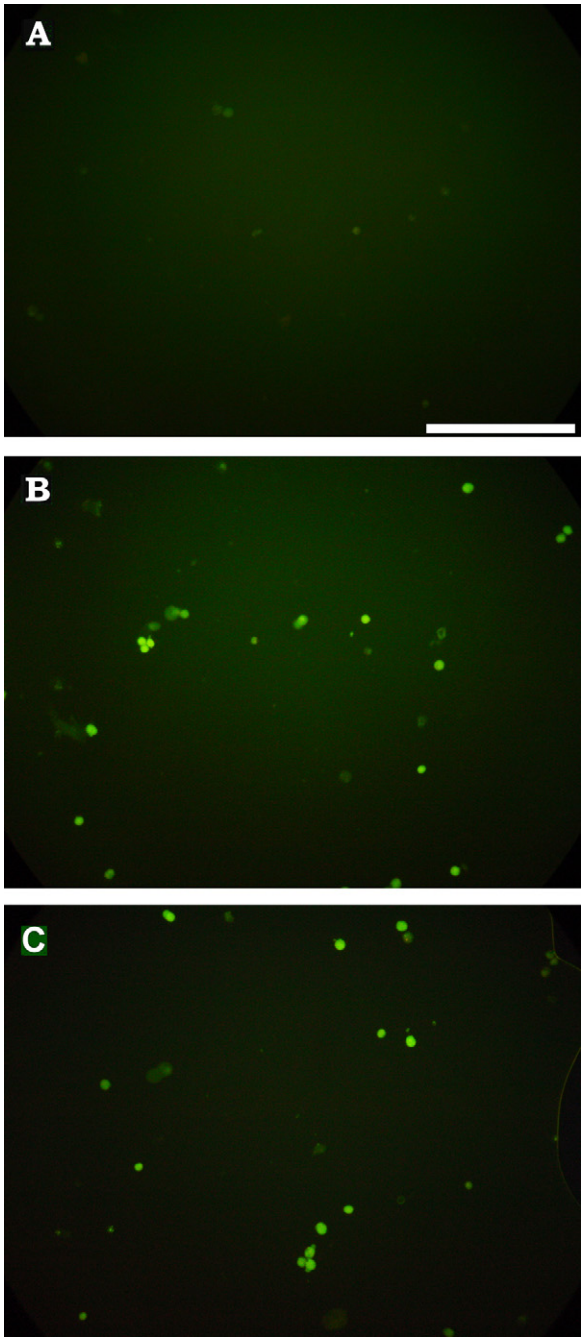

Fig. 5. Changes (expressed as differences in fluorescence intensity) of the cytoplasmic calcium ion concentration in hybrid Vero cells, membrane-engineered with TRV specific antibody against TRV (A), CMV (B) and a virus-free solution (saline buffer 0.5% (w/v)) (C). A considerable reduction in cytoplasmic Ca^{2+} concentration was monitored only and after the addition of the homologous virus (TRV). Bar in (A) represents 250 μm.

up to 96 tests in approximately 70 min and to detect the target virus in all relevant experiments. Thus, BERA-HTP appeared to be more rapid and efficient than conventional immunological methods, lateral flow tests or Loop-Mediated Isothermal Amplification (LAMP) method without sacrificing the level of sensitivity (Boubourakas et al., 2009; Mumford, 2006). This high sample processing capacity of the BERA-HTP, together with its simplicity are characteristics essential in applications where a large number of plant-samples need to be examined simultaneously for a series of plant viruses or virus serotypes like TRV.

In agreement with the previous findings, cell membrane hyperpolarization after treating membrane-engineered cells with homologous viruses was associated with changes in cytoplasmic Ca^{2+} concentration, which seems to be a common mechanism accompanying receptor-like interactions between molecules on the cell surface and target analytes (Moschopoulou and Kintzios, 2006; Moschopoulou et al., 2008; Perdikaris et al., 2009; Whelan and Zare, 2003).

5. Conclusions

The present study is an important step towards the development of a portable plant virus detection system suitable for field applications and based on biosensor technology. Further experiments are required, possibly in combination with Artificial Neural Networks, in order to define the linear range of the response and to achieve a quantitative detection of plant viruses. Future tests using a wider range of plant viruses and their respective host species are necessary in order to confirm the lack of interference of heterologous viruses on the selectivity of the sensor. Finally, sensor sensitivity could be improved by increasing the concentration of immobilized cells per sensor or the concentration of electroinserted antibodies onto the cell surface. Upon optimization, the novel biosensor could comprise a high throughput screening assay useful in routine virus diagnosis.

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