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Analytical Methods

Assessment of a direct hybridization microarray strategy for comprehensive monitoring of genetically modified organisms (GMOs)

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ABSTRACT

Detection of GMO material in crop and food samples is the primary step in GMO monitoring and regulation, with the increasing number of GM events in the world market requiring detection solutions with high multiplexing capacity. In this study, we test the suitability of a high-density oligonucleotide microarray platform for direct, quantitative detection of GMOs found in the Turkish feed market. We tested 1830 different 60 nt probes designed to cover the GM cassettes from 12 different GM cultivars (3 soya, 9 maize), as well as plant species-specific and contamination controls, and developed a data analysis method aiming to provide maximum throughput and sensitivity. The system was able specifically to identify each cultivar, and in 10/12 cases was sensitive enough to detect GMO DNA at concentrations of $\leqslant$1%. These GMOs could also be quantified using the microarray, as their fluorescence signals increased linearly with GMO concentration.

1. Introduction

Genetically modified crops continue to increase globally, both in terms of cultivated land area and event/trait diversification. According to the latest ISAAA report, a total of 27 GM crops comprising 336 different GM events have been granted regulatory approval by one or more national authorities for food/feed use and/or planting worldwide (James, 2013). However, public concern over possible health and environmental risks associated with the production and consumption of GMOs has led many countries, especially those in the EU, to restrict the import of GM foods (Zhang & Guo, 2011), and introduce GMO labelling regulations for consumer information. For instance, a compulsory labelling threshold of 0.9% authorized GM material has been defined in the European Union (European Commission, 2003a,b) and Turkey (Anonymous, 2009). In Turkey, 19 GM events from maize (16) and soybean (3) have been approved for use in feed, but not food products, although 2 of the maize events have subsequently had their approval suspended pending a High Court investigation (Global Agricultural Information Network, 2014).

With the increasing diversity both of GM crops and regulations regarding their use in different countries, the detection of GMOs in food and feed products has become a very complex issue (Jensen, 2009). A robust analytical method is a prerequisite for monitoring GMOs and meeting legislative labelling requirements (Querci, Van den Bulcke, Zel, Van den Eede, & Broll, 2010), while the differing approval status of GMO varieties across the world trade network has created a demand for tools meeting the specific requirements of each legislative authority. Conceptually, these needs could either be met by developing tools tailored to each country's laws or by a universal method covering the majority of known GM events in a single quantitative, sensitive, and cost-effective test (Jensen, 2009; Prins et al., 2008; Ruttink et al., 2010).

To date, the polymerase chain reaction is the most widely applied method for GMO monitoring, with its high sensitivity,

Abbreviations: AOCS, American Oil Chemists' Society; CaMV, Cauliflower Mosaic Virus; CGH, comparative genomic hybridization; CRM, certified reference material; DNA, deoxyribonucleic acid; EST, expressed sequence tag; FE, Feature Extraction; gDNA, genomic DNA; GM, genetically modified; GMO, genetically modified organism; IQR, Inter-Quartile Range; ISAAA, International Service for the Acquisition of Agri-biotech Applications; NAIMA, NASBA implemented microarray analysis; nt, nucleotides; PCR, polymerase chain reaction; RNA, ribonucleic acid.

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specificity, low cost, and easy handling. In particular, real-time PCR offers the potential for quantification, provided that the reactions are carefully controlled and optimized (Jensen, Rønning, Løvseth, & Berdal, 2003; Kamle & Ali, 2013). Various PCR-based methods have been described for qualitative (Hernandez et al., 2005; Matsuoka et al., 2001) and quantitative (Brodmann, Ilg, Berthoud, & Herrmann, 2002; Hernandez et al., 2004; Ronning, Vaitilingom, & Berdal, 2003) detection of specific GM elements in plant samples. Multiplex PCR is the most common strategy for monitoring of multiple GMOs simultaneously, but the intrinsic interference and competition between PCR primers severely limits the number of targets that can be amplified in a single reaction (Shrestha, Hwu, Wang, Liu, & Chang, 2008). For this reason, systems designed to screen for most known GMOs by PCR typically require a large number of single reactions (e.g. Querci et al., 2009), or a moderate number of multiplex PCRs to be carried out in parallel (Cottenet, Blancpain, Sonnard, & Chuah, 2013), leading to high cost and labour per sample. Other researchers have addressed the multiplex problem by physically dividing PCR reactions into micro-droplets (Guo et al., 2011) or across a micro-well chip (Shao et al., 2013). In these approaches, a high-throughput analysis method is then used to characterize the pooled PCR products.

The DNA microarray is a well-established high-throughput analysis technology, allowing sequence-specific, parallel detection of a large number of genetic elements from complex DNA samples. Accordingly, microarray analysis has also been applied to GMO detection (Zhang & Guo, 2011). To date, several different methods based on multiplex PCR followed by hybridization of the PCR products to a low-density DNA microarray have been reported for the detection of GM events (Hernandez et al., 2005; Bordoni, Germini, Mezzelani, Marchelli, & De Bellis, 2005; Xu et al., 2006; Leimanis et al., 2006; Kim, Kim, Lee, Kim, & Kim, 2010), including one that has been developed into a commercial product (Leimanis et al., 2008). However, the limited multiplexing capacity of PCR still restricts the number of different GM events that can be detected in parallel. PCR amplification prior to microarray detection also makes reliable quantification problematic, as different samples may amplify with different efficiencies. Thus, methods that modify or substitute the PCR amplification step with other nucleic acid amplification techniques have also been proposed, such as multiplex quantitative DNA array-based PCR (Rudi, Rud, & Holck, 2003), NASBA implemented microarray analysis (Morisset, Dobnik, Hamels, Zell, & Gruden, 2008), padlock probe ligation in combination with microarray detection (Prins et al., 2008; Ujhelyi et al., 2012), and SNPlex (Chaouachi et al., 2008). Microarray analysis has also been used to characterize PCR products amplified in parallel on a micro-well chip (Shao et al., 2013).

In a few cases, a microarray-based method for GMO detection has been described in which target DNA is hybridized to the array without an initial amplification step. In principle, this has potential for GMO quantification, as the amount of DNA bound to the microarray should be proportional to the copy number of the element under scrutiny. Cansiz et al. (2012) tested a sandwich-type array platform with fluorescent detection, concluding that this could give sufficient sensitivity for direct detection of GMO DNA. High-density microarrays can be printed with many thousands of oligonucleotide probes; a probe set representing all possible sequences has been proposed as a means of detecting unknown GM events (Nesvold, Kristoffersen, Jensen, & Berdal, 2005). This concept was further refined by Tengs et al. (2007) using overlapping probe sequences covering all the vectors and the majority of trait genes currently used in GM plants. It was demonstrated that this approach could be used accurately to characterize the GMO cassette inserted in different GM soya and maize lines, even for an event where no prior sequence data were available (Tengs et al., 2010).

The goal of this study was to develop a DNA microarray as a screening method for GMOs approved for use in feed products in Turkey. To this end, we adopted a direct hybridization strategy using a Comparative Genomic Hybridization microarray platform. CGH has the capacity to screen thousands of DNA elements simultaneously and the sensitivity to detect unit changes in the copy number of individual elements in a large genome (Baumbusch et al., 2008). The only previous study using a CGH platform for GMO detection (Tengs et al., 2010) demonstrated that it could characterize GM cassettes, but only 100% GMO DNA was tested. Our aims here differed in that we wanted to focus on specific GMOs, assess the sensitivity of the system, and determine whether it could be used for GMO quantification. Therefore, we designed a microarray including 1830 overlapping 60-mer oligonucleotide probes covering the insertion sites and GMO cassettes from 3 soya and 9 maize GMO cultivars, as well as species-specific controls, and developed an analysis procedure for GMO detection and quantification. To our knowledge this is the first report of direct gDNA hybridization to a high-density microarray being assessed for quantitative detection of GM crops.

2. Materials and methods

2.1. Plant Materials

Certified Reference Materials for soya (*Glycine max*) GMO cultivars A2704-12 (AOCS 0707-B), GTS40-3-2 (IRMM-410), MON89788 (AOCS 0906-B), and maize (*Zea mays*) cultivars bt11 (ERM-BF412), bt176 (ERM-BF412), DAS1507 (ERM-BF418), DAS59122 (ERM-BF424), GA21 (ERM-BF414), MON810 (ERM-BF413k), MON88017 (AOCS 0406), MON89034 (AOCS 0906-E) and NK603 (ERM-BF415) were obtained from Sigma–Aldrich (St. Louis, MO, USA) or the American Oil Chemists' Society (Urbana, Il, USA). All CRMs used and their GMO content are listed in Table S1.

2.2. gDNA extraction

Total DNA was isolated with the Foodproof GMO Sample Preparation Kit (Biotecon Diagnostics GmbH, Potsdam, Germany) as described in the manufacturers' instructions; purified DNA was eluted in 50 μl of Elution Buffer. The DNA yield and purity were evaluated by measuring UV absorbance at 230, 260 and 280 nm using a NanoDrop 2000c UV/Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA) blanked with Elution Buffer. An A_{260}/A_{280} ratio of >1.75 was taken as an indicator of adequate DNA purity. All samples were also checked for degradation by agarose gel electrophoresis (Sambrook & Russell, 2001). Briefly, 100 ng of each purified DNA sample was loaded onto a gel, consisting of 1% (w/v) agarose in 0.5× TBE buffer and containing ethidium bromide at a final concentration of 0.2 μg/ml. Electrophoresis was carried out at 100V for 30–45 min, and the gels were then visualized on a UV transilluminator (Gel Doc system; Bio-Rad Laboratories, Hercules, CA, USA). All samples were extracted in triplicate and extracted DNA was stored at −20 °C for subsequent steps.

2.3. Microarray design and production

Microarray probes, along with the final array, were designed using tools on Agilent Technologies' (Santa Clara, CA, USA) microarray design website (https://earray.chem.agilent.com), except where stated below.

2.3.1. Probe design and selection

DNA sequences of the GM cassettes of interest were obtained from the websites of Genbank (https://www.ncbi.nlm.nih.gov)

and the GMO Detection Method Database (https://www.http://gmdd.shgmo.org). Most were complete, but only partial sequences were available for the GM cassettes introduced into *G. max* cultivar A2704-12, and *Z. mays* cultivars bt176, GA21 and NK603.

All the available GM cassettes along with flanking plant DNA sequences were used to generate 60-mer probe candidates by a simple tiling approach, starting a new probe every 30 nt. In addition, the Cauliflower Mosaic Virus genome and the coding sequences of *G. max* lectin *Le1* (Genbank: K00821.1) and *Z. mays invertase* (gb: HQ263133.1) and *zein-2* (gb: M23537.1) genes were similarly tiled, to act as controls for contamination and non-GM plant genes, respectively, giving a total of 2758 probe candidates. These candidates were then filtered according to quality criteria recommended for Agilent arrays (Agilent Technologies, 2013): any probes containing homopolymers of >6 bases (144 probes), outside the acceptable range of GC content (15–60% GC; 479 probes), or rated as 'POOR' by the e-array probe design software (26 probes), were eliminated. Probes covering the 35S promoter region in the CaMV genome were also deleted, as this region is present in the GM cassettes.

The insertion sites of each GM cassette are particularly useful for determining which cultivar a GM element originated from. Therefore, where available, a 100 nt sequence centred on each insertion site was extracted, and 60-mer probe candidates were generated as described above, but starting a new probe every 5 nt to maximise the chance of finding a highly specific probe sequence that gives good hybridization.

To avoid over-representation of elements found in several GM cassettes, which could lead to increased noise and dilution of the fluorescence signal after array hybridization, all the GM cassettes were searched using blastn (Camacho et al., 2009) for regions with 95% or greater sequence identity over at least 50 bp. These regions were delineated (Table S2) and in each case the probes from only one occurrence of each element (generally the longest) were selected, with all duplicates being deleted.

2.3.2. Microarray design

After these filtering steps, a test set of 1830 probes (147 from insertion sites, 1683 from the rest of the screened elements) remained, to which were added 2000 probes each previously designed from ESTs of *G. max* and *Z. mays* from the Agilent probe database. For efficient sample throughput, the 8 × 60,000 probe CGH array format was chosen, and 10 copies of each probe were distributed in dispersed locations across the array. The 10 replicates should allow the uniformity of each hybridization across the array, and the consistency of each probe to be assessed. This filled a total of 58,300 array features; an additional 997 Agilent control features were included, and the remaining empty array positions were filled with single copies of further *G. max* EST probes from the Agilent database (https://earray.chem.agilent.com).

2.4. gDNA labelling, hybridization and detection

Sample labelling and hybridization were carried out according to the array manufacturer's Enzymatic Labelling for Array CGH protocol, v. 7.2 (Agilent Technologies, July 2012). In this study, 400 ng of isolated gDNA was used as the input for each restriction digest; generally, GMO-containing samples were labelled with Cy3, and co-hybridized with Cy5-labelled GMO-free reference gDNA from the same species. Species-specific Cot-1 DNA was not added to the hybridization mix as the probes on the array should not coincide with repetitive regions. Following the manufacturer's recommendations, microarrays were hybridized for 24 h and washed using Wash Procedure B for environments with >10 ppb ozone. Labelled arrays were scanned immediately after washing at 2 μm resolution using a Nimblegen MS 200 microarray scanner (Roche Diagnostics, Indianapolis, USA), and then stored under vacuum in the dark.

2.5. Data analysis

Microarray image files were loaded into Feature Extraction v11.5 software (Agilent Technologies), and first inspected visually. Any visible large-scale anomalies were noted and arrays re-washed and scanned if appropriate. Fluorescence intensity values for each probe were extracted using the default protocol for CGH applications; spatial trends in fluorescence intensity were corrected, and global differences between the Cy3/Cy5 signals on each array (dye bias) were normalized by linear regression. Corrected fluorescence intensity values, expressed in fluorescence units on a scale from 0 (no signal) to 65502 (saturated) were collated from the FE output files using custom Perl scripts. From the 10 replicates of each probe, any outliers (defined as fluorescence values falling outside the inter-quartile range by >1.42 × IQR; Agilent Technologies, 2012) were eliminated.

2.6. Statistical methods

Statistical analysis of each individual fluorescence data point (testing for significance above background, normalizing trends and biases specific to each hybridization, and removal of outliers) were carried out by the Agilent FE software as described above. Fluorescence data tables were then imported into Microsoft Excel 2010 for further analysis. For each probe, the mean and standard deviation of the fluorescence value for all non-outlier replicates on each array were calculated. The standard deviation and sample size of each set of replicates were used to calculate 95% confidence intervals (Neyman, 1937; Excel "CONFIDENCE" function, $\alpha = 0.05$) which were then added to and subtracted from the mean. Descriptive statistics were calculated using the appropriate Excel functions, as was linear regression where required (LINEST and SLOPE functions). These statistical functions were applied as described in Section 3.2 and summarized in Fig. 2.

3. Results and discussion

3.1. GMO elements are detected by differential hybridization to the microarray

Genomic DNA was isolated from CRMs of 3 different GM soya, and 9 different GM maize lines (see Material and Methods). These lines (with the addition of 8 further maize events generated by trait stacking of the lines used here) include all GMOs approved for feed use in Turkey (Global Agricultural Information Network, 2014) and also bt176, which is a non-approved event in both Turkey and the EU. For most of the lines, several different CRM samples with varying GMO contents (from 0% to 10%) were available (Table S1). However, for A2704-12, MON89788, MON88017 and MON89034, the CRM contained 100% GMO material; in these cases, the purified gDNA was mixed with GMO-free DNA from the same species to give DNA samples with 10%, 5%, 1% and 0.5% GMO content.

Following the typical array-CGH approach in which test samples are co-hybridized with a reference on the same array, 40 GMO samples, including one or more concentrations of all 12 CRMs, were co-hybridized with a GMO-free control. Fluorescence signals for each probe were compared and any not significantly above local background were eliminated. For the remainder, the GMO sample fluorescence signal was divided by that of the GMO-free control, and the base 2 logarithm of this ratio ($\log_2$ratio)

calculated. For each individual hybridization, a cut-off value for 'noise' was defined as the mean $\log_2$ratio of all non-GMO probes on the array plus 2 standard deviations.

When using 100% GMO DNA, the majority of GMO-specific probes gave signals above this cut-off; results for 3 of the CRMs (maize MON88017 and MON89034, and soya MON89788) are shown in Fig. 1. As expected, probes that were not GMO-specific (e.g. the flanking plant DNA from MON89034 and MON89788 cassettes) had $\log_2$ratios clustered around zero, while those for the maize *hsp70* intron found within the MON89034 cassette clustered around 1, indicating that the genome concentration of this element is doubled in the GMO (one copy in the cassette, and a second native copy elsewhere in the genome). However, the majority of GMO-specific probes were derived from unrelated plant or bacteria species, and these gave much stronger signals, although the magnitude varied greatly both between and within GM elements. This variation depends on two factors; firstly, a naturally occurring sequence in the maize or soya genome closely resembling one of the GM elements would give some fluorescence in the GMO-free control, reducing the positive signal for individual probes. This could explain the relatively weak signal for some probes for the chloroplast transit peptide CTP2, as the sequence of transit peptides is functionally constrained and so relatively well conserved between species. Secondly, the hybridization efficiency of each individual probe depends on the probe sequence; those which hybridize more efficiently will give a stronger signal above background.

In summary, these results confirmed that the GM events tested can be specifically detected and characterized by direct array hybridization of 100% GMO DNA, as was previously demonstrated for 3 other GMOs by Tengs et al. (2010). However, the authors of that study noted that this approach may not be sensitive enough to detect lower concentrations of GMO gDNA. We also observed that at lower GMO concentrations, the number of probes giving signals above the cut-off dropped rapidly. However, the observation that some probes give much stronger signals than others suggested an alternative approach, in which we use the microarray to screen probe sequences and select those with the best sensitivity and specificity for each GMO event.

3.2. Optimal probes allow accurate detection of low GMO concentrations

An analysis strategy was developed in which each probe is treated as a separate experiment and the most sensitive and specific probes identified for each GMO, along with control probes for hybridization quality and plant genomic DNA (summarized in Fig. 2). For a potential GMO screening tool, it is also desirable to increase sample throughput/reduce sample cost; therefore, we explored whether two test samples (one each labelled with Cy3 and Cy5) could be hybridized to the same array, and the two colour channels analyzed individually. To facilitate this, the mean fluorescence intensity of 10 different negative control hybridizations (1 or 2 replicates each of 6 different GMO-free CRMs) was calculated for each probe, and these values were used to define the range of signal intensities observed in the absence of GMO DNA. One method for doing this is to calculate 95% confidence intervals (Neyman, 1937). If the 95% confidence intervals for two sets of data do not overlap, this provides evidence that the values are different. Comparing confidence interval ranges is reported to be less prone to false positives than other standard significance tests (Payton, Greenstone, & Schenker, 2003). It also has the advantage when dealing with a large amount of data that only 2 pieces of information for each probe – the mean and the confidence interval for all replicates – are needed to compare the negative control and test values, whereas conducting e.g. a *t*-test would need the entire dataset. Accordingly, for each GMO hybridization, the fluorescence intensity of every probe's 10 replicates on the array was collated, and 95% confidence intervals calculated. A probe was considered statistically positive if the lowest bound of its 95% confidence interval was greater than the upper bound of the 95% confidence interval for the negative control hybridizations of the same probe. Using this relatively conservative test should give fewer false positives than calculating a p value <0.05 (Payton et al., 2003). Taking advantage of this approach, a further 16 GMO samples were co-hybridized in pairs on 8 arrays, and the Cy3 and Cy5 fluorescence signals analyzed separately. The data from the 40 GMO samples hybridized previously were also re-analyzed similarly, and the performance of each probe across all the samples was evaluated empirically.

3.2.1. Selection of hybridization control probes

For the control probes, the results from a representative dataset of 10 GMO samples and 4 GMO-free controls were compared. After eliminating a small number of probes that showed dye bias or a high degree of variability between samples, some of the soya and maize EST probes were observed to show no species-specificity, presumably corresponding to genes that are highly conserved between the two species. From this group, the 10 brightest and 10 faintest probes respectively were selected as positive and negative hybridization controls. The mean fluorescence values observed for these probes were then compared across all samples (Supplementary Fig. S1), showing a high degree of consistency between samples, and no bias towards either species or dye for these probes. In a small number of samples (6/56) these confidence intervals did not overlap for 6 or more of the hybridization control probes, indicating that there was a statistically significant difference in the overall hybridization quality of these samples. Visual inspection of these 6 samples had already suggested that they were unusually faint or had high background. Any fluorophore-based microarray system is sensitive to variations in sample quality and environmental conditions such as sunlight and atmospheric ozone (Redon, Fitzgerald, & Carter, 2009). The high consistency of the hybridization controls between ~90% of samples demonstrates that the methods and feature extraction procedure used here are generally effective at minimizing such variations. For the 6 samples that were significantly different for the reference set, useful results were still obtained by applying statistical normalization to the data (Section 3.2.3).

3.2.2. Selection and validation of species-specific probes

Detection of plant species-specific markers in an unknown DNA sample is a pre-requisite for accurate GMO identification. From the representative dataset, plant gene/EST-specific probes that gave fluorescence values $\geqslant$10-fold brighter in one species than the other, were not highly variable between samples, and were considerably brighter than the negative hybridization controls were selected as species-specific probes (Fig. 2). A total of 823 maize-specific probes and 473 soya-specific probes met these quality criteria. At the same time, the probes designed against the CaMV genome were assessed; 29 that gave signals significantly brighter than background (defined as having 95% confidence intervals that did not overlap with those for negative control hybridizations) were eliminated as false positives, as there was no CaMV present in the reference samples, leaving 199 suitable for use as contamination controls.

From these useful probes, the 200 most specific for each plant species, the first 80 CaMV-specific probes, and the 20 hybridization controls, were incorporated into a Microsoft Excel-based data analysis tool. The microarray fluorescence data from an unknown sample can be pasted into this tool to obtain an immediate assessment of hybridization quality and the species present; example data are

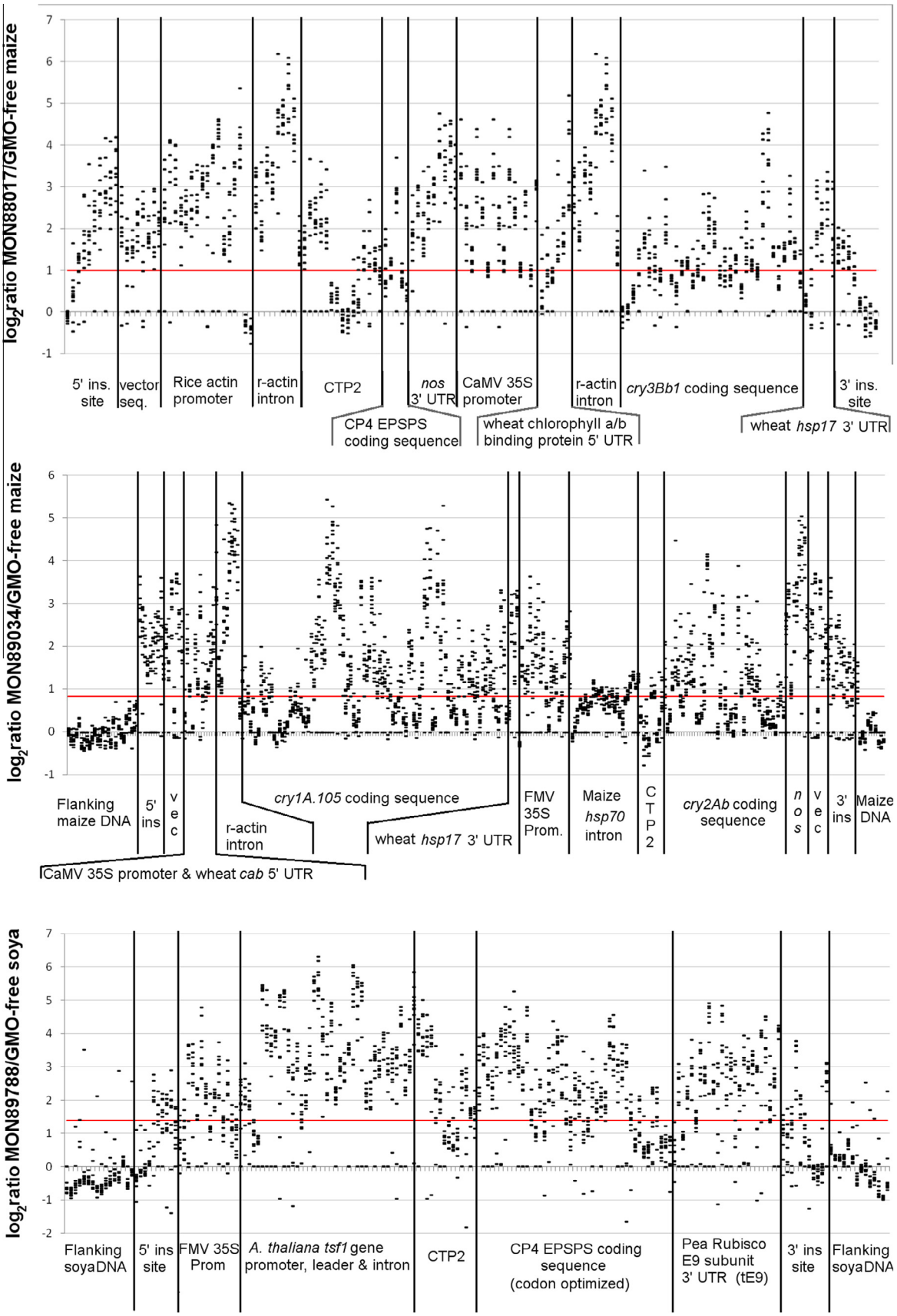

Fig. 1. Differential hybridization of labelled GMO DNA to the microarray. For each GMO (indicated by the y-axis label) the $\log_2$ratio of all GMO-specific probes are plotted along the x-axis in the order (5′–3′) in which their sequences appear in the GMO cassette. Each division of the X-axis represents a specific probe sequence, and values for the 10 replicates of that probe in a single hybridization are indicated by the vertically stacked points on the plot. The red lines indicate the noise-value cut-off for each hybridization (average $\log_2$ratio of all non-GMO probe signals + 2 Standard Deviations). Specific GMO elements are indicated below the axis. Vector seq./vec – *A. tumefaciens* T-DNA borders; CTP2 – *A. thaliana* chloroplast transit peptide; 5′/3′ ins. – probes for 5′ and 3′ insertion sites.

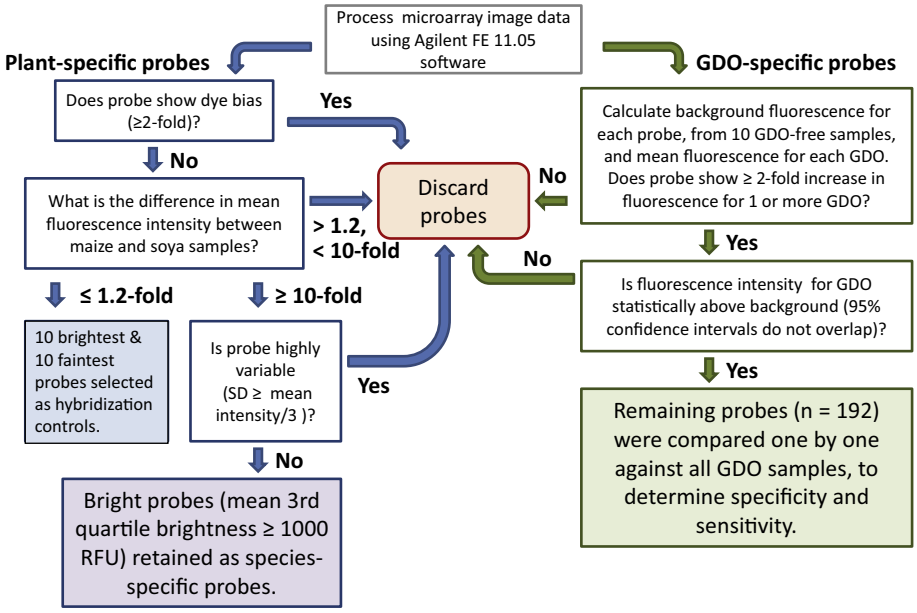

Fig. 2. Flow chart of the evaluation and selection procedure for control, species-specific and GMO-specific microarray probes.

shown in Fig. S2. Briefly, plant-specific probes were sorted in descending order of brightness, and their mean fluorescence and 95% confidence intervals calculated from the representative dataset, for both positive and negative samples of each species. Data from test samples were then compared with these values (Fig. S2). All soya gDNA samples gave fluorescence signals significantly above negative controls for the soya-specific probes but not for the maize-specific probes, while the reverse was true for the maize samples. For all the samples tested, both maize and soya, none gave signals significantly above background for the CaMV-specific probes, showing that there was no CaMV contamination in the samples tested. Sequences and details of the control probes are given in Supplementary Table S3.

These data again showed high consistency between hybridizations with the fluorescence of the positive probes typically falling within the 95% confidence intervals of the mean positive values observed in the representative dataset. Even the 6 samples that had significant differences in the hybridization controls were still accurately defined as either maize or soya, although there were quantitative differences in the brightness of some of the species-specific probes. This demonstrated that the direct hybridization method is highly robust for detection of different plant species. Existing GMO detection methods, either by RT-PCR or microarray, use individual well-defined reference genes as species-specific markers (Prins et al., 2008; Querci et al., 2009). Probes based on 3 such reference genes, soya *Le1* and maize *inv* and *zein-2,* were also included in this study and confirmed to be highly species-specific in this system. We also validated several hundred more EST-based probes as species-specific, with the inclusion of multiple species-specific markers increasing the robustness of the analysis. Although not tested in the scope of this study, it would be straightforward to replace some maize- and soya-specific probes with those for other crop species, thereby creating a universal array platform suitable for use with all GM crops. Additionally, if a sample containing gDNA from multiple species is applied to the array, the fluorescence signal for each species-specific probe is expected to be proportional to the concentration of that species' gDNA. Therefore, it would be possible to estimate that relative abundance of genetic material from each species in the sample, although further experiments are required to establish the accuracy of quantification.

3.2.3. Defining GMO-specific probe hybridization patterns

Initial analysis of different GMO samples co-hybridized to the same array with a GMO-free reference demonstrated that some probes were specific for each GMO (Fig. 1). To increase sample throughput, and confirm that results were comparable between arrays, all the data were re-analyzed using the negative reference derived from 10 different GMO-free hybridizations as described in Section 3.1. The 6 samples with poor hybridization quality were first normalized by plotting the fluorescence values measured for all non-species specific probes against those found in the reference, fitting a linear regression to this plot, and transforming the data using the equation of the linear regression.

All the probes designed to be GMO-specific were then assessed, and only those which showed a fluorescence signal >2-fold greater in magnitude and significantly above that of the GMO-free reference (defined as non-overlapping 95% confidence intervals) in samples containing 10% or less GMO gDNA were retained (Fig. 2). Many probes did not pass these criteria, demonstrating the importance of testing hybridization probes empirically; a previous array based on tiling of GM elements also observed that only a minority of probes scored as true positives (Tengs et al., 2007). After filtering, the remaining 192 probes were then grouped by GMO element and compared one by one with all the GMO samples. Sequences of all the GMO-specific probes, along with their targets, are listed in Supplementary Table S4. While some probes were highly specific for 1 GMO, others were found in multiple lines, as expected from the elements shared between them. Therefore the most specific and sensitive probes, including at least 2 for each GMO under test, were selected and a pattern-matching approach developed to determine which GMO(s) were present in a given sample (Fig. 3). This group of 33 probes represented the minimum number required to distinguish between the 12 GMOs tested in this study, and included probes specific for promoters, terminators and genes used in genetic modification of plants, as well as event-specific insertion sites and vector sequences. Sensitivity is of prime importance for GMO detection, so the ability of these probes to detect different dilutions of each GMO was also assessed. The maximum sensitivity of each probe measured is listed in Table 1. For 10/12 of the GMOs tested, one or more probes were sensitive enough to detect the GMO when present as 1% or less of the gDNA sample. The only two GMOs not detected at this level were bt176 and

GMO events	Promoters										Terminators					
	CaMV 35S		Rice actin				Other				*nos*		Ta *hsp17*		Other	
	Probe1	Probe2	Probe1	Probe2	Probe3	Tsf1	FMV 35S	TS7-CTP2	CTP4	Wheat peroxidase	Probe1	Probe2	Probe1	Probe2	ORF25	tE9
Maize																
bt11	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
bt176	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DAS1507	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-
DAS59122	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
GA21	-	-	++	++	-	-	-	-	-	-	++	-	-	-	-	-
MON810	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MON88017	-	-	+	+	++	-	-	-	-	-	+	+	+	-	-	-
MON89034	-	-	-	-	-	-	-	-	-	-	-	++	+	+	-	-
NK603	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Soya																
A2704-12	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
gts40-3-2 (RRS)	-	-	-	-	-	-	-	-	++	-	-	+	-	-	-	-
MON89788	-	-	-	-	-	++	++	++	-	-	-	-	-	+	-	++

GMO events	Protein-coding genes								Other DNA sequences								
	cry gene family						*pat*		Insertion sites					bt176 DNA		MON810	
	cry1A.105	cry1F	cry2Ab	cry34Ab1	cry3Bb1	Probe1	Probe2	CS8-CP4-EPSPS	A2704-12 ins	bt11 ins	gts40-3-2 ins	MON88017 ins	NK603 ins	Probe1	Probe2	Probe1	Probe2
Maize																	
bt11	-	-	-	-	-	-	++	-	-	+	-	-	-	-	-	-	-
bt176	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
DAS1507	-	++	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
DAS59122	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
GA21	x	x	x	x	x	-	-	-	-	-	-	-	-	-	-	-	-
MON810	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+
MON88017	-	-	-	-	++	-	-	-	-	-	-	++	-	-	-	-	-
MON89034	++	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NK603	-	-	-	-	-	-	-	-	-	++	-	-	++	-	-	-	-
Soya																	
A2704-12	-	-	-	-	-	++	+	-	+	-	-	-	-	-	-	-	-
gts40-3-2 (RRS)	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
MON89788	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-

Fig. 3. Pattern matching for GMO-specific probes in the 12 different lines tested in this study. A positive signal was defined as one where the 95% confidence intervals of probe fluorescence in the test sample did not overlap with those for the GMO-free controls ($p < 0.05$). Probes matched are grouped by element class. Symbols used are as follows: +Probe detects GMO at 10% concentration or less; ++probe detects GMO at 1% concentration or less. xThese probes should be negative to distinguish GA21 from other GMOs that otherwise contain the same elements.

DAS59122, which were detected at a minimum concentration of 5% or 10% respectively. The majority of GMOs could be also be detected at 0.5% concentration, thereby surpassing the EU labelling threshold of 0.9%; the same is expected to be true of DAS1507 and RRS/gts40-3-2, but a 0.5% CRM was not available for these two lines, and so was not tested.

These sensitivities are comparable to those reported in some previous microarray studies (Bordoni et al., 2005; Prins et al., 2008) although inclusion of an optimized amplification step would improve sensitivity to 0.1% (Leimanis et al., 2008; Morisset et al., 2008; Ujhelyi et al., 2012). Up to the detection limits observed, the pattern-matching approach employed here allowed the characterization of mixtures of different GMOs in a single sample (Leimanis et al., 2008).

3.3. Direct hybridization reveals sequence-level differences between GMO lines

In the majority of commercially-available GMO detection kits, single probes or PCR primer pairs are used to detect elements that are common to several GMOs, such as the CaMV 35S promoter and the *nos* terminator. However, the inclusion of multiple probes for each element on our array revealed that in many cases these elements differ between lines at the sequence level. This observation is illustrated by the case of the CaMV 35S promoter, in comparison with several of the lines tested in this study (Fig. 4). The entire promoter region is ~600 nt in length, and our array included 9 probes spanning this region. In the conditions tested in this study, probes designed from the 35S promoter showed low sensitivity, usually only detected at high GMO concentrations. However, these results demonstrate the effects of 3 different types of sequence variation: (A) Copy number. Soya line A2704-12 contains 4 copies of its GM expression cassette; also, the smaller size of the soya genome (1.15 Gb) compared to maize (~2.5 Gb), means that there are approximately twice as many genome copies in the same mass of gDNA. Accordingly, line A2704-12 gave 5 to 10-fold higher signals for CaMV probes than maize lines containing the same element, and could be detected down to 0.5% concentration using a CaMV-specific probe. (B) Element truncation. Only the core of the 35S promoter is required for efficient gene expression, so some lines do not contain the entire region. This difference is clearly observed between maize lines MON88017 and MON89034, for which the

Table 1
Details of the minimum set of 33 probes able to discriminate between all 12 GMOs tested in this study.

Probe ID	GDO Element	Detected by	Sensitivity[a]	Quantitative range tested[a]
CUST_28_PI429010589	NK603 Insertion site	NK603	0.50%	0.5–5%
CUST_31_PI429010589	A2704-12 Insertion site	A2704-12	5%	5–100%
CUST_37_PI429010589	bt11 Insertion site	bt11	10%	na
CUST_50_PI429010589	Gts40-3-2 Insertion site	RRS/gts40-3-2	10%	na
CUST_92_PI429010589	MON88017_insertion site	MON88017	1%	1–100%
PRID2265426614	cry34Ab1 gene	DAS59122	10%	na
PRID2265426648	Wheat P peroxidase	DAS59122	10%	na
PRID2265426765	*pat* gene	bt11	1%	1–10%
PRID2265426769	*pat* gene	A2704–12	1%	1–100%
		DAS1507, DAS59122	10%	na
PRID2265426830	ZmDNA	MON810	0.50%	0.5–10%
PRID2265427138	nos terminator-plasmid	GA21	0.50%	0.5–4.3%
PRID2265427163	cry3Bb1 gene	MON88017	0.50%	0.5–100%
PRID2265427165	CTP4 (chl. transit peptide)	RRS/gts40-3-2	1%	1–10%
PRID2265427481	Rice actin promoter	MON88017	0.50%	0.5–100%
PRID2265427485	Rice actin promoter	GA21	0.10%	0.1–4.3%
		MON88017, NK603	5%	na
PRID2265427754	ZmDNA	bt176	5%	na
PRID2265427768	ZmDNA	bt176	5%	na
PRID2265427792	L-Ta.lhcbl	MON88017, MON89034	10%	na
PRID2265427804	Rice actin promoter	GA21	0.50%	0.5–4.3%
		MON88017, MON89034	5%	na
PRID2265427901	cry1A.105 gene	MON89034	1%	1–100%
PRID2265427931	Wheat *hsp17* terminator	MON88017	5%	5–100%
PRID2265427938	Wheat *hsp17* terminator	MON89034, MON89788	10%	na
PRID2265428037	cry2Ab gene	MON89034	1%	1–100%
PRID2265428064	nos terminator	MON88017	5%	5–100%
		MON89034	1%	1–100%
		RRS/gts40-3-2, NK603	10%	na
PRID2265428180	P4-FMV-*Tsf1*	MON89788	0.50%	0.5–100%
PRID2265428188	I6-Tsf1	MON89788	0.50%	0.5–100%
PRID2265428219	CS8-cp4-*epsps* gene	MON89788	5%	na
PRID2265428265	T9-E9	MON89788	1%	1–100%
PRID2265428337	MON810 flanking region	MON810	10%	na
PRID2265428396	*cry1F* fragment	DAS1507	1%	1–10%
PRID2265428638	TS7-CTP2	MON89788	1%	1–100%
PRID2265428647	ORF25 polyadenylation signal	DAS1507	1%	1–10%
PRID2265429035	CaMV 35S promoter	bt11	0.50%	0.5–10%
PRID2265429141	CaMV 35S promoter	A2704-12	0.50%	0.5–100%
		MON88017	5%	5–100%

[a] Expressed as the lowest/highest detectable %GMO concentration (w/w) among the samples tested.

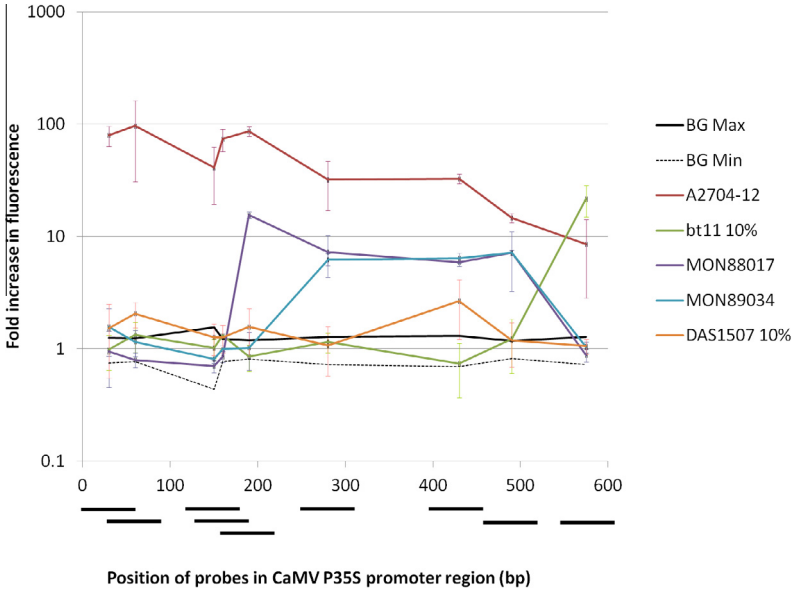

Fig. 4. Fluorescence signals from different GMO hybridizations demonstrate differences between the CaMV 35S promoter sequences found in each GMO. The positions of 10 60 nt probes along the CaMV 35S promoter sequence are shown as black bars under the X-axis, and the fluorescence signals observed for different GMO lines marked above the X-axis, directly above the centre of the relevant probe. The relative fluorescence is expressed as the fold increase over background (mean fluorescence signal obtained from 10 GMO-free samples). The lines labelled BG max/min show 1 Standard Deviation above and below the GMO-free background fluorescence. Except where indicated in the key, test samples are 100% GMO DNA. Error bars show Standard Deviation.

latter is more truncated and does not detect the 5′-most probe detected by the former. Similarly, the 35S promoter found in bt11 extends further at its 3′ end than either MON88017 or MON89034, and the probe closest to the 3′ end of the element proved to be both sensitive and specific for this maize line (Fig. 3). (C) Point mutations. DAS1507 also includes the 3′ end of the 35S promoter, but has 2 single-nucleotide substitutions, a single-nucleotide insertion, and a 6 nt deletion relative to the bt11 sequence. These differences are sufficient to eliminate binding to the probe.

These variations are usually ignored in GMO detection approaches that employ a small number of probes, as they necessarily focus on the most conserved regions of each element, while PCR amplification steps obscure differences in copy number. In contrast, the direct hybridization approach allows discrimination between these minor variations, which can facilitate accurate GMO identification. Similarly, using an array tiling known GM elements with probes every 7 nt, it was possible to reconstruct the sequence of elements present in a GMO sample (Tengs et al., 2010).

3.4. Quantification of GMO elements

Furthermore, as the DNA samples were directly labelled and hybridized without PCR amplification, this method should be suitable for quantification of each GMO. To test the viability of this approach, series of hybridizations with 100%, 10%, 5%, 1% and 0.5% of lines A2704-12, MON88017, MON89034 and MON89788 were compared. Using 3–4 probes specific for each line, the fold increase in fluorescence was plotted against GMO concentration (Fig. 5). In each case, a linear regression fitted well to the data ($R^2 > 0.96$), showing that the fold increase in fluorescence over background is proportional to the concentration of each GMO. Therefore, the linear regression equations for each line can be used to estimate the concentration of each GMO in samples screened using the same hybridization conditions. Similarly, equations for estimating concentration were established for each of the other GMOs, apart from bt176 and DAS59122. The quantitative range for each probe, determined by the range of concentrations of the available CRMs, is given in Table 1. It was observed that quantification estimates were only valid for the GMO in which they were defined; for example, the rice *actin* promoter probe (PRID2265427804) that is useful for quantification of GA21 binds the same element in MON88017 and MON89034, but with lower affinity, giving an underestimate of their concentration. Therefore it is first necessary to qualitatively determine which GMOs are present by the pattern-matching described above, before selecting the relevant probes for quantification. The only previously reported quantitative microarray-based GMO detection system is NAIMA (Morisset et al., 2008), which uses an elegant multiplex RNA amplification method mimicking retroviral replication to substitute for PCR amplification. Compared with our approach, NAIMA showed higher sensitivity in triplex reactions, but its performance in detecting larger numbers of elements simultaneously has not been reported.

3.5. Accuracy and effectiveness of direct hybridization for GMO detection

Accuracy is of great importance for any GMO detection method, as food and crop products that test positive may have to be destroyed, which is costly both for governments and the food industry. In our detection protocol we wanted to minimize the risk of false positives, so we used a statistical significance test to establish whether GMO samples gave a signal higher than background (Section 3.2). At a p-value of 0.05, it might be expected that on average 1 or 2 (5%) of our 33 optimal probes for GMO identification would appear to be positive due to random fluctuations in the data for any given sample. For this reason, we chose a more conservative test (requiring the 95% confidence intervals of the data and negative controls to be non-overlapping) as this method typically gives a lower false positive rate than p-value predictions (Payton et al., 2003). Also, requiring multiple probes to be positive to detect a GMO greatly reduces the risk of errors, as the probability of all of them giving false positives decreases exponentially with the number of probes (for 2 probes, $0.05^2 = 0.0025$; for 3 probes, $0.05^3 = 0.000125$, etc.) For these reasons, we believe that the method described here is at least as robust against spurious detection of GMOs as established approaches.

Conversely, false negatives also need to be minimized as they could result in propagation of undetected GMOs. In a previous study using the same microarray platform as reported here (Tengs et al., 2010), the authors were unsure whether the system would be sensitive enough to detect GMOs diluted with non-GMO DNA. Our array was designed with the aim of maximizing sensitivity: all probes to GMO sequences were included in 10 replicates to increase the statistical power to distinguish a weak positive signal from a negative one. Strikingly, the sensitivity of probes for low ($\leqslant$5%) GMO concentrations varied widely, sometimes even between overlapping probes, for reasons discussed above (Section 3.1). Bearing this in mind, we used our initial hybridizations to identify the probes that gave the least background and highest specificity for each GM element (Fig. 3 and Table 1), and were then able to detect 10/12 GMOs tested at $\leqslant$1% concentration. This is an important threshold, as food and feed products containing >0.9% GM material are required to be labelled as containing GMO by Turkish and EU law (Anonymous, 2009; European Commission, 2003b). For event bt176, which was only detectable at $\geqslant$5% concentration, relatively few probes were included on the array, both because only partial sequence of the GM cassette was available to design probes, and also because probes designed against the GC-rich bacterial *bar* gene found in this cassette were rejected at the array design stage, as >60% GC sequences are predicted to have poor hybridization characteristics. In future work, it would be valuable to test some of these probes as they may give more specific detection of bt176, even if their hybridization is sub-optimal.

It should be noted that for some of the GM events tested here, only one of the probes provided maximum sensitivity. For example, for NK603 the insertion site probe (CUST_28_PI429010589) could detect 0.5% GMO DNA, but the other 2 probes included in the optimal set only gave a positive signal at 5% or 10% GMO concentration (Table 1). Therefore it would be difficult to be confident that a weak positive signal for the single probe CUST_28_PI429010589 demonstrates the presence of low levels of NK603, rather than a false positive. In future development of this array system, it will be important to identify more probes and optimize the hybridization conditions to ensure that all the GM events can be determined with high confidence even at low concentrations.

Our strategy used direct gDNA detection without amplification, simplifying sample preparation while preserving quantitative relationships between elements, similar to the universal GMO element array described previously by Tengs et al. (2007, 2010). In both cases a similar approach of tiling probes against known GM elements was used. The earlier study tiled single-copy probes at 7 nt intervals, whereas ours were tiled at 30 nt intervals but with 10 replicates of each. The tiling distance was increased to avoid having too many overlapping probes competing for the same pieces of labelled DNA in the hybridization, which could dilute the fluorescence signal (Agilent Technical Support, personal communication). Notably, most of the insertion site probes (which were tiled at 5 nt intervals) gave relatively low amplitude

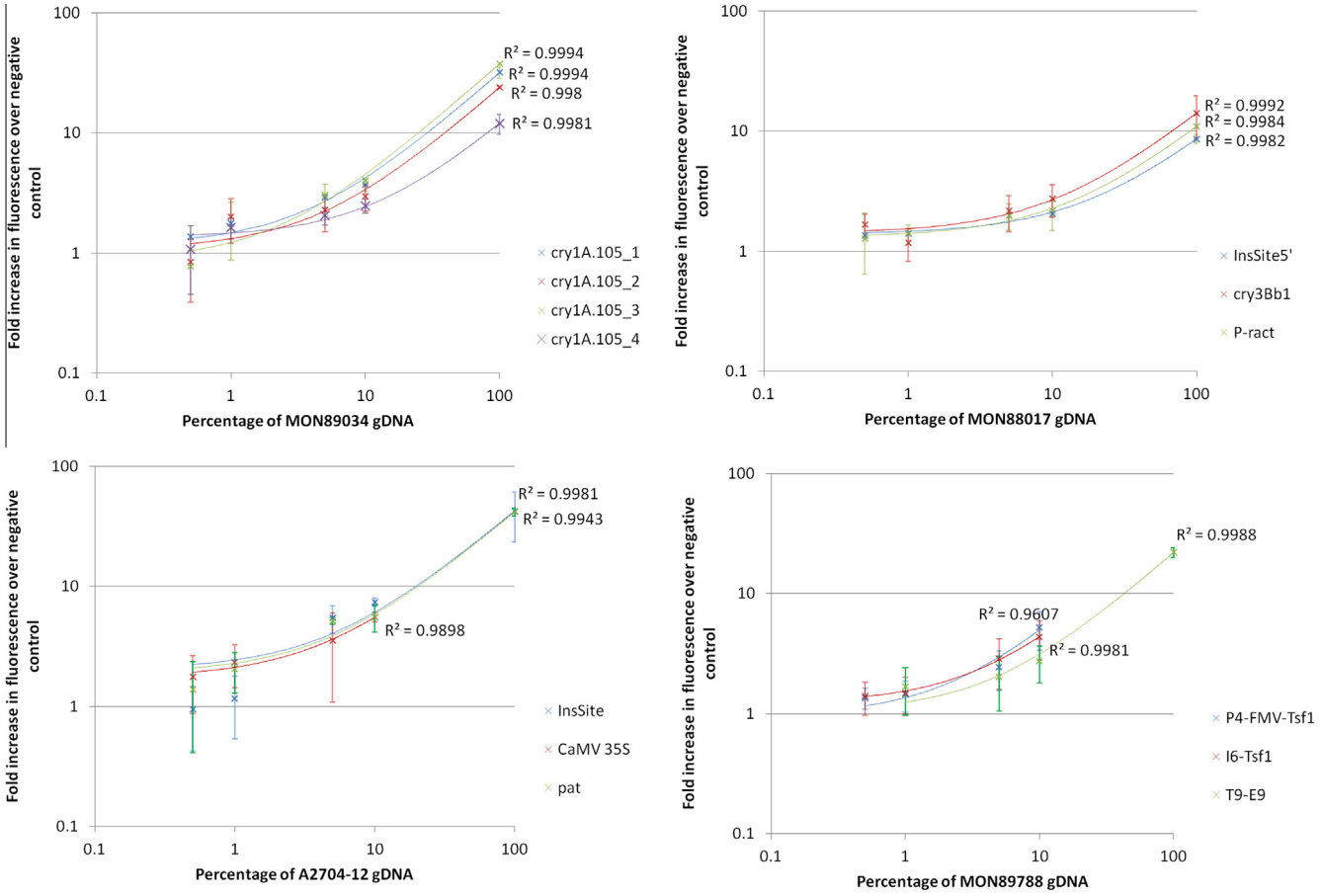

Fig. 5. Fluorescence signals obtained using probes selected for quantification are proportional to GMO concentration, for four different GMOs (indicated in the x-axis of each graph). Signals are expressed as the mean fold increase in fluorescence over background, where the background is the mean fluorescence observed from 10 GMO-free hybridizations. The data are plotted on a log scale and error bars show ± standard deviation, with the key indicating the identity of the probes displayed on each graph. Trend lines show the best fit to each set of data points by linear regression.

fluorescence signals, indicating that some signal dilution may have occurred. However, several of them still gave sufficient sensitivity to be used for GMO detection (Table 1).

The number of GM events approved for use somewhere in the world increases every year. One of the major advantages of a high-density microarray platform is its capacity – up to 60,000 features in this example or the capacity to detect up to 2000 different targets on the basis of 10 replicates of 3–5 optimum probes for each target. Apart from microarrays, the most wide-ranging GMO detection platforms described to date have the capacity to detect 47 (Cottenet et al., 2013) and 91 (Shao et al., 2013) elements simultaneously. From this perspective, a microarray like this one remains the best prospect for universal detection of GMOs in a single test.

4. Conclusions

In this study, we describe the first application of direct genomic DNA hybridization to a high-density microarray for quantitative detection of GMO crops. The microarray designed in this study was successful in distinguishing between 12 different GMOs found in the Turkish feed market, in most cases down to the <1% GMO concentration required for labelling regulations. Additionally, increasing labour- and cost-effectiveness, our strategy allows 2 samples labelled with different fluorophores (Cy3 and Cy5) to be hybridized to each array, allowing 16 samples to be tested on each 8-plex microarray slide. Therefore, this study serves as proof of principle that a microarray can be used to detect GM elements in gDNA samples without a PCR amplification step, simplifying analysis and allowing quantification of each GMO detected. Using the results from this study, it is expected that further optimization of sample preparation, probe design and replicate number on the array will provide additional improvements in sensitivity.

We also demonstrated that the fluorescence signals obtained from these hybridizations increased linearly with GMO DNA concentration, allowing straightforward quantification of the GMOs tested, which is important to enable compliance with EU and Turkish labelling legislation. Moreover, sequence-level variations in the GM elements present in different GM events could be detected by their effect on probe hybridization, increasing the confidence with which GMOs containing similar events could be distinguished.

There is room for improvement in the system proposed, as microarray hybridization requires technical expertise and may be too expensive for routine GMO detection. However, we were able us to screen a large number of probes for each element, and define a minimum set of 33 probes that could distinguish between all the GM events tested. These probe sequences could be used as the basis of a cost-effective, targeted GMO detection product for the Turkish market.

Conflict of interest

The authors declare no known conflict of interest in this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.08.030.

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