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REVIEW

Advances in molecular techniques for the detection and quantification of genetically modified organisms

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Abstract Progress in genetic engineering has led to the introduction of genetically modified organisms (GMOs) whose genomes have been altered by the integration of a novel sequence conferring a new trait. To allow consumers an informed choice, many countries require food products to be labeled if the GMO content exceeds a certain threshold. Consequently, the development of analytical methods for GMO screening and quantification is of great interest. Exponential amplification by the polymerase chain reaction (PCR) remains a central step in molecular methods of GMO detection and quantification. In order to meet the challenge posed by the continuously increasing number of GMOs, various multiplex assays have been developed for the simultaneous amplification and/or detection of several GMOs. Classical agarose gel electrophoresis is being replaced by capillary electrophoresis (CE) systems, including CE chips, for the rapid and automatable separation of amplified fragments. Microtiter well-based hybridization assays allow high-throughput analysis of many samples in a single plate. Microarrays have been introduced in GMO screening as a technique for the simultaneous multianalyte detection of amplified sequences. Various types of biosensors, including surface plasmon resonance sensors, quartz crystal microbalance piezoelectric sensors, thin-film optical sensors, dry-reagent dipstick-type sensors and electrochemical sensors were introduced in GMO screening because they offer simplicity and lower cost. GMO quantification is performed by real-time PCR (rt-QPCR) and competitive PCR. New endogenous reference genes have been validated. rt-QPCR is the most widely used approach. Multiplexing is another trend in this field. Strategies for high-throughput multiplex competitive quantitative PCR have been reported.

Keywords Genetically modified organisms · GMO · Molecular techniques · DNA hybridization · Biosensors · Microarrays · Capillary electrophoresis · Real-time PCR · Competitive PCR

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Introduction

The rapid progress of biotechnology has enabled the introduction of exogenous sequences that confer new characteristics, such as herbicide tolerance, resistance to insects, etc into the plant genome. The foreign DNA comprises a transcription promoter, a coding sequence and an expression terminator. Most transgenic plants contain the promoter of the 35S subunit of ribosomal RNA of the cauliflower mosaic virus and the terminator of nopaline synthase gene from *Agrobacterium tumefaciens*. Examples of transgenic plants are the soy Roundup Ready, the maize MaisGard and the tomato Flavr Savr.

In recent years there has been an ongoing debate on and increasing research efforts evaluating the risks associated with the introduction of GMO into agriculture, their use in the diet and their spread in the environment. Several countries, including EU countries, Japan, Australia, New Zealand, Thailand and China have implemented mandatory labeling for foods derived from transgenic plants. In the EU, food labeling is mandatory in cases where more than 0.9% of the food ingredients (considered individually) are of GMO origin. Consequently, the development of reliable methods of GMO detection, identification, tracing and quantification has become increasingly important.

GMO analysis is carried out by either detecting the inserted foreign DNA or detecting the novel protein that is specifically expressed in transgenic plants. The proteins are detected by immunochemical assays (ELISA). These assays are simple, specific and quantitative. However, protein-based assays are not suitable for processed foods because of the loss of epitopes during processing. Thus, DNA is the preferred analyte for both raw ingredients and processed food.

The present review article will focus on the latest developments (from the last 3–4 years) in molecular techniques for GMO detection (screening) and quantification.

GMO screening

Electrophoretic methods

The classical approach to GMO screening involves amplification of the GMO-specific promoter and/or terminator sequences followed by the sizing of the PCR products by agarose gel electrophoresis (AGE) and ethidium bromide staining. The method is not automatable and provides no sequence confirmation. The current trend is towards the development of multiplex assays and the replacement of AGE by capillary electrophoresis.

Hernandez et al. developed a multiplex PCR method for the simultaneous identification of four GM maize lines: Bt11, Mon810, T25 and GA21. The PCR products were analyzed by agarose gel electrophoresis. The detectability was 0.05%. The transferability of the method between laboratories was also demonstrated in this work [1].

A method for GMO detection was reported that is based on PCR clamping by peptide nucleic acid (PNA). Specific PNA fragments were designed that inhibit PCR either by competing with the primer for annealing to target sequences, hybridizing adjacent and downstream of the primer, or hybridizing somewhere in the middle of the amplified sequence. The observed PCR inhibition by PNA reveals the particular GMO-related sequence in the sample and provides semi-quantitative estimation of the GMO content [2].

A multiplex PCR followed by capillary gel electrophoresis (CGE) with laser-induced fluorescence (LIF) detection was reported for the simultaneous detection of five transgenic maizes (Bt11, T25, Mon810, GA21 and Bt176) and a reference gene (zein) in a single run. YOPRO1 was employed as a fluorescent intercalating dye and hydroxyethylcellulose as the sieving medium. The separation time was 30 min. The detectabilities were about 0.05%, which is well below the EU threshold value of 0.9%. It was shown that CGE could detect nonspecific amplification products that are undetectable by agarose gel electrophoresis [3].

A microfabricated, inexpensive, reusable capillary electrophoresis chip was developed in-house for the rapid separation of amplified GMO-specific sequences. The chip was composed of two glass plates, each 25×76 mm, thermally bonded together to form a close structure. Photomasks with a cross topology were constructed rapidly using polymeric material instead of chrome plates. The channels were etched to a depth of 30 μm. The widths of the injection and separation channels were 30 and 70 μm, respectively. The effective separation length was 4.5 cm. (Hydroxypropyl)methyl cellulose was added to the separation buffer along with the intercalating dye SYBR Green I, which is selective for dsDNA. A home-built laser-induced fluorescence system was employed for the detection of the separated fragments. The system was applied to the separation and detection of PCR-amplified 35S promoter (195 bp), NOS terminator (180 bp) and lectin gene sequence (181 bp). The separation and detection was complete in less than 60 s. As little as 0.1% GMO was detectable [4].

A microchip-based CGE method was developed using programmed field strength gradients, a double-T microchip and a poly(ethylene oxide) sieving matrix for GMO detection in soybean. PCR products were analyzed within 11 s [5].

Nadal et al. reported an event-specific multiplex (pentaplex) PCR for Bt11, GA21, Mon810, NK603 and Zea mays L. alcohol dehydrogenase (a species-specific reference gene). The primers flanked the plant genome/transgene junction region. More interesting is that the analysis of amplified product was performed by a CGE method that combined identification by size and color (CGE–SC method). All of the targets were labeled during PCR by using suitable fluorescent dyes such as 6-carboxyfluorescein, tetrachloro-6-carboxyfluorescein and hexachloro-6-carboxyfluorescein. Amplicons of similar sizes were labeled with different dyes. The limit of detection (LOD) was 0.1% for each GMO [6].

The ligation-dependent probe amplification (LPA) method was applied to the simultaneous event-specific detection and relative quantification of two GMOs. The principle of the LPA technique is as follows. Two oligonucleotide probes hybridize to adjacent positions of the target sequence and are ligated by DNA ligase. Besides the target recognition sequence, the probes contain universal segments that allow subsequent annealing of PCR primers. The ligation product

is amplified by PCR using universal primers. Spacer sequences are placed between the target hybridization and primer annealing sites in order to generate PCR products varying in size. The PCR products are analyzed by capillary electrophoresis. In contrast to classical multiplex PCR, which requires a pair of primers per target DNA, LPA only employs a common pair of primers for multiplex amplification [7].

Microtiter well-based hybridization assays

Microtiter wells allow the analysis of many samples in parallel in a single plate. Consequently, microtiter well-based assay formats are automatable and suitable for high-throughput GMO screening. A highly sensitive bioluminometric hybridization assay was reported for the detection of GM soy. The Ca^{2+}-dependent photoprotein aequorin was used as a reporter. A universal detection reagent was produced through the conjugation of aequorin with the oligonucleotide $(dA)_{30}$. Biotinylated PCR products from 35S promoter, NOS terminator and lectin were captured on streptavidin-coated wells and one strand was removed by NaOH treatment. The immobilized single-stranded DNA was then hybridized with oligonucleotide probes containing a target-specific segment and a poly(dT) tail. All hybrids were then determined by the addition of the aequorin-$(dA)_{30}$ universal reagent. The bound aequorin was measured by injecting Ca^{2+} solution and integrating the light emission for 3 s. As little as 2 pM of amplified 35S promoter, NOS terminator and lectin sequences were detected with a signal-to-background ratio of 2. GMO was detectable at the 0.05% level with a signal-to-background ratio of 8.2 [8].

Biosensors

Biosensor development aims at making molecular tests simpler, faster and less costly. DNA biosensors based on optical, electrochemical and piezoelectric transducers have been developed for the detection of amplified GMO-related sequences. The capture of target DNA by a specific oligonucleotide probe (recognition layer) that is attached to the surface of the sensor is the most widely used approach to biosensor design. Probe immobilization process should ensure that the oligonucleotide retains its ability to hybridize with the target DNA.

Optical biosensors

A number of papers describe the development of DNA biosensors based on surface plasmon resonance (SPR). Hybridization of the target DNA with the immobilized probe on the sensor surface results in a change in the refractive index of the solution near the surface. The change in the refractive index is linearly related to the mass of hybridized target DNA. SPR sensors allow the real-time monitoring of hybridization without labeling.

Two approaches for immobilizing the probe to the gold-coated surface of the sensor were studied extensively [9]. The first involved coating the gold layer with streptavidin and capturing the biotinylated probe. The surface was treated with mercaptoundecanol followed by epichlorohydrin and carboxylated dextran. The dextran surface was activated with *N*-hydroxysuccinimide (NHS) and carbodiimide (EDAC) before adding streptavidin. Mercaptohexanol was used for blocking prior to the immobilization of the biotinylated probe. The second approach involved the direct coupling of a thiolated probe onto the crystal. The latter method is much faster and gives similar results.

Considerable effort has been put into optimizing the sample treatment for the generation of single-stranded DNA prior to detection with the SPR sensor.

Denaturation of target DNA is required prior to hybridization. However, reannealing of the strands results in a decrease of the signal. The following approaches were compared:

(a) The PCR product is biotinylated at one end using the appropriate primer. The DNA is captured on streptavidin-coated magnetic beads and the nonbiotinylated strand is released by NaOH followed by application to the sensor.
(b) Use of a 5′ phosphorylated primer and post-PCR digestion of the phosporylated strand by lambda-strendase, which recognizes the phosphorylated end.
(c) Thermal denaturation.
(d) Thermal denaturation in the presence of blocking oligonucleotides that hybridize to various regions of the target that do not overlap with the hybridization site of the specific probe. Hybridization of the blocking oligos prevents the reassociation of the target DNA strands. Denaturation at a high-temperature followed by short (1 min) incubation with oligonucleotides that prevent strand reannealing gave the best results [10].

Wang et al. compared two methods for probe immobilization: direct coupling of thiol-modified DNA probe to the gold surface; coating of the gold surface with dextran, the attachment of streptavidin to the dextran layer and the immobilization of biotinylated oligonucleotide probes. The detection limits were 2.5 nM and 20 nM, respectively [11].

SPR imaging of multiple hybridization assays was carried out by immobilizing oligonucleotide probes on photolithographically patterned gold substrates. However, more work is required in order to improve the detectability of this system [12].

Kalogianni et al. reported the first DNA biosensor that allows visual and rapid (within minutes) detection of GMO-related DNA sequences without the need for an instrument.

The biosensor was designed in a dry-reagent disposable dipstick format. Oligonucleotide-functionalized gold nanoparticles served as reporters, which constitute an integral part of the sensor. Biotinylated PCR-amplified fragments for 35S promoter and NOS terminator were hybridized (7 min) in solution with probes bearing an oligo(dA) tail at the 3′-end. The solution was applied to the biosensor followed by immersion in the appropriate buffer. Migration of the buffer along the sensor strip by capillary action rehydrates the gold nanoparticles conjugated to oligo(dT), which hybridize with the oligo(dA) tails. The hybrids are captured by immobilized streptavidin at the test zone of the sensor, giving a characteristic red line due to the accumulation of the nanoparticles. The excess nanoparticles are bound at the control zone of the strip by immobilized oligo(dA) strands. Amplified products are detectable at 0.16 nM. The biosensor can detect 0.1% GM soybean. The dry-reagent dipstick format minimizes the requirements for the preparation of solutions and the training of qualified personnel [13].

A silicon-based optical sensor was reported comprising a silicon wafer coated with a silicon nitride (Si_3N_4) layer that served as the optical layer. The sensor surface was modified with hydrazine to enable coupling of oligonucleotide probes carrying an aldehyde group at the 5′-end. Biotinylated PCR products were denatured and hybridized with the immobilized probes. The hybrids were detected by reacting with horse radish peroxidase (HRP)–antibiotin conjugate. Tetramethylbenzidine was used as a substrate. Precipitation of the converted substrate causes a color change of the sensor surface from gold to blue/purple. The sensor allows visual detection of the amplified DNA without instrumentation. The targets detected were: (a) the endogenous genes lectin (soybean), invertase (maize), ACC synthase gene (canola) and stearoyl-ACP (fiber-specific acyl carrier protein) desaturase gene (cotton); (b) the promoter, marker gene and terminator such as CaMV 35S promoter, nptII, GUS and NOS terminator; (c) trait genes such as Roundup herbicide-resistance gene from Agrobacterium strain CP4 encoding EPSPS gene (cp4-epsps), the BAR gene encoding phosphinothricin acetyl transferase (pat) and Bt toxin genes (CrylAb, CrylAc). The sensor detects about 0.1 fmol of target [14].

Piezoelectric biosensors

Piezoelectric biosensors detect the increase in mass due to hybridization on the sensor surface. The increase in the mass causes a decrease in the resonance frequency. Quartz crystal microbalance (QCM) sensors consist of a quartz surface to which gold electrodes are attached. The gold layer facilitates probe immobilization. QCM-based sensors allow real-time monitoring of the hybridization reaction without the need for labeling of the probe or target DNA sequence.

Extensive studies aimed at optimizing the coating of the gold quartz crystal surface for probe immobilization were carried out using two immobilization procedures, i.e., the direct immobilization of thiolated probes or the capture of biotinylated probes from a streptavidin-coated sensor surface. The sensitivity was found to be higher for the thiolated probes, whereas the dynamic range was greater with biotinylated probes [15].

A QCM DNA biosensor with immobilized probe on a streptavidin-coated gold surface was applied to the detection of Cry1A(b) gene, which is derived from *Baccilus thuringiensis* and confers resistance to insects in maize. The resonance frequence shift was linearly related to %GMO in the range of 0.1–5% of GM Mon810 maize flour [16].

The QCM biosensor was also applied to the detection of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene that is introduced in the Roundup Ready soybean genome and confers resistance to the herbicide glyphosate. The avidin–biotin interaction was employed for probe immobilization on the surface of the sensor. The sensor was applied to both PCR-amplified and unamplified plant genomic DNA. It was found that 30% GMO was detectable without amplification using 3.6 μg of genomic DNA [17]. Although this is a very high GMO content compared to the 0.9% threshold set by the EU, these efforts demonstrate the state-of-the-art of QCM sensors and call for future improvements [18].

Electrochemical biosensors

Enzyme-based electrochemical sensors were developed that employ disposable oligonucleotide-modified screen-printed gold electrodes. The probe carries an -SH group at the 5′-end for attachment to the gold surface. The PCR product was denatured and hybridized, in solution, with a biotinylated probe. The solution was then pipetted on the electrode and allowed to hybridize with the immobilized probe. The sensor was washed and a streptavidin–alkaline phosphatase conjugate was added. The enzyme catalyzes the hydrolysis of naphthyl phosphate substrate to the electroactive naphthol, which is detected by differential pulse voltammetry. Alternatively, the BCIP/NBT substrate mixture can be added, leading to the formation of a precipitate that acts as an insulator between the gold surface and a solution containing the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox pair. Detection is accomplished by impedance spectroscopy. The sensor was applied to the analysis of samples from soy and maize containing 1% and 5% GMO [19, 20].

Another sensor was reported in which the oligonucleotide probe was immobilized on a screen-printed carbon electrode surface by applying a potential. Heat-denatured PCR product was pipetted directly onto the electrode surface. The electrode was immersed in a solution containing methylene blue, which

interacts with guanine residues. The accumulation of methylene blue on the surface was measured by square wave voltametry [21].

An electrochemical sensor was reported in which the capture of target DNA from immobilized peptide nucleic acid (PNA) probe on a glassy carbon electrode results in the electrostatic interaction of positively charged $[Co(NH_3)_6]^{3+}$ complex ions with the negatively charged phosphate backbone of the DNA strands. The accumulation of $[Co(NH_3)_6]^{3+}$ on the sensor surface leads to an increase in the electrical current. The GMO-related sequences were amplified by asymmetric PCR that generates single-stranded DNA fragments, thus avoiding the denaturation step as well as the decrease in the signal due to strand reassociation of double-stranded PCR products. The sensor was tested with soya containing 5% GMO [22].

Microarrays

Microarray technology has been applied to the parallel multianalyte detection of several PCR products in a single run. The arrays consist of oligonucleotide probes that are immobilized on a glass support. The probes are suitable for GMO-specific sequences or reference genes.

Germini et al. developed a PNA microarray (immobilized 15-mer PNA probes) for the detection of four types of transgenic maize, one type of transgenic soybean and two endogenous controls; that is, the zein gene for maize and lectin gene for soy. Multiplex PCR was performed and one primer of each pair was labeled with the fluorescent dye Cy5. The LOD was 0.25% for each GMO after two rounds of amplification. The LOD of the PNA microarray hybridization assay was evaluated with complementary 15-mer DNA oligonucleotides and found to be 1 nM [23].

Xu et al. designed three types of microarrays for GMO detection. One is a screen chip comprising probes for promoter, reporter and termination sequences that are widely found in transgenic plants. These sequences include the 35S promoter, 35S terminator, NOS promoter, NOS terminator, Npt11 terminator and the FMV 35S promoter. Another type of microarray consists of probes designed for the detection of specific gene inserts, and the third type of array contains species-specific probes. All probes carried an $–NH_2$ group at the 5′-end and were immobilized on aldehyde-activated glass slides. The method was applied to commercial GM soybean, cotton and rapeseed. Genomic DNA was amplified by multiplex PCR. Amplified fragments were labeled with Cy5-dCTP during PCR. The reaction products were hybridized to the array followed by fluorometric scanning. The detectability was 0.5% and 1% for soybean and maize, respectively [24].

A DNA microarray was reported for simultaneous detection of nine GMOs from five plant species and three screening sequences (35S promoter, NOS terminator and the npt11 gene). Controls for the detection of CaMV were also included in the chip. The amplified products were biotinylated using biotin-dATP and the hybrids were detected colorimetrically. The detection was based on streptavidin-conjugated gold nanoparticles. The LOD was lower than 0.3% [25].

More recently, multiplex event-specific-PCR was combined with a microarray for the detection of GM soybean and six maize events. Amplified fragments were labeled with Cy5-dCTP. To ensure specificity, the oligonucleotide probes (immobilized on aldehyde-activated glass slides) contained a host-specific and an insert-specific segment. Similarly, in each pair of PCR primers, one primer recognized the host genome whereas the other hybridized to the inserted gene. LOD was 0.5–1% [26].

GMO quantification

Quantification of GMO has provided the next challenge after the development of methods for their detection in real samples. Just like detection methods, the main step is exponential amplification by PCR. The accumulation of amplified DNA during PCR can be expressed by the following equation:

$$P_n = P_0 \times \prod_{i=1}^{n} (1 + E_i)$$

where P_n is the amount of PCR products after n cycles, P_0 is the starting amount of template and E_i is the efficiency of DNA synthesis during each cycle [27]. The efficiency is not known and may be affected by several parameters, such as variations in reaction conditions and the presence of inhibitors (e.g., proteins, lipids, polysaccharides, polyphenols, etc.) at various concentrations in the real samples. Because of the exponential mode of amplification, small differences in PCR efficiency lead to large changes in the amount of amplification product.

GMO quantification requires the determination of two target DNA sequences, i.e., a GMO-specific sequence and a plant-specific reference gene. The reference gene permits compensation for differences in the amount and integrity of isolated genomic DNA between samples. The ratio of the copies of the two sequences, expressed as a percentage, gives the relative GMO content of the sample. Both the development and validation of quantitative PCR methods are carried out by analyzing certified reference materials.

Competitive PCR

One approach used to compensate for variations in the amplification efficiency involves co-amplification of the target

sequence with a constant amount of a synthetic DNA internal standard (competitor) that closely resembles the target DNA and has the same primer binding sites. This approach is named competitive PCR. Any variation in amplification efficiency caused by changes in reaction conditions or the presence of inhibitors affects the amplification of target and competitor equally, so that the ratio of their PCR products is constant through the exponential and plateau phase of PCR and gives the ratio of the initial amounts of the two fragments in the sample. The discrimination of competitor and target sequences after PCR can be accomplished by introducing an insertion or deletion into the competitor to allow electrophoretic separation. Alternatively, a small region (about 25 bp) of the target sequence is replaced by another sequence of the same size, thus allowing differential determination of the amplified products by hybridization. Quantification of the reference gene via a second competitive PCR is also needed [28].

Size-dependent discrimination of amplified fragments from target and competitor is usually accomplished by agarose gel electrophoresis. Capillary electrophoresis (CE), however, provides shorter separation times, automation, lower detection limits and an extended linear dynamic range, through the use of laser-induced fluorescence detection. CE allows the discrimination of target and internal standard amplified fragments differing by only 10 bp, thereby reducing the effect of the size difference on the amplification efficiency [29]. Double-competitive PCR, which involves two competitive PCRs for the determination of the GMO-specific gene and a reference, has been combined with CE for the determination of Bt event-176 maize in real samples. The second approach to constructing competitors, the replacement of an internal sequence by a new one, allows specific recognition of the two products by hybridization. The equal sizes of the fragments ensures the same amplification efficiency. PCR products are detected by a heterogeneous hybridization assay performed in microtitration wells [30]. Both amplification products are labeled with biotin at one end during PCR using a 5′-biotinylated primer. The fragments are captured on the surfaces of the wells through hybridization to immobilized probes. The hybrids are quantified by a bioluminometric assay using a streptavidin–aequorin conjugate. The method has been evaluated by analyzing real samples containing Roundup Ready Soybean. The limit of quantification for the 35S promoter was 24 copies. Compared to electrophoresis, the microtiter well-based hybridization assay of amplified DNA offers sequence confirmation, higher detectability and easy automation. Also, the cost of a microplate luminometer is lower than that of a laser-induced fluorescence detector. However, because only one reporter is employed (the photoprotein aequorin), the amplified target and competitor must be quantified in separate wells. Consequently, four wells are required for the determination of amplified products from the GMO-specific gene and the reference gene along with the corresponding competitors. Very recently, a method was reported that enables (a) the co-amplification of the GMO-specific gene and the reference gene and their competitors in the same reaction mixture (performance of a single PCR instead of two reactions) and (b) the simultaneous determination of the four amplification products by a quadruple hybridization assay using four chemiluminescent reporters in a single microtiter well [31]. Biotinylated amplified fragments are captured on streptavidin-coated wells. The nonbiotinylated strand is removed by NaOH treatment and the immobilized strands hybridize with a mixture of four probes. The first probe carries a poly(dA) tail at the 5′-end to allow hybridization with aequorin-$(dT)_{30}$. The second probe is 3′-labeled with fluorescein to bind the antifluorescein–horseradish peroxidase conjugate. The third probe consists of a segment complementary to the target and a region complementary to an oligonucleotide that is conjugated to beta galactosidase. The fourth probe is 3′-labeled with digoxigenin for linkage with the antidigoxigenin–alkaline phosphatase conjugate.

Currently a major challenge in quantitative competitive PCR is the development of multiplex assays for the quantification of several targets in the same sample. Multiplexing provides higher throughput, lower cost and lower consumption of sample and reagents compared to single-target assays. A multiplex quantitative competitive PCR was reported that is based on a multianalyte hybridization assay performed on spectrally encoded microspheres [32]. Commercially available microspheres stained with precise amounts of two fluorophores were used. Various sets of microspheres were coupled with oligonucleotide probes specific for DNA targets and competitors. Biotinylated PCR products were heat-denatured and hybridized with the microsphere sets. The hybrids were determined using a streptavidin–phycoerythrin conjugate. The microspheres were then analyzed by a flow cytometer. Each microsphere was interrogated by two laser beams, at 635 nm and 532 nm. The first line is used to excite the fluorophores within each microsphere, thus allowing classification of the microspheres. The second line was used to excite phycoerythrin, and the fluorescence signal was related to the amount of target DNA. Given that 100 distinguishable sets of microspheres are commercially available, the method could potentially be extended to the quantification of 50 target DNA sequences along with the corresponding 50 competitors.

Other multiplex end-point analysis approaches for the determination of GMO have been published, which aim to keep the efficiency constant between co-amplified products using bipartite primers. These primers contain a universal region at one end, whereas the other end functions as a typical PCR primer. Initially, four PCR cycles [33] or 20 cycles of oligonucleotide ligation reaction [7] are performed

and the products act as templates for the next PCR round, by using only one primer as both forward and backward or just one new pair of primers, respectively. The small number of cycles at the first reaction ensures a common efficiency for all targets, whereas the use of one pair of primers at the second PCR provides the characteristics of competitive PCR mentioned above for the reaction. In the first study, the need for a multianalyte technique for the detection of ten inserted DNA elements plus one reference gene and a synthetic internal reference target (for the determination of seven different GM maize events) is covered by DNA arrays that enable multiple detection. The amplicons of the ligase reaction can be discriminated by size, by adding different size spacers between the universal and the target-complemented regions in the primers of the first reaction, so that the final products, two reference and equal number of GMO-specific genes, were separated by CE and detected by laser-induced fluorescence.

Real-time PCR

End-point PCR for GMO quantification has the drawback of requiring separate steps for DNA amplification and assay of the products. In contrast, real-time PCR allows continuous monitoring of the amplification products by a homogeneous fluorometric assay. The elimination of the need for post-PCR manipulation has rendered quantitative real-time PCR methods the most widely used approach in GMO testing. The information obtained, i.e., the amplification curves, can be used to quantify the initial amount of template. Quantification is carried out in the logarithmic phase of PCR, by relating the number of amplification cycles required to reach a preset threshold fluorescence signal to the number of target DNA copies in the sample. The assays may be based on the use of fluorescent intercalating dyes (such as SYBR Green I). Alternatively, the products can be detected by hybridization with fluorophore-labeled probes, exploiting the phenomenon of fluorescence resonance energy transfer (FRET). The design of multichannel instruments and the surmounting of limitations, such as the suppression of low-abundance target amplification by high-abundance ones [34], has allowed the development of multiplex DNA quantification.

rt-QPCR assays (TaqMan principle) have been developed for Mon863 [35, 36], Mon15985 and Mon88913 maize [37]. The LOD was 0.05% by agarose gel electrophoresis and rt-PCR. rt-QPCR assays (TaqMan) were also reported for four alternative reference genes for maize, including Adh1, hmga, ivr1 and zein. The methods were evaluated by four laboratories [38].

Plasmid DNA constructs containing cloned transgenic DNA sequences are increasingly being promoted as standards (calibrators) for GMO quantification. Quantitative assays for Bt176 maize, Bt11 maize, GA21 maize and GT73 canola using event-specific primers, TaqMan probes and plasmid DNA calibrators were developed. Molecular characterization of the transgenic events was carried out. Standard curves were constructed based on either plasmid DNA or genomic DNA standards in order to demonstrate the suitability of plasmid DNA calibrators [39]. A novel plasmid containing the junction sequences of nine GM maize events was introduced as a calibrator for the event-specific quantification of nine GM maizes by rt-QPCR (TaqMan) [40].

The cotton-specific Sad1 gene (stearoyl-acyl carrier protein desaturase) was validated as an endogenous reference gene using rt-QPCR (TaqMan). No amplification product was obtained with other species, such as *Gossypium*, *Arabidopsis thaliana*, maize and soybean, thus demonstrating the specificity of Sad1 for cotton. Furthermore, the reference gene Sad1 was combined with a GMO-specific gene (Cry1A(c)) for the development of duplex quantitative PCR [41]. TaqMan rt-QPCR was applied to the quantification of three varieties of insect-resistant cotton: Mon531, GK19 and SGK321. A plasmid containing segments of both the GMO-specific Cry1A(c) gene and the Sad1 reference gene was constructed for standardization purposes [42]. A similar method was reported for the quantification of herbicide-tolerant Mon1445 and insect-resistant Mon531. For standardization, instead of reference materials, a plasmid containing Mon1445, Mon531 and Sad1 sequences was used [43].

The development of rt-QPCR for the *Brassica napus*-specific gene BnACCg8 as a reference gene for GM canola was reported. A locked nucleic acid TaqMan probe was designed to ensure high specificity [44]. The rice (*Oryza sativa*)-specific gene sucrose phosphate synthase was validated as an endogenous reference gene for rt-QPCR [45]. An rt-QPCR method was developed for GM potato. The method was based on the determination of the transgene Cry3A and an endogenous UDP-glucose pyrophosphorylase gene of potato [46]. A tomato (*Lycopersicon esculentum*) species-specific gene LAT52 was validated as a reference gene for rt-QPCR of transgenic tomatoes [47].

A recent study assessed the factors affecting Mon810 corn quantification in an interlaboratory setup using rt-QPCR. Three genomic DNA isolation procedures were compared. The DNA extraction method had a significant influence on the results when construct-specific rt-QPCR was used instead of event-specific PCR. The use of plasmid DNA calibrators as opposed to reference materials also affected the results [48].

Conclusions

The recent advances in molecular techniques for GMO screening reveal the following trends. For GMO screening, the classical agarose electrophoretic techniques are being replaced by capillary electrophoresis, which allows rapid

separation and automation. CE chips are particularly promising in this area. Electrophoretic techniques do not provide sequence confirmation. Microtiter well-based hybridization assays offer high sample throughput for a relatively small number of GMO-related sequences. Microarrays, on the other hand, enable the screening of a large number of GMO-related sequences per sample, but the sample throughput is low. We anticipate that biosensors will play a leading role in the low-cost, rapid and simple screening of GMO in cases where high throughput and automation are not required. Disposable biosensors that provide visual detection of GMO without instrumentation are particularly attractive. In regards to GMO quantification, there are a steadily growing number of validated endogenous reference genes. Plasmid DNA constructs are becoming the material of choice for constructing calibration graphs. Real-time PCR, based on homogeneous fluorometric hybridization assays, is the most widely used technique. However, the latest advances in quantitative competitive PCR have allowed high throughput and multiplexing ability. Rapid, simple and automatable methods for DNA extraction from raw materials and food products are still in high demand.

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