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Detection of nonauthorized genetically modified organisms using differential quantitative polymerase chain reaction: application to 35S in maize

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ABSTRACT

Detection of nonauthorized genetically modified organisms (GMOs) has always presented an analytical challenge because the complete sequence data needed to detect them are generally unavailable although sequence similarity to known GMOs can be expected. A new approach, differential quantitative polymerase chain reaction (PCR), for detection of nonauthorized GMOs is presented here. This method is based on the presence of several common elements (e.g., promoter, genes of interest) in different GMOs. A statistical model was developed to study the difference between the number of molecules of such a common sequence and the number of molecules identifying the approved GMO (as determined by border-fragment-based PCR) and the donor organism of the common sequence. When this difference differs statistically from zero, the presence of a nonauthorized GMO can be inferred. The interest and scope of such an approach were tested on a case study of different proportions of genetically modified maize events, with the P35S promoter as the Cauliflower Mosaic Virus common sequence. The presence of a nonauthorized GMO was successfully detected in the mixtures analyzed and in the presence of (donor organism of P35S promoter). This method could be easily transposed to other common GMO sequences and other species and is applicable to other detection areas such as microbiology.

In a decade of use of genetically modified organisms (GMOs)[3], planting of GM crops has consistently increased and areas sown with GM crops exceeded 100 million hectares in 21 countries during 2005 [1]. Over 100 genetically modified plants have already been approved by regulatory agencies in different countries [2]. Due to public perception of GMOs as controversial, legislation requires traceability and detection of GMOs in some countries. In more than 15 countries, compulsory labeling is required for products that contain GMOs or derived product above a certain threshold [3]. The list of approved GMOs differs from country to country (asynchronous approval) and the control of unapproved GMOs is necessary to monitor the presence of GMOs that are not authorized. Several recent unintentional releases of nonauthorized GMOs (Bt10 maize, Shanyou63 rice, LLRice601) clearly indicate the need for a detection method for unapproved GMOs. In the European Union (EU), legislation strictly imposes a zero tolerance level for nonauthorized GMOs [4,5]. This problem is not restricted to the EU because occurrence of GMOs authorized elsewhere in food supplies can be expected due to worldwide trade (for instance, the release of Chinese Shanyou63 rice into the U.S. market).

In addition to GMOs authorized elsewhere, the source of unapproved GMOs that enter the food supply chain can also be those that have been authorized only for feed or industrial use. The first well-documented occurrence of such a case was in October 2002 when Starlink maize was found in the U.S. food supply chain, halting the U.S. trade toward the EU. Starlink maize was not approved for human consumption because the inserted protein Cry9C was suspected to be allergenic. Starlink maize was therefore withdrawn from the market at considerable expense [6,7].

Due to the commercialization of the new generations of GMOs, more GMOs that will not be approved for human consumption, such as those intended for industrial processes, bioremediation, or the production of pharmaceuticals [8,9], will enter the market

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[3] *Abbreviations used:* GMOs, genetically modified organisms; GM, genetically modified; Q-PCR, quantitative polymerase chain reaction; dQ-PCR, differential Q-PCR; *CaMV*, Cauliflower Mosaic Virus; CT, cycle threshold.

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as observed in the USA. Therefore, traceability of such organisms or verification of their absence in food will need to be assured.

To ensure traceability of GM products and to prevent the entry of unapproved GMOs in to the market, methods to detect nonauthorized GMOs are thus needed. The problems of detection, characterization, and quantification of unapproved GMOs present analytical challenges for GMO detection laboratories because the data on inserted constructs and nucleotide sequences are usually proprietary and reference materials are not available [10], which hampers the development of specific tests for nonauthorized GMOs.

Currently, no strategy has been fully described and internationally accepted for the detection of nonauthorized GMOs, but several theoretical approaches have been proposed (e.g., the "matrix approach" proposed by INRA in 1999 for the GMochips program http://www.gmochips.org retaken in the European research program Co-Extra http://www.coextra.eu). The so-called differential qualitative PCR, also called the "indirect subtractive approach," is based on the presumption that screening tests being positive and no authorized GMO being found in the sample provide indirect evidence for the presence of (a) nonauthorized GMO(s) [11] and has been used for instance in 2000 by the French frauds repression Services for detecting unapproved GMOs. Additionally, the DNA flanking the screening elements can be sequenced, which enables elucidation of the GM construct and design of specific tests for the nonauthorized GM line. Such an approach was used to design PCR tests to detect nonauthorized GM rice varieties [12]. Alternatively, profiles obtained by anchored PCR may differentiate approved and unapproved GMOs.

In the matrix approach that INRA proposed several years ago, the idea was extended to screening of many targets that are present in the constructs and to detection of authorized and unauthorized GMOs by, e.g., PCR and hybridization (see the validated DualChip http://biotech.jrc.it/home/documents/report-JRC-EAT.pdf) or a SNPlex-based method. From the combination of positive and negative amplifications and the hybridization results compared to approved GMO patterns, presence of nonauthorized GMOs can be inferred. Due to the large number of analyses that would need to be performed in such approaches, the microarray technique has been proposed [11], (see DualChip validation). GMO detection microarrays have already been prepared [13–15] but are not yet widely used in GMO detection laboratories. An extension to the detection on microarrays of sequences of the plasmidic vectors and inserted sequences has also been studied (A. Holst-Jensen et al., unpublished).

Another theoretical approach was proposed by Nesvold et al. [16], with a rationalized design for a set of synthetic oligonucleotide probes that would cover the entire plant genome of the species in question. By hybridizing the whole genomic DNA of the sample and of the wild-type plant, any differences in the genomes would be made evident. The approach is tempting because it does not require prior knowledge of inserted sequences, but the whole genome sequence of the plant in question and information about inter cultivar variability must be available before such tests can be used, unless hybridization is carried out with vectors' and inserted sequences.

The "profiling" approach is based on the majority of the insert containing (a) common sequence(s) flanked by specific DNA sequences that allow for discrimination among GM events. Anchored PCR, where genomic DNA is cut by a restriction enzyme prior to adaptor-ligation, was proposed to detect unknown GMOs [11]. Subsequently, common sequence-specific and adaptor-specific primers are used to amplify the DNA and each GMO is represented by a specific fragment size. Fragments of unexpected length are therefore considered indicative of the presence of nonauthorized GMOs.

In this article, we propose a new approach for detecting nonauthorized GMOs which is based on differential quantitative PCR (dQ-PCR) and is an extension of the qualitative differential PCR already in use in French enforcement laboratories. We statistically test the hypothesis that the quantity of a common sequence, present in different GM lines, equals the sum of quantities of approved event-specific sequences. When the sum of quantities of DNA copies of authorized GM events differs statistically from the quantity of DNA copies of the common sequence, the presence of a nonauthorized GMO can be inferred. This approach has been tested on a case study of GM maize events. Promoter P35S originating from *Cauliflower mosaic virus* (CaMV) was chosen as the common sequence because it is the most commonly introduced promoter in currently approved genetically modified plants. A statistical model was developed to reliably determine the presence of the nonauthorized GMOs. Effects of the DNA extraction procedure, the replicate analysis, and the operator extracting DNA on quantitative analyses by real-time PCR were evaluated and taken into account when setting up the model. The presence of P35S promoter does not necessarily imply the presence of genetically modified plants but could be due to presence of CaMV [17,18]. Therefore, an additional control was introduced to account for the presence and quantity of P35S originating from the virus. This paper is the first published application of dQ-PCR to the problem of detecting nonauthorized GMOs.

Its major interest lies in the use of existing equipment and reagents without the need for additional personnel training in contrast to the above-cited other detection methods of unapproved/unknown GMOs. Such characteristics should confer it a clear advantage over alternative methods that are directly applicable.

Materials and methods

Materials

Heterozygous seeds from the GM maize event T25 were provided by Aventis. Heterozygous Bt176 maize seeds were obtained from Novartis and Mon810 event seeds were provided by Monsanto Co. Seeds were obtained through the Direction Générale de l'Alimentation of the French Ministry of Agriculture. Seeds were grown in a greenhouse and leaves were collected and stored at −20 °C. Non transgenic DNA was isolated from leaves of maize (*Zea mays* L.; hybrid LG-2447) obtained from Limagrain Co. (Chappes, France).

pBR322 plasmid with the inserted whole genome of the *Cauliflower mosaic virus* (strain CabbS) was provided by the Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, Strasbourg, France [19].

Isolation of DNA

DNA was isolated from 1 g of plant leaves ground in liquid nitrogen. A DNA Midi Kit for blood and cell culture (Qiagen, Valencia, CA) was adapted for isolation of the DNA from large starting materials of plant tissue. Ten milliliters of digestion buffer G2 was added to 1 g of ground plant material and vortexed. One hundred microliters of α-amylase (10 mg/ml; Sigma, St. Louis, MO) and 100 μl of RNase (10 mg/ml; Amresco/Interchim, Montluçon, France) were added, and samples were incubated at 65 °C for 30 min. Two hundred microliters of Qiagen Proteinase K (20 mg/ml) was added, and samples were incubated at 65 °C for 1 h. Samples were centrifuged at 16,000 g for 5 min. The supernatant was also passed through a Miracloth filter (Merck Eurolab, Darmstadt, Germany) to avoid clogging of the isolation columns. The supernatant was then applied to the QBT-buffer-equilibrated Qiagen columns and the washing and elution steps were performed as described by the manufacturer (genomic-tip protocol). DNA was precipitated using isopropanol at 4 °C and centrifugation at

8400 g for 6 min. The DNA pellet was washed with 70% ethanol, transferred to a 1.5-ml Eppendorf tube, dried with a speedvac for 12 min at 30 °C and dissolved in 100 μl of TE buffer. Samples were stored at 4 °C overnight to fully dissolve and transferred to −20 °C.

For the three genetically modified maize varieties, samples were extracted twice (in five independent parallel procedures) by two different operators. Five parallel extractions of each GMO were joined and the other five were left separated. This enabled us to study the effect of the operator and the variability between DNA isolations performed concurrently. Blank DNA isolations (isolations from cell-culture-grade water; Biological Industries, Beit Haemek, Ashrat, Israel) were performed concurrently with the sample DNA isolations to test for cross contamination during the DNA extraction procedure.

DNA quantification

Isolated DNA was electrophoresed on an 0.8% agarose gel concurrently with 10–30 ng of phage λ DNA (MBI Fermentas, Vilnius, Lithuania). Gel images were analyzed by Bio-1 D v. 99.06 software (Vilber Lourmat Biotechnology, Marne-la-Vallée, France) and DNA concentration was determined by comparison of the ethidium bromide fluorescence of DNA to the λ DNA standard curve.

Additionally, a real-time PCR was performed with maize species-specific primers (*Adh*; see below) for three dilutions of each DNA extract containing approximately 20,000 2,000, and 200 copies of genomic DNA. DNA concentrations were equalized according to the obtained Ct values. DNA extracts were tested for PCR inhibition by assessing PCR efficiency calculated from the standard curve obtained.

Real-time PCR primers and probes used in this study

Real-time PCR probes and primers used in this study were previously published [18,20–23] and are presented in Table 1. For genetically modified organisms, probe and primer sets were chosen to specifically amplify the border fragments of the GMOs, thereby ensuring no possible cross-reactivity between different GMOs.

Real-time PCR

Real-time PCRs were run using 200 nM probe and 300 nM primer concentrations for all assays using either 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) or 1 × Q-PCR MasterMix (Eurogentec Co., Liege, Belgium). Both PCR mastermixes contain uracil *N*-glycosylase enzyme to prevent carryover contaminations and ROX as a passive reference dye; the mixes were previously checked for equality of performance (data not shown). PCRs were run either in 20 μl reaction volume on an ABI Prism 7900HT platform (Applied Biosystems) or in 25 μl reaction volume on an ABI Prism 7700 instrument (Applied Biosystems). The MasterMix and PCR instrument were not varied within one experimental design (see Table 2). Universal thermal cycling conditions were used for all amplicons: 2 min at 50 °C, 10 min at 95 °C, with 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The baseline and threshold were adjusted manually after each real-time PCR run. Equal baseline and threshold were used for all amplicons within one experimental design to enable comparisons of results from different PCR plates.

Experimental designs

Five different experimental designs were consecutively executed to test the performance of the dQ-PCR technique. The designs are summarized in Table 2. The first objective was to experimentally confirm the copy number of P35S promoter per GMO as described in the literature (designs 1 and 2). The variation in results that occurs due to extraction, the plate effect, and the operator was studied in design 3. The dQ-PCR test was performed in design 4 on samples containing individual GM maize events and mixtures of events Bt176, Mon810, and T25 maize using different copy numbers. Additionally, a plasmid containing CaMV virus was introduced in to DNA mixtures design 5 to assess the influence of the presence of CaMV.

Statistical analysis

The aim of the dQ-PCR is to detect nonauthorized GMOs in a DNA mixture containing multiple GM events. The quantity of a common element present in all events used in the study was measured by real-time Q-PCR as was the quantity of authorized GM events by amplification of its specific border fragment. When the sum of the quantity of authorized events substantially differs from the quantity of the common element, the presence of a nonauthorized GMO can be inferred. Therefore, the sum of quantities of authorized events is subtracted from the total amount of the common genetic element, as shown in the following equation:

Table 1
Primers and probes used in the study

Event	Orientation	Location	Primers and probe names	Sequence 5′ –> 3′	Length (bp)	References
Bt176	forward primer	transgene	PBt176F	GAACTGGCATGACGTGG	17	C. Collonnier et al.
	reverse primer	plant genome	PBt176RM	GAAGGGAGAAACGGTCG	17	unpublished data
	fluorogenic probe	on the border	PBt176-pr	CCTGCCCGTCACCGAGATCTGATGT	25	
Mon810	forward primer	plant genome	PMo810FM	CCTTCATAACCTTCGCCCG	19	20
	reverse primer	transgene	PMo810R	AATAAAGTGACAGATAGCTGGGCA	24	
	fluorogenic probe	on the border	Mo810-pr	ACGAAGGACTCTAACGTTTAACATCCTTTGCCA	33	
T25	forward primer	transgene	PT25F	ACAAGCGTGTCGTGCTCCAC	20	21
	reverse primer	plant genome	PT25RM	GACATGATACTCCTTCCACCG	21	
	fluorogenic probe	on the border	T25-pr	TCATTGAGTCGTTCCGCCATTGTCG	25	
Adh	forward primer	internal *Adh*	ADH-F3	CGTCGTTTCCCATCTCTTCCTCCT	24	22
	reverse primer	internal *Adh*	ADH-R4	CCACTCCGAGACCCTCAGTC	20	
	fluorogenic probe	internal *Adh*	ADH1	AATCAGGGCTCATTTTCTCGCTCCTCA	27	
35S	forward primer	internal P35S	SF	CGTCTTCAAAGCAAGTGGATTG	22	23
	reverse primer	internal P35S	SR	TCTTGCGAAGGATAGTGGGATT	22	
	fluorogenic probe	internal P35S	35S	TCTCCACTGACGTAAGGGATGACGCA	26	
CaMV ORFIV	forward primer	internal ORFIV	CaMVF	GGCCATTACGCCAACGAAT	19	18
	reverse primer	internal ORFIV	CaMVR	ATGGGCTGGAGACCCAATTTT	21	
	fluorogenic probe	internal ORFIV	CaMV-MGB	TTCTCCGAGCTTTGTC	16	

ORF, open reading frame.

Table 2
Overview of experimental designs

	Verification of P35S copy number per GMO		Extract effect	Application of differential Q-PCR test	Differential P-PCR test: introducing CaMV control
Design	1	2	3	4	5
PCR performed	P35S / Bt176 / Mon810 / T25	P35S / Bt176/ Mon810 / T25 / Adh	P35S / Bt176 / Mon810 / T25 / Adh	P35S / Bt176 / Mon810 / T25	P35S / Bt176 / Mon810 / T25 / CaMV (ORFIV)
GMO quantities	1000, 5000 copies (Bt176 and T25) 2000, 10,000 copies (Mon810)	4000, 40,000 copies	100, 400, 1600 copies	0,100, 400, 1600 copies	0, 100, 1000 copies
Parameters	Mixtures of GMO	Curve for each GMO	2 experimenters 5 extracts for each GMO = 10 extracts per each one	26 mixtures of GMOs	16 mixtures of GMOs + CaMV
Statistical analysis	Nonlinear regression	ANOVA	ANOVA	Differential Q-PCR test	Differential Q-PCR test

$$\mu = \text{Quantity}_{\text{common element}} - \text{Quantity}_{\text{authorized GMOs}}$$

When μ substantially differs from 0, it can be assumed that the sample contains nonauthorized GMOs. A statistical test of hypothesis, called the dQ-PCR test, was developed to determine the presence or absence of nonauthorized GMOs with significance levels of 5% (for all examples). The significance level of a statistical hypothesis test is a fixed probability of wrongly rejecting the null hypothesis H_0 if it is in fact true.

The principle of approach is first to estimate target quantity (copy number) with a separate model for Q-PCR analysis and second to execute a dQ-PCR test (see also Appendix 1 in Supplementary material for more details on both models).

Model for estimation of the target quantity in Q-PCR

Quantification of each event-specific target was performed using the model $Ct = \alpha - \beta \log N$, where α and β are the ordinate and the slope of the standard curve, respectively. This model is defined for absolute copy numbers applied to a calibration curve [24]. The regression curve is obtained through linear regression by the least square method. Calibration curves must be calculated for each of the target amplicons. With the estimators of calibration curve, the copy number of an unknown sample, denoted by $N^{(u)}$, can be calculated with the equation:

$$\hat{N}^{(u)} = 10^{\left(\frac{Ct^{(u)} - \hat{\alpha}}{\hat{\beta}}\right)},$$

where $Ct^{(u)}$ is the observed Ct for the unknown sample and $\hat{\alpha}$ and $\hat{\beta}$ are the estimators of α and β.

dQ-PCR test

In this case, the method was tested on a maize model system where P35S was used as a common element of the three GM maize events Bt176, Mon810, and T25. So μ can be expressed as

$$\mu = Quantity_{P35S} - Quantity_{Bt176} - Quantity_{Mon810} - Quantity_{T25}.$$

This equation can be extended when the CaMV is present as

$$\mu = Quantity_{P35S} - Quantity_{Bt176} - Quantity_{Mon810} - Quantity_{T25} - Quantity_{CaMV}.$$

The test, $\mu = 0$, can be separated into two hypotheses: the null hypothesis (H_0) $\mu = 0$, which is initially assumed to be true, against the alternative hypothesis $\mu > 0$. Confirmation of the null hypothesis would indicate the absence of a nonauthorized GMO in the sample. In our study, one of the GMOs was considered nonauthorized.

The dQ-PCR test algorithm was based on the "Delta method" [25]. The Delta method is a method of approximating the expected value as a function of random variables. For this purpose, this function is considered a Taylor series. In our case, the asymptotic normality of the parameters can be proven; therefore, the normal distribution was used to test the null hypothesis. The Delta method allows us to show that $\hat{\mu} - \mu \sim N(0, \sum^2)$, where $\sum^2$ depends on several parameters (estimated quantities...). The test statistic used, T, can be written as $\hat{T} = \frac{\hat{\mu}-\mu}{\sum}$, where $\hat{\mu}$ is the estimated value calculated according to the observed data and μ is the theoretical value; i.e., $\mu = 0$. As shown previously, this statistic is a standard normal random variable. The obtained value was compared with the 5th percentile of normal distribution (i.e., 1.64). When the estimated value of T is lower than 1.64, the null hypothesis cannot be rejected; therefore, the presence of nonauthorized GMO in the sample cannot be determined. When the estimated value of T is higher than the 5th percentile of the normal distribution, the null hypothesis can be rejected with a significance level of 5% and the presence of nonauthorized GMO in the sample is accordingly assumed.

R statistical freeware [26] was used to analyze real-time Q-PCR experimental data and to test the null hypothesis.

Results and discussion

As a first step, the number of P35S promoter copies per GMO maize event used in the study was experimentally determined and compared to the information of the BATS database on the GMO constructs [27]. Then, the influences of DNA extraction and operators on the results of Q-PCR were assessed. The dQ-PCR test was applied to determine the GMO percentage range in which the presence of nonauthorized GMO can be assessed. Finally, the CaMV virus (which is the donor organism of P35S promoter) was included in the DNA mixtures. The presence of CaMV virus and the reliability of the dQ-PCR test when the virus is present were assessed to avoid false positive results.

Verification of P35S copy number per GM maize

Before testing the performance of our model, the copy number of P35S promoter per GM event was experimentally confirmed by a previously developed quantitative PCR (sF/sR primers), [23]. According to the BATS database [27], the P35S enhancer copy numbers detected by sF/sR primers are two copies of P35S per Bt176 genome, two copies of P35S per Mon810 genome and one copy of P35S per T25 genome.

Several experiments were carried out to validate our Q-PCR test against the literature data. The first experiment was based on experimental design 1 (Table 2). In this design, the real time Q-PCR tests were performed on eight DNA mixtures containing high copy numbers of Bt176, Mon810, and T25 events. Different amounts of each GMO were used to prepare these mixtures: 5000 or 1000 haploid genome copies of Bt176 and T25 and 10,000 or 2000 haploid genome copies of Mon810. Standard curves

were also made for each GMO and a P35S standard curve was prepared using the Mon810 event. To estimate the P35S copy number, a non linear model was considered (see Appendix 2, in [28]). The value 2 was fixed to the Mon810 coefficient to decrease the number of parameters in the model. After calculating, the coefficients were defined as 2 for Mon810, 2.22 with SD of 0.17 for Bt176, and 0.60 with a SD of 0.11 for T25. Our data were close to the bibliographic data in that the quantity of P35S copies detected for Mon810 and Bt176 was the same, although the T25 result was lower than expected. To complete and confirm these results, experimental design 2 (Table 2) was further tested.

This experiment was based on the comparison between P35S regression curves of each GMO. Real-time Q-PCR tests were performed on one sample for each Bt176, Mon810, and T25 event at 40,000 and 4000 haploid genome copies. Three regression curves were tested with an ANOVA to look for their equality using two steps: testing (i) the equality of slopes of regression curves and (ii) the equality of ordinates. The hypothesis of equality of slopes was tested by comparing two models: model 1, where both the slopes and the ordinates associated with the different GMOs differ and model 2, where a common slope for all GMOs and different ordinates are associated with regression curves of different GMOs. When the hypothesis of slope equality was accepted, the equality of the ordinates was further tested by comparing model 2 with model 3, where a common slope and ordinate for all GMOs are calculated.

Calculation allowed us to accept the hypothesis of slope equality ($p = 0.37$) and to reject the hypothesis of ordinate equality ($p < 10^{-6}$). So the three regression curves had the same slope but could not be considered equal. Accordingly, the Q-PCR test had the same efficiency for all targets, but the ordinates differed.

Relative P35S quantity of GMOs was then compared. This calculation was performed for both P35S and *Adh* quantities. The obtained ratio between the P35S promoter quantity in the Mon810 event and that in the Bt176 event was 1.15, indicating an equal P35S copy number in these two events. The ratio of the P35S promoter quantity in the T25 event and that in the Bt176 event equaled 0.48, indicating that the Bt176 event contains twice as many P35S copies as the T25 event. The obtained results confirmed the literature data and coefficients 2, 2, and 1 were introduced to the differential quantitative PCR model for maize events Bt176, Mon810, and T25, respectively. The following equation was therefore used for determination of presence of unknown GMOs:

Table 3
Ratio of Bt176 and *Adh* content in 10 Bt176 extracts relative to the value of the first extract

Extract	Relative quantity of Bt176	Relative quantity of *Adh*	Ratio
1	1	1	1
2	1.06	1.40	0.76
3	1.37	1.39	0.99
4	0.95	0.93	1.02
5	2.71	2.44	1.11
6	0.76	0.79	0.97
7	0.97	1.20	0.81
8	1.17	1.11	1.05
9	0.84	0.94	0.90
10	0.84	0.90	0.93

$$\mu = N^{(u)}{}_{P35S} - 2 \times N^{(u)}{}_{Bt176} - 2 \times N^{(u)}{}_{Mon810} - N^{(u)}{}_{T25}.$$

The introduction of these coefficients in the test modified only the calculation of variance.

Effect of DNA extraction and operators on Q-PCR results

The influence of extract DNA quantity and quality on accuracy of real-time PCR results has been observed in many cases. Three experiments were performed to test the influence of these effects on Q-PCR results. Because the three experimental designs provided similar results, the results presented below refer only to the experimental design 3 (see Table 2). Standard regression curves were made for each DNA extract with 100, 400, and 1600 haploid genome copies (see Fig. 1), and a comparison of these curves was performed. An ANOVA was carried out on all curves to determine whether they could be considered equal. We confirm the equality of slopes of regression curves for Bt176 Q-PCR identification ($p = 0.28$). The equality of ordinates was rejected ($p < 10^{-13}$). The same results were obtained for the *Adh* target. The distance between parallel lines was similar for these two Q-PCR targets as shown in Fig. 1. Further, the correlation between ordinates of Bt176 and *Adh* curves was determined as 94.9%. The relative quantities for each independent extract of Bt176 and *Adh* are given in Table 3, which shows that the ratio between Bt176 and *Adh* extract quantities are close to 1 for all studied DNA extracts. As expected, the results show both DNA extraction and operator effects on the absolute number of target copies. However, these effects disappear

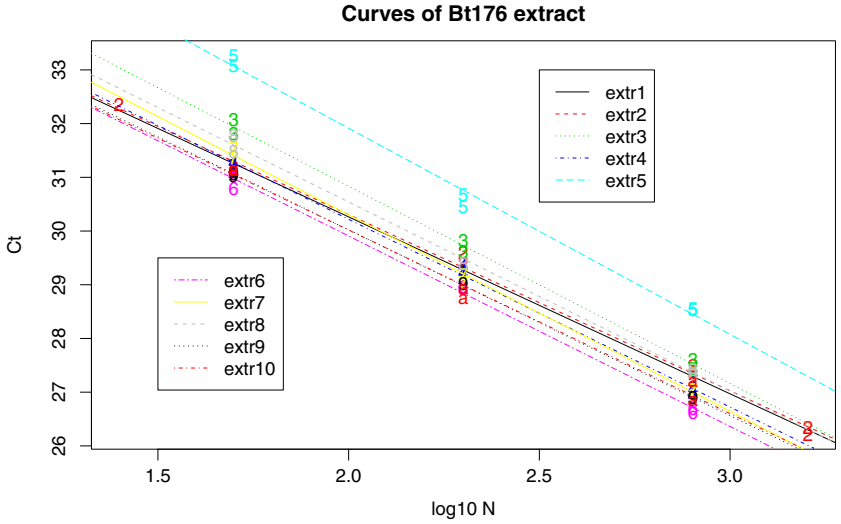

Fig. 1. Calibration curves for determination of extract effect for event Bt176. Standard curves of Bt176 extract for border fragment of Bt176. (Ct, cycle threshold, *N*, haploid genome copy number). Number of each extract is used as plotting symbol (except for extract 10 represented by 'a'), and different line types are used to differentiate each extract.

when a relative quantification method is used and the event-specific quantity is normalized using a single-copy maize endogenous gene. Similar results (data not shown) were obtained for T25 and Mon810 DNAs.

To eliminate this DNA effect on absolute genome copy number, all following experiments were carried out using the same DNA of each GMO for the calibration curve and the mixtures.

Performance of the dQ-PCR test at low copy numbers of target amplicons

The performance of the dQ-PCR method was tested practically on DNA mixtures containing GM maize events. To establish the lowest applicability limit and to simulate the situation where trace amounts of GM materials are present in the samples (a quite common situation in enforcement laboratories), the performance of the dQ-PCR method was tested on mixtures of GM events containing low haploid genome copy numbers (experimental design 4; see Table 2). Real-time PCR tests were performed on 26 mixtures (among 64 possibilities) of Bt176, Mon810, and T25 events, containing 0, 100, 400, or 1600 haploid genome copies of individual GM events. The 26 mixtures were chosen to have P35S ratios (defined below) ranging 15–50% for one GMO at least.

The numbers of total P35S copies and GM-specific copies were determined experimentally and compared to the actual haploid genome copy number added to the reactions. Good correlations between the estimated and the actual numbers of the target sequences were obtained for all tested amplicons as shown in Fig. 2. Correlation coefficients were calculated for all targets: $\mathrm{cor}(\mathrm{Bt176}_{\mathrm{calculated/true}}) = 99.9\%$, $\mathrm{cor}(\mathrm{Mon810}_{\mathrm{calculated/true}}) = 99.5\%$, $\mathrm{cor}(\mathrm{T25}_{\mathrm{calculated/true}}) = 99.7\%$, and $\mathrm{cor}(\mathrm{P35S}_{\mathrm{calculated/true}}) = 99.2\%$.

The significance level of our asymptotic test was set to 5%. In design 4, only sample 3 wrongly rejected the null hypothesis H_0 (which explains the detection of Bt176 when the quantity equaled 0; see Fig. 3); the empirical value of the significance level is 1/26 = 3.8%. Due to cost-effective detection practice, the number of repetitions for a single test in GMO detection is very often less than five. Therefore, simulations were carried out to determine whether the significance level is really close to 5%. These simulations were based on the observed data and normal distribution hypothesis. Levels for each mixture were simulated and all results were close to the value of 5% with a mean of 4.8%. As expected, the significance level of our test, i.e., the probability of wrongly rejecting the null hypothesis, is therefore equal to 5%.

Finally, the determination of an unknown GMO in the mixture was assessed experimentally. Our aim was to detect nonauthorized GMOs in a DNA mixture containing multiple GM events. Hypothesis testing was performed with one of the GM maize lines used in the experiment considered a nonauthorized GMO and the other two maize GM lines considered authorized GM events. The total quantity of the P35S promoter was compared to the quantity of the authorized GM events. When the sum of the quantity of authorized events substantially differed from the quantity of the common element, the presence of a nonauthorized GMO could be inferred (Table 4).

The power of the statistical test, i.e., the probability of truly rejecting the null hypothesis, was further assessed. First, the power of the statistical test was shown to be dependent on the absolute quantity of the nonauthorized GMO in the reaction. At the levels of 1600 and 400 haploid genome copies of nonauthorized GMO in the DNA mixture, the nonauthorized GMO events were detected in 100% of cases. When using 100 haploid genome copies, reliability of the test varied by GM event (33% for T25 and 83% for Bt176 and Mon810; Table 5).

The power of the statistical test thus clearly increased with higher GM event copy number quantity per reaction. At very low copy number (100 haploid genome copies), the detection of unknown GM events is less reliable, probably due to the measurements being taken close to the limit of quantification for real-time PCRs.

For the same reason as for the significance level, some simulations were carried out to determine the value of the power of our test. For the Bt176 event the mean values for the power of the test were 68% at the level of 100 haploid genome copies of Bt176 per reaction, 95% at the level of 400 haploid genome copies per reaction, and 100% at the level of 1600 haploid genome copies

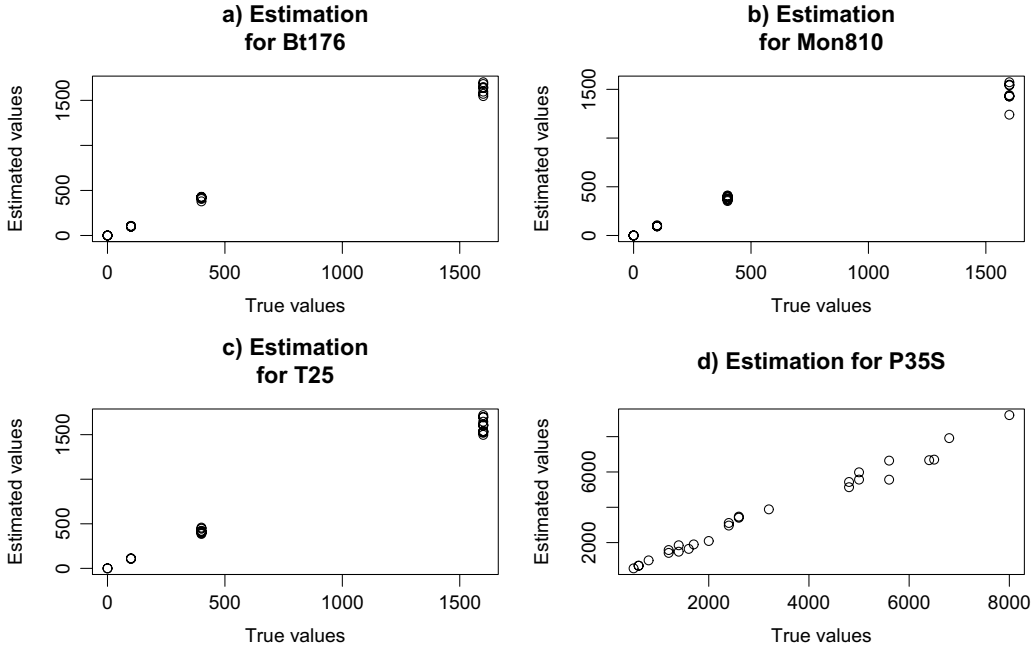

Fig. 2. Correlation of true and estimated copy numbers of target sequences. Correlations of experimentally estimated target copy numbers and DNA copy numbers added to the reaction for (a) Bt176 event, (b) Mon810 event, (c) T25 event, and (d) P35S promoter.

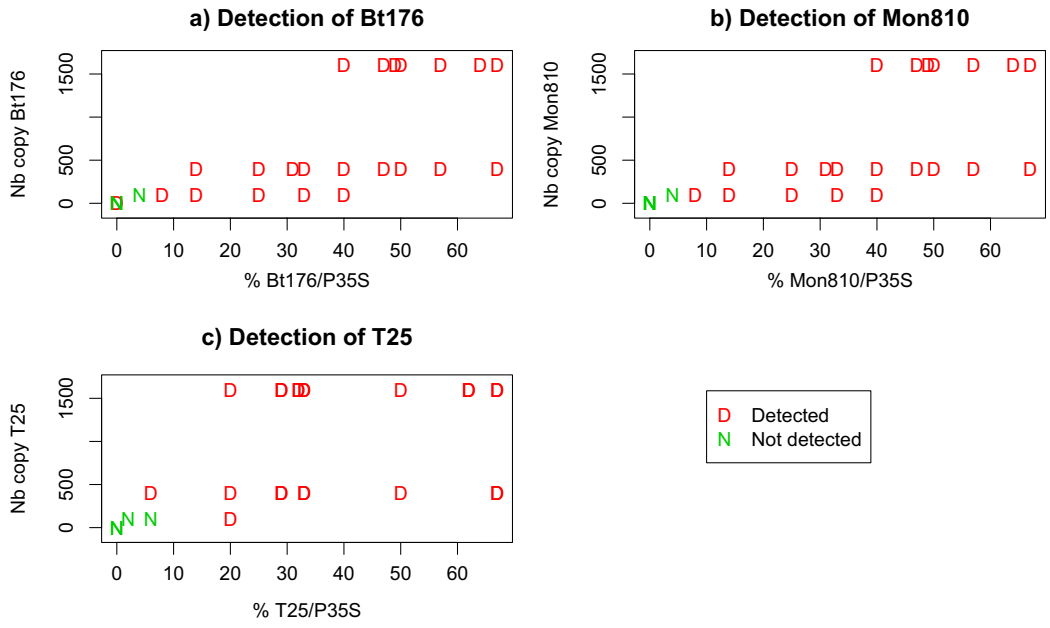

Fig. 3. Detection of a nonauthorized GMO as a factor of P35S content. The success of detection of a nonauthorized GMO depended on the haploid genome copy number of individual GM events per reaction (*y* axis) and the percentage of P35S promoter originating from this event relative to total P35S amount (*x* axis).

Table 4
Detection of a nonauthorized GMO in a mixture at low genome copy number

Sample	P35S		Bt176				Mon810				T25			
	A	E	A	E	T	%	A	E	T	%	A	E	T	%
1	600	687	0	0	N	0.0	100	98	D	33.3	400	407	D	66.7
2	1200	1418	0	0	N	0.0	400	390	D	66.7	400	458	D	33.3
3	2400	3111	0	0	D	0.0	400	375	D	33.3	1600	1613	D	66.7
4	4800	5136	0	0	N	0.0	1600	1548	D	66.7	1600	1521	D	33.3
5	600	691	100	98	D	33.3	0	0	N	0.0	400	421	D	66.7
6	500	541	100	100	D	40.0	100	94	D	40.0	100	114	D	20.0
7	800	993	100	99	D	25.0	100	96	D	25.0	400	416	D	50.0
8	1400	1485	100	104	D	14.3	400	372	D	57.1	400	409	D	28.6
9	2600	3407	100	107	D	7.7	400	354	D	30.8	1600	1600	D	61.5
10	5000	5568	100	101	N	4.0	1600	1426	D	64.0	1600	1645	D	32.0
11	1200	1577	400	425	D	66.7	0	0	N	0.0	400	444	D	33.3
12	2400	2964	400	423	D	33.3	0	0	N	0.0	1600	1699	D	66.7
13	1400	1848	400	429	D	57.1	100	95	D	14.3	400	386	D	28.6
14	2600	3473	400	423	D	30.8	100	93	D	7.7	1600	1539	D	61.5
15	1600	1644	400	405	D	50.0	400	366	D	50.0	0	0	N	0.0
16	1700	1896	400	378	D	47.1	400	407	D	47.1	100	106	N	5.9
17	2000	2091	400	418	D	40.0	400	375	D	40.0	400	410	D	20.0
18	3200	3882	400	420	D	25.0	400	397	D	25.0	1600	1540	D	50.0
19	5600	5562	400	426	D	14.3	1600	1240	D	57.1	1600	1719	D	28.6
20	4800	5433	1600	1681	D	66.7	0	0	N	0.0	1600	1497	D	33.3
21	5000	5980	1600	1701	D	64.0	100	105	N	4.0	1600	1529	D	32.0
22	5600	6642	1600	1636	D	57.1	400	410	D	14.3	1600	1605	D	28.6
23	6400	6667	1600	1648	D	50.0	1600	1541	D	50.0	0	0	N	0.0
24	6500	6698	1600	1547	D	49.2	1600	1573	D	49.2	100	109	N	1.5
25	6800	7916	1600	1597	D	47.1	1600	1440	D	47.1	400	398	D	5.9
26	8000	9219	1600	1573	D	40.0	1600	1429	D	40.0	1600	1693	D	20.0

A, actual haploid genome copy number added to the reaction; E, experimentally estimated haploid genome copy number; %, the percentage of P35S promoter originating from GM event relative to total P35S amount; T, result of statistical test of detection of unknown GM line, where successful detection is marked by D and unsuccessful detection by N.

per reaction. Thus results of simulations were close to empirical values calculated on observed data (Table 5).

Secondly, successful detection of the P35S originating from the unknown GMO depended on the ratio of the P35S quantity of the unknown's GMO to the total amount of P35S copies ($N^{(u)}_{Unknown\ GMO}/(2\times N^{(u)}_{Bt176}+2\times N^{(u)}_{Mon810}+N^{(u)}_{T25})$. For example, in the mixtures where 100 haploid genome copies of Bt176 were added to the reaction, the test's power was determined to be 98.3% when the P35S promoter originating from the Bt176 event constituted 25% of the total P35S quantity. However, the Bt176 event was successfully detected in only 8.3% of the cases when the ratio of P35S from the Bt176 event compared to total P35S content equaled 4%. The P35S ratios used in this experiment 4–66.67% for Bt176 and Mon810 (2 P35S copies per genome) and 1.5–66.67% for T25 (see Table 4). Better reliability of detection of the unknown GMO was in all cases achieved at the highest percentage of P35S originating

Table 5
Results of detection of a nonauthorized GMO for design 4

GMO	DNA quantity (copy number)	Number of samples	Number of samples detected	% Samples detected	Simulation's results of the power of the test (%)
Bt176	100	6	5	83	67.4
	400	9	9	100	95
	1600	7	7	100	100
Mon810	100	6	5	83	61
	400	9	9	100	90.8
	1600	7	7	100	99.8
T25	100	3	1	33	41.6
	400	9	9	100	89.3
	1600	12	12	100	94.2

from the unknown sample (Fig. 3). The limit of detection for nonauthorized GM events was shown to be 10–20% of the total P35S content, except in reactions where only 100 copies of the haploid genome of unknown GMO were added. The limit of detection for unknown GM lines in this case is higher because detection of unknown GM events is observed when the percentage of its common element equaled 20% or more of the total amount of the common element (see Fig. 3).

Lower success of detection was also observed for GM event T25. At 400 haploid genome copies of target amplicon, the Bt176 and Mon810 events were successfully detected at the 10% P35S content level and the T25 event was detected at the 10–20% level. The lower success of detection of the T25 event results from its content of one P35S copy per haploid genome compared to two copies of P35S in Bt176 and Mon810 events, as explained previously.

We have therefore confirmed that the dQ-PCR can be reliably used to detect the presence of nonauthorized GMOs in a mixture of GM events, in particular at low copy numbers.

Detection of unknown GMOs in presence of CaMV

The positive result of the screening method for P35S promoter can originate from either the presence of a GMO event or the presence of CaMV in the sample; therefore, a plasmid containing the entire CaMV genome was added to DNA mixtures in further experiments in combination with DNA of maize GM events Bt176, Mon810 and T25. The performance of the dQ-PCR method to detect the presence of the virus in a sample was first tested. Real-time PCR was performed on 16 mixtures that contained 0, 100, and 1000 copies of the CaMV plasmid and haploid genomes of Bt176, Mon810, and T25 (Table 2). The null hypothesis was tested, as described before, with the additional estimation of CaMV genome copies. Q-PCRs were performed at low target copy numbers to evaluate the performance of the method at the limit of detection because no or very few virus contents are observed in enforcement laboratories (virus non-host plants or diseased host plants with low kernel production as a result of agricultural practices).

Good correlation between the experimentally estimated copy numbers and the true target copy number per reaction was observed (see Fig. 4). Correlations of 99.2% were calculated for Bt176 event, 99.3% for Mon810 event, 99.8% for T25 event, 97.9% for the P35S promoter, and 89.1% for CaMV target.

In the second step, the hypothesis testing was performed by successively considering each GMO maize line and the CaMV target sequence as a nonauthorized GMO. The results of the hypothesis testing are summarized in Table 6. At 1000 GMO copies of haploid

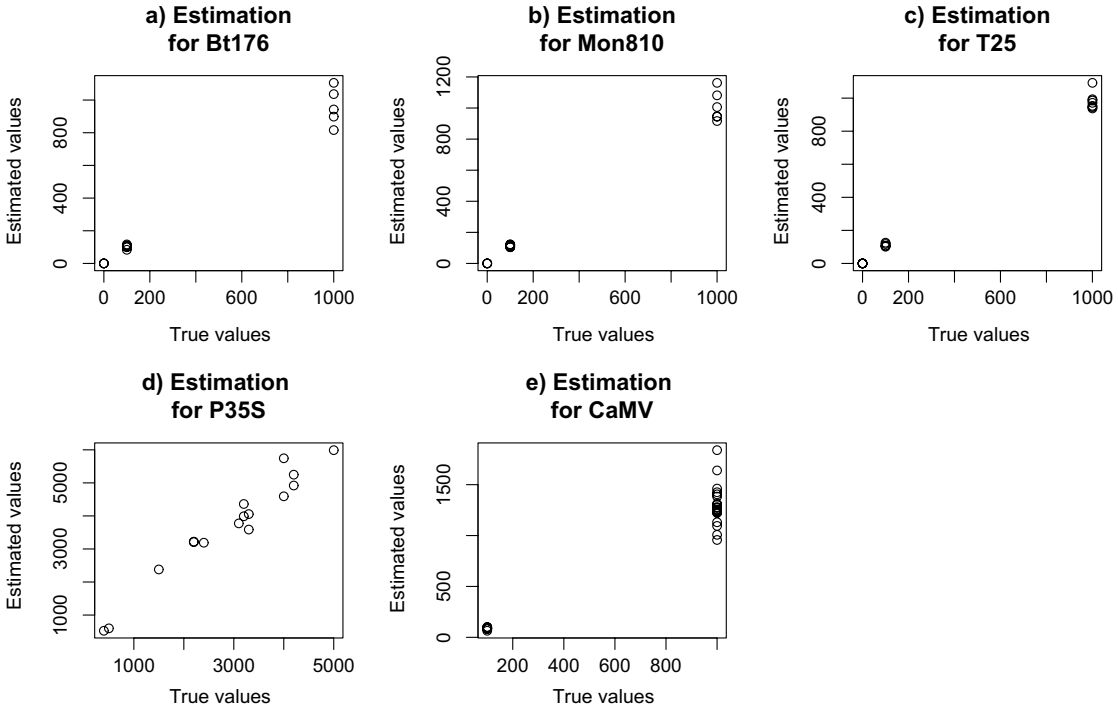

Fig. 4. Correlations of true and calculated copy numbers of target sequences in mixtures of GMOs and CaMV. Correlations of experimentally estimated target copy numbers and DNA copy numbers added to the reaction for (a) Bt176 event, (b) Mon810 event, (c) T25 event, (d) P35S promoter, and (e) CaMV virus.

Table 6
Detection of a nonauthorized GMO in a mixture of GMOs and CaMV

Sample	P35S		Bt176				Mon810				T25				CaMV			
	A	E	A	E	T	%	A	E	T	%	A	E	T	%	A	E	T	%
1	2200	3089	0	0	N	0.0	100	123	N	9.1	1000	953	D	45.5	1000	1576	D	45.5
2	3100	3625	0	0	N	0.0	1000	1162	D	64.5	100	126	N	3.2	1000	1317	D	32.3
3	4000	4398	0	0	N	0.0	1000	1082	D	50.0	1000	972	N	25.0	1000	1768	N	25.0
4	400	521	100	111	D	50.0	0	0	N	0.0	100	106	D	25.0	100	88	D	25.0
5	2200	3102	100	115	N	9.1	0	0	N	0.0	1000	985	D	45.5	1000	1340	D	45.5
6	500	592	100	109	D	40.0	100	112	D	40.0	0	0	N	0.0	100	70	N	20.0
7	1500	2304	100	107	N	13.3	100	120	N	13.3	100	118	N	6.7	1000	1491	D	66.7
8	2400	3073	100	105	N	8.3	100	116	N	8.3	1000	938	D	41.7	1000	1333	D	41.7
9	3200	3828	100	114	N	6.2	1000	944	D	62.5	0	0	N	0.0	1000	1379	D	31.2
10	3300	3448	100	89	N	6.1	1000	916	D	60.6	100	107	N	3.0	1000	1219	D	30.3
11	4200	5013	100	123	N	4.8	1000	1006	D	47.6	1000	1092	N	23.8	1000	1414	D	23.8
12	4000	5478	1000	1093	D	50.0	0	0	N	0.0	1000	946	D	25.0	1000	1086	D	25.0
13	3200	4179	1000	814	D	62.5	100	105	N	6.2	0	0	N	0.0	1000	1180	D	31.2
14	3300	3891	1000	936	D	60.6	100	115	N	6.1	100	101	N	3.0	1000	1404	D	30.3
15	4200	4705	1000	893	D	47.6	100	104	N	4.8	1000	993	D	23.8	1000	1031	D	23.8
16	5000	5709	1000	1026	D	40.0	1000	946	D	40.0	0	0	N	0.0	1000	1515	N	20.0

A, actual haploid genome copy number added to the reaction; E, experimentally estimated haploid genome copy number; %, the percentage of P35S promoter originating from GM event relative to total P35S amount; T, result of statistical test of detection of unknown GM line or CaMV, where successful detection is marked by D and unsuccessful detection by N.

Table 7
Detection results of a nonauthorized target GMO and transgenic donor organism CaMV in samples

GMO	DNA quantity (copy number)	Number of samples	Number of samples detected	% Samples detected
Bt176	100	8	2	25
	1000	5	5	100
Mon810	100	7	1	14
	1000	6	6	100
T25	100	5	1	20
	1000	7	5	71
CaMV	100	2	1	50
	1000	14	12	86

genome and 1000 copies of plasmid CaMV, the unknown target was reliably detected in 100, 100, 71, and 86% of the DNA mixtures for Bt176, Mon810, T25, and CaMV, respectively (see Table 7). The detection of unknown target was not as reliable at 100 haploid genome copies, as observed also in design 4.

The experiment was designed to assess the success of CaMV detection at a wide range of CaMV percentages in the DNA mixtures (20–66.7% of the total amount of P35S). As expected from the study in design 4, the ability to reliably detect an unknown target depended on the relative content of P35S originating from the unknown target vs the total amount of P35S (Fig. 5). We could reliably detect the presence of the CaMV virus when it constituted at least 20–30% of the total P35S content.

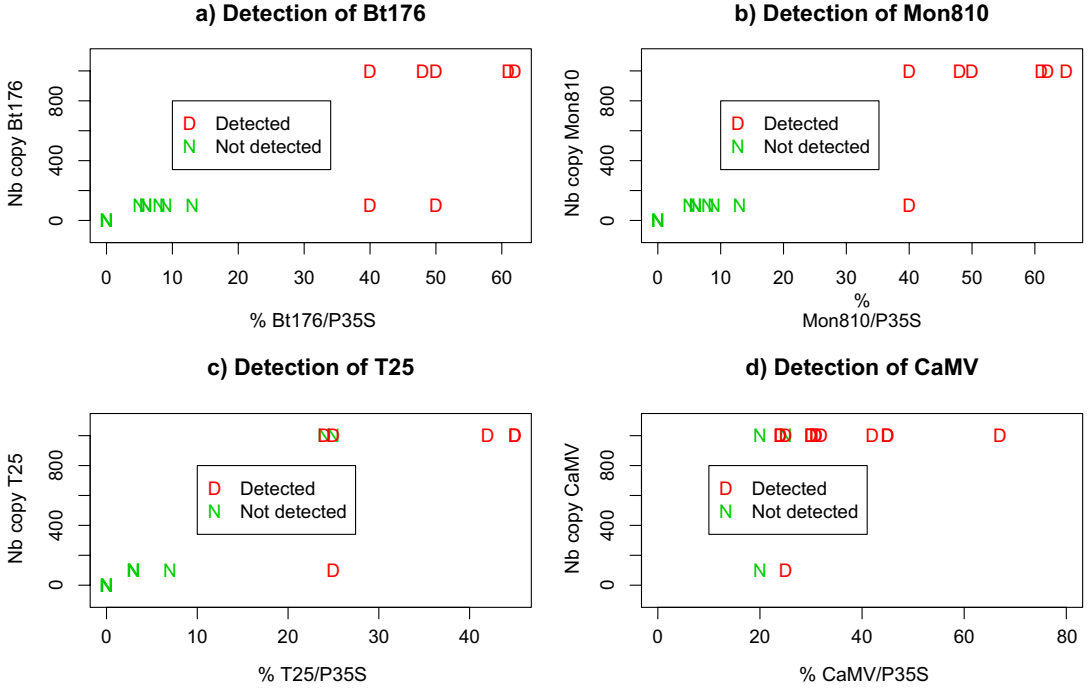

Fig. 5. Detection of a nonauthorized GMA as a factor of P35S content in design 7. The success of detection of a nonauthorized GMO depended on the haploid genome copy number of individual GM events or CaMV per reaction (*y* axis) and the percentage of P35S promoter originating from this event relative to total P35S amount (*x* axis).

Also, in this experiment, the number of P35S copies per haploid genome influenced the level of detection (T25 and CaMV vs Bt176 and Mon810). Indeed, Bt176 and Mon810 events (which carry two copies of the target amplicon) were more likely to be detected because they contributed 40% of the total P35S at the 1000 haploid genome level, where as T25 and CaMV contributed only 20–30% at the same copy number. Results here are a little higher than results in design 4, but in this case the study was focused on CaMV detection and its relative quantity of P35S.

Limits of dQ-PCR performance

Our study shows that the presence of nonauthorized GMO can be successfully inferred by comparing absolute quantities of amplicons and thus of targeted sequences. The application of a statistical model to assess Q-PCR results is justified here and ensures reliable test results.

The level of detection for sequences of unknown origin obtained in our case study depends on both the relative and the absolute quantities of targeted sequences. The limit of detection observed is in accordance with the limits of detection and quantification inherent to real-time Q-PCR tests.

Although our experiments were performed using a range of low copy numbers of target amplicons, we can assume that the dQ-PCR test would perform reliably using a range of higher copy numbers that are in the linear range of Q-PCR tests. The choice of performing the experiments at low copy numbers tests the model under the usual routine conditions that laboratories experience in the search for trace amounts of GMO in samples.

As previously shown, an effect of DNA extraction and/or DNA quantification interferes with the performance of the real-time PCR method and consequently with the performance of the dQ-PCR. These effects could be accounted for by data normalization with an endogenous reference gene (i.e., *Adh* in case of maize). However, the introduction of additional endogenous reference gene data in our statistical test would consequently also increase the variability of our test statistic and potentially decrease the performance of the test. Further work should therefore be focused on incorporation of an endogenous gene into the statistical model. Furthermore, as the number of marketed genetically modified organisms with different common elements increases, the model should be extended to include multiple common elements per study.

Our results also show that the absolute number of GMO elements inserted into the plant genome can be accurately determined by statistical testing of Q-PCR results, as we have shown with the determination of P35S copy number in the maize GM events. A potential application could be determining the number of construct insert copies in newly released GM events. Such tests could be used to determine the presence of additional sequences due to the plant transformation located outside the main GMO insert and thus could be useful for risk assessment purposes and dossier approvals.

Conclusions

Detection of unapproved GMOs in samples is urgently needed as evident since the last unintended release of GMOs such as LLRice601 and Shanyou 63 rice to the market. Several techniques to assess the presence of unapproved GMOs are currently examined by the Co Extra European research program (http://www.coextra.eu/). However, all methods studied for detection of nonapproved GMOs require an extensive change of the techniques used in the analytical laboratories and personnel training. In addition, without statistical methods, they so far generally do not provide means to assess the quantity (absolute or relative) of unapproved GMOs. An exception could be observed for qualitative methods using control plans with multiple attributes (see for instance the OPACSA software: URL: http://www.coextra.eu/researchlive/reportage851.html).

This paper presents the fastest, simplest, and most directly applicable approach to the detection of nonauthorized GMOs taking into account both the methods currently in use in analytical labs and the need to provide quantitative data in a cost- and time-effective way. Among the several detection strategies of unknown GMOs (matrix approach, profiling, etc.), we favored the approach whose low costs should facilitate its practical implementation in laboratories by using currently available methods and equipment. The method enables the detection of nonauthorized GMOs that contain common sequences in mixed samples. The method requires a previous knowledge of possible common sequences present but does not require specific target sequence knowledge of the potential nonauthorized GMOs being screened. The applicability of this strategy was successfully tested in the case of maize with the very commonly used P35S screening method. However, the statistical model can be easily transposed to other screening sequences such as tNOS terminator and genes of interest (e.g., *epsps, cry*).

We must emphasize that, according to the matrix approach (as defined in 1999 by Y. Bertheau in the GMOchips European research program), the absence of statistically different numbers of sequences copies, even the most frequently used, does not prove the absence of unapproved/unknown GMOs because numerous sequences are used in the commercial GMOs.

Application of the differential quantitative PCR required a development of a statistical model using an R-based program, which will be made available later through the Co-Extra web site. The developed program provides both the results on content of known GMO in the sample and the statistical interpretation of the result (i.e., the indication of the presence of nonauthorized GMOs). The acceptability level can be easily changed according to the aim of the analysis and the nature of the suspected GMO (e.g., according to the expected safety risk level or severity of the authorities). Different levels could for example be set for GMOs authorized in other countries for nonauthorized GMOs for which the safety was not assessed. The dQ-PCR method could also easily be applied to a wider scope of testing than GMO detection, for example, to the detection of pathogens, allergens, or mycotoxin–producing organisms which could easily be targeted by our detection strategy.

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Appendix A:. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2008.02.013.

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