Food Chemistry 201 (2016) 110–119

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Development and applicability of a ready-to-use PCR system for GMO screening

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ARTICLE INFO

Article history:
Received 10 June 2015
Received in revised form 9 November 2015
Accepted 3 January 2016
Available online 6 January 2016

Keywords:
GMO
PCR
Screening
Ready-to-use
Multi-target
Decision Support System
Detection

ABSTRACT

With the growing number of GMOs introduced to the market, testing laboratories have seen their workload increase significantly. Ready-to-use multi-target PCR-based detection systems, such as pre-spotted plates (PSP), reduce analysis time while increasing capacity. This paper describes the development and applicability to GMO testing of a screening strategy involving a PSP and its associated web-based Decision Support System. The screening PSP was developed to detect all GMOs authorized in the EU in one single PCR experiment, through the combination of 16 validated assays. The screening strategy was successfully challenged in a wide inter-laboratory study on real-life food/feed samples. The positive outcome of this study could result in the adoption of a PSP screening strategy across the EU; a step that would increase harmonization and quality of GMO testing in the EU. Furthermore, this system could represent a model for other official control areas where high-throughput DNA-based detection systems are needed.

1. Introduction

Since the first GMO approval in 1996, adoption of GM technology has been growing exponentially and the number of GM crops introduced to the market has been increasing. The International Service for the Acquisition of Agri-Biotech Applications (ISAAA) reported a record of 181.5 million hectares of biotech crops grown in 2014 in a total of 28 countries (James, 2014). While the vast majority of these crops are cultivated outside of Europe, the EU is nevertheless faced with the consequent introduction of GM products to its market and must be able to respond as foreseen by its legislation.

The European Union established a strict regulatory framework to trace GMOs "from the farm to the fork". All GMOs and derived products undergo an authorisation process that aims to guarantee safety for human, animal and environmental health (European Parliament & Council of the European Union, 2001, 2003a,b). As part of this regulatory framework, a mandatory labelling of any GMO-derived or GMO-containing food or feed has been introduced, intending to ensure consumers' freedom of choice. Because adventitious or technically unavoidable contamination might occur along the supply and production chains, a labelling threshold of 0.9% of the GM ingredient was established for all products (European Parliament & Council of the European Union, 2003a). In addition, a "Minimum Required Performance Limit" of 0.1% was established for feed containing GMOs already approved elsewhere and for which an application for authorisation in the EU had been requested (European Commission, 2011). Based on these regulations, EU control laboratories must be able to detect low amounts of GM materials, evaluate their authorisation status and, when appropriate, quantify the GM content to check the compliance with legal provisions. At the time of publication, 78 GMOs (single or stacked GM events) had to be traced along the food and feed supply chains (European Commission, 2003).

GMO detection, identification, and quantification follow a complex multistep procedure that most often relies on real-time PCR (RTi-PCR), a DNA-based technique that has proved to be the most reliable and versatile (Holst-Jensen et al., 2012; Miraglia et al., 2004) and is used in all EU control laboratories.

In the first step, a screening phase reveals whether samples actually contain any GM material; as it is known which GMOs contain which screening elements, screening already provides an indication of the potential identity of any GMOs detected. This step uses assays that target regulatory sequences, genes or constructs

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http://dx.doi.org/10.1016/j.foodchem.2016.01.007
0308-8146/

that are widely used in plant transformation. Screening is important in terms of analysis cost and time optimisation, especially when the presence of a potentially large number of GMOs must be investigated (Novak et al., 2009), a situation that is increasingly found in feed products, or when the rate of positive sample is very small. The optimal GMO screening strategy should (1) detect all authorised and authorisation-pending events; (2) reduce the number of samples that need further investigation; (3) potentially detect unknown/unauthorised GM events.

When the presence of GM material is detected, the identity and authorization status of the GMOs must be assessed. The assays used in this step are GM event-specific since they target unique sequences generated during the insertion of the exogenous DNA in the plant genome. Lastly, for the authorised GMOs or GMOs with pending authorisation, the third step requires quantification. For non-authorised GMOs, no further action is needed as their presence violates the zero tolerance rule applied in the EU.

Testing laboratories have so far developed their own methodologies, and a vast range of screening and identification strategies exist. Among them, multi-target approaches combined with Decision Support Systems (DSS) are widely acknowledged as the most practical tools to improve time and cost-effectiveness of GMO analysis (Barbau-Piednoir et al., 2014; Bundesamt für Verbraucherschutz und Lebensmittelsicherheit., 2012; Scholtens et al., 2013; Van den Bulcke et al., 2010; Waiblinger, Grohmann, Mankertz, Engelbert, & Pietsch, 2010). However, the higher the number of assays to be performed, the more complex the setup of all reactions. In addition, building a comprehensive DSS requires intensive effort in terms of laboratory testing and data collection from publicly available sources (Block et al., 2013; Gerdes, Busch, & Pecoraro, 2012a).

Ready-to-use PCR plates, known as pre-spotted plates (PSP), consist of plastic supports for RTi-PCR (e.g. 96-well plates) in which primers and probes from chosen assays are pre-dispensed. PSP facilitate multi-target analyses because a single reaction mixture per sample, containing all reagents except primers and probes, is prepared and then loaded in the appropriate wells. This approach speeds up laboratory activity while reducing the risk of mistakes. In 2009, the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) at the Joint Research Centre (JRC) of the European Commission developed the first application of PSP for GMO detection (Querci et al., 2009) and later demonstrated its applicability to processed food and feed matrices (Kluga, Folloni, Van den Bulcke, Van den Eede, & Querci, 2012). This "identification" PSP enabled the specific recognition of 39 GM events in seven crop species simultaneously, and relied on the qualitative use of event-specific quantitative assays validated by the EURL GMFF according to international standards (Horwitz, 1995; International Organization for Standardization, 1994). Since this first release, other studies have shown that ready-to-use systems can cope with different GMO testing needs in both the official and private sectors, as well as for different legal frameworks (Cottenet, Blancpain, Sonnard, & Chuah, 2013; Gerdes, Busch, & Pecoraro, 2012b; Mano et al., 2009; Randhawa, Singh, Sood, & Bhoge, 2014).

With the aim of providing official EU testing laboratories with updated and improved tools for GMO detection as required by its mandate (European Parliament, & European Council, 2004), the EURL GMFF established a new dual component screening approach combining PSP with a dedicated DSS. First, a screening PSP was developed with the potential to screen for the presence of all GMOs listed in the EU register (European Commission, 2003) in a single experiment. To do so, the screening PSP combines element-, construct-, taxon- and event-specific methods, for a total of 16 assays. All the assays are from the EU reference methods database, also known as the GMOMETHODS database (Bonfini, van den Bulcke, Mazzara, Ben, & Patak, 2012), and selected to guarantee maximum GMO coverage. These methods were standardised for use on the PSP and then reassessed in-house to ensure there was no loss in molecular specificity and sensitivity. Subsequently, it was assessed whether combining a screening PSP with a Decision Support System, the JRC GMO-Matrix (Angers-Loustau et al., 2014), would build a more powerful analytical strategy. The JRC GMO-Matrix offers a straightforward interpretation of the screening PSP reactions by providing, based on the analysis of the pattern of positive/negative results, an exhaustive list of GMOs that are to be tested in the identification phase. The combination of the screening PSP and JRC GMO-Matrix allows for an optimal experimental design, limiting the number of samples and identification assays to be tested to a minimum. Finally, the screening system was successfully tested by 20 official GMO testing laboratories.

A general implementation of this screening strategy would enhance the harmonisation and the efficiency of GMO testing throughout the EU.

2. Materials and methods

2.1. Development of the screening pre-spotted plates

2.1.1. Selected RTi-PCR assays

A total of 7 taxon-, 5 element-, 1 construct- and 3 event-specific assays (Table 1) were selected from the GMOMETHODS database (http://gmo-crl.jrc.ec.europa.eu/gmomethods/) (Bonfini et al., 2012) as to obtain maximum coverage of authorised GMOs using the least number of assays. The chosen taxon-specific assays were those used in the first PSP release (Querci et al., 2009); element- and construct- specific assays were chosen, using the JRC GMO-Matrix, to maximise the number of GM events detected; event-specific assays were chosen to cover a gap of 3 GM events not detected by the other screening assays.

2.1.2. In silico molecular specificity analyses

The molecular specificity of the 16 assays, initially verified in the respective original validation studies, was re-assessed in silico using an internal version of the JRC GMO-Matrix, which simulates the RTi-PCR reactions using a combination of the methods' primers and probes against the DNA sequences of >80 GM events, plant genomes (including soybean, *Brassica oleracea*, Polish canola, sugar beet, cotton progenitor, potato, rice, wheat and maize) and >60 CaMV strains contained in a restricted database (Patak, 2011).

2.1.3. Plant materials

Certified reference materials (CRMs) from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and from the American Oil Chemists' Society (AOCS, IL, USA) were used for the experimental molecular specificity analyses, corresponding to 52 GM events (Table 2). Unless not available, the CRM was used at a 1% nominal level. Where only an alternative nominal level was available, samples were diluted to 1% GM content using non-GM material.

Genomic DNA (gDNA) from maize CRMs was extracted using the Nucleospin® Food Kit (Macherey-Nagel GmbH, Düren, Germany); gDNA from soybean, rapeseed, rice and sugar beet CRMs was extracted using a modified CTAB extraction protocol (Murray & Thompson, 1980); and gDNA from cotton CRMs was extracted using the Foodproof® GMO Sample Preparation Kit (Biotecon Diagnostics GmbH, Potsdam, Germany) according to the manufacturers' instructions. Wild type gDNA from all crops were extracted from retail samples or blank levels of CRMs (Table 2). Each sample was extracted in duplicate. gDNA was quantified by fluorescence detection using the PicoGreen® ds DNA quantitation kit

Table 1
Screening pre-spotted plate methods and LOD_{10} observed on the pre-spotted plates. The reference codes are those from the GMOMETHODS database. LOD_{10} values represent the minimum copy number (cp) at which all replicates provided positive results. Ct values were obtained on ABI7900 instruments and given values represent the means of 10 replicates.

	Method (target)	Reference	LOD_{10} (cp)	Ct ± SD
Taxon-specific	hmg (maize)	QT-TAX-ZM-002	5	37.04 ± 0.84
	lec (soy)	QT-TAX-GM-002	5	37.36 ± 0.87
	cruA (rapeseed)	QT-TAX-BN-012	20	37.28 ± 0.44
	sah7 (cotton)	QT-TAX-GH-016	5	38.40 ± 0.70
	ugp (potato)	QT-TAX-ST-010	5	37.01 ± 0.84
	pld (rice)	QT-TAX-OS-017	5	38.33 ± 0.84
	gs (sugar beet)	QT-TAX-BV-013	5	37.97 ± 0.64
Element-specific	p35S	QT-ELE-00-004	5	37.21 ± 0.77
	tNos	QL-ELE-00-013	10	36.82 ± 0.43
	CTP2-EPSPS	QL-CON-00-008	5	37.09 ± 0.62
	pat	QT-ELE-00-002	5	35.51 ± 0.61
	bar	QL-ELE-00-014	5	37.60 ± 0.86
	cry1Ab/Ac	QL-ELE-00-016	5	36.46 ± 1.04
Event-specific	DAS-40278 (maize)	QT-EVE-ZM-004	10	36.45 ± 0.49
	CV127 (soybean)	QT-EVE-GM-011	5	36.69 ± 0.77
	DP-305423 (soybean)	QT-EVE-GM-008	5	37.54 ± 0.53

(Invitrogen: Molecular Probes, Eugene, OR, USA) and a Bio-Rad VersaFluor™ Fluorometer Reader according to the manufacturers' instructions and was examined on an agarose gel to verify its integrity. All gDNA samples were diluted in sterile distilled water and subjected to inhibition tests to assess DNA quality (Žel et al., 2008).

2.1.4. Experimental molecular specificity analyses and RTi-PCR setup

All CRM DNA samples were tested in duplicate against the 16 chosen methods in RTi-PCR experiments. The experimental conditions anticipated to be used in the PSP were simulated in the molecular specificity analyses. Methods were tested on a 96-well plate in a volume of 25 μL containing 100 ng DNA, 1× TaqMan® Universal PCR Master Mix (Life Technologies), 600 nM of each primer and 200 nM of the corresponding FAM/TAMRA-labelled probe. Thermocycling consisted of a 2 min UNG step at 50 °C and a 10 min denaturation step at 95 °C, followed by 45 cycles of a denaturation step at 95 °C and an annealing/elongation step of 1 min at 60 °C; acquisition data was set at 60 °C annealing/elongation step. RTi-PCR runs were performed using the ABI 7900 and 7500 Real-Time PCR Systems Life Technologies) and data analysed using the SDS 2.4 and 7500 software v2.0.6, respectively. Baseline and thresholds were set manually.

2.1.5. Update of the identification PSP

The identification PSP previously developed by the EURL GMFF (Kluga et al., 2011; Querci et al., 2009) was updated to include assays detecting all authorised GM events from crops commonly found positive for GM materials in the last few years by control laboratories (maize, soybean, canola and cotton, unpublished data). In particular, 9 new assays were added following the methodology described in Querci et al. (2009) while the ones targeting crops and events not expected to be frequently found in samples (potato, rice and sugar beet reference gene and events, and maize LY038 event) were removed. The targets now include the reference genes for maize (hmg), soybean (lec), rapeseed (cruA) and cotton (sah7), 17 maize events (3272, 98140, Bt11, Bt176, DAS-40278, DAS-59122, GA21, MIR162, MIR604, MON810, MON863, MON87460, MON88017, MON89034, NK603, T25, TC1507), 9 soybean events (A2704, A5547, CV127, DP-305423, DP-356043, FG72, GTS40-3-2, MON87701, MON89788), 8 rapeseed events (GT73, Ms1, Ms8, Rf1, Rf2, Rf3, T45, Topas 19/2) and 10 cotton events (281-24-236, 3006-210-23, GHB119, GHB614, LLCotton 25, MON1445, MON15985, MON531, MON88913, T304), all of which are validated assays listed in the GMOMETHODS database (Bonfini et al., 2012).

2.1.6. PSP procurement and quality assessment

Screening PSP production was outsourced to Eurogentec SA (Liège, Belgium) and identification PSP production to Life Technologies (Carlsbad, CA, USA). Both PSP types were submitted to a quality control by the EURL GMFF by loading a total of six plates with (1) a non-target DNA sample (Lambda DNA, Life Technologies, 100 ng/well), (2) a gDNA taxon-specific control sample and (3) a solution of gDNAs covering all targets. All tests provided the expected results (data not shown).

2.1.7. Limit of detection (LOD)

The sensitivity of the assays selected for the screening PSP was first tested on freshly prepared reaction mixtures and later confirmed on PSP.

Two series of mixed gDNA samples, targeting respectively taxon-specific assays and GM target assays, were prepared to contain 6, 4, 2, 1 and 0.2 copies/μL of each target. To do so, the initial copy number of the individual DNA samples was estimated by running a droplet digital PCR (ddPCR) in quadruplicate. 1–50 ng DNA (except potato: 0.03 ng) were quantified in a 20 μL reaction mix containing 2X QX200™ ddPCR SuperMix for Probes (no dUTP) (Biorad 186-3023), 600 nM of each primer and 200 nM of the fluorescently-labelled probe. Droplet generation and reading were performed on the Biorad QX200™ Droplet Digital PCR System and PCRs were run on the Biorad C1000 Touch™ Thermal Cycler according to manufacturer's instructions. Data were analysed using the QuantaSoft Software version 1.6.6. All samples were diluted in 10 ng/μL of non-target DNA (λ DNA, Life Technologies).

Method sensitivity was then assessed loading 5 μl per reaction, corresponding to 30, 20, 10, 5 and 1 copie(s) per assay, of the two series of mixed gDNA samples in 10 freshly prepared RTi-PCR replicates and on 10 screening PSP (Eurogentec SA, Liège, Belgium).

The LOD_{10} was determined as the last dilution level at which no negative result was observed (i.e. the level at which 95% of replicates provides positive response).

2.1.8. Decision Support System (DSS)

The DSS consists of a specific interface for the JRC GMO-Matrix web application (http://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/). In short, a pre-selected array of assays, reflecting the methods used for the screening PSP, was uploaded to a dedicated webpage and

Table 2
Summary of specificity tests. T/E: theoretical (bioinformatics)/experimental evaluation of the presence/absence of the target. Unless specified by "*", T and E always matched. –: no signal expected nor observed, +: positive signal expected and observed. *Unexpected weak positive amplification signal observed (Ct > 36).

Plant material	Taxon-specific assays							Screening assays						Event-specific assays		
	hmg (maize) T/E	lec (soy) T/E	cruA (rapeseed) T/E	sah7 (cotton) T/E	ugp (potato) T/E	pld (rice) T/E	gs (S. beet) T/E	p35S T/E	tNos T/E	CTP2 EPSPS T/E	pat T/E	bar T/E	cry1 Ab/Ac T/E	DAS-40278 T/E	CV127 T/E	DP-305423 T/E
Maize																
Wild type	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3272 (SYN-E3272-5)	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
98140 (DP-098140-6)	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
Bt11 (SYN-BT011-1)	+	–	–	–	–	–	–	+	+	–	+	–	+	–	–	–
Bt176 (SYN-EV176-9)[1]	+	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–
DAS-40278 (DAS-40278-9)	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
DAS-59122 (DAS-59122-7)	+	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–
GA21 (MON-00021-9)	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
MIR162 (SYN-IR162-4)	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
MIR604 (SYN-IR604-5)	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
MON810 (MON-00810-6)	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
MON863 (MON-00863-5)	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–
MON87460 (MON-87460-4)	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–
MON88017 (MON-88017-3)	+	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
MON89034 (MON-89034-3)	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–
NK603 (MON-00603-6)	+	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
T25 (ACS-ZM003-2)	+	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–
TC1507 (DAS-01507-1)	+	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–
Soybean																
Wild type	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A2704-12 (ACS-GM005-3)	–	+	–	–	–	–	–	+	–	–	+	–	–	–	–	–
A5547-127 (ACS-GM006-4)	–	+	–	–	–	–	–	+	–	–	+	–	–	–	–	–
CV127 (BPS-CV127-9)	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–
DAS-68416-4 (DAS-68416-4)	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–
DP-305423 (DP-305423-1)	–	+	–	–	–	–	–	–*	–*	–	–	–	–	–	–	+
DP-356043 (DP-356043-5)	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–
FG72 (MST-FG072-3)	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–
GTS40-3-2 (MON-04032-6)	–	+	–	–	–	–	–	+	+	–	–	–	–	–	–	–
MON87701 (MON-87701-2)	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–
MON87705 (MON-87705-6)	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–
MON87708 (MON-87708-9)	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MON87769 (MON-87769-7)	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MON89788 (MON-89788-1)	–	+	–	–	–	–	–	–*	–*	+	–	–	–	–	–	–
Rapeseed																
Wild type	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
GT73 (MON-00073-7)	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–
DP-73496 (DP-073496-4)	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Ms1 (ACS-BN004-7)	–	–	+	–	–	–	–	–	+	–	–	+	–	–	–	–
Ms8 (ACS-BN005-8)	–	–	+	–	–	–	–	–	+	–	–	+	–	–	–	–
MON88302 (MON-88302-9)	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–
Rf1 (ACS-BN001-4)	–	–	+	–	–	–	–	–	+	–	–	+	–	–	–	–
Rf2 (ACS-BN002-5)	–	–	+	–	–	–	–	–	+	–	–	+	–	–	–	–
Rf3 (ACS-BN003-6)	–	–	+	–	–	–	–	–	+	–	–	+	–	–	–	–
T45 (ACS-BN008-2)	–	–	+	–	–	–	–	+	–	–	+	–	–	–	–	–
Topas 19/2 (ACS-BN007-1)	–	–	+	–	–	–	–	+	–	–	+	–	–	–	–	–

(*continued on next page*)

Table 2 (*continued*)

Plant material	Taxon-specific assays							Screening assays						Event-specific assays		
	hmg (maize) T/E	lec (soy) T/E	cruA (rapeseed) T/E	sah7 (cotton) T/E	ugp (potato) T/E	pld (rice) T/E	gs (S. beet) T/E	p35S T/E	tNos T/E	CTP2 EPSPS T/E	pat T/E	bar T/E	cry1 Ab/Ac T/E	DAS-40278 T/E	CV127 T/E	DP-305423 T/E
Cotton																
Wild type	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
281 × 3006 (DAS-24236-5 × DAS-21Ø23-5)[2]	–	–	–	+	–	–	–	–*	–	–	+	–	–	–	–	–
GHB119 (BCS-GH005-8)	–	–	–	+	–	–	–	+	+	–	–	+	–	–	–	–
GHB614 (BCS-GH002-5)	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
LL25 (ACS-GH001-3)	–	–	–	+	–	–	–	+	+	–	–	+	–	–	–	–
MON1445 (MON-01445-2)	–	–	–	+	–	–	–	+	+	+	–	–	–*	–	–	–
MON15985 (MON-15985-7)[3]	–	–	–	+	–	–	–	+	+	–*	–	–	+	–	–	–
MON531 (MON-00531-6)	–	–	–	+	–	–	–	+	+	–*	–	–	+	–	–	–
MON88913 (MON-88913-8)	–	–	–	+	–	–	–	+	–*	+	–	–	–*	–	–	–
T304-40 (BCS-GH004-7)	–	–	–	+	–	–	–	+	+	–	–	+	–	–	–	–
Potato																
Wild type	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
EH92-527-1 (BPS-25271-9)	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–
Rice																
Wild type	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
LLRICE62 (ACS-OS002-5)	–	–	–	–	–	+	–	+	–	–	–	+	–	–	–	–
Sugar beet																
Wild type	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
H7-1 (KM-000H71-4)	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–

[1] Sequence assembled from gene cassette structure information.
[2] Sequence information obtained by merging sequences from single events DAS-24236-5 and DAS-21Ø23-5.
[3] Sequence information obtained by merging sequences from single events MON-15985-7 and MON-00531-6.
* In MON1445 and in MON88913 traces of the MON531 were detected. In MON15985 and MON531 traces of the event MON1445 were detected. In 281-24-236 × 3006-210-23, MON89788 and DP-305423 traces of GTS40-3-2 were detected.

then used for the interpretation of screening PSP results. By filling in the pattern of positive/negative results obtained, the algorithm provides the list of GM events potentially present in the analysed sample (see Event Finder Functionality in Angers-Loustau et al. (2014)).

2.2. Inter-laboratory study

To assess the applicability of the PSP-based screening strategy, 20 control laboratories from 17 countries were selected among the members of the European Network of GMO Laboratories (ENGL) that had shown an interest in participating in the study and had met pre-determined criteria (i.e. accredited for ISO17025, successful in the completion of previous comparative testing rounds, availability of complex samples and availability of compatible RTi-PCR instruments). They were entrusted with the task to re-analyse 7 real-life samples, namely official samples from control plans and known to contain GM events from maize, soybean, rapeseed and cotton. Each laboratory was provided with 8 screening PSP, master mix and a practice sample of known GM content (feedstuff flour containing soybean event GTS40-3-2 and maize event MON88017). In addition, 8 identification PSP were distributed in order to ensure that all laboratories followed an identical identification strategy allowing for a comparable assessment of the screening PSP performance. RTi-PCR instruments used by participants were the ABI 7300 (2), ABI 7500 (6), ABI 7700 (1), ABI 7900 (7), Stratagene MX3005p (2) ABI 7900HT Fast (1) and Biorad CFX 96 (1).

Participating laboratories were asked to first perform a preliminary test, analysing the practice sample to ensure they were familiar with the tool. Then, each sample was analysed by the laboratories in duplicate. DNA extraction and assessment of its quality and quantity were performed by each laboratory according to its own procedures. Analysis using the screening PSP/JRC GMO-Matrix and the identification PSP followed according to the protocols provided. A baseline ranging from cycles 3 to 15 and a threshold of 0.2 Rn were applied manually for the analysis settings of the RTi-PCR run and amplification signals with a Ct > 40 were considered negative. If samples were analysed differently (e.g. using automatic determination of baseline and threshold), then raw data was requested and re-analysed. Results obtained from the screening PSP were compared with the GM events detected by the identification PSP. This evaluation was done by ascertaining that the detection of one of the targets in the screening PSP was confirmed by the presence of at least one GM event carrying the target or, for taxon- and event-specific assays, that the detection of the target itself was confirmed. For each method of the screening PSP, comparison results were recorded in a contingency table and analysed for their concordance, sensitivity (i.e. absence of false negatives) and specificity (i.e. absence of false positives) according to the formulae described in Table 3. Methods for the 3 event-specific assays were not reported on the contingency table due to insufficient data (lack of positive samples for these events). Results for the rice, sugar beet and potato taxon reference genes methods were not reported as the identification PSP used for counter analysis did not contain methods for these taxa.

The performance of the JRC GMO-Matrix in predicting, on the basis of the screening PSP results, which GM events are present, was also assessed by comparing the results returned by the software to those obtained experimentally using the identification PSP.

Finally, identified GM events in the course of this study were used to gain an insight into the state of play of GMOs in EU food and feed. Samples were classified as containing a GM event when both replicates were positive. Inconclusive results (i.e. one replicate positive and one negative) were not considered for this last analysis; however, in these instances, the positive signal occurred always at high Ct value (Ct $\approx$ 38).

3. Results and discussion

3.1. Development and single-laboratory validation of the screening PSP

The screening PSP developed and validated in the present study allows the detection in a single PCR experiment of 74 out of the 78 plant GM events listed in the EU register (European Commission, 2003) through the combination of 16 assays. The 4 GMOs not detected are 1 authorised GM cotton (GHB614) and 3 GM events that were added to the EU register during the course of the study (2 authorized GM soybeans, MON87769 and MON87708, and 1 rapeseed GM event listed under the LLP regulation (European Commission, 2011), DP-73496). The assays (7 taxon-specific, 5 element-, 1 construct- and 3 event-specific assays) were distributed on a 96-well PCR plate, allowing the analysis in parallel of 4 DNA extracts plus the positive and negative controls (Fig. 1).

The 16 methods were successfully adapted and standardised for the PSP conditions (i.e. change in master mix, reagent concentrations, reaction volume and cycling conditions). In silico specificity tests confirmed that no cross-reactivity was to be expected between the chosen assays and the other GM events or entire crop genomes from flax, barley, common wheat, polish canola, *B. oleracea*, cotton progenitor, sugar beet, soybean, common rice, potato and maize. Similarly, molecular specificity was confirmed under the new reaction and cycling conditions for each method and amplification was observed for all expected positive assays (Table 2). As shown in Table 2, unexpected weak positive amplification signals (Ct > 36) occurred in 7 samples. These samples were further analysed on the identification PSP and the presence of another GM event, carrying the element for which weak amplifications were observed, was in each case identified. Presence of contaminants in certified reference materials is not uncommon and these signals were therefore not considered as false positive results

Table 3
Concordance of results between the identification pre-spotted plate (expected) and the screening assays (test outcome). n/a: not applicable (limited number of true positive or negative samples).

Expected	Test outcome	Variables and formulas	hmg	lec	cruA	sah7	p35s	tNOS	CTP2-EPSPS	pat	bar	cry1Ab/Ac
Pos	Pos	a	231	231	125	14	263	240	173	105	14	62
Neg	Pos	b	4	4	11	1	3	3	13	20	10	15
Pos	Neg	c	5	1	2	3	0	3	6	2	2	2
Neg	Neg	d	30	34	132	252	4	24	78	143	244	191
Sensitivity (%)		100 × (a/(a + c))	97.9	99.6	98.4	n/a	100.0	98.8	96.6	98.1	n/a	96.9
Specificity (%)		100 × (d/(b + d))	88.2	89.5	92.3	99.6	n/a	88.9	85.7	87.7	96.1	92.7
Concordance (%)		100 × ((a + d)/(a + b + c + d))	96.7	98.1	95.2	98.5	98.9	97.8	93.0	91.9	95.6	93.7

Results for event-specific assays from the screening PSP are not displayed as no positive target was detected for DAS-40278 and CV127 and only 1/270 tests was positive for DP-305423.

Fig. 1. Screening pre-spotted plate layout. 16 assays (7 taxon-specific, 5 element-specific, 1 construct-specific and 3 event-specific assays) are pre-spotted in 6 replicates throughout a 96-well PCR plate. The chosen assays and layout allow for the detection of GM material presence in 2 samples in duplicate plus a positive and negative control.

caused by the methods (Grohmann, Brünen-Nieweler, Nemeth, & Waiblinger, 2009; Trapmann, 2006; Waiblinger, Ernst, Anderson, & Pietsch, 2008).

Estimations of the limit of detection for both freshly prepared assays (data not shown) and screening PSP (Table 1) were in line with the minimum performance requirements for analytical GMO detection methods as defined by the EURL GMFF guidance document (ENGL, 2015) specifying that the LOD should be less than 25 copies.

In addition to the detection of the EU-authorised GM events, screening assays, and thus the screening PSP can be used to design a knowledge-based detection strategy for unauthorised GMOs (Holst-Jensen et al., 2012). As the restricted database of GM events sequences (Patak, 2011) used by the JRC GMO-Matrix not only includes sequences for all authorized (and with pending authorization) GMOs but also for some known unauthorized GMOs, the screening PSP was shown (in silico) to allow the detection, among others, of unauthorized rice GM events (e.g. Golden Rice 2, TT51-1, LLRICE601, T1c-19 and Bt-ZJ22), potato events (e.g. Amadea and BPS-PH048-1) and rapeseed event Oxy-235. However, although screening results can be indicative of the presence of unauthorized GMOs, further investigations are always needed for confirmation (see for example EURL GMFF webpage dedicated to detection of unauthorized events: http://gmo-crl.jrc.ec.europa.eu/emerg-unauth.html).

3.2. Inter-laboratory study

3.2.1. Evaluation of the screening PSP

Participating laboratories performed the analyses between April and May 2014. Nineteen out of the 20 laboratories performed the preliminary analysis and all obtained the expected positive results for the GM screening and taxon-specific targets, i.e. hmg, lec, cruA, p35s, tNOS and CTP2-EPSPS. All other targets tested negative with the exception of some unexpected amplifications at high Ct values for ugp (9/38 repetitions) and gs (1/38 repetitions).

Results from a total of 135 real-life samples (hence 270 DNA extracts) were taken into consideration for the performance analyses. Real-life sample results obtained with the screening PSP complied well with those observed in the identification PSP, showing a level of concordance between 91.9% and 98.9% (Table 3). More specifically, the measure of sensitivity, which indicates the rate of detected screening elements among all expected positives as identified by the identification PSP, was above 96.6% for all targets except for sah7 and bar. In the case of these 2 assays, the total number of positive targets was too limited to provide an accurate assessment of the sensitivity value (17/270 and 16/270, respectively). Values for the specificity (i.e. the rate of negative screening results among all expected negatives as determined by the identification PSP) varied between 85.7% and 99.6%. Specificity rate for p35s was not assessed since only 7 tests were expected to be negative. The sensitivity and specificity values observed in this study were slightly lower than values found in previous collaborative validation studies for the same assays (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2012; Grohmann et al., 2009; Waiblinger et al., 2008; Weighardt et al., 2004). However, in these cases, laboratory-prepared DNA mixtures were used instead of real-life samples.

In this study, most of the discrepancies observed arise from samples for which the presence of element(s) on the screening plate was not associated with the identification of GM event(s). Such cases however always showed weak positive amplification signals (Ct > 36) suggesting that GM events were indeed present at a concentration close to the limit of detection, as it was often confirmed or suspected based on the data provided by the participants of this inter-laboratory study (e.g. reports of non-quantifiable GM event presence, data not shown). Discrepancies could also partially be explained by the difference in sensitivity

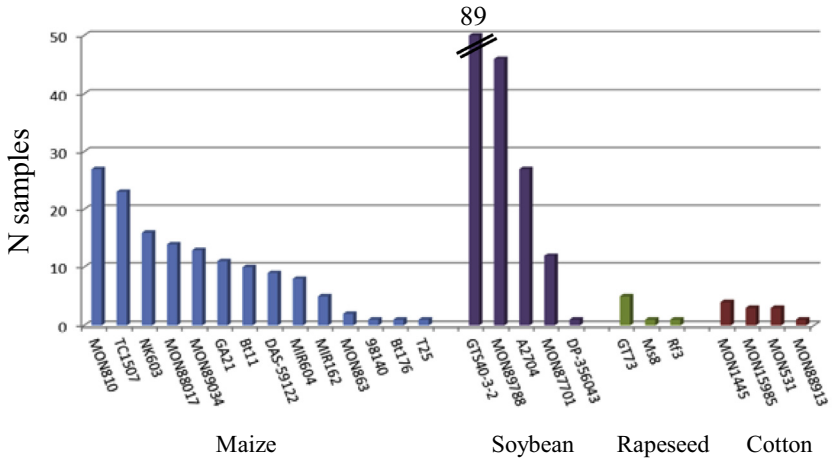

Fig. 2. GM events profile in 2014. Events detected in samples analysed in the inter-laboratory study.

between the screening and event-specific assays that were used for the counter analysis. Screening assays would be expected to be more sensitive as genetic elements can be present in more than one copy (e.g. NK603 contains two copies of p35S, CTP2-EPSPS, tNOS), while the single junction sequence targeted by event-specific assays is by definition present in a single copy. The difference in sensitivity between the two types of assays would become particularly evident when one or more GM events - carrying the same screening elements – are present at a very low concentration. Another explanation for inconsistencies might also simply be that the GM event was not detectable by the identification PSP, as would be the case for unauthorized GM events or events from crops other than those included in the identification PSP (i.e. rice, potato, sugar beet). Only one case was identified for which an event (MON89788) was detected with a low Ct (i.e. 31 in both replicates) but its corresponding screening element (CTP2-EPSPS) was not. However, the screening target was correctly detected by the screening PSP in the other 45 samples containing MON89788, thus excluding a flaw of the PSP.

Another study in which a multi-target GMO screening approach had been tested on real-life samples also reported sporadic signals at high Ct for screening assays without further identification of the corresponding GM events (Scholtens et al., 2013), confirming the challenges often presented by the analysis of this type of samples.

3.2.2. DSS (JRC GMO-Matrix) evaluation

Results obtained by the screening PSP were processed using the JRC GMO-Matrix DSS. The comparison between results predicted and results obtained experimentally on the identification PSP showed that, in a total of 842 events-specific assays that resulted positive in the 135 samples, 806 were successfully predicted by the decision support system. The unpredicted 36 positive assays (false negatives) correspond to de minimis presence of GM events detected by the identification PSP only, and are never associated with a flaw in the DSS algorithm. The web-based JRC GMO-Matrix, combined with the screening PSP, thus succeeded in predicting the events present in the analysed samples in 95.7% of the cases.

By providing an exhaustive list of GM events that can be present in a sample based on its screening results, this user-friendly matrix reduces the time and effort needed to interpret the screening results and relieves laboratories from the need to develop their own matrices. The main advantage of the JRC GMO-Matrix over other existing DSS is that it relies on in silico analyses at the level of actual GMO events' DNA sequences from the EURL GMFF restricted database. With this feature, the matrix is able to differentiate GMO elements bearing different sequences but annotated with the same name, or synthetic elements for which only partial sequences are used and renamed (e.g. the synthetic promoter SCP1, containing only a portion of the CaMV 35S promoter and used to transform the event DP-356043 (ILSI Research Foundation Washington D.C, 2012).

3.2.3. State of play of GMOs in EU food and feed

This wide inter-laboratory study provided an insight into the profile of the GM events found across the EU in 2014 (Fig. 2). A total of 14 maize, 5 soybean, 4 cotton and 3 rapeseed GM events were identified by the 20 laboratories. In maize, MON810 (27), TC1507 (23) and NK603 (16) were the most frequent GM events detected. These were also the first three GM events found in the 2011 PSP applicability study (Kluga et al., 2012) that analysed 64 commercial types of corn chips. Among soybean GM events, the GTS40-3-2 (89) was first on the list followed by MON89788 (46) and A2704 (27). Only 5 and 4 samples were found to contain rapeseed or cotton GM events, respectively. The observed GMOs thus indicate a prevalence of maize and soybean GM events in food and feed across the EU.

4. Conclusions

The proposed screening strategy for GMO detection, combining the PSP and its DSS, has been challenged in a real-life routine scenario and has proved to perform successfully with regard to results, reliability and ease-of-use.

To our knowledge, this is the first example of a multi-target PCR-based system and its associated DSS developed using a combination of laboratory and bioinformatics approaches. Indeed, the 16 methods have been selected among the EU reference methods using a bioinformatics decision support tool and their specificity predicted in silico and, afterwards, confirmed experimentally. Moreover the GMO screening system has been tested by 20 laboratories on a large number of real-life samples (135). We therefore believe that this system is a good candidate for providing a reference for screening food and feed for GMO presence in the EU.

Although the testing strategy cannot be tailored to each sample when using PSP, their use provides clear advantages for laboratories in terms of analysis time and costs, while increasing laboratory capacity. Considering that laboratories often screen for a more limited array of GM targets (Food and Veterinary Office, 2014), the widespread use of screening PSP would improve the likelihood of

detecting all the GMOs present while, at the same time, further harmonising the implementation of GMO testing.

The system here described can be easily adopted by official laboratories without the need for in-house validation of the assays or development of a decision support system. The EURL GMFF will moreover regularly update the system by including targets for the detection of newly authorized GMOs.

Finally, given the success of the PSP-based strategy for GMO analysis, the PSP tool could very well represent a model for other official control areas where high-throughput DNA-based detection systems are needed, such as for species identification, food allergens or the detection of food-borne pathogens.

Acknowledgments

This work was funded by the Joint Research Centre institutional budget and by DG SANTE of the European Commission. We thank all laboratories that have participated to the inter-laboratory study: Austrian Agency for Health and Food (Vienna, AT), Environment Agency Austria (Vienna, AT), National Centre of Public Health Protection (Sofia, BG), State General Laboratory of Cyprus (Nicosia, CY), State Office for Agriculture, Food Safety and Fishery (Rostock, DE), National Centre for Food, Spanish Food Safety Agency and Nutrition (Madrid, ES), Service Commun des Laboratoires du MINEFI (Illkirch, FR), National Food Chain Safety Office, Food and Feed Safety Directorate, (Budapest, HU), Italian National Institute for Health (Roma, IT), Veterinary Public Health Institute for Lazio and Toscana Regions (Roma, IT), National Health Laboratory (Dudelange, LU), The Netherlands Food and Consumer Product Safety Authority (Wageningen, NL), RIKILT Institute of Food Safety (Wageningen, NL), National Research Institute of Animal Production (Szczecinie, PL), Institute for Diagnosis and Animal Health (Bucharest, RO), National Food Agency (Uppsala, SE), National Institute of Biology (Ljubljana, SI), Central Control and Testing Institute of Agriculture (Bratislava, SK), National Food Reference Laboratory (Ankara, TR), LGC Limited (Teddington, UK). We thank S. Pecoraro and L. Gerdes from the Bavarian Health and Food Safety Authority (LGL) for their valuable comments on the improvement of the decision support system. We thank L. Vidmar, L. Dascalu, R. Brustio and S. Price of the Joint Research Centre for their support.

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