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ORIGINAL PAPER

Two quantitative multiplex real-time PCR systems for the efficient GMO screening of food products

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Abstract According to the EU and Swiss food legislation, only deregulated traits of transgenic plants are allowed to be imported and sold to the consumer. In order to control imports of soya and maize products from retailers, efficient and reliable methods for the detection and quantification are a prerequisite. The screening for specific DNA elements characteristic of transgenic plants is crucial for further analysis and has a major impact on the efficiency of the whole analysis workflow. To allow laboratories to efficiently and reliably screen food products for transgenic plant products, two novel multiplex real-time polymerase chain reaction (PCR) systems were developed and validated. One system determines DNA contents from maize, soya, cauliflower mosaic virus (CaMV) 35S promoter (P35S), NOS terminator from Agrobacterium tumefaciens and CaMV, and the second PCR system simultaneously detects DNA sequences from figwort mosaic virus promoter (PFMV), from bar gene of S*treptomyces hygroscopicus, from* gene coding for phosphinothricin acetyltransferase (PAT) and from a DNA construct of enolpyruvyl shikimate phosphate synthase gene (CP4-EPSPS) and *Arabidopsis thaliana* (*CPT2*). The tests exhibit good specificity and a limit of detection of at least 0.1 % for all analytes.

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Introduction

Ever since the introduction of the first transgenic plant, the Flaver Savr^{TM} tomato, many other transgenic or genetically modified plants (GMO) have been developed and to some extent successfully commercialized. The number of commercially available transgenic plants is constantly rising. Hence, the variety as well as the number of all possible target sequences that need to be detected is steadily increasing, which represents immense analytical effort and great challenge.

According to the EU and Swiss legislation for food, only deregulated traits of transgenic plants are allowed to be imported and sold to the consumer. If the product contains more than 0.9 % of a deregulated transgenic plant material, it has to be labelled. The lion's share of all GMO traits still remains among the crops maize and soya. In order to control imports of soya and maize products from retailers, efficient and reliable methods for the detection and quantification are a prerequisite. Most of the transgenic plants contain constructs with sequences of the cauliflower mosaic virus 35S promoter (PFMV) and/or the NOS terminator from Agrobacterium tumefaciens (Tnos). Therefore, these gm constructs are suitable as detection gene markers in screening assays and for the quantification of GMO fraction in food. It is important to estimate the overall GMO content of the tested sample in the initial screening step in order to determine whether further analysis is required. Historically, many different single PCR systems are applied for the purpose of testing raw and processed products on the presence of GMO. They are laborious and material consuming. Additionally,

after the analysis, the results have to be collected and charted, which is prone to mistakes. Therefore, there was a need for a more comprehensive approach, enabling the maximum yield of information out of the same amount of extracted DNA. Multiplex PCR systems have already been developed. Some of them require visualization by gel electrophoresis, thus providing only qualitative results [1–5]. Others are composed by a multiplex PCR followed by post-PCR analyses like capillary electrophoresis [6–10] or microarray based [11–15], which offer only qualitative insights. Or they use pre-portioned single systems requiring high amount of isolated DNA and consumables like DNA polymerase [16, 17]. All these systems need post-PCR analysis which have an intrinsically contamination potential and are time-consuming and/or need expensive instrumentation.

One of the first quantitative multiplex PCR systems was designed for the determination of four transgenic maize lines [18]. Several other systems were designed for other transgenes or screening elements [19–24]. Screening methods have become increasingly important to minimize the cost of an analysis and the analytical effort and to offer an exact preselection for further analysis. Earlier publications often presented methods only for the transgenic markers without including housekeeping genes to determine the DNA species, making it impossible to calculate a relative amount of GMO. But this is a prerequisite for the decision if further analysis is required.

Here, we present the validation data of two respective systems—a pentaplex and a tetraplex quantitative real-time PCR system—for GMO screening in commodities, food and feed products.

The pentaplex system enables the simple and sensitive and quantitative estimation of the proportion of transgenic plants containing the cauliflower mosaic virus 35S promoter (P35S) and/or the NOS terminator from Agrobacterium tumefaciens (Tnos) in maize and soya samples. The tetraplex system provides a simultaneous quantitative screening for DNA sequences from figwort mosaic virus 34 S promoter (PFMV), from bar gene of *streptomyces hygroscopicus,* from *a* synthetic gene coding for phosphinothricin acetyltransferase (PAT) and from a DNA construct spanning the junction between enolpyruvyl shikimate phosphate synthase gene (CP4-EPSPS) from A*grobacterium tumefaciens* ssp. strain 4 and from a chloroplast transit peptide signal sequence from *Arabidopsis thaliana (CPT2).*

Materials and methods

Plant material and DNA samples

Reference material was obtained from Sigma-Aldrich (Buchs, Switzerland), European Reference Material (ERM, Geel, Belgium). Further plant materials for specificity testing were collected from the market. Materials from proficiency tests organized by FAPAS (Sand Hutton, UK) and USDA Grain Inspection, Packers & Stockyards Administration (GIPSA) were used for further validation. Bt176 (ERM BF411f) served as source for the transgenic marker sequences of bar; for PAT, Bt11 (ERM 412f); for CP4-EPSPS, NK604 (ERM BF415f); and for PFMV, the transgenic potato NewLeaf plus (Russet Burbank, not certified). A concentration of 20 ng/µl was assigned as 100 %. As source for the cauliflower mosaic virus DNA, an amplicon from a single PCR was taken from a broccoli sample from the market. The PCR amplicon was confirmed by P35S-specific PCR and diluted 1 to 1E8 in PCR-grade Water. This dilution was assigned to be 10 % (or 2 ng/µl). As virus contents and transgenic content cannot be compared, this arbitrary definition seemed acceptable and practicable.

DNA extraction

DNA extraction from all sample matrices was performed using a Wizard Plus Minipreps DNA purification system (Promega, Madison, USA). In parallel, extractions were performed also using Nucleospin Food kit (Macherey–Nagel, Düren, Germany), leading to comparable results. Usually, 200 mg of grinded sample material was extracted, and DNA was eluted in 50 µl elution buffer, according to the producer's manual. If possible, the DNA amount was determined photospectrometrically and diluted to 20 ng/µl using PCR-grade water.

Primers and probes

The primers and probes were taken either from published single PCR systems or established in "in-house" PCR systems. For all in-house designed (by Beacon Designer 5.1 software) PCR systems, specificity was first tested without probes in the single SYBR green format. This is useful to detect and avoid PCR systems amplifying unspecific sequences. After an additional first check of specificity, probes were designed according to the channels of the Rotorgene 6000. We choose FAM-, Tet-, ROX-, Cy5- and DY681-labelled probes (for details, see Tables 1 and 2).

Real-time PCR procedure

DNA extracts (5 µl) were added to 20 µl of reaction mix containing QuantiFast multiplex PCR NoROX Mastermix (Qiagen AG, Hilden, Germany), primers and probes (for final concentration, see Tables 1 and 2). All primers and probes were synthesized by Microsynth AG (Balgach, Switzerland). PCR was performed on the Rotorgene 6000 real-time system (Corbett, Australia/Qiagen), according to

Table 1 Multiplex (pentaplex) qPCR system AllGVOScB for the simultaneous determination of DNA sequences from maize high-mobility group protein gene, soya lectin gene, CaMV35S promoter, NOS terminator and cauliflower mosaic virus

Primer/probe	Final conc. (μM)	Sequence	Amplicon (bp)	GenBank acc. no./source/labelling
Maize, major high-mobility group protein gene				
MaiJF2 F	0.2	TTG GAC TAG AAA TCT CGT GCT GA	79	[25]
Mhmg-R	0.2	GCT ACA TAG GGA GCC TTG TCC T		
MhmgCy5	0.08	CAA TCC ACA CAA ACG CAC GCG TA		Cy5/BHQ2
Soya lectin gene				
Lectin F	0.4	TCC ACC CCC ATC CAC ATT T	81	This work
Lectin R	0.4	GGC ATA GAA GGT GAA GTT GAA GGA		K00821
TMPLec_Rox	0.03	AAC CGG TAG CGT TGC CAG CTT CG		Rox/BHQ2
P35S				
35S-F	0.8	GCC TCT GCC GAC AGT GGT	82	[26]
35S-R	0.8	AAG ACG TGG TTG GAA CGT CTT C		
TMP35Stet	0.03	CAA AGA TGG ACC CCC ACC CAC G		Tet/BHQ1
Tnos				
NOS 154F	0.8	CAT GAC GTT ATT TAT GAG ATG GGT TT	237	This work
NOS 237R	0.8	CGC TAT ATT TTG TTT TCT ATC GCG TAT		
TMPNOS182	0.03	ATG ATT AGA GTC CCG CAA TTA TAC ATT T		Fam/BHQ1
Cauliflower mosaic virus				
CaMV F	0.4	TGA AAT CCT CAG TGA CCA AAA ATC	152	[27]
CaMV R	0.4	TAC AAG GAC AAT CAT TGA TGA GC		
CaMV DY681	0.08	AAG CCG TTG CAG CGA AAA TCG TTA ATG A		DY681-BHQ2

Table 2 Multiplex (tetraplex) qPCR system AllGVOScC for the simultaneous determination of sequences from FMV promoter, bar gene, CTP2-CP4EPSPS construct and synthetic pat gene

Primer/probe	Final conc. (μM)	Sequence	Amplicon (bp)	GenBank acc. no./source/labelling
PFMV				
FMV F	0.3	CAAAATAACGTGGAAAAGAGCT	78	[30]
FMV R	0.3	TCTTTTGTGGTCGTCACTGC		
FMV Fam	0.08	CTGACAGCCCACTCACTAATGC		Fam-BHQ1
Bar				
RapB F1	0.1	ACA AGC ACG GTC AAC TTC C	60	[31]
RapB R1	0.1	GAG GTC GTC CGT CCA CTC		
RapB Joe	0.08	TAC CGA GCC GCA GGA ACC		Fam-BHQ1
CTP2-CP4EPSPS				
GT73 F	0.1	TCC CGC TCT AGC GCT TCA AT	88	[32]
GT73 R	0.1	TCG AGC AGG ACC TGC AGA A		
GT73Rox	0.08	CTG AAG GCG GGA AAC GAC AAT CTG		Rox BHQ2
Pat				
pat141 F	0.4	GCA AAA AAG CGG TTA GCT CCT	108	[33]
pat248 R	0.4	ATT CAG GCT GCG CAA CTG TT		
Pat193Cy5	0.08	CGG TCC TCC GAT CGC CCT TCC		Cy5/BHQ2

the following cycling protocol: for the pentaplex system "AllGVOScB": initial step of 5 min at 95 °C, followed by 45 cycles of 5 s at 95 °C and 45 s at 60 °C, and for the tetraplex system "AllGVOScC": initial step of 5 min at 95 °C, followed by 45 cycles of 5 s at 95 °C and 45 s at 62 °C. In parallel, the tests were conducted on Mx3005P

(Stratagene, USA), showing that one thermoprofile may be used for both systems: initial step 10 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 45 s at 62 °C and 30 s at 72 °C, where single fluorescence measurement is made.

Results and discussion

Design of the multiplex real-time PCR systems

Specificity

All primers and probes were successfully checked for lack of relevant homologies by BLAST nr search within GenBank databases. DNA from a wide range of plants and other food ingredients was isolated. The success of isolation was tested by photospectrometrically determination of the DNA content (100 ng was used as template per reaction). DNA of the following organisms was isolated and tested in the multiplex PCR system AllGMOSc1: beef, trout, white beans, lentils, kidney beans, mung beans, borlotti beans, chickpeas, peas, runner beans, wheat, tomato, potato, rice, plum, apricots, peanuts, hazelnut, almonds, walnut, white lupine, blue lupine, onion, garlic, carrot, celery, parsley, nutmeg, white pepper, cinnamon, aniseed, coconut and paprika. The only cross-reactivity observed was for lupines. They showed a cross-reactivity of maximum 0.6 % in the lectin system (soya). This must be considered when examining samples containing material from lupine. To demonstrate the detectability of GMO, the following transgenic plants were tested: maize (Bt176, Bt11, MON810, GA21, T25, StarLink, MON863, TC1507, MON89034, MON88017, NK603, 59122, MIR604), rice (LL601, LL62, KMD1), soya (GTS40-3-2 (RoundUp Ready), 2704, 5547) and rapeseed (Oxy235). All of them gave the expected signals according to the screening list [29].

Table 3 Multiplex row of standards for AllGVOSc1 in the range from 0.032 % to 3.2 % and 1 to 100 % (100 % = 20 ng/µl)

No.	mhmg (%)	Tnos (%)	CaMV35S (%)	Lectin (%)	CaMV (%)
AllGVOScB					
1	1	0.032	0.032	100	0.032
2	100	0.1	0.1	1	0.1
3	3.2	0.32	0.32	32	0.32
4	10	1	1	10	1
5	32	3.2	3.2	3.2	3.2

As source of DNA for maize (mhmg), CaMV35s promoter and Tnos the European Reference Material Bt-11 ERM BF410a was used. For Soja (lectin) GMO-free soya flower the European Reference Material RoundUp Ready ERM BF412f was used. For the detection of CaMV virus, a PCR mix containing the CaMV amplicon was diluted 1 to 1E8 in PCR grade water and arbitrarily assigned as 10 %. The asymmetric set-up serves to assess whether competitive effects between the template concentrations in this multiplex PCR system take place. No such effects were observed

Sensitivity, precision and measurement uncertainty

To evaluate the sensitivity, DNA extracts with a concentration of 20 ng/µl were diluted in herring sperm DNA solution (20 ng/µl) down to a transgenic concentration of 0.032 % transgenic concentration (equal to 6.4 pg/µl). Amplicons were used in dilutions as described. These single analyte dilution rows were mixed according to Tables 3 and 4 to produce multiplex standards, simulating samples containing all four and five target sequences. Each data point was analysed six times ($N = 6$) over a period of

Table 4 Multiplex row of standards for AllGVOScC in the range from 0.0032 % to 1 %

No.	Bar (%)	CTP2-CP4EPSPS	Pat	PFMV
AllGVOScC				
1	0.32	0.0032	0.032	1
2	0.1	1	0.01	0.32
3	0.032	0.32	0.0032	0.1
4	0.01	0.1	1	0.032
5	0.0032	0.032	0.32	0.01
6	1	0.01	0.1	0.0032

The asymmetric set-up serves to assess whether competitive effects between the template concentrations in this multiplex PCR system take place. No such effects were observed. However, to get these analytes independently apart from the asymmetric dilution row, different GMO species were used (for details, see "Materials and methods")

Table 5 Performance of the AllGVOScB

	mhmg	Tnos	CaMV35S	Lectin	CaMV
AllGVOScB					
Amplification efficiencies	0.85	0.93	1.07	1.14	0.84
Correlation R^2	0.99	0.97	0.95	0.98	0.92
Precision ± %	18	39	42	20	33
Accuracy ± %	6	13	9	16	32
Performance %	0.83	0.79	0.77	0.83	0.77

The multiplex serial dilution was taken as calibrator to assess the amplification efficiencies (E), correlation (R^2), precision and accuracy. The precision was calculated by averaging the individual precisions (relative standard deviations) of each dilution point (see Tables 3, 4). The difference between true value and the individual measurement (every dilution) was calculated (in % of the true value). All these differences (%) were averaged, and the resulting value was taken as estimation of the accuracy. The amplification efficiencies were taken from the Rotorgene algorithm directly. The numbers shown here represent mean values from nine single experiments. The performance (P) was calculated according to an earlier publication [28] by the formula: $P = R^2 * (1 - \mathrm{ABS}(E - 1))$

1 month. All samples showed 100 % positive signals down to 0.032 % concentrations of the analytes. None of the six negative controls showed a positive signal. The quantitative data were collected, and the relative standard deviation (rSD) was calculated for the estimation of the precision. The mean deviation from the true value served for the estimation of the accuracy (Tables 5, 6).

The quantification of GMO by quantitative PCR based on screening elements is only possible for samples enclosing one single GMO trait in conjunction with the same GMO trait as calibrator, due to the different relations of marker and housekeeping genes in different GMO traits. Therefore, in practice, this quantitative approach leads only to a rough estimation of the GMO content. But this is already important to be able to divide GMO free or slightly contaminated samples from substantial mixtures of GMO.

Analysis of samples from proficiency testing programs

Table 7 shows the true values and the values measured by the system "AllGVOSc1". Correlation between measured and assigned values was poor. This reflects mainly the problem of calibration. Samples can only be quantified using the corresponding reference material, as different insertion numbers of the measured screening elements and/or copy numbers of the housekeeping genes have a great impact on the result. As it is unknown at the moment of analysis of unknown samples, this prerequisite is impossible to be fulfilled. In addition, products made from transgenic plants often include a mixture of transgenes. In consequence, it is impossible to choose a single corresponding transgene as calibrator.

Table 6 Performance of the AllGVOScC

	Bar	CTP2-CP4 EPSPS	Pat	PFMV
AllGVOScC				
Amplification efficiencies	1.16	1.18	1.27	1.1
Correlation R^2	0.98	0.99	0.98	1
Precision ± %	17	16	29	11
Accuracy ± %	2	0.4	7	17
Performance %	0.84	0.84	0.77	0.9

The multiplex serial dilution was taken as calibrator to assess the amplification efficiencies, correlation, precision and accuracy. The precision accuracy, correlation and amplification efficiencies were calculated as described in Table 5

Conclusions

Herein, we showed GMO screening methods for the reliable detection of four and five target sequences, respectively, in multiplex real-time PCRs, each performed in one tube. In this way, we could accommodate the detection of up to all eight target DNA sequences in each tested sample. Furthermore, combining two multiplex systems by a common thermoprofile on one of the tested thermocyclers, we propose a way for a high throughput screening of all eight target sequences at once.

Table 7 Determination of proportions of P35S and Tnos sequences

Source of the sample	True value P35S %	Measured value P35S %	True value Tnos %	Measured value Tnos %
Fapas GeMMA round 20 1.	2.4	3.4	2.4	2.4
Fapas GeM C11	1.6	1.4	1.6	5.4
Fapas GeM MU01	2.6	1.9	1.3	2.7
Fapas GeM SU34A	1.0	2.7	N	3.2
Fapas GeM SU35A	2.0	7.5	1.8	5.0
GIPSA October 2012-S1	1.0	1.5	1.5	2.0
GIPSA October 2012-S2	0.0	0.0	0.0	0.0
GIPSA October 2012-S3	0.6	1.0	0.5	0.4
GIPSA October 2012-S4	0.7	1.3	1.2	1.7
GIPSA April 2013-S1	1.1	1.9	1.4	2.8
GIPSA April 2013-S2	0.0	0.0	0.0	0.0
GIPSA April 2013-S3	1.7	3.3	1.0	3.2
GIPSA April 2013-S4	0.3	0.7	N	N
GeM MU22	0.68	1.1	N	1.1

Comparison with results of maize and soya samples from proficiency tests of maize and/or soya (N = not assigned). The % values are normalized versus soya (lectin). As calibrator for P35S and Tnos, material from maize event Bt11 and for soya, a certified transgene-free soya was used. At the moment, no quantitative proficiency tests for the other screening elements are available. The weight-by-weight value as described in the proficiency test report was taken as true value. For GeMMA 20 and GeMC11, the spiked RoundUp Ready content was taken as true value. For GeMMU 01, the sum of the Mon810 and NK603 was taken as true value of P35S content, and for Tnos, only the NK603 content was taken

Additionally, two of these analytes in system "AllGVOSc1" were present in a roughly 100 times higher concentration. Nevertheless, it was possible to determine the minor components with high accuracy. This shows that when using optimal primers and concentrations, the parallel amplification of multiple target sequences in a balanced multiplex real-time PCR is possible without reciprocal interference. In conjunction with the here proposed two multiplex standard rows, maize and soya samples can be analysed in a very efficient manner. Cross-contaminations of maize samples with soya and inverse can easily be detected in raw and processed products, making the interpretation clearer and hence further analysis more straightforward. Furthermore, the detection of the plant-derived DNA sequences (lectin and/or mhmg) can serve as an internal control of the DNA quality, especially if DNA is derived from processed and refined sample material. The size of the amplicons is adjusted to detect the targets even in strongly fragmented DNA, common in highly processed food samples. Additionally, the maize- and soya-specific assay comprises an internal amplification control to detect inhibitions. Rare transgenic traits can be herewith detected and characterized, and they are in accordance with the compilation of the relevant GMO traits [29]. This thorough screening step is also one possible analytical strategy to detect non-approved GMOs for which no specific assays, reference materials or even the sequences are available. However, the quantification should still be done by transgene-specific PCR systems due to different insertion numbers of the here used screening elements and/or copy numbers of the housekeeping genes.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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