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An innovative and integrated approach based on DNA walking to identify unauthorised GMOs ☆

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ABSTRACT

In the coming years, the frequency of unauthorised genetically modified organisms (GMOs) being present in the European food and feed chain will increase significantly. Therefore, we have developed a strategy to identify unauthorised GMOs containing a pCAMBIA family vector, frequently present in transgenic plants. This integrated approach is performed in two successive steps on Bt rice grains. First, the potential presence of unauthorised GMOs is assessed by the qPCR SYBR®Green technology targeting the terminator 35S pCAMBIA element. Second, its presence is confirmed via the characterisation of the junction between the transgenic cassette and the rice genome. To this end, a DNA walking strategy is applied using a first reverse primer followed by two semi-nested PCR rounds using primers that are each time nested to the previous reverse primer. This approach allows to rapidly identify the transgene flanking region and can easily be implemented by the enforcement laboratories.

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1. Introduction

Rice (*Oryza sativa*), one of the most important crops in the world, is a staple food for more than three billion people. In addition, this cereal grain is also used in animal feed. The majority of rice is grown and consumed in Asia, particularly in China (Chen, Shelton, & Ye, 2011; Datta, 2004; James, 2009; Kathuria, Giri, Tyagi, & Tyagi, 2007). On the European (EU) market, most of the rice is currently imported from Asia (Stein & Rodriguez-Cerezo, 2009). In order to provide food to the growing worldwide population (approximately eight billion in 2020), rice production should increase significantly (25–40%). To this end, genetically modified (GM) rices are developed to ensure sufficient rice production in spite of the lack of arable land. According to the scientific literature on GM rice, the research in laboratories mainly target improving biotic (insect, virus, fungi, bacteria) and abiotic (drought, salinity, cold) tolerances (Ahmad et al., 2012; Chen et al., 2011;Datta, 2004; High, Cohen, Shu, & Altosaar, 2004; Kathuria et al., 2007; Yu, Wang, & Wang, 2012). The development of GM rice is highly supported by the Chinese government (Chen et al., 2011; Xia et al., 2011). Since 2009, two insect resistant GM rices (Huahui-1 and Bt Shanyou 63) are cultivated on a large scale for commercialisation in China. In addition, other insect resistant (Tarom molaii) and herbicide tolerant (CL121, CL141, CFX51, IMINTA-1, IMINTA-4, PWC16, LLRICE62, LLRICE06 and LLRICE601) GM rices are nowadays commercialised worldwide (Biosafety scanner; CERA; Chen et al., 2011; Tan, Zhan, & Chen, 2011; Wang, Zhu, Lai, & Fu, 2012; Xia et al., 2011). On the EU market, the introduction and the control of genetically modified organisms (GMOs) in the food and feed chain are submitted to the EU legislation in order to guarantee the freedom of choice to the consumer (Reg. EC no. 1829/2003; Reg. EC no. 1830/2003). However, the continuous enforcement of this legislation is complex for several reasons. First of all, the number (around 30 to 120 GMOs) and the diversity (2 to 15 genes) of commercialised GMOs will increase significantly in the 5 coming years (Stein & Rodriguez-Cerezo, 2009). Moreover, numerous vectors will be used, such as the pBin19, pBI121, pPZP and pCAMBIA families (Komori et al., 2007). Second, in addition to genes conferring insect resistance or herbicide tolerance, a larger range of traits will

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be developed (e.g., abiotic stress tolerance, disease resistance and nutritional allegations). Third, the present commercialised GM crops are principally developed by American and EU companies which have a major interest in being authorised to commercialise their products on the EU market. Nevertheless, in 2015, more and more GMOs intended for local consumption will be developed by Asian technological centres. These GM crops are unlikely to be submitted for EU approval. Thus, it is very likely that the frequency of unauthorised GMOs on the EU market will significantly increase by their accidental (or adventitious) presence in raw materials and processed food or feed (Stein & Rodriguez-Cerezo, 2009). In addition, the possible escape of GMOs from field-trials or during development stages could also be another source of unauthorised GMOs (Holst-Jensen et al., 2012; Zapiola, Campbell, Butler, & Mallory-Smith, 2008). Indeed, although the presence of GM rice is to date not authorised on the EU market, the herbicide tolerant LLRice601, in 2004, and the insect resistant Bt Shanyou 63, in 2006, and KeFeng-6, in 2010, have been detected in food products imported from China (Commission Implementing Decision No 2011/884/EU; Stein & Rodriguez-Cerezo, 2009; Wang, Zhu, Lai, & Fu, 2011). In 2012, more than 50% of the GMOs detected in food/feed matrices, reported in the RAPID Alert System Database, concerned unauthorised GM rices imported from Asia, mainly China (RASFF portal). To address the increasing number of alerts, the EU commission decided to implement "Emergency measures regarding unauthorised genetically modified rice in rice products originating from China and repealing Decision 2008/289/EC" (Commission Implementing Decision no. 2011/884/EU).

To ensure an efficient GMO detection in food and feed products on the EU market, several screening methods have been developed, mainly based on the most common recombinant elements in GM crops like transcription-regulating sequences. These elements are p35S (Cauliflower mosaic virus (CaMV) 35S promoter) and tNOS (*Agrobacterium tumefaciens* nopaline synthase terminator) (Barbau-Piednoir et al., 2010). The majority of these methods have been performed with the SYBR®Green and TaqMan® technologies (Barbau-Piednoir, Botteldoorn, Yde, Mahillon, & Roosens, 2012b; Barbau-Piednoir et al., 2010; Broeders, De Keersmaecker, & Roosens, 2012b; Kluga et al., 2012; Mbongolo Mbella et al., 2011; Reiting, Grohmann, & Mäde, 2010). However, the detection of elements derived from natural organisms (viruses and bacteria) can be misinterpreted. One of the most common examples is a p35S positive signal which could also mean the identification of the host CaMV in *Brassica* species (Broeders, Papazova, Van den Bulcke, &

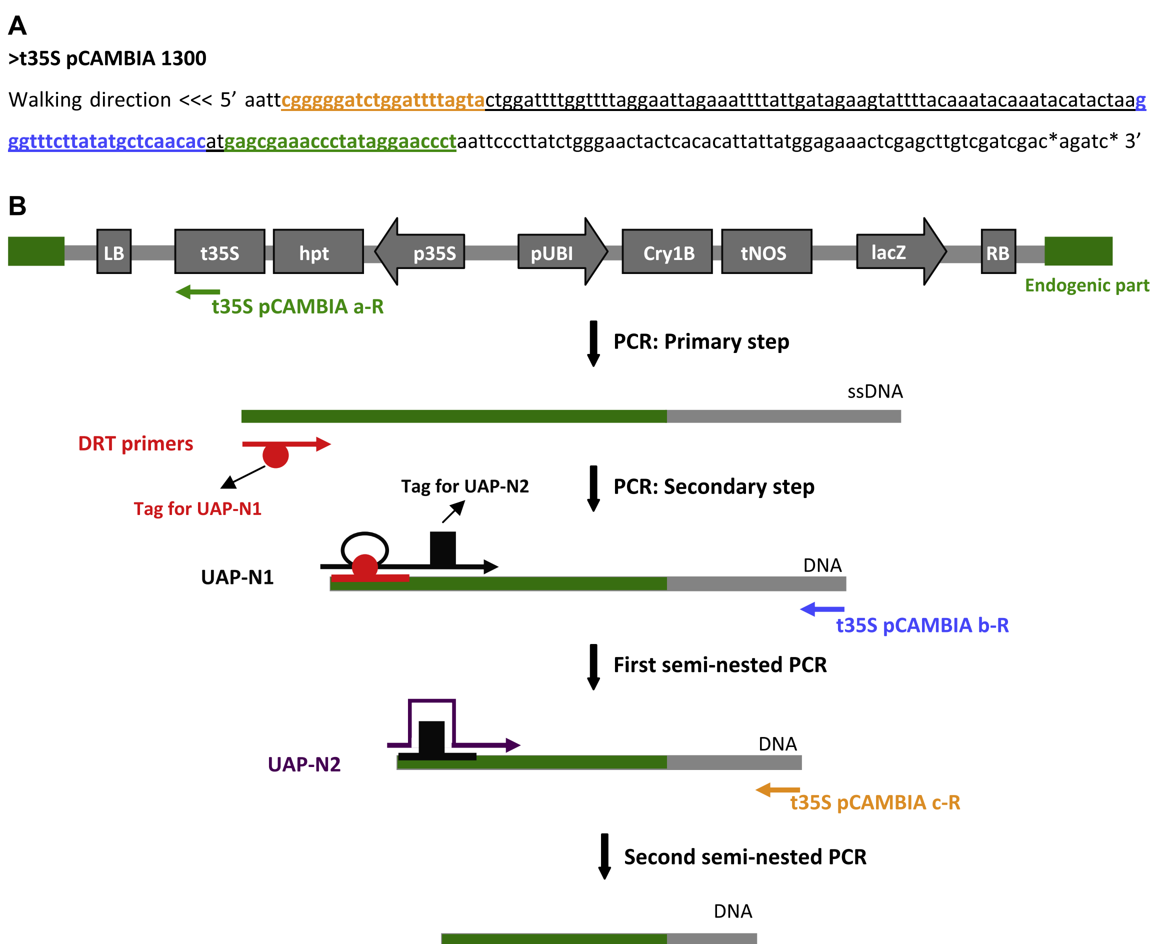
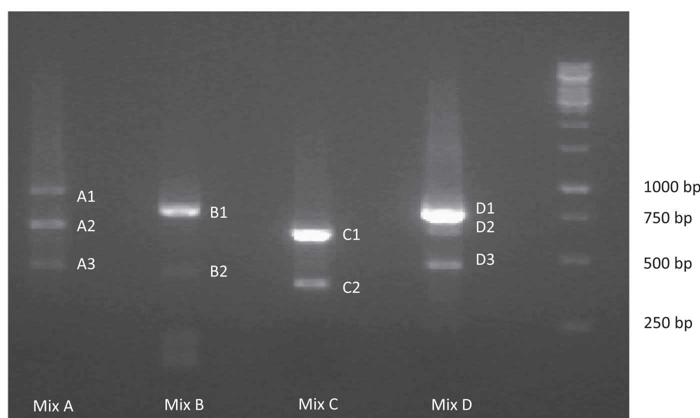


Fig. 1. DNA walking strategy. (A) Designed primer position of t35S pCAMBIA a-R (green), t35S pCAMBIA b-R (blue) and t35S pCAMBIA c-R (orange) to target t35S pCAMBIA sequence (highlighted) from t35S pCAMBIA 1300 sequence. This sequence is identical for all pCAMBIA vectors (1200, 1201, 1281Z, 1291Z, 1301, 1302, 1303, 1304, 1380, 1381Xa, 1381Xb, 1381Xc, 1381Z, 1390, 1391, 1391Xa, 1391Xb, 1391Xc and 1391Z except for 2200, 2201, 2300, 2301 which are lightly shorter at the 3′ end*). (B) Principle of DNA walking approach based on a double semi-nested PCR. Three reactions are carried out to amplify the targeted sequence. First, a two-step PCR taking place in one tube is carried out. In the first step, single strand DNA (ssDNA) fragments are produced by a single primer extension reaction using t35S pCAMBIA a-R primer (green). This reaction is repeated four times in four individual tubes. In the second step, four different DRT primers (A–D) (red) are immediately added individually to the four reaction tubes. Second, the first semi-nested PCR is obtained in combining t35S pCAMBIA b-R primer (blue) and a long universal tagging primer (UAP-N1) (black). Finally, the second semi-nested PCR is carried out by the combination of t35S pCAMBIA c-R primer (orange) and a short universal tagging primer (UAP-N2) (purple) in order to increase the yield of the specific product.

Roosens, 2012a, 2012b). Therefore, additional markers have been developed to discriminate the presence of the transgenic crop or the natural organism, such as CRT (targeting the transcriptase gene of CaMV virus) used for routine analysis in-house and CaMV (targeting the ORFIII of CaMV virus) (Broeders et al., 2012a, 2012b, 2012c; Chaouachi et al., 2008).

However, the strategy described above is merely an indirect proof of the potential GMO presence in food matrix. Direct proof can only be supplied by the characterisation of the junction between the transgenic integrated cassette and the plant genome. To get this crucial information, DNA walking methods have been used to identify this unknown nucleotide sequence flanking already known DNA regions in any given genome (Leoni, Volpicella, De Leo, Gallerani, & Ceci, 2011; Volpicella et al., 2012). Classically, three classes of strategies exist: (a) restriction-based methods, involving a preliminary restriction digestion of the genomic DNA (Jones & Winistorfer, 1992; Leoni et al., 2011; Shyamala & Ames, 1989; Theuns et al., 2002; Triglia, Peterson, & Kemp, 1988); (b) extension-based methods, defined by the extension of a sequence-specific primer and subsequent tailing of the resulting single-strand DNA molecule (Hermann et al., 2000; Leoni et al., 2011; Min & Powell, 1998; Mueller & Wold, 1989); and (c) primer-based methods, coupling various combinatorial (random or degenerate) primers to sequence-specific primers (Leoni et al., 2011; Parker, Rabinovitch, & Burmer, 1991). Up to now, some studies have been published about the junction characterisation of transgenic plants such as thale cress (*Arabidopsis thaliana*) (Ruttink et al., 2010; Windels, De Buck, Van Bockstaele, De Loose, & Depicker, 2003b), potato (*Solanum tuberosum*) (Cullen, Harwood, Smedley, Davies, & Taylor, 2011; Côte, Meldrum, Raymond, & Dollard, 2005), rice (*O. sativa*) (KeFeng-6, KeFeng-8, LLRICE62, Bt Shanyou 63 (TT51-1)) (Cao et al., 2011; Spalinskas, Van den Bulcke, Van den Eede, & Milcamps, 2012; Su, Xie, Wang, & Peng, 2011; Wang et al., 2011, 2012), maize (*Zea mays*) (MON810, MON863, MON88017, NK603, LY038, DAS59122-7, T25, 3272, Bt11, BT176, CHB351, GA21) (Collonnier et al., 2005; Holck, Va, Didierjean, & Rudi, 2002; Raymond et al., 2010; Rønning, Vaïtilingom, Berdal, & Holst-Jensen, 2003; Spalinskas et al., 2012; Taverniers et al., 2005; Trinh et al., 2012; Windels et al., 2003a; Yang et al., 2005b), cotton (*Gossypium hirsutum*) (MON1445) (Akritidis, Pasentsis, Tsaftaris, Mylona, & Polidoros, 2008), canola (*Brassica napus*) (GT73) (Taverniers et al., 2005) and soybean (*Glycine max*) (MON89788, GT40-3-2) (Raymond et al., 2010; Trinh et al., 2012; Windels, Taverniers, Depicker, Van Bockstaele, & De Loose, 2001). However, most of the methods in these studies cannot easily be used in routine analysis by the enforcement laboratories: techniques are laborious and complex (finger-

A



B

>Chromosome II

GGAAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGAACACGCGTCGTTTACCTTCGGGGCGTCATCCTCGACGACTTCGACGACG
GCGACGCCAAAGTTCCTTCGCCTAGTCGTCAAACGCCAAGGCCAAAACTTACACGCCAAGAAGAGCTAGCAGCAGATCATTAAACAAAA
CGGCACACCTCACCGACAATCCAATACTCCTACGAAATACCGATGCGAGGATTATACTAGATCCCACCGGGCCCACACGTCAGCGACCCT
CGTGCGACCCCAAAGGCGAGGAGCGGAGGCCGCCGCCCTCCGGCGGTGTGCTCTCCTCGGCCTTGCGCGAAAAGAAGATGGCAGGATT
AAAATACTCTAGGATATTCCGGAGCCCCCTTATCCAGTTCTCGTCACTGGCACGTGGGCCCCACCGTGGGCCCCACGGGAGGAAAAGGA
TGGGGGACCGCACGAGTTTTTACACTCTCACCCGTAACATTACACGTAATAAATTTCACATATACCCCTGTGGGGGTTTTTACACGCAGCC
CCCTCTACGGCTCCCCCGCGAGCGTGGCCCGCGCCGCTGACACGTGGGCCCCCAGCCGACTTAATGGCCCAACAACGCCGAATTAATT**CG**
GGGGATCTGGATTTTAGTA

>Chromosome III

GGAAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGAACACGCGTCGTTTACCTCCGGGGACCCCCTGACATCAAAACAAAAGCAG
CACCAATTTAAATTTTCCCTATTTCAGCCAAGAAATGAAACTGCAATGTTGCAATGCAATGCCATTTGGTATGTGCAGTGTGTGCAGGAG
GAGGAGACACGAGCGGCAGCTATAGCCGGAGATTTAACCTTGATGAAGGTGAGTCACTTACTACAATGCAGTACTTGCTGCTGCGGACG
TAGCTGCTGTGGATGACCTGCACGAACACCGGGTTCCCGGGCTGAACCGGCACGGGGACGCGCGAGATCTCCTCCCTGTCTTCCTCCCTC
AGGTTGTACTCGGCCACCCGTCCGTGCCTGCTGAAGTAGTACTCCCCGTAGCGCCCGCCTTCCGCGGCCGCGACAACATCTTCATCTTCCC
CATCGTTTTCTTTCGCTTCTGCAGGTGGCAGACACGCATATAAGTTACAATGCCGATTTGGGAGCTTTTAGCTGCTGCATATTCTACCACAT
TGTTACCCAGATTCTCCTAATATGTAGTTGTATAATCCAGAAAATAAACTAGAACCAGAAGCTAGAAAACCTAGCTTCTCCAAATTCTTAG
AAGCTGACTTCCAAACAGCGGATTCTCATCTTAAGCTCACCAAACAGGGCCTATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAAT
AACACATTGCGGACGTCTTTAATGTACTGAATTAACGCCGAATTAATT**CGGGGGATCTGGATTTTAGTA**

Fig. 2. Characterisation of the junction between the integrated transgenic pCAMBIA cassette and the rice genome. (A) Visualisation of the amplicons obtained with the different DRT mixes (A–D) on a 1% agarose gel. (B) Amplicon sequences presenting the junction between the pCAMBIA 1300 vector (underlined) and the rice genome identified on the chromosome II and the chromosome III, respectively. The t35S pCAMBIA c-R (in bold) and the UAP-N1/UAP-N2 primers are dotted-underlined. These sequences were obtained by classic sequencing of the plasmids.

printing by capillary electrophoresis, genomic DNA library via (unpredictable) restriction enzyme) with regard to a method exclusively based upon PCR, require a lengthy procedure with generally multiple steps to get results, or present a lack of specificity, yield or data concerning the compatibility with a low amount of target.

The aim of the present study is to supply an integrated approach to identify unauthorised GMOs: A first real-time PCR screening allows the detection of the terminator 35S (t35S) of the pCAMBIA family vectors to indicate the potential presence of unauthorised GMOs in food matrices (Fig. 3). Then, an appropriate DNA walking method, anchored on the sequence used for the screening followed by two semi-nested PCRs to identify the junction, confirms the GMO presence.

2. Materials and methods

2.1. Plant material

Grains of transgenic Bt rice (*O. sativa* L. *Japonica cv Ariete*) and its wild-type (WT) were used in this study to develop the methodology (Breitler et al., 2004). This transgenic rice was transformed by *A. tumefaciens* with the binary vector pCAMBIA1300, which contains the synthetic Cry1B gene from *Bacillus thuringiensis* conferring insect resistance. The Certified Reference Materials (CRM) in the form of seeds powders or genomic DNA (gDNA) were obtained from the American Oil Chemists' Society and the Institute for Reference Materials and Measurements and were used to test the specificity (AOCS, Urbana, USA; IRMM, Geel, Belgium). These materials were characterised as previously described (Broeders et al., 2012c). The list of all plant material is shown in Table 1.

2.2. DNA extraction, concentration and purity

Bt rice grains were ground to obtain a homogenous powder. DNA was extracted using a CTAB-based procedure (ISO 21571) in combination with the Genomic-tip20/G (QIAGEN, Hilden, Germany). This DNA extraction method, adapted from the EU-RL GMFF validated method, is composed of four main successive steps: (1) Extraction of proteins, polysaccharides and organic components, (2) Precipitation of DNA in presence of C-hexadecyl-Trimethyl-Ammonium-Bromide (CTAB), (3) Purification of DNA using a tip20 column and (4) Precipitation of DNA with isopropa-

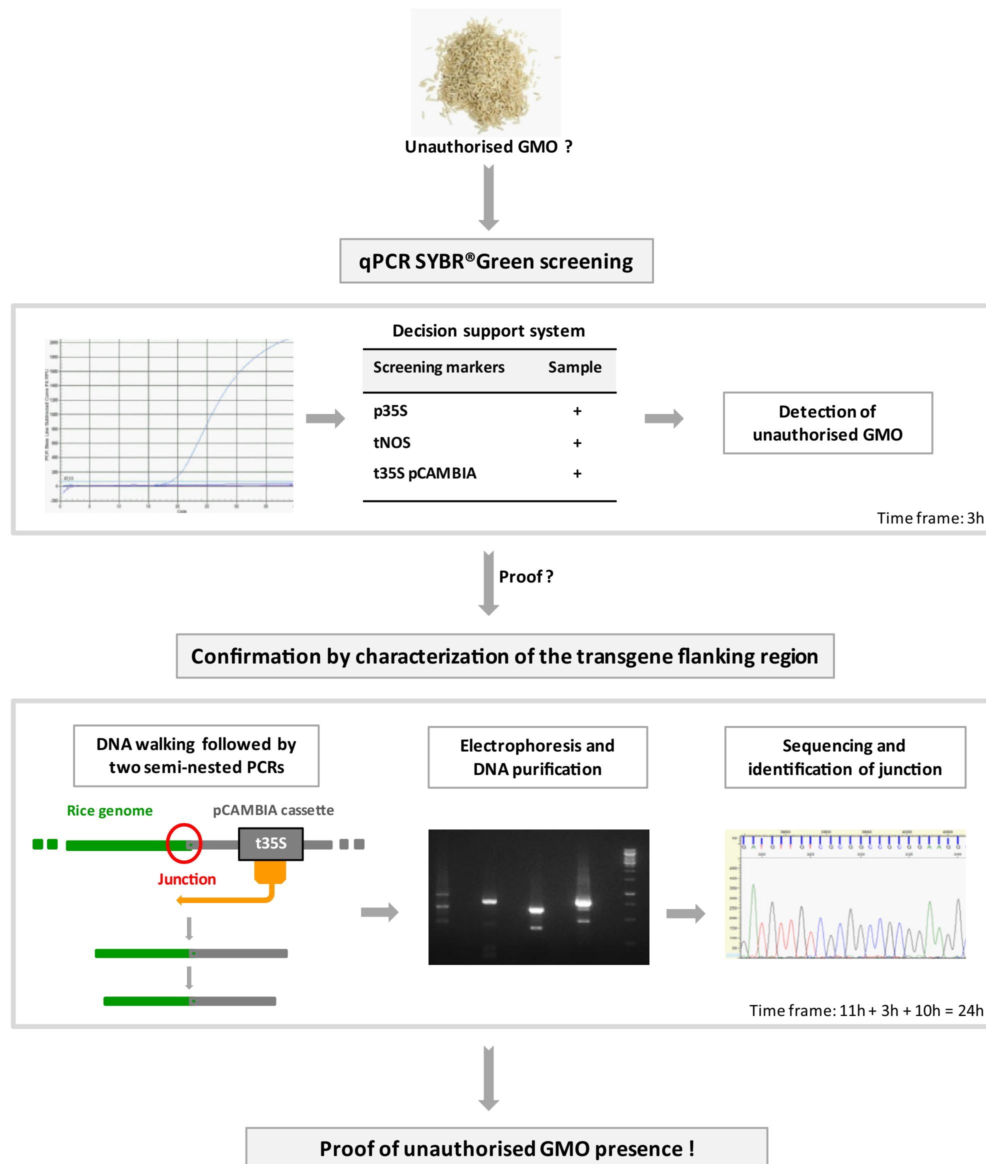


Fig. 3. Workflow of the present integrated approach.

Table 1
Specificity of t35S pCAMBIA marker tested on plant materials observed *in silico* and *in vitro* by qPCR SYBR®Green assay. The positive and the negative signals obtained are indicated by + and –, respectively.

Species	Plant materials	GMO% (m/m)	*In silico*	*In vitro*
Maize (*Zea mays*)	WT[a]	0	–	–
	MON 810[a,c]	1	–	–
	MON 89034[a]	1	–	–
	MON 863[a,c]	1	–	–
	MON 88017[a,c]	1	–	–
	Bt176[a,c]	1	–	–
	3272[a,c]	10	–	–
	DAS59122[a,c]	9.87	–	–
	TC1507[a,c]	1	–	–
	T25[a,c]	100	–	–
	Bt11[a,c]	1	–	–
	GA21[a,c]	1	–	–
	NK603[a,c]	1	–	–
	MIR604[a,c]	1	–	–
	MIR162[a,c]	1	–	–
	98140[a]	1	–	–
Soybean (*Glycine max*)	WT[a]	0	–	–
	GTS40-3-2[a,c]	10	–	–
	A2704-12[a,c]	100	–	–
	A5547-127[a,c]	100	–	–
	MON 89788[a]	1	–	–
	305423[a]	1	–	–
	356043[a]	1	–	–
	MON 87701[a]	1	–	–
Oilseed rape (*Brassica napus*)	WT[a]	0	–	–
	RT73[a]	1	–	–
	MS8[a,c]	100	–	–
	Rf3[a,c]	100	–	–
	T45[a,c]	100	–	–
	Ms1[a,c]	1	–	–
	Rf1[a,c]	1	–	–
	Rf2[a,c]	1	–	–
	Topas 19/2[a,c]	1	–	–
Cotton (*Gossypium hirsutum*)	WT[a]	0	–	–
	MON 1445[a,c]	1	–	–
	MON 15985[a,c]	100	–	–
	MON 531[a,c]	100	–	–
	LL25[a,c]	100	–	–
	GHB614[a]	100	–	–
	GHB119[a]	10	–	–
	281-24-236 x 3006-210-23[a]	1	–	–
	MON 88913[a]	1	–	–
Potato (*Solanum tuberosum*)	WT[a]	0	–	–
	EH92-527-1[a,c]	1	–	–
Sugar beet (*Beta vulgaris*)	WT[a]	0	–	–
	H7-1[a]	100	–	–
Rice (Oryza sativa)	WT[a]	0	–	–
	LLRICE62[a,c]	1	–	–
	Bt rice	100	+	+
Plasmid	Sybricon t35S pCAMBIA[b]	/	+	+

[a] CRM (Certified Reference Materials).
[b] Plasmid pUC18 containing t35SpCAMBIA amplicon.
[c] GMOs containing the p35S and/or tNOS elements (Barbau-Piednoir et al., 2010).

nol (European Union Reference Laboratory, 2006; International Standard ISO 21571, 2005). DNA concentration was measured by spectrophotometry using the Nanodrop® 2000 (ThermoFisher, DE, USA) device and DNA purity was evaluated by the A260/A280 and A260/A230 ratios. DNA extraction, concentration and purity of CRMs were carried out as previously described (Broeders et al., 2012c).

2.3. Development and assessment of oligonucleotide primers

The oligonucleotide primers were designed to target the t35S sequence of the pCAMBIA vector (Fig. 1). To get universal oligonucleotide primers detecting all pCAMBIA vectors, all t35S pCAMBIA sequences were compared via the software "ClustalW2". The oligonucleotide primers were thus designed manually on the conserved region of all pCAMBIA vectors. To be convenient for the DNA walking approach, these oligonucleotide primers were chosen at the nearest extremity of the walking direction. Note that the t35S pCAMBIA element is the starting position and the walking direction is defined on the rice genome through the left border of the transgenic cassette (Cambia, Canberra, Australia; ClustalW2). Via a different combination, the same oligonucleotide primers were usable for qPCR assays. The oligonucleotide primers and the obtained amplicon sequences are indicated in Table 2 and Fig. 1. The specificity of oligonucleotide primers was initially evaluated *in silico* using the program "wprimersearch" from the software "wEMBOSS", which mimics PCR amplification (Barbau-Piednoir et al., 2012b; wEMBOSS) (Table 1).

2.4. qPCR SYBR®Green technology

As previously described, for all qPCR assays, a standard 25 µl reaction volume was applied containing 1× SYBR®Green PCR Mastermix (Diagenode, Liège, Belgium), 250 nM of each primer and 5 µl of DNA (10 ng/µl). The qPCR cycling program consisted of a single cycle of DNA polymerase activation for 10 min at 95 °C followed by 40 amplification cycles of 15 s at 95 °C (denaturing step) and 1 min at 60 °C (annealing–extension step). The program for melting curve analysis was performed by gradually increasing the temperature from 60 to 95 °C in 20 min (±0.6°/20 s) (Barbau-Piednoir et al., 2010; Broeders et al., 2012c). All runs were performed on an iQ™5 real-time PCR detection system (BioRad, Hemel Hempstead, UK) or an ABI 7300 qPCR system (Applied Biosystems, CA, USA) for the specificity assessment and the rest of the analysis, respectively.

Concerning the qPCR method acceptance parameters, evaluation of specificity, sensitivity and inter-run repeatability was carried out as previously described (Broeders et al., 2012c). In brief, the specificity of the t35S pCAMBIA c-F and the t35S pCAMBIA a-R primers was tested on several WTs, GMOs and LLPs (Low Level Presence) by qPCR SYBR®Green method using C_t and T_m values as criteria (Tables 1 and 2) (Reg. EC no. 619/2011). Sensitivity and repeatability were determined for t35S pCAMBIA primers on Bt rice using the qPCR SYBR®Green method on serial dilutions going from 2000 to 0.1 haploid genome equivalents (HGEs) (Tables 2 and 4). From these serial dilutions, the PCR efficiency and linearity (R^2) were estimated.

2.5. Cloning, sequencing and plasmid registration

The t35S pCAMBIA amplicon was cloned into a pUC18 plasmid (INVITROGEN, CA, USA) to obtain the t35S pCAMBIA Sybricon as previously described (Barbau-Piednoir et al., 2010; Broeders et al., 2012c; Sambrook & Russell, 2001). Briefly, the t35S pCAMBIA amplicon was first subcloned into the pCR®2.1-TOPO® Vector using the TOPO TA Cloning® Kit (INVITROGEN, CA, USA) according to the manufacturers' instructions. After EcoRI restriction, the correct amplicon was then cloned into the vector pUC18 (INVITROGEN, CA, USA). The plasmid was sequenced via a Genetic Sequencer 3130XL using the Big Dye Terminator Kit v3.1 (Applied Biosystems, CA, USA) and was tested using the qPCR reaction conditions and the specific primers as indicated in point 2.4. The t35S pCAMBIA Sybricon plasmid was registered under "Safe Deposit" at the "Belgian Culture Collection for Micro-organisms" in the "Plasmid and DNA Library Collection" (BCCM/LMBP, Gent, Belgium; BCCM number: LMBP 8352). Authenticity was assessed by the BCCM/LMBP prior to acceptance and certification (Barbau-Piednoir et al., 2010; Broeders et al., 2012c).

2.6. DNA walking approach

2.6.1. DNA walking and double semi-nested PCR

The assay was performed using 100 ng of 100% Bt rice DNA (Fig. 1). Degenerated random tagging (DRT) and Universal tagging primers (UAP-N1 and N2) were provided by APAgene™ GOLD Genome Walking Kit (BIO S&T, Montréal, Canada). Recombinant *Taq* DNA Polymerase (10342; INVITROGEN, CA, USA) was used to synthesise DNA. The three gene-specific primers for t35S pCAMBIA were designed as described above (Section 2.3). The t35S pCAMBIA a-R primer was used to perform the DNA walking and then the t35S pCAMBIA b-R and the t35S pCAMBIA c-R primers were applied in the first and the second semi-nested PCR rounds, respectively. PCR mixes and conditions were carried out according to the manufacturers' instructions. The final PCR product was separated by electrophoresis on a 1% agarose gel (INVITROGEN, CA,

Table 2
Oligonucleotide sequences used for qPCR assays, DNA walking approach and PCR amplifications. In the purpose of an integrated approach, the same t35S pCAMBIA oligonucleotides are used for the qPCR as well as for the DNA walking. PLD marker (Phopholipase D gene from rice (*Oryza sativa*)) is used as the rice endogene control.

Method	Oligonucleotide name	Oligonucleotide sequence	Product size (bp)	Reference
SYBR®Green qPCR	p35S F p35S R	AAAGCAAGTGGATTGATGTGATA GGGTCTTGCGAAGGATAGTG	75	Barbau-Piednoir et al. (2010)
SYBR®Green qPCR	tNOS F tNOS R	GATTAGAGTCCCGCAATTATACATTTAA TTATCCTAGKTTGCGCGCTATATT	69	Barbau-Piednoir et al. (2010)
SYBR®Green qPCR	t35S F t35S R	Data not shown Data not shown	107	In-house, Broeders et al. (2012b)
SYBR®Green qPCR	PLD F PLD R	GCTTAGGGAACAGGGAAGTAAAGTT CTTAGCATAGTCTGTGCCATCCA	80	Mbongolo Mbella et al. (2011)
SYBR®Green qPCR	t35S pCAMBIA c-F t35S pCAMBIA a-R	CGGGGGATCTGGATTTTAGTA AGGGTTCCTATAGGGTTTCGCTC	137	This study
DNA walking	t35S pCAMBIA c-R t35S pCAMBIA b-R t35S pCAMBIA a-R	TACTAAAATCCAGATCCCCCG GTGTTGAGCATATAAGAAACCC AGGGTTCCTATAGGGTTTCGCTC	/	This study
PCR	Rice chromosome II-F pCAMBIA-R	CGAAAAGAAGATGGCAGGAT CTGTCGATCGACAAGCTCGAGT	490	This study
PCR	Rice chromosome III-F pCAMBIA-R	TTTCTTTCGCTTCTGCAGGT CTGTCGATCGACAAGCTCGAGT	515	This study

Table 3
Analysis of Bt rice identity *in silico* and *in vitro* by qPCR SYBR®Green assay. The positive and the negative obtained signals are indicated by + and –, respectively.

	p35S		tNOS		t35S		t35S pCAMBIA		PLD	
	In silico	*In vitro*	*In silico*	*In vitro*	*In silico*	*In vitro*	*In silico*	*In vitro*	*In silico*	*In vitro*
WT Bt rice	–	–	–	–	–	–	–	–	+	+
Bt rice	+	+	+	+	–	–	+	+	+	+

Table 4
LOD_6 (Limit Of Detection with 6 repeats) of t35S pCAMBIA screening marker for 100% Bt rice in SYBR®Green qPCR assay obtained at 5 HGEs (Haploid Genome Equivalent) (in bold). The inter-run repeatability is determined as the relative standard deviation of the test results (RSD_r %).

Dilution	HGE/well	Final DNA concentration (ng/µl)	Final DNA concentration/well (ng)	Dilution factor	RSD_r (%)
Stock		4	/		
S1	2000	0.2	1	20	1.5
S2	1000	0.1	0.5	2	1.7
S3	400	0.04	0.2	2,5	1.1
S4	100	0.01	0.05	4	0.9
S5	50	0.005	0.025	2	1.8
S6	20	0.002	0.01	2,5	0.9
S7	10	0.001	0.005	2	2.0
S8	**5**	**0.0005**	**0.0025**	**2**	**2.2**
S9	2	0.0002	0.001	2,5	
S10	1	0.0001	0.0005	2	
S11	0.1	0.00001	0.00005	10	

USA) (100 V, 400 mA, 60 min). The amplicons were retrieved by excising the specific band from the gel and were purified using the QIAEX® Agarose Gel Extraction Kit (QIAGEN, Hilden, Germany).

2.6.2. Cloning and sequencing

Two sequencing strategies have been used. On the one hand, the purified amplicons were directly sequenced using the t35S pCAMBIA c-R primer to get information on the sequences including the junction between the transgenic integrated cassette and the plant genome (direct sequencing). On the other hand, each purified amplicon was cloned into the pCR®2.1-TOPO® Vector using the TOPO TA Cloning® Kit (INVITROGEN, CA, USA) according to the manufacturers' instructions. A PCR was carried out on colonies using PCR™2.1-TOPO® and t35S pCAMBIA c-R primers and analysed by electrophoresis on a 1% agarose gel (INVITROGEN, CA, USA) (100 V, 400 mA, 60 min). The colonies possessing a fragment of the correct size were further cultured. The plasmids were extracted, using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) according to manufacturers' manual, to be sequenced (classic sequencing). All sequencing reactions were performed on a Genetic Sequencer 3130XL using the Big Dye Terminator Kit v3.1 (Applied Biosystems, CA, USA) (Broeders et al., 2012c; Sambrook & Russell, 2001). The obtained sequences were aligned via the software "ClustalW2" and then analysed using the software "Nucleotide BLAST NCBI" (ClustalW2; Nucleotide BLAST NCBI).

2.6.3. Verification of the transgene flanking regions by PCR amplification

The transgene flanking regions identified by DNA walking were verified by PCR amplification. The PCR was carried out using a reverse primer designed in downstream to the t35S pCAMBIA a-R primer on the pCAMBIA construct and a forward primer designed on the rice chromosome II or III (Table 2). These oligonucleotide primers were initially evaluated *in silico* using the program "wprimersearch" from the software "wEMBOSS" (wEMBOSS). A standard 25 µl reaction volume is applied containing 0.625 U of DreamTaq™ DNA Polymerase (Fermentas, CA, USA), 1× DreamTaq™ Buffer (Fermentas, CA, USA), 0.2 mM of dNTPs, 250 nM of each primer and 5 µl of DNA (10 ng/µl). The PCR program consisted of a single cycle of 10 min at 95 °C (initial denaturation) followed by 35 amplification cycles of 30 s at 95 °C (denaturation), 30 s at 60 °C (annealing) and 1 min at 72 °C (extension) and finishing by a single cycle of 10 min at 72 °C (final extension). The run was performed on an iQ™5 real-time PCR detection system (BioRad, Hemel Hempstead, UK). The PCR products were analysed by electrophoresis on a 1% agarose gel (INVITROGEN, CA, USA) (100 V, 400 mA, 60 min). The PCR products were purified using USB® ExoSAP-IT® PCR Product Cleanup (Affymetrix, CA, USA) according to the manufacturers' instructions. All sequencing reactions were performed on a Genetic Sequencer 3130XL using the Big Dye Terminator Kit v3.1 (Applied Biosystems, CA, USA) (Broeders et al., 2012c; Sambrook & Russell, 2001). The obtained sequences were analysed using the software "Nucleotide BLAST NCBI" (ClustalW2; Nucleotide BLAST NCBI).

3. Results and discussion

3.1. Selection of the t35S pCAMBIA screening marker and development of a qPCR SYBR®Green assay

Considering the high diversity of genetic elements integrated in GM rices, our attention was focused on rice transformation vectors. Because of its presence in 30% of transgenic plants and, more particularly, in 65 and 53 peer reviewed publications on GM rices in 2011 and 2012, respectively, the pCAMBIA family vector was considered as a strategic target to detect a large spectrum of unauthorised GMOs (Ahmad et al., 2012; Kathuria et al., 2007; Komori et al., 2007; Scopus; Yu et al., 2012). The t35S pCAMBIA screening marker was thus developed to identify unauthorised GMOs containing a pCAMBIA family cassette. The t35S pCAMBIA-specific SYBR®Green screening method, generating an amplicon of 137 bp, was performed for integration in to the CoSYPS (Combinatory SYBR®Green qPCR Screening) for GMO detection, composed of 18 SYBR®Green methods (RBCL, LEC, ADH, CRU, PLD, SAD1, GLU3, p35S, tNOS, pFMV, pNOS, t35S, Cry1Ab/Ac, Cry3Bb, pat, bar, epsps and CRT), which is able to run in a single 96-well plate (Barbau-Piednoir et al., 2010; Barbau-Piednoir et al., 2012a; Broeders et al., 2012a, 2012b, 2012c; European Union Reference Laboratory for GMFood & Feed, 2006; Mbongolo Mbella et al., 2011; Van den Bulcke et al., 2010; Vaïtilingom, Pijnenburg, Gendre, & Brignon, 1999; Yang et al., 2005a).

3.1.1. Analysis of Bt rice in silico and in vitro

The general structure of pCAMBIA vector is composed notably of p35S, tNOS and t35S elements (Cambia, Canberra, Australia). Bioinformatics studies have shown that the common methods, including those described in the compendium of reference methods for GMO analysis, allow detection of p35S and tNOS (Data not shown, Table 3) (Barbau-Piednoir et al., 2010; Corbisier et al., 2005; Fernandez et al., 2005; Höhne, Santisi, & Meyer, 2002; Joint Research Centre, 2011; Reiting, Broll, Waiblinger, & Grohmann, 2007; Waiblinger, Ernst, Anderson, & Pietsch, 2008). Concerning the t35S element of the pCAMBIA family vectors, its sequence was slightly different at the 5′ end compared to the authorised GMOs and LLPs events containing a t35S element (A2704-12, A5547-127, Bt11, Bt176, DAS59122, GHB119, LLRICE62, T25, TC1507 and Topas-19-2). Therefore, this element was not detected by the t35S SYBR®Green detection method developed previously in-house (Broeders et al., 2012b; Personal

communication). All these bioinformatics data were confirmed *in vitro* by qPCR SYBR®Green assay (Table 3).

3.1.2. Development of the t35S pCAMBIA screening marker

In order to discriminate unauthorised GMOs containing pCAMBIA family vectors, the t35S pCAMBIA screening marker was developed. To this end, the sequence of the t35S pCAMBIA element was analysed. The majority of the pCAMBIA vectors (1200, 1201, 1281Z, 1291Z, 1300, 1301, 1302, 1303, 1304, 1380, 1381Xa, 1381Xb, 1381Xc, 1381Z, 1390, 1391, 1391Xa, 1391Xb, 1391Xc, 1391Z, 2200, 2300, 2301), except 0380 and 0390, possessed the t35S element. Its sequence was practically identical (slightly shorter by 5 bp at the 3′ end for 2200, 2201, 2300 and 2301). The t35S pCAMBIA a-R and t35S pCAMBIA c-F primers were designed manually in the conserved region of the pCAMBIA family vector to discriminate exclusively this element (Tables 1 and 2). The specificity of this marker was tested initially *in silico* with the software wEMBOSS.

3.1.3. qPCR SYBR®Green assay

To develop the t35S pCAMBIA marker, the specificity of t35S pCAMBIA c-F and a-R primers was tested *in vitro* on all authorised GMOs and LLPs events by the qPCR SYBR®Green assay (Tables 1 and 2). As expected, only the Bt rice, containing a pCAMBIA cassette, was detected after 40 cycles with a C_t value at 22.70 and a T_m value at 73 °C, indicating that the screening marker was specific. All the other WT, GMO and LLP materials tested did not give a signal after 40 cycles. Then, the sensitivity of this marker was determined via the limit of detection with 6 repeats (LOD_6). The LOD_6 is defined as the amplicon copy number that affords a positive PCR result (expressed as C_t-value) upon six-fold measurement of the target sequence in the same DNA sample (Table 4). To this end, DNA from 100% Bt rice was diluted to 4 ng/μl and 4 independent dilution series were prepared (in nuclease-free water) starting from this concentration. The dilution series (from 1 to 0.00005 ng/μl of DNA) were prepared prior to setting up each of the qPCR runs. For each assay, a range from 2000 to 0.1 HGEs was tested in a qPCR SYBR®Green assay. The HGE content of the DNA extracts was calculated according to the size of the rice genome (0.5 pg) (Arumuganathan & Earle, 1991). The LOD_6 was obtained at 5 HGEs (corresponding to 0.0025% of unauthorised GMOs) with a mean C_t value of 35.28 Ct and a mean T_m value of 72.52 °C. Because the LOD_6 was observed below a HGE of 20, the t35S pCAMBIA screening marker was considered as being sensitive. The experiment was also evaluated as repeatable according to the RSD_r percentage (relative standard deviation, RSD, of the test results). In addition, the PCR efficiency (80%) and the linearity (R^2 = 0.9954) were assessed as acceptable.

3.2. DNA walking approach

Following a positive signal observed in qPCR SYBR®Green assay, the second step was to characterise the junction between the transgenic cassette and the plant genome to confirm the presence of pCAMBIA unauthorised GMOs in food/feed matrices (Fig. 3). Therefore, a DNA walking approach has been developed.

3.2.1. In silico study

In order to supply an integrated approach, an additional oligonucleotide primer, named t35S pCAMBIA b-R, was designed manually, on the conserved region of the t35S pCAMBIA sequence, localised between the t35S pCAMBIA a-R and t35S pCAMBIA c-R primers previously used for the qPCR SYBR®Green assay (Fig. 1 and Table 2). The specificity of this primer was confirmed *in silico* via the software wEMBOSS (data not shown).

3.2.2. Characterisation of the junction

The amplicons resulting from the double semi-nested PCR were visualised on a 1% agarose gel. For each kind of DRT primers mixes (A–D), amplicons were observed with an approximate size of 300 bp up to 1000 bp (Fig. 2A). The identity of the amplicons was confirmed by direct sequencing of the purified PCR products. The sequencing of the plasmids containing these amplicons allowed identifying the t35S pCAMBIA c-R and UAP-N1/UAP-N2 primers and determining the exact size of the amplicons (408–944 bp) (Fig. 2). All analysed amplicons presented a sequence including the junction between the pCAMBIA vector and the rice genome. Two transgenic insertions have been detected. For the majority of the amplicons (A2, A3, B1, C2, D1, D2 and D3), the pCAMBIA cassette was integrated on a genomic sequence (OSJNBb0111B07) from the chromosome III of *O. sativa* japonica Group coding for a putative uncharacterised protein. For the three other amplicons (A1, B2 and C1), the transgene flanking region was localised on a genomic sequence (OSJNBa0016G10) from the chromosome II of *O. sativa* japonica Group coding for a putative uncharacterised protein. These transgene flanking regions present a shorter left ends compared to the pCAMBIA cassette situated on the chromosome III. This variability of length could be explained by the fact that a left end integrates less precisely than a right end (Gheysen, Villarroel, & Van Montagu, 1991; Krizkova & Hrouda, 1998). To confirm the two chromosomal insertions, a PCR amplification using primers annealing to the pCAMBIA construct and the rice chromosome II or III was carried out (Table 2). The presence of PCR amplification as well as the sequencing of these amplicons allowed verifying properly the transgene flanking regions (data not shown).

As all the obtained amplicons provided the transgene flanking regions, these results demonstrated the high efficiency, specificity and reliability of the present integrated approach. In addition, it's interesting to note that the two different transgene flanking regions were not identically detected in function of the DRT primers used. Indeed, all the types of DRT primers allowed identifying the junction on the chromosome III while only the A, B and C DRT primers have detected the junction on the chromosome II. This system using four different DRT primers thus presents the advantage to increase the likelihood to detect unauthorised GMOs, independently of the tested matrices.

The proposed strategy is based on the presence of known transgenic elements. Consequently, the success of this integrated approach is limited to the knowledge level of transgenic elements making up unauthorised GMOs. Therefore, in spite of the good performance of this method, the strategy is not appropriate to detect GMOs constituted of only unknown elements. To this end, other technologies are more suitable such as "Next Generation Sequencing" methods. However, this last technique is at the present time not easily implementable in GMO routine analysis due to its high cost and its long time frame for data processing.

4. Conclusion

Considering the numerous unauthorised GM rices detected in food/feed matrices on the EU market listed in 2012, as well as their expected increase in the coming years, this study supplies to the enforcement laboratories a strategy to ensure the unauthorised GMO detection in the food and feed chain in semi-routine analysis (RASFF portal; Stein & Rodriguez-Cerezo, 2009).

The proposed integrated approach is composed of two main steps (Fig. 3). On the one hand, the potential presence of unauthorised GMOs, containing a pCAMBIA family vector, in food/feed matrices is detected via the qPCR SYBR®Green technology. The key choice to target the pCAMBIA family vector, via its element t35S, will allow detection of a large spectrum of unauthorised GMOs. The t35S pCAMBIA marker was developed to be specific,

sensitive, efficient, repeatable and to be integrated into the CoSYPS. On the other hand, once this marker is indicated as positive for a given food/feed matrix, the potential presence of unauthorised GMOs, containing a pCAMBIA vector, is demonstrated by the characterisation of the junction between the integrated cassette and the plant genome using a DNA walking method starting from the t35S pCAMBIA a-R primer. This method is then followed by two semi-nested PCR rounds using the t35S pCAMBIA b-R and t35S pCAMBIA c-R primers, respectively. With regard to the previous articles describing methods characterising the junction sequences of GMOs, the present DNA walking approach possesses the substantial advantage to be easily implementable in semi-routine use thanks to the simplicity of a method exclusively based on PCR. In addition, its short time frame to get the results (less than three days including DNA walking method, DNA purification and direct sequencing) and its relatively low cost clearly represent a crucial benefit for the enforcement laboratories.

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