Novel Strategies for Genetically Modified Organism Detection

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Genetically Modified Organisms in Food

Production, Safety, Regulation and Public Health

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Production, Safety, Regulation and Public Health

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Chapter 12

Novel Strategies for Genetically Modified Organism Detection

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INTRODUCTION

The introduction of genetically modified organisms (GMO) with desirable agronomic traits has allowed improving the yield and quality of crops, as well as the nutritional properties of plants. In line with health concerns and with political and economic interests, a legal basis has been established at global scale to facilitate the production/commercialization of GMO. To comply with most legislation requirements, great efforts have been devoted to the development of highly reliable methods for GMO detection, identification, tracing, and quantification. Currently, polymerase chain reaction (PCR)-based methods are generally used for GMO screening and identification, being real-time PCR the technique of choice for GMO quantification (Mafra, 2011). However, to face the steadily increasing cultivation area of GM crops and the number of (un) authorized GM events, efforts have been focused on the development of simple, low-cost, and user-friendly tools to rapidly generate data on GMO detection.

The interest in DNA biosensors (genosensors) for GMO detection has been growing due to their possibility for automation and microfabrication based on simple and portable detection systems, such as visual or electrochemical devices. One of the major challenges in GMO analysis concerns the simultaneous detection of several events. With this goal, applications of DNA microarrays have emerged as new multitarget platforms for the simultaneous detection of several construct elements, allowing high-throughput GMO diagnostics (Michelini et al., 2008).

One major limitation of applying genosensors or microarrays for GMO testing is the need for previous DNA amplification owing to the required sensitivity of target transgenic elements in a background of genomic plant DNA. This is currently performed by PCR technology that, despite its numerous advantages, has some limitations such as the lack of true multiplexing properties and the need of specific equipment. To mitigate the drawbacks linked to PCR technology, alternative nucleic acid amplification methods with promising characteristics have been developed and applied to GMO testing (Morisset et al., 2008a). Loop-mediated isothermal amplification (LAMP) methods have emerged as promising amplification alternatives to PCR, without the need for thermal cycling equipment.

This chapter intends to provide an overview on the most recent advances regarding the novel biosensing and alternative amplification technologies applied to GMO testing.

BIOSENSORS

DNA biosensors (genosensors) are analytical devices that result from the integration of a sequence-specific probe (usually a short synthetic oligonucleotide) and a signal transducer. Therefore, the presence of GMO is detected by hybridization of introduced DNA (target DNA sequence) with GMO-specific probes that are immobilized onto the transducer surface. In general, the genosensor construction involves the following steps: (1) immobilization of the DNA probe onto the electrode surface; (2) hybridization with the target sequence; (3) evaluation of labeling marks and detection methods (Lucarelli et al., 2004; Manzanares-Palenzuela et al., 2015a). The optimization of these steps is critical to improve the performance of these devices. Transducers that can detect nucleic acid hybridization are classified into electrochemical, optical (surface plasmon resonance, SPR), and piezoelectric (quartz crystal microbalance, QCM).

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Electrochemical Biosensors

Electrochemical biosensors are based on the electroactive analyte oxidation or reduction on the working electrode surface, which is submitted to a fixed or varying potential. The electrochemical signal is generated by the variation on the electron fluxes, being measured by an electrochemical detector. There are several platforms for DNA electrochemical sensing: direct and indirect DNA electrochemistry, DNA-specific redox indicator detection, nanoparticle-based electrochemistry amplification, and DNA-mediated charge transport (conductive polymers, specific redox reporters, intercalators, redox dyes, and nanoparticles) (Drummond et al., 2003; Viswanathan et al., 2009). In Figure 1, an example of an electrochemical DNA platform to detect Roundup Ready® (RR) soybean is presented.

Identical to optical biosensors, most of the electrochemical sensors target expression elements, namely 35S promoter and *nos* terminator, making them excellent alternatives for GMO screening (Table 1). Electrochemical biosensors targeting other sequences have also been described, namely *pat* (inducing tolerance to glufosinate herbicide), *cp4epsps* (inducing tolerance to glyphosate herbicide), *cry1A(b)* (inducing insect resistance) and *nptII* (responsible for antibiotic resistance) genes, among others. All the systems have presented high specificity and sensitivity, highlighting their relevance for GMO analysis.

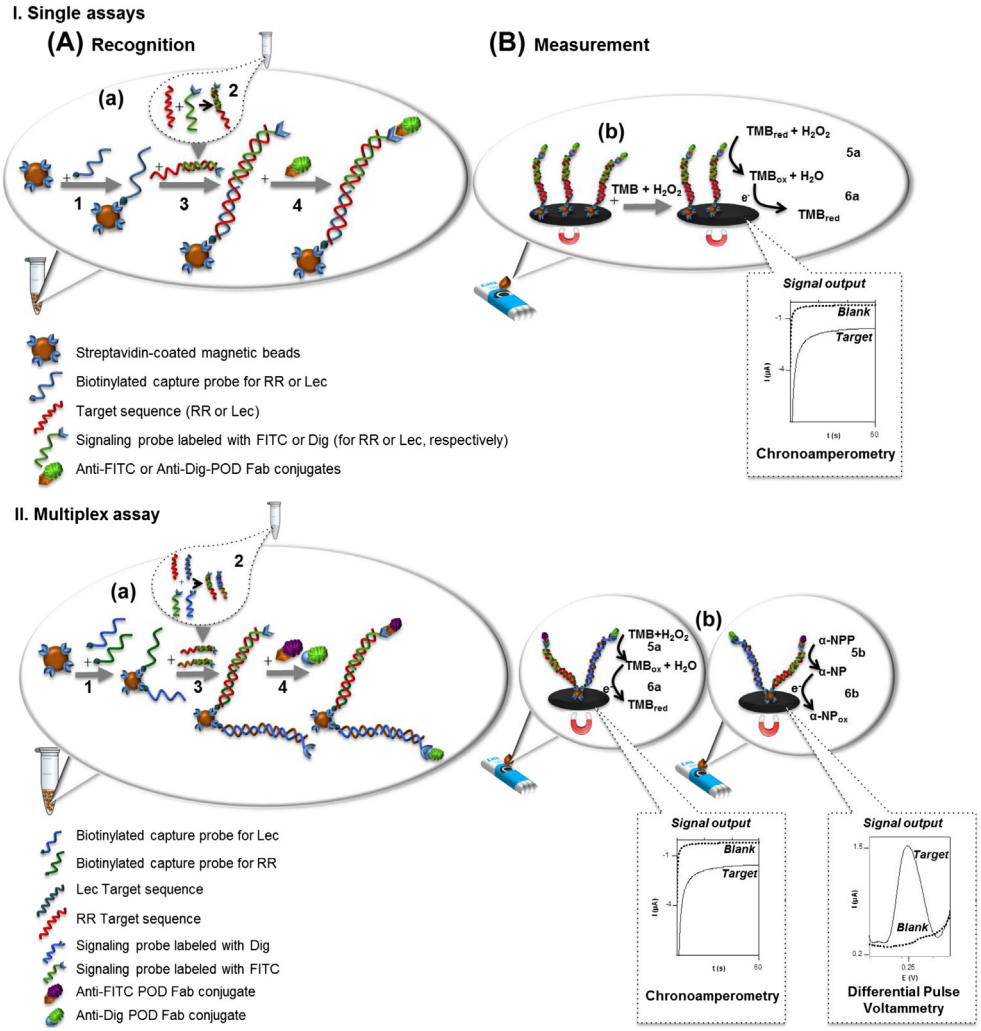

FIGURE 1 Scheme of the (I) single assays (valid for either RR or Lec detection) and (II) multiplex assay (simultaneous detection of RR and Lec). The assays are divided into two steps, recognition (A) and measurement (B): (1) attachment of capture probe(s) to the surface of magnetic beads; (2) homogeneous hybridization between a labeled-probe and target sequence; (3) heterogeneous hybridization with capture probe bound to the beads; (4) addition of the Fab-enzyme conjugate; (5a–6b) enzymatic reactions occurring after adding the enzymatic substrate (TMB/α-NPP); (6a) chronoamperometric measurement of TMBox reduction at the electrode surface; (6b) voltammetric measurement of naphthol oxidation current at the electrode surface. *Reprinted with permission from Manzanares-Palenzuela et al. (2015b). Copyright 2015, Elsevier.*

TABLE 1 Electrochemical Genosensors for GMO Detection

Electrode Type	Target	DNA Immobilization Strategy	Detection Method	Linear Range (nM)	Detection Limit (nM)	References
SPE-Au	35S	SAM	DPV with enzymatic amplification	0–24.6 (synthetic) 0–120 (amplicons)	0.25 (synthetic) 1 (amplicons)	Carpini et al. (2004)
	35S	SAM	EIS with enzymatic amplification	0.012–12 (synthetic)	0.0012 (synthetic)	Lucarelli et al. (2005)
GCE	35S	Covalent attachment with ethyl-enediamine	DPV with MB as indicator	5–120	–	Xu et al. (2006)
	35S	Adsorption	DPV with $[Co(NH_3)_6]^{+6}$ as indicator	–	–	Kerman et al. (2006)
	35S	Adsorption on Pt nanoparticles	SWV with $[Co(phen)_3]^{+3}$ as indicator	2.14–214 (synthetic)	1 (synthetic)	Wang et al. (2008)
	35S	SAM onto Au electrodes	DPASV using PbS nanoparticles onto GCE	0.012–48	0.00438	Sun et al. (2008)
	NOS	SAM onto Au electrodes	DPASV using CdS- nanoparticles onto GCE-CV with MB as indicator	0.008–4	0.00275	Sun et al. (2007)
	PAT	Adsorption onto nanogold/ nanoPANI-chitosan	EIS (label-free)	0.001–1000	3.1×10^{-4}	Feng et al. (2008)
	PAT	Adsorption onto nanogold/PDC	EIS (label-free)	0.1–10,000	0.024	Yang et al. (2007a)
	PAT	Potential-controlled adsorption on ZrO_2/nanogold	DPV with MB as indicator	0.1–1000	0.031	Zhang et al. (2008)
	PAT	Adsorption on nanoPANI-ZrO_2/ Tyrosine	EIS (label free)	0.0001–1000	2.68×10^{-5}	Yang et al. (2012a)
	PAT, NOS	Adsorption on ZrO_2/SWNT/PDC	EIS (label-free)	0.01–1000 (PAT)	0.00138 (PAT)	Yang et al. (2007b)
	PAT, NOS	PDDA/PDC-SWNTs films	DPV with MB as indicator	0.01–1000 (PAT)	0.0026 (PAT)	Yang et al. (2008)
	PEP gene	Adsorption on nano(Au–Pt) polytyramine	EIS (label-free)	0.001–100	3.6×10^{-4}	Yang et al. (2012b)
CPE	35S	Adsorption of the DNA in the PbSe/chitosan composite	DPV with Methylene violet as indicator	0.05–5000	0.016	Xie et al. (2008)
	nptII	Adsorption by controlled potential	SWV with MB as indicator	–	–	Ligaj et al. (2003)
	Bar	Covalent attachment with ethyl-enediamine	SWV with $Co(bpy)_3$ as indicator	–	–	Ligaj et al. (2006)

Continued

TABLE 1 Electrochemical Genosensors for GMO Detection—cont'd

Electrode Type	Target	DNA Immobilization Strategy	Detection Method	Linear Range (nM)	Detection Limit (nM)	References
	Bar, cp4epsps	Adsorption onto aluminum films	DPV with MB as indicator	100–100,000 (bar) Not quantitative for amplicons	22.5 (bar) Not reported for amplicons	Ren et al. (2005)
	PAT, NOS	Potential-controlled adsorption on poly lysine/SWNT	EIS (label-free)	0.001–100 (PAT) Not quantitative for amplicons	3.1×10^{-4} (PAT) Not reported for amplicons	Jiang et al. (2008)
	PAT, NOS	Adsorption on PANI-MWNT/chitosan	EIS (label-free)	0.0001–100 Not quantitative for amplicons	2.7×10^{-5} (PAT) Not reported for amplicons	Yang et al. (2009)
	PAT, NOS	Potential-controlled adsorption on nanogold-CNT/nanoPANI	EIS (label-free)	0.001–1000 (PAT)	5.6×10^{-4} (PAT) Not reported for amplicons	Zhou et al. (2009)
	MON810	Adsorption on CILE/p-ERG film	DPV with MB as indicator	0.01–1000 Not quantitative for amplicons	0.00452	Sun et al. (2014)
	A2704-12 soybean	Ionic liquid modified and partially reduced graphene. SAM	DPV with MB as indicator	0.001–2000 Not quantitative for amplicons	2.9×10^{-4}	Sun et al. (2013)
Au	35S and NOS	SAM	SWV with MB as indicator	–	–	Tichoniuk et al. (2008)
	NOS	SAM	Cyclic voltammetry with MB as indicator	50–100,000 (synthetic)	36 (synthetic)	Zhu et al. (2008)
	nptII	SAM	SWV with enzymatic amplification (aniline polymerization)	0.1–1 (synthetic) 0.2–1.0 (amplicons)	0.1 (synthetic) 0.2 (amplicons)	Wang et al. (2009)
	Bt	SAM	Solid state voltammetry using Ag nanoparticles	0.001–1000	10^{-5}	Jiang et al. (2011)
	PAT	Potential-controlled adsorption onto a SiO_2 PATP	EIS (label-free)	0.01–1000	0.0015	Ma et al. (2008)
	ivr, SSIIb, MON810	SAM	SWV with $[OsO_4(bipy)]$ as indicator	25–200 (ivr and MON810)	10	Duwensee et al. (2009)
	Cry1a/b	SAM	SWV with $[OsO_4(bipy)]$ as indicator	–	0.6%	Mix et al. (2012)

TABLE 1 Electrochemical Genosensors for GMO Detection—cont'd

Au (array)	35S, G1b1, pat, cp4epsps, SSIIb, cordapaA, lectin	SAM	Chronoamperometry with enzymatic amplification	Up to 20,000 total DNA, 0–5% GMO	22.5	Liao et al. (2013)
Au interdigitated microelectrodes	35S	Electrocopolymerization with PPy/MWCNT	EIS (label-free)	0.025–0.08	–	Lien et al. (2010)
SPCE	35S	Covalent attachment to succinimide-functionalized acrylic microspheres onto the AuNP/SPCE	DPV with anthraquinone-2-sulfonic acid monohydrate sodium salt as indicator	2.0×10^{-6}–2.0	7.79×10^{-7}	Ulianas et al. (2014)
	NOS	Adsorption by controlled potential	SWV with MB as indicator	–	2400	Meric et al. (2004)
	Lectin, RR soybean (multiplex)	Streptavidin-coat magnetic beads	Chronoamperometry with peroxidase and DPV with alkaline phosphatase	0.0020–0.250	0.65×10^{-3}	Manzanares-Palenzuela et al. (2015b)
SPE	Bt maize	Covalent attachment with –COOH	EIS with Ag signal amplification	0.00001–0.002	0.72×10^{-4}	Bonanni et al. (2009)
SPE, 3D GNEE	MON810	SAM	Chronoamperometry	0.25–10 (synthetic SPGE) 0.25–5 (synthetic 3D GNEE)	0.25 (synthetic) 367–1832 copies MON810	Barroso et al. (2015)

bar or pat, phosphinothricin *N*-acetyltransferase (PAT) enzyme; 35S, cauliflower mosaic virus promoter; cp4epsps, 5-enolpyruvulshikimate-3-phosphate synthase; CILE, carbon ionic liquid electrode; CNT, carbon nanotube; Cry1A(b), Cry1A(c) or Cry2A2, delta-toxin; CV, coefficient of variation; 3D GNEE, three-dimensional gold nanoelectrode ensembles; DPASV, differential pulse anodic solid voltammetry; DPV, differential pulse voltammetry; EIS, electrochemical impedance spectroscopy; ERG, electrochemically reduced graphene; GCE-CV, glassy carbon electrode coupled with cyclic voltammetry; GUS, β-D-glucuronidase enzyme; IVS2, intron from the maize alcohol dehydrogenase gene; MB, methylene blue; MWCNT, multi-walled carbon nanotube; nptII, neomycin phosphotransferase II enzyme; PANI, polyaniline; PANI-MWNT, polyaniline-multiwalled carbon nanotube; PATP, *p*-aminothiolphenol; pBI121, expression vector for plant transformation; PDC, 2,6-pyridinedicarboxylic acid; PDC-SWNT, 2,6-pyridinedicarboxylic acid single-wall nanotubes; PDDA, poly(diallyldimethyl ammonium chloride); PEP, phosphoenolpyruvate carboxylase promoter; PPy, polypyrrole; RR, Roundup Ready; SD, standard deviation; SPCE, screen printed carbon electrode; SPE, screen printed electrode; SPR, surface plasmon resonance; SSIIb and ivr, taxon-specific gene for maize; SWNT, single-wall nanotubes; SWV, square wave voltammetry; NOS, *Agrobacterium tumefaciens* nopaline synthase terminator.

Optical Biosensors

Optical biosensors offer a number of advantages like high sensitivity and specificity, isolation from electromagnetic interference, possibility of multiplexing by carrying signals of different wavelengths for multiparameter detection, compact design and minimally invasive, and the possibility of remote monitoring in hazardous/inaccessible spots (Narsaiah et al., 2012). Among optical biosensors, the SPR-based DNA biosensor has been applied to GMO detection.

SPR detects and quantifies changes in the refractive index at the metal–liquid interface caused by the hybridization of the target DNA with the immobilized probe on sensor surface. Changes in reflectivity give a signal (increase) that is proportional to the mass of the target bound to the surface. SPR is considered a label-free method because it can detect the binding of the analyte on a surface without any label (Sassolas et al., 2008). Since the first application of an SPR-based biosensor for GMO detection (Mariotti et al., 2002), other works have been reported and are summarized in Table 2.

Most of the described SPR biosensors target gene expression elements, promoter 35S (cauliflower mosaic virus 35S) and terminator *nos* (*Agrobacterium tumefaciens* nopaline synthase), making them excellent screening methods. With an increased

TABLE 2 Optical Biosensors for GMO Detection

Methods	Target Sequence/ Gene	Application	Detection Limit	Reproducibility (CV)	Linearity Range (nM)	References
SPR Biacore X™	P35S, T-NOS	CRM from soybean powder (2% RR)	1 nM	<3%	1–125 nM 1–100 nM	Mariotti et al. (2002)
	P35S	Synthetic oligonucleotides, CRM from soybean powder (2% RR), pBI121 plasmid, maize from animal feed	2.5 nM	≤5%	≥25 nM	Giakoumaki et al. (2003)
	P35S	GM maize	2.5 nM	1%	0–25 nM	Wang et al. (2004a)
	P35S	Synthetic oligonucleotides, and GM maize	2.5 nM	1%	0–25 nM	Wang et al. (2004b)
SPR Spreeta™	P35S	GM maize	10 nM	6%	–	Wang et al. (2004a)
Nanoparticle-based DNA biosensor	P35S, T-NOS	CRM from soybean powder (0, 0.1, 0.5, 1, 2 and 5%)	0.16 nM (0.8 fmol)	2.6–12.2% (SD)	0–25 fmol	Kalogianni et al. (2006)
Electrochemiluminescence	P35S, T-NOS	Tobacco	5 nM	–	5–5000 nM	Zhu et al. (2010)
Chemiluminometric immunosensor array	Epsps, nptII, pat	Soybeans, red pepper leaves, rice leaves	0.2% (epsps), 2.16% (nptII), 2.6% (pat)	9.7% (epsps); 15.4% (nptII), 6.4% (pat)	0–10%	Jang et al. (2011)
SERS spectroscopy	cry1A(b), cry1A(c)	Rice	0.1 pg/mL	–	0.1 pg/mL–10 ng/mL	Chen et al. (2012a)
	P35S	Bt176 maize	11 nM	–	25–100 nM	Guven et al. (2012)

pat, phosphinothricin *N*-acetyltransferase (PAT) enzyme; P35S, cauliflower mosaic virus promoter, epsps, 5-enolpyruvulshikimate-3-phosphate synthase; CRM, certified reference material; Cry1A(b), Cry1A(c) or Cry2A2, delta-toxin, CV, coefficient of variation; nptII, neomycin phosphotransferase II enzyme; pBI121, expression vector for plant transformation; SD, standard deviation; SERS, surface-enhanced raman scattering; SPR, surface plasmon resonance; T-NOS, *Agrobacterium tumefaciens* nopaline synthase terminator.

level of specificity, other SPR-based sensors have also been developed to target gene coding regions, such as Cry1Ab delta-endotoxin or cp4epsps, among others. Most of the systems allow the detection of raw plant material, such as certified reference materials (CRM; e.g., maize, soybean, cotton) or synthetic oligonucleotides, with high sensitivity and specificity (Table 2).

Piezoelectric Biosensors

QCM is a simple technique with high resolution, based on the piezoelectric effect that consists of applying mechanical forces on the surface of a piezoelectric material. This causes the appearance of electrical charges, but the reverse effect also occurs, which corresponds to the mechanical deformation by the application of an electric charge. Piezoelectric quartz crystal devices are very useful for direct measurements of biologically active molecules without the need for labeling or use of additional chemicals. In QCM sensors, the gold surface of the quartz crystal is coated with the DNA probe(s) able to hybridize with the complementary target(s) present in the analyte. Immobilization strategies of probes via thiol (Karamollaoglu et al., 2009; Mannelli et al., 2003a,b), biotin (Mannelli et al., 2003a,b; Minunni et al., 2001), and amino groups (Minunni et al., 2001) have been used for GMO screening. The QCM sensors have been applied to detect the 35S promoter and *nos* terminator in RR soybean (Mannelli et al., 2003b), the coding regions for Cry1A(b) in maize (Passamano and Pighini, 2006), and *epsps* in RR soybean (Stobiecka et al., 2007). These devices have shown promising results for real-time, label-free, and direct detection of DNA for GMO analysis (Karamollaoglu et al., 2009).

MICROARRAYS

The use of DNA microarrays has greatly increased as they offer promising multitarget platforms able to detect numerous DNA sequences. Additionally, these methods can be reusable and allow continuous, fast, sensitive, and selective detection of DNA hybridization. DNA microarrays (also called gene-chips, DNA-chips, or biochips) usually rely on the immobilization of a single-stranded DNA probe onto a surface to recognize its complementary strand. They result from the assembly of numerous (up to a few 1000) DNA biosensors onto the same detection platform, which consist of glass supports containing specific oligonucleotide-capture probes immobilized on their surface. They allow parallel detection and analysis of the patterns of expression of thousands of genes in a single assay, which is possible because of the high degree of miniaturization, offering an advantage over other methods (Elenis et al., 2008).

Several microarray platforms have been proposed for GMO analysis, with the possibility of simultaneously detecting several expression elements (e.g., P-35S, T-NOS) and/or specific genes (e.g., *nptII*, *cp4epsps*, *cry1A(b)*), allowing the retrieval of a great amount of information in a single assay (Bai et al., 2007, 2010; Dobnik et al., 2010; Lee, 2014; Li et al., 2015; Morisset et al., 2008b; Shao et al., 2014).

ALTERNATIVE DNA AMPLIFICATION METHODS

Although perfectly feasible in most well-equipped laboratories, PCR cannot be performed in the field. To overcome this drawback, different isothermal amplification techniques have been attempted avoiding the need of thermal cycles. So far, most of the published methodologies relying on the isothermal amplification of DNA have been developed for molecular diagnosis purposes, such as pathogenic bacteria and virus identification (Gill and Ghaemi, 2008). Some of these techniques, namely strand displacement amplification, nicking-enzyme amplification reaction, rolling circle amplification, loop-mediated isothermal amplification (LAMP), and helicase-dependent amplification (HDA) have already been used for GMO testing (Morisset et al., 2008a). However, only LAMP and HDA have given interesting results for GMO analysis (Zahradnik et al., 2014), LAMP being the most used isothermal amplification technique.

LAMP requires the use of a DNA polymerase with strand displacement activity (generally the thermostable *Bst* DNA polymerase large fragment) and two sets of specifically designed primers (inner and outer primers) to recognize a total of six distinct sequences of the target DNA. First proposed by Notomi et al. (2000), LAMP is initiated by the annealing of an inner primer containing sequences of both the sense and antisense strands of the target DNA. After inner primer extension, the outer primer binds upstream the inner primer and is extended by the polymerase, while strand displacement DNA synthesis leads to the release of a single-stranded DNA. This displaced strand forms a stem–loop structure at 5′ end and serves as a template for DNA synthesis, now primed by the second inner and outer primers that hybridize on the other end of the target. This produces a dumbbell-structured DNA that enters cycle amplification. The final products of LAMP are stem–loop DNA with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand (Morisset et al., 2008a; Notomi et al., 2000). Figure 2 shows the principle of LAMP applied to GMO detection.

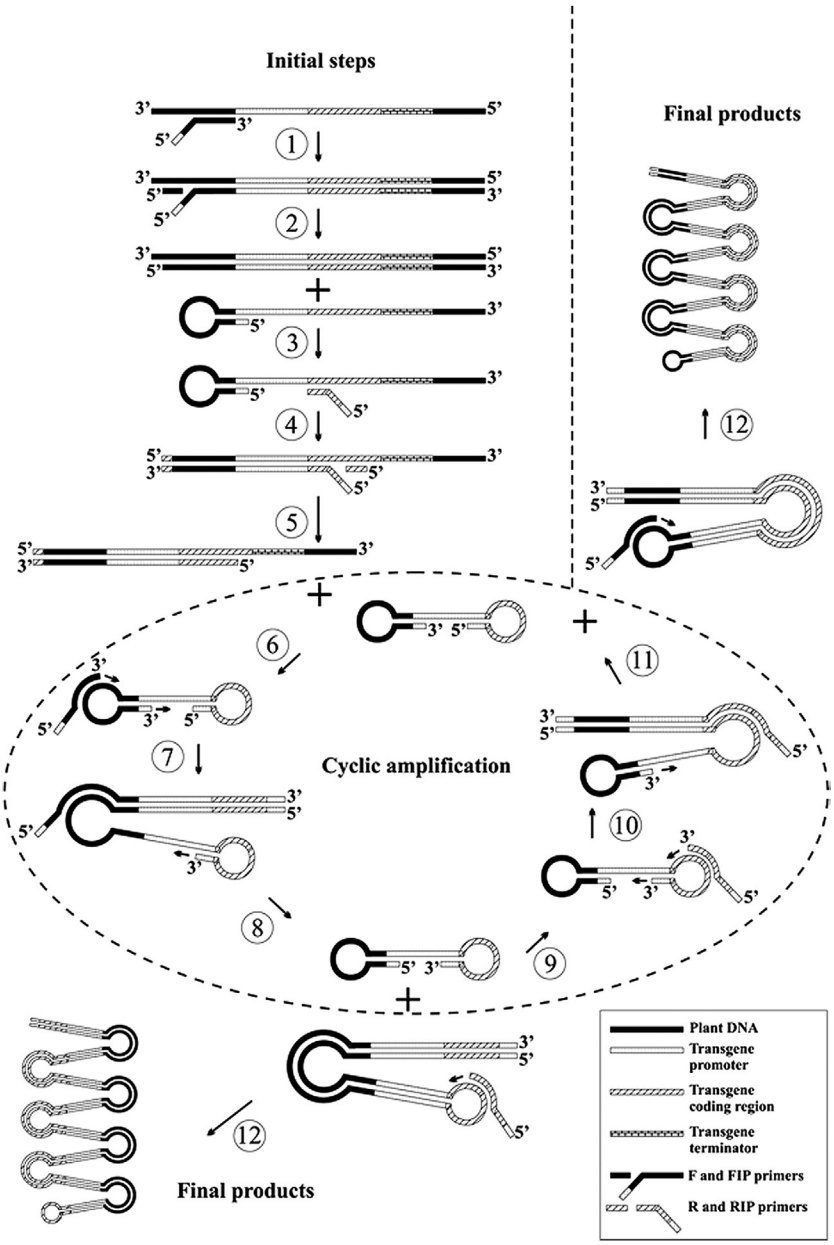

FIGURE 2 Schematic representation of loop-mediated isothermal amplification (LAMP). Two inner primers (termed FIP and RIP) and two outer primers (termed F and R) binding on six different region of the target sequence are used in LAMP. In the initial steps, the reaction starts with the annealing of the FIP primer on the target sequence. The FIP primer is then extended due to the strand displacement activity of the DNA polymerase (1). The outer F primer binds upstream of the FIP primer and is extended by the polymerase while displacing the FIP extended product (2). The released FIP-extended product forms a loop due to the hybridization of complementary regions from the target DNA and the FIP primer (2). The inner RIP anneals on this FIP-extended product (3) and is extended by the polymerase. The outer R primers binds immediately upstream of the RIP primer and its extension leads to displacement of the RIP-extended product (4). A double-stranded product is then obtained; the single-stranded RIP-extended product is released and will serve for the cycle amplification phase of LAMP (5). In that phase, the RIP-extended product forms a double loop also termed dumb-bell form. While this dumbbell structure starts self-primed DNA extension, the FIP primer binds on its complementary region (6) and is extended (7). This FIP-extended product is released by the strand displacement of the self-primed extended product, which forms a stem-loop DNA (8). The FIP-extended product, that also harbors a dumbbell form, starts a self-primed extension while the RIP primer binds on its complementary sequence (9) and starts primer extension (10). The simultaneous extension of RIP primer and FIP-extended product leads to the release of another stem–loop DNA and the initial dumbbell-shaped RIP-extended product (11), that will be used for another LAMP cycle. Both stem–loop DNA products released after steps 8 and 11 are used as templates for primer RIP and FIP extension, as well as self-primed extension of the resulting products. The final LAMP products are stem–loop DNA of various sizes (12). *Reprinted with permission from Morisset et al. (2008a). Copyright 2015, Springer.*

TABLE 3 Overview of LAMP Application for GMO Detection

Target	LAMP Conditions	Monitoring Conditions	Sensitivity	References
Oilseed rape MS8/RF3 (P-35S, P-NOS, T-NOS, event-specific junction)	55 °C/2 h	Agarose gel electrophoresis	0.01% GMO (T-NOS, P-35S)	Lee et al. (2009)
Maize CBH351 (SSIIb, event-specific junction)	65 °C/60 min	Electrochemical	3×10^2 copies/reaction	Ahmed et al. (2009)
RR soybean (P-35S, *epsps* gene)	65 °C/45 min	Visual (SYBR green); Agarose gel electrophoresis	Up to 10^{-5} dilution (~5 copies)	Liu et al. (2009)
Rice KMD1, TT51-1, KF6 (event-specific junction)	63 °C/60 min	Visual (SYBR green or with hydroxy naphthol blue)	0.005% (KF6) 0.01% (KMD1, TT51-1)	Chen et al. (2012b)
Rice KMD1(cry1Ab gene)	65 °C/60 min	Visual (precipitate after centrifuging; SYBR green); Agarose gel electrophoresis	3×10^2 copies of pMD19-cry1Ab plasmid DNA	Li et al. (2013)
Maize T25 (pat gene)	65 °C/45 min	Real-time turbidimeter; visual (SYBR green)	5 g/kg GMO	Xu et al. (2013)
Transgenic sugarcane (cry1Ac gene)	65 °C/60 min	Visual (precipitation; Calcein/Mn^{2+} complex under UV light; SYBR green)	43.1 copies of plasmid, 1.0 ng/mL sugarcane genomic DNA	Zhou et al. (2014)
Maize BVLA 430101 (phytase gene)	65 °C/60 min	Real-time turbidimetry	30 copies of phytase gene	Huang et al. (2014)

Cry1Ab, cry1Ac, delta-toxins; epsps, 5-enolpyruvulshikimate-3-phosphate synthase; pat, phosphinothricin *N*-acetyltransferase (PAT) enzyme; P35S, cauliflower mosaic virus promoter; RR, Roundup Ready; P-NOS, *Agrobacterium tumefaciens* nopaline synthase promoter; SSIIb, taxon-specific gene for maize; T-NOS, *Agrobacterium tumefaciens* nopaline synthase terminator.

LAMP allows visual monitoring, making this technique inexpensive, simple, and suitable for field applications. During DNA amplification, large amounts of pyrophosphate, produced as a reaction byproduct, react with magnesium and form a white precipitate that can be used to visually detect positive results (Zhang et al., 2014). Naked eye monitoring of LAMP can also be performed by means of DNA-binding fluorescent dyes, such as SYBR Green I that turns from orange to green when binding to double stranded DNA. Table 3 summarizes different LAMP-based strategies applied to GMO detection. Although SYBR Green I has been reported to increase sensitivity, compared with visual turbidity measurements, it increases the reaction cost and the risk of contamination due to the addition of dye at the end of LAMP (Zhang et al., 2014). To overcome this shortcoming, Zhang et al. (2013) developed a system for GMO screening and identification (rice, soybean, and maize), which included a microcrystalline wax bead encapsulating SYBR green fluorescent dye. The bead was destroyed by incubation at 85 °C after LAMP, liberating the dye that allowed visual detection of color and simultaneously avoided dye inhibition and cross-contamination (Zhang et al., 2013). The simplicity and low cost of visual detection are determinant features of in-field applications, but providing only qualitative results, a limitation in GMO analysis. Other described LAMP monitoring strategies include gel electrophoresis, real-time turbidimetry, real-time fluorescence, and electrochemical biosensors (Table 3). Agarose gel electrophoresis of LAMP products generate a characteristic multiple band pattern that allows unequivocal identification of positive results, but without quantification. Real-time turbidity measurements of LAMP performed with simple equipment can be used for quantitative purposes (Mori et al., 2004). LAMP with fluorescence has also been described as a possibility for real-time monitoring, allowing the quantification of target genes (Huang et al., 2014; Zhang et al., 2014), though, to the best of our knowledge, GMO quantitative applications are still very scarce. The most commonly cited disadvantage of LAMP regards the complicated design of multiple primers to cover six regions of the target DNA.

FINAL REMARKS

In response to the growing diversity of GMO on the market, the need for screening and specific methods has led to new analytical advances. To address the requirement for real-time and high-throughput GMO monitoring, biosensors, in particular,

electrochemical genosensors have demonstrated their usefulness. Biosensors can provide rapid, low-cost, sensitive, and specific measurements suitable for in-field analysis. The efficiency of GMO diagnostics could be improved by analyzing several targets simultaneously, which is presently being exploited using the microarray platforms. The ability to multiplex greatly expands the power of genosensor analysis. Therefore, there is a vast potential market for biosensor applications that has just began to be exploited.

Although a remarkable success in biosensor technology for GMO testing has been reached, true applicability to CRM or real food samples is still at a preliminary stage as they mostly rely on synthetic DNA recognition. Besides the reported low detection limits, much effort is also required to increase actual sensitivity that depends on PCR efficiency. As promising alternatives to conventional PCR, isothermal amplification strategies such as LAMP are especially suitable for in-field use and are low-cost, enabling visual and electrochemical detection.

Despite the advantages of the described novel approaches, one major drawback regards the lack of true quantitative analysis as GMO content should be determined in relation to a taxon-specific gene and not simply as an absolute estimation of marker sequences.

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