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ORIGINAL ARTICLE

DNA microarray-based detection and identification of bacterial and viral pathogens of maize

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Abstract We describe the construction and evaluation of DNA microarray for simultaneous detection and identification of five microbial pathogens of maize: *Pantoea ananatis*, *P. agglomerans*, *Enterobacter cloaceae* subsp. *dissolvens*, *Maize dwarf mosaic virus* (MDMV) and *Sugarcane mosaic virus* (SCMV). Unlike other DNA microarrays described, our microarray comprises probes targeting the whole genomes of the tested pathogens. Control probes are complementary to genomes of closely related microorganisms that are nonpathogenic to maize and against maize and human genome sequences in order to avoid the potential false-positive results. Obtained results indicate that the fluorescence signals from pathogen and control probes are well distinguished in all performed experiments. The microarray's performance was compared with classical PCR-based pathogen detection method, and the versatility of the assay was tested *in silico*.

Keywords DNA microarray · Maize pathogen detection · *Pantoea ananatis* · *P. agglomerans* · *Enterobacter cloaceae* subsp. *dissolvens* · *Maize dwarf mosaic virus* (MDMV) · *Sugarcane mosaic virus* (SCMV)

Introduction

The use of microarrays is highly appreciated for parallel detection and identification of microbial pathogens. DNA microarrays have been used for pathogen detection in environmental samples (Call et al. 2003), in wastewater (Kelly et al. 2005), for detecting waterborne pathogens (Lee et al. 2010), specific groups of microorganisms in complex systems (Sahm et al. 1999) and for identification of specific pathogen species like *Enterobacter sakazakii* (Wang et al. 2009) or *Escherichia coli* (Wang et al. 2010). Despite their broad potential, comparing to RT-PCR- or ELISA-based methods, the microarray techniques are still underexploited in phytopathology (Hadidi et al. 2004). The DNA microarrays were used for detection of potato viruses (Bystřická et al. 2003), cucurbit-infecting tobamoviruses (Lee et al. 2003), plum pox virus (Pasquini et al. 2008), grapevine viruses (Nicolaisen 2011) and for detection of a number of plant viruses in a multiplex assay (Engel et al. 2010). Other microarray studies involve both complex detection of a wide range pathogens (Zhang et al. 2013) and targeted identification of pathogens in a particular plant host, e.g., tomato (Tiberini et al. 2010) or potato (Fessehaie et al. 2003). Nevertheless, there is no specific DNA microarray available for detection of microbial pathogens

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of maize (*Zea mays*). The assay described here is an initial study of using a high-density DNA microarray for diagnosis of five maize microbial pathogens. Maize is infected by numerous bacterial and viral pathogens, which can significantly decrees its crop (Lapierre and Signoret 2004; Frederiksen and Odvody 2000). We focused on five (two viral and three bacterial) pathogens that were detected in Poland during five-year survey (Krawczyk et al. 2010; Trzmiel 2009; Trzmiel and Jeżewska 2008). Also, to our knowledge, no other maize bacterial or viral pathogen detection was reported in Europe (Janse 2012).

We constructed and initially evaluated a high-density DNA microarray (MaizePath microarray) for simultaneous detection of mentioned maize pathogens. Unlike other DNA microarrays, the MaizePath comprises species-specific probes targeting the entire genomes of the tested pathogens. To our knowledge, this is the first case of using a high-density DNA microarray in phytopathology.

Materials and methods

Bacterial and viral strains

American Type Culture Collection (ATCC) and laboratory bacterial and viral strains (a collection of Institute of Plant Protection-National Research Institute), isolated from maize plants, were grown under appropriate conditions. Virus isolates were maintained in infected plants grown under greenhouse conditions. Stock bacterial cultures were maintained on the LB (Luria-Bertani broth) medium supplemented with 50% (wt/vol) glycerol at –20 °C.

Probe design

A 4 × 180 K Agilent CGH microarray with 60-mer oligonucleotide probes complementary to genomes of 3 bacterial (*P. ananatis, P. agglomerans, E. cloaceae subsp. dissolvens*) and 2 viral (SCMV, MDMV) maize pathogens was designed (Genotypic Technology Private Limited). The microarray was manufactured according to Agilent Technology Custom *Maize, 4x180 K* protocol. Control probes were complementary to genomes of several commonly occurring microorganisms related to the target species but not found on maize. Similarly, *Sorghum mosaic virus* (SrMV) and Johnsongrass *mosaic virus* (JGMV) are related to SCMV and MDMV, but not infecting maize. *E. coli*, sequences were used as representative sequences for the *Enterobacteriaceae* family in particular because a significant number of potential human pathogens, e.g., *E.coli*, *Klebsiella pneumoniae*, or *E. cloaceae* subsp. *cloaceae,* have been identified on the phylloplane of maize (data not shown). Hence, to avoid false-positive results, the negative control set included probes targeting both maize (*Z. mays*) and human (*Homo sapiens*) genomes. The probes were designed to target the full genomes of the viruses (ca 10 kb) and the genomes of *P. agglomerans, P. ananatis, E. cloaceae subsp. dissolvens* (ca 5 Mb). To increase the specificity of MaizePath, additional probes were designed to target highly conserved 16S rRNA genes and internal transcribed spacers (ITS). Probes were chosen with an average spacing of 40 bp (viruses) and 2500 bp (bacteria), respectively, to obtain 60-nt-long oligonucleotides. Both pathogen and control probes were selected to have a similar GC content between 20 and 60% with a majority of probes having 40–60% GC bases. The Tm distribution of control probes is in range 70–100 °C.

DNA, RNA extraction and hybridization

The chromosomal DNA of the bacterial strains was extracted from cultures grown for 24 h on TSA (Tryptic Soy Agar) medium using the QIAamp kit (Qiagen, Hilden, Germany). The DNA was eluted in 0.2 ml of preheated (65 °C) sterile water, and concentrations were measured on a spectrophotometer (NanoDrop, Thermo Fisher Scientific, Wilmington, USA) prior to storage at −20 °C. Total plant RNA was isolated from 100 mg of fresh leaf material of virus-infected maize. Isolation was carried out using a NucleoSpin RNA Plant Kit (Macherey-Nagel, Duren, Germany), following the protocol supplied. The RNA was eluted with 40 µl RNase-free water and stored at −20 °C. First-strand cDNA synthesis was done using SuperScript TM III First-Strand Synthesis SuperMix (Invitrogen, USA) with oligo(dt)20 following the instructions of the manufacturer. Both bacterial DNA and viral cDNA samples were prepared together for the hybridization step. Preparation of DNA and cDNA samples included digestion with restriction enzymes and staining with the fluorescent dye (Cy3 or Cy5) strictly following the Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis, Protocol version 7.1, December 2011. Similarly, all hybridization and posthybridization processing steps were conducted with Agilent reagents and strictly following the protocols. For each microarray, 0.8 µg of fluorescently labeled cDNA was placed in appropriate buffer from Oligo aCGH/ChIP-Chip Hybridization Kit and hybridized to slides. After 24 h of hybridization in 65 °C, microarrays were washed with Oligo aCGH/ChIP-on-Chip Wash Buffer Set reagents, drained and scanned using Agilent's G2505C microarray scanner.

Microarray data analysis

All microarray data have been deposited to Gene Expression Omnibus under accession GSE69895. Microarray images were processed in Agilent Feature Extraction

software (v. 10.7.3.1), using standard procedures of CGH 1010 Sep 10 full protocol. Feature extraction was done using default settings, i.e., no background subtraction and spatial detrend and "ranked consistent" probe methods" were applied for normalization. Quality check was done using both ArrayQuality (Paquet and Yang 2010) and limma R/Bioconductor packages. For data analysis, we applied methods used for expression profiling (Smith et al. 2009). We used "median" normalization from limma package (Smyth and Speed 2003), as we noticed that "loess" normalization resulted in on average ~10% overestimation of probe signals. To evaluate the specificity and sensitivity of the MaizePath microarray and for verification, if gene expression analysis methods can be applied for identification of maize pathogens, we designed three independent experiments (A–C) addressing specific problems. A log-2 Cy5/Cy3 intensity ratio (*M* value) for each probe was determined across all microarrays tested, and *M* values for pathogen and control probes were averaged across all microarrays.

Sequence homology searches

Since it is known that other agriculturally important plants like rice (*Oryza sativa*) and sorghum (*Sorghum vulgare*) are affected by the three tested, maize bacterial pathogens: *P. ananatis*, *P. agglomerans* and *E. cloaceae subsp. dissolvens* (Morales-Valenzuela et al. 2007), we used exactly the same parameters as those described for investigating probe specificity.

A comparison of DNA microarray with standard PCR pathogen detection method

Using a touch-down PCR technique (TD-PCR) (Korbie and Mattick 2008), we tested three *P. ananatis*-specific primer pairs in one assay: (1) PanITS1/EC5 (Walcott et al. 2003), (2) PanITS1/EC5-Gi (Gitaitis et al. 2002) and (3) Pan16S/Pan16AS (De Boer 2003), using the strains hybridized to the microarray: M241, M304, M408, M471 and ATCC 33244. The sensitivity of the PCR detection was tested for each primer pair and bacterial strain on a series of a tenfold dilution of genomic DNA, starting with 50 ng/µl. The performance of PCR was also assessed by spiking maize genomic DNA with DNA of the pathogens.

Results

Construction of the MaizePath *microarray*

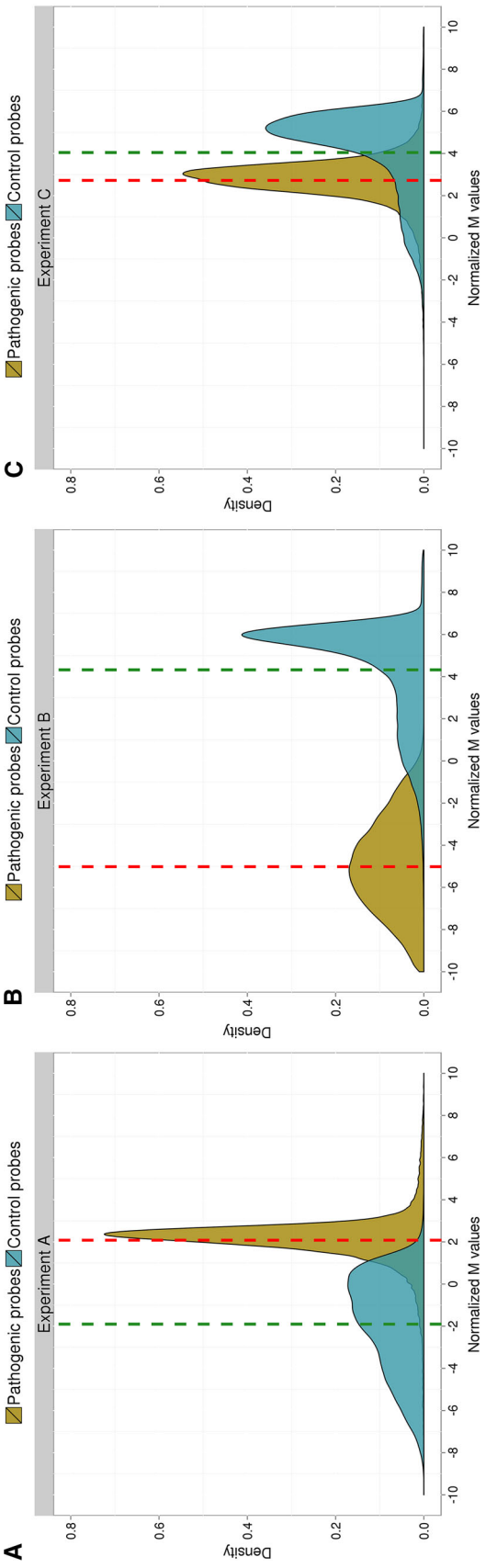
The DNA microarray developed contains 180,880 probes with 69,510 (~38%) pathogen and 107,930 (~60%) control probes and the 3440 (~2%) standard Agilent control features (Dumousseau et al. 2012). The number of probes for each group is representative of the genome sizes of pathogen and control organisms with an average probe coverage for *P. ananatis* 46.65%, *P. agglomerans* 40.15%, *E. dissolvens* 1.1%, MDMV 98.37%, SCMV 30.43%; controls: JGMV 55.83%, SrMV 40.86%, *E. coli* 1.37%, *Z. mays* 1.46% and *H. sapiens* 2.18%.

Data normalization

The data quality assessment from each experiment (A–C) using the limma R/Bioconductor package showed image plots indicating a uniform background for both channels: red (Cy5) and green (Cy3). Since the proportion of background was low for all microarrays, only a simple background correction (subtract method, limma package) was required prior to data normalization. Employing "median" normalization was sufficient to obtain comparable signal patterns, expressed with normalized *M* values, for all microarrays within each of three experiments (Fig. 1).

To assess the specificity of the microarray, both pathogen and control probes were mixed and hybridized to the microarray and investigated in three experiments (A–C). In experiment A (Fig. 1a), a global increase of the fluorescence signals for pathogen sequences was recorded which is reflected by the distribution and shift of normalized *M* values toward positive values on the *x*-axis (mean $M = 2$). The *M* values from probes targeting particular pathogens revealed a global increase of Cy5 fluorescence for the bacteria. This effect was slightly lower for viruses and could be explained by the lower number of virus probes in the array. However, the increased fluorescence obtained for bacterial probes can also be due to cross-hybridization of probe sequences with sequences of related species (e.g., *P. agglomerans* with *P. ananatis* detection probes). The analysis of control probes (*E. coli*, SrMV, JGMV, *H. sapiens*) showed on average lower *M* values compared to the target sequences (*P. ananatis*, *P. agglomerans*, *E. dissolvens*, SCMV, MDMV) (Fig. 1a). The *M* value distribution for the controls was shifted toward negative values with a mean *M* value equal to −1.9 (Fig. 1a).

The second experiment (experiment B, 4 microarrays) involved a comparison of control probes (including maize) labeled with Cy5 and a group of pathogens labeled with Cy3. The fluorescence signal recorded was significantly stronger for the group of control species probes (including maize) and discriminated well from the signal of the target pathogen probes. A massive increase in fluorescence was recorded for controls with an average $M = 4.3$ (Fig. 1b) and a reduction of *M* values for maize pathogens (average $M = -5$) (Fig. 1b). The *M* values from probes targeting



◀**Fig. 1** Specificity of MaizePath microarray. A graphic presentation of fluorescence signals recorded for microarrays pathogen and control probes, expressed as distribution of normalized *M* values, averaged across microarrays. Experiment A Hybridization comparison of both pathogen and control organisms (without maize); Experiment B hybridization comparison of negative controls and reference pathogen strains; Experiment C hybridization comparison of both pathogen and control organisms (including maize). *Red* and *green dashed lines* indicate mean *M* value

particular pathogens indicate no differences for pathogen probes across species. For control probes, a peak ($M = 6$) was observed for maize, while other controls are more uniformly distributed across wider range of normalized *M* values (from -2 to 10). We cannot exclude the possibility of cross-hybridization inside both control and pathogen groups; nevertheless, the signals from pathogen and control species were well distinguished.

In experiment C, we tested whether the hybridization of target pathogen molecules to specific probes was not affected by the presence of DNA of maize and other control samples. The experiment C (8 microarrays) involved a comparison equivalent to the one described for experiment A, but with an addition of maize DNA and the DNA templates of additional control organisms: 3 plant pathogenic bacteria (*P. carotovorum* subsp. *carotovorum, E. amylovora, A. tumefaciens*) and 1 virus (*Potato mosaic virus Y,* PVY), which were not targeted by the microarray. All DNA templates were mixed with genomic maize DNA (1:2, v:v). Despite the presence of maize DNA, the comparison, similarly to experiment A, indicates positive *M* values for pathogen probes (Fig. 1c). The same effect can be seen for controls, also showing high positive *M* values with similar mean $M = 4$ (and 4.3 for experiment B). Moreover, the shape of distribution of *M* values from probes targeting particular pathogen is exactly the same for all species as the one presented in experiment A for pathogens and in experiment B for controls (Fig. 1).

The MaizePath microarray in cross-species studies

The BLAST analysis revealed that only 24.6 and 14.6% of total number of maize probes are complementary to sorghum and rice genomes, respectively. Also, the sequence similarity between pathogen probes and both sorghum and rice genomes is very low. The results of BLAST search with sequences of 69,510 target pathogenic and 107,930 control probes as an input query, were the following: **(a)** *Sorghum* genome [GenBank: CM000760.1-CM000769.1] showed the highest similarity with over 20,000 probes matching to maize probes (24.6%, $E \leq 10^{-8}$) and the lowest similarity to target, pathogen probes matching only

34 probes (0.05%, $E \leq 10^{-3}$). **(b)** For rice genome [GenBank: NC_008394.4, NC_008395.2-NC_008405.2] much lower similarity was observed with more than 12,000 (14.6%, $E \leq 10^{-6}$) maize probes and 107 (0.15%, $E \leq 10^{-4}$) pathogenic probes matching rice genome.

A comparison of DNA microarray with standard PCR pathogen detection method

A specificity and sensitivity of pathogen detection were tested for three pairs of *P. ananatis*-specific PCR primers and compared to microarray results. In PCR's specificity testing, all three primer pairs gave the positive results in *P. ananatis* detection. However, only the primers PanITS1/EC5-Gi enabled the detection of all *P. ananatis* strains used in the test (M241, M304, M408, M471 and ATCC 33244). Using the pair: PanITS1/EC5 we were not able to detect the M471 strain, and the pair: Pan16S/Pan16AS did not detect the reference strain ATCC33244 of *P. ananatis*. In PCR's sensitivity testing, we were able to detect the following amounts of pure pathogen DNA: using the pair ITS/EC5: 0.5–50 ng; ITS1/EC5-Gi: 0.5 ng; Pan16S/Pan16AS: 5–50 ng, while for the microarray this number was higher (73.6 ng) (Table 1). In case when the tested species DNA was pooled and mixed with maize DNA (1:2, v:v), both PCR and microarray technique were able to detect the same amount of DNA: 39.52 ng of *P. ananatis*. The developed microarray gave the positive fluorescence signal also when other four target pathogens were hybridized: *P. agglomerans*: 61.56 ng, *E. cloaceae* subsp. *dissolvens*: 71.82 ng, SCMV: 10.26 ng and MDMV: 10.26 ng. The conclusion is that in this particular assay, the microarray's performance is comparable to standard PCR; however, the real detection sensitivity of the microarray can be defined only after performing a series of hybridizations of each pathogen separately.

Discussion

When designed, a DNA microarray can be used routinely as a test for the presence of various phytopathogens (Zhang et al. 2010; Tiberini and Barba 2012). However, to our knowledge, none of the described DNA microarrays is suitable for the detection of microbial, maize pathogens. Moreover, maize is infected by closely related microorganisms (e.g., *P. agglomerans* and *P. ananatis*) and their reliable detection and identification require a high-resolution analysis.

What is special to our microarray is that it consists of probes targeting the whole genomes of five microbial pathogens of maize and five other, control species. In total, it gives more than 180 K probes; thereby, it is classified as a high-density microarray and is one of the most complex microarrays for the phytopathogen detection. For example, a DNA chip designed to detect and identify thirteen genera of plant viruses contained only 345 probes targeting 169 phytopathogen species (Zhang et al. 2010). For plant viroid detection, only 103 probes were used to detect 37 different species (Zhang et al. 2010). Other authors (Engel et al. 2010; Nicolaisen 2011) have also used a small number of probes designed only for a chosen region of genome of the tested microorganism. All mentioned microarrays have also significantly smaller number of control probes in comparison with our microarray. To analyze a such big amount of data, we chose a microarray-based comparative genomic hybridization (array-CGH, aCGH) technique, which is mainly used for high-throughput, genome-wide screening of copy number variations, but can be also applied to cross-species studies (Vallée et al. 2006) and identification of specific, genomic regions, e.g., sequences of phytopathogens. The main advantage of this technique is its ability to investigate thousands of genomic loci in a high-resolution manner.

Table 1 The comparison of sensitivity detection of *P. ananatis* using species-specific PCR primers and MaizePath microarray

Method used	Template DNA	
	Pathogens genomic DNA (1)	Pathogens pooled genomic DNA + maize genomic DNA (1:2) (2)
PCR (ITS1/EC5)	0.5–50 ng	39.52 ng
PCR (ITS1/EC5-Gi)	0.5 ng	39.52 ng
PCR (Pan16S/Pan16AS)	5–50 ng	39.52 ng
DNA microarray (MaizePath)	73.6 ng (a)	39.52 ng (b)

Legend: Calculations: (a) 800 ng of the pooled DNA of the 22 pathogen strains and aliquot of human DNA was hybridized to the microarray. Four of 23 strains was *P. ananatis*, so: 800 ng / 23 strains * 4 *P. ananatis* strains = 73.6 ng. (b) One-third of the pooled DNA was pathogens. In total, 26 pathogen strains and aliquot of human DNA were hybridized and 4 of them was *P. ananatis*, so: 800 ng/3 = 266.7. And 266.7 ng/27 strains * 4 *P. ananatis* strains = 39.52 ng

Obtained results indicate that the fluorescence signals from pathogen probes are well distinguished from the control probes, in all performed experiments. The presence of additional DNA did not hamper the microarray's performance, and the shape of distribution of *M* values from pathogen targeting probes is repeatable between experiments (Fig. 1). However, to assess the exact specificity and sensitivity of the developed microarray, a series of hybridizations of individual pathogens and controls are needed.

The results of theoretical experiment (BLAST analysis of probes) performed suggest that the developed microarray has a versatile character and could be used also to detect tested bacterial pathogens in other plant hosts including sorghum and rice. Specially, that the used probe length (60nt) minimizes sensitivity to single nucleotide mismatches, which are common during cross-species hybridization, thus guarantees high hybridization specificity. However, the actual tests have to be performed yet.

A classic PCR using species-specific primers is the simplest and most common molecular technique used for screening for the presence of phytopathogens. However, due to natural genetic variation one needs to be aware that the primers described in the literature as species specific may not be specific to all its strains and thus cause a false-positive or false-negative results. The latter case was described above, when only one, out of three tested primer pairs specific for *P. ananatis*, enabled the detection of all *P. ananatis* strains hybridized to the microarray. This is the main drawback of this technique, which is not the case in the microarray due to a large number of probes targeting the whole genome of the pathogen. The results obtained indicate that the fluorescence signal from pathogenic probes is well distinguished from the control probes across all microarrays tested. A further tests need to be performed in order to exclude the possibility of cross-hybridization effect of closely related species (e.g., *P. ananatis* and *P. agglomerans*). However, the designed microarray has the potential to become a useful diagnostic tool that, unlike the species-specific PCR, enables the detection and identification of more than one pathogen in one assay.

To sum up, a novelty for science in this assay is first, using the high-density microarray for plant pathogen detection, second, the whole genome approach for probe design and, third, including the probes targeting host plant into the microarray's controls. The advantage of such approach is a potential versatility of the designed microarray, which, as we showed *in silico* by BLAST probes analysis, could be used for detection and identification of the same pathogens (*P. ananatis*, *P. agglomerans* and *E. cloaceae* subsp. *dissolvens*) also in rice and sorghum plants. The microarrays are available from the authors on a cost-reimbursement basis, to academic and nonprofit institutions.

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