Research Article

Quantitative-competitive polymerase chain reaction coupled with slab gel and capillary electrophoresis for the detection of roundup ready soybean and maize

The aim of the present study was to develop a quantitative-competitive PCR (QC-PCR) method to detect DNA from transgenic herbicide-resistant (roundup ready, RR) soybean and maize. Since no QC-PCR system for the quantification of RR maize had been published at the time of writing, a specific competitor DNA for transgenic event was developed. For the QC-PCR of RR-soybean, a commercially available competitor was employed. These internal standards were calibrated by coamplifying with mixtures containing RR-soybean and maize DNAs. The calibrated QC-PCR systems were applied to certified RR-soybean and maize flour mixtures in order to demonstrate their suitability not only for the quantification of the glyphosate resistance traits in DNA matrices, but also in practically relevant samples. In addition, a special focus of the present work was to compare the detection of QC-PCR products by slab gel and CGE with UV detection. CGE permitted the precise detection of transgenic events also below the equivalence points; while in slab gel electrophoresis, due to the low sensitivity the quantification of genetically modified DNA was allowed only at the equivalence point.

Keywords: Capillary gel electrophoresis / Competitive polymerase chain reaction / Competitor / DNA / Transgenic crops DOI 10.1002/elps.200500397

1 Introduction

The cultivation of genetically modified (GM) plants is becoming increasingly important worldwide. In 2003, GM crops covered a surface of approximately 68 million of hectares: they were grown mostly in the USA (42.8 million ha), Argentina (13.9 million ha), and Canada (4.4 million ha) [1]. The main transformed crops were soybean, maize, cotton, and canola with two main traits (insect resistance and herbicide tolerance). In 2003, according to the ISAAA report [1], the increase of acreage covered with transgenic crops was equal to 15%, with a 40-fold increase with respect to 1996.

The competent authorities of the European Union have dictated several directives for the commercialization of foodstuff containing genetically modified organisms (GMOs). The Novel Food Regulation [2] imposes the labeling for foods and ingredients containing or consisting in GMOs, while according to the directive 49/2000 [3] any foodstuff containing more than 1% of authorized GMO must be labeled as transgenic. Finally, the recent directive 641/2004 [4] restricts the need of labeling for products containing authorized GMOs above 0.9% and for products containing unauthorized GMOs above 0.5%. Considering the low threshold limits (0.5–0.9%) for the proportion of GMOs to be tolerated in an ingredient without the need for declaration and the adverse impact on market of food products labeled as “GMO-containing”, the surveillance of food containing GMOs requires reliable methods for the detection of genetic alterations. Quantitative-competitive PCR (QC-PCR), widely applied in the fields of clinical diagnosis and expression analysis, has also been demonstrated to be a suitable method for GMO analysis and quantification [5]. This approach is based on coamplification of the target sequence and defined amounts of an internal DNA standard (compet-
itor) carrying the same primer-binding sites. Given identical amplification efficiencies of the target and competitor DNA, the ratio of the amounts of the two amplicons (usually determined by slab gel electrophoresis) represents the ratio of target DNA and competitor present in the sample before the amplification [5]. Using slab gel electrophoresis for the detection of PCR products, the quantification is precise only at the equivalence point (molar ratio of amplified target versus competitor equals to one) [6]. This is a consequence of poor reproducibility and accuracy of slab gel electrophoresis procedure which usually include the separation of amplicons by agarose gel, the staining of the gel with ethidium bromide, the recording of the image by a digital imaging device, and the quantification of DNA fragments with specialized software.

CE has been successfully applied in these last years for the separation and detection of a huge number of compounds assuring several advantages such as speed of analysis, automation, and quantitative determinations. In particular, CE in polymer solutions has been demonstrated to be a powerful analytical tool for the separation of charged biomolecules (proteins, single- and double-stranded DNA fragments) [7]. Recent papers have evidenced the potentialities of the combined use of PCR and CE for the detection of transgenic DNA in different matrices [8, 9]. In addition, CE is shown to be a good alternative to improve the quantitative capabilities of QC-PCR [10].

In the present study, the development of a QC-PCR method for the quantification of DNA from transgenic herbicide-resistant (roundup ready, RR) maize was described. Since no QC-PCR system for the quantification of RR maize had been published at the time of writing, a specific competitor DNA for this transgenic event was developed. In order to assess the feasibility of developed QC-PCR method in detecting and quantifying RR maize, a well-established QC-PCR system for RR-soybean [11] was considered as reference method. For the QC-PCR of RR-soybean, a commercially available competitor was employed. A special focus of the studies was to compare the separation of QC-PCR products by slab gel electrophoresis and by CE with UV detection. For this purpose, the same QC-PCR conditions were employed for both the separation techniques. In particular, QC-PCR conditions were set up in order to obtain an equivalence point approximately equal to 1 and 5% w/w for RR-soybean and maize, respectively. The potentialities of CE in the definition of GMO percentages independently from the equivalence point were also evidenced by quantifying material with defined RR-soybean and maize.

2 Materials and methods

2.1 Soybean and maize samples

Seeds of GM RR-soybean and maize (Monsanto, USA) as well as nonmodified varieties (soybean cv. “Pacific” and maize cv. “Vertice”, SIS-Bologna-Italy) were supplied by the producers. Fifty seeds from each accession and cultivar (transgenic and conventional) were germinated in a growth chamber following the usual practices. At four-leaf stage, 1 g of leaves from each accession and cultivar was sampled, frozen in liquid nitrogen, and stored at −80°C until analysis. Certified reference materials (CRMs), produced by IRMM (Geel, Belgium), for RR-soybean (IRMM-410S-0; IRMM-410S-2; IRMM-410S-3; IRMM-410S-4; IRMM-410S-5) and RR maize (IRMM-414-0; IRMM-414-2; IRMM-414-3; IRMM-414-4; IRMM-414-5) were purchased from Fluka (Germany). These were freeze-dried powders containing different mass fractions of the GMO soybean (0, 0.5, 1, 2, and 5% w/w) and maize (0, 0.5, 1, 1.7, and 4.3% w/w).

2.2 Extraction and purification of genomic DNA

The DNA from GM and non-GM leaf samples as well as from CRMs was extracted according to the procedure described in [12]. The quality and efficiency of DNA extraction were controlled by agarose gel electrophoresis. The concentration of DNA in solution was measured by spectrophotometer (Beckman) at 260 nm, and its purity was evaluated by the ratio of absorbance at 260 and 280 nm.

2.3 LOD of PCR assays

To determine theoretical sensitivity levels decreasing amounts of target DNA with/without conventional (background) DNA were subjected to PCR [13]. In the experiments with background DNA, the template reaction mix was achieved after mixing varying volumes of pure GMO DNA with non-GMO DNA. Decreasing quantities of GMO DNA with no background DNA were obtained by serially diluting GMO DNA in double-distilled water in order to achieve GMO mass fractions in solution according to Table 1. Additionally, Table 1 shows the number of genome copies that were theoretically expected after serial dilution. The basis of the calculation for the number of genome copies in the dilution steps was a genome size for soybeans (haploid) of 1.55×10^9 bp [14], and for maize (haploid) of 5.00×10^9 bp (DOGS, Database of Genome Size, http://www.cbs.dtu.dk.databases/DOGS/index.html).
2.4 PCR

Oligonucleotides used were purchased from Genenco-M-Medical (Milan, Italy). The primer pair GM07/GM08 was specific for the detection of genetic modifications in RR-soybean and amplified a 169 bp segment [15], while the primer pair GA21/1–5’/GA21/1–3’ specifically identified the transgenic event in RR maize [16].

QC-PCR was carried out on a Whatman Biometra (Germany) T-gradient Thermal Cycler. Each reaction mixture contained 200 ng of total target DNA, 1 μL of DNA competitor (amounts ranging from 100 pg to 1 ng), 2.5 mM magnesium chloride (MgCl₂) (MBI Fermentas), PCR buffer 1× (MBI Fermentas), 200 μM (dNTPs), 0.5 μM for each primer (Genenco), and 0.4 U of Taq Polymerase (Taq Hot Start-MBI Fermentas) for a final volume of 20 μL.

The samples were subjected to PCR with the following temperature profiles: (i) PCR with soybean. After initial denaturation (95°C for 12 min), 40 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 25 s, there was a final extension step of 10 min at 72°C. (ii) PCR with maize. Initial denaturation at 95°C for 12 min, followed by 40 cycles of amplification at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The reaction was concluded at 72°C for 10 min.

2.5 Amplification kinetics

A critical factor in ensuring the accuracy of QC-PCR is to demonstrate that coamplifications of the target and internal control are equivalent [6]. In order to compare target and competitor amplification kinetics, equivalent amounts of these molecules were coamplified at increasing cycle numbers (between 35 and 50 cycles). Specifically 500 ng of a template DNA mixture containing 0.1% (w/w) of RR DNA were coamplified with 0.5 ng of DNA competitor at the PCR conditions previously described. After separation by CGE, the measured peak areas of target and competitor products were expressed as logarithms to produce the corresponding PCR kinetics. This can be done by plotting the log of target and log of internal competitor peak areas versus cycle numbers. These assays allowed the determination of the exponential range of PCR and comparison of the relative amplification efficiencies of target and competitor templates for each transgenic event. The slopes of both regression lines were essentially identical (0.65 and 0.67; $R^2 = 0.996$ for both RR-soybean and maize), demonstrating that the amplification efficiencies of the target and internal competitor templates were indistinguishable. Product accumulation proceeded exponentially up to cycle 43, after which it approached the plateau phase (data not shown). Based on these data, 40 cycles were chosen for all QC-PCR analyses.

2.6 Competitor for RR-soybean and maize

For the quantitative determination of RR-soybean, a commercial competitor supplied by Sigma Aldrich was employed (here thereafter named CSY). Expected size of amplified products of target DNA and competitor were equal to 169 and 220 bp, respectively.

As for RR maize no commercial competitor is available, it was constructed by employing the “Competitive DNA Construction Kit” supplied by Takara. Synthesized competitor (here thereafter named CMZ) had the same amplification efficiency as target DNA (270 bp) and had an expected size equal to 370 bp. Primers nucleotide sequences were not disclosed due to confidentiality reasons linked to patent approval.

2.7 Calibration of the QC-PCR

DNA mixtures (20 ng/μL) containing 0.5, 1, 2.5, 5, 10, and 50% w/w of RR-soybean or maize DNA and corresponding amounts of conventional DNA (99.5, 99, 97.5, 95, 80, and 50%) were prepared. A 500 ng of the mixtures containing 1% of RR-soybean and 5% of RR maize were coamplified with different amounts of competitor DNA (1 μL of 100 pg-1 ng/μL). The limit of 1% RR-soybean was chosen as value close to the threshold limit imposed by the directive 641/2004 [4]. For the setting up of a valuable QC-PCR system for RR maize, the limit of 5% GMO was
arbitrarily selected. Conditions for the QC-PCR were as described above. The equivalence point was calculated by the linear regression between the logarithm of the ratios of the amounts of amplification products and the logarithm of the initial amount of target (GMO DNA) [6].

2.8 Slab agarose gel electrophoresis

Amplification products (10 μL each) were separated by 1.8% agarose gel electrophoresis in 0.5 × TBE (Tris-borate-EDTA) buffer. A standard 100-bp (MBI Fermentas), containing 14 bands from 100 to 3000 bp, was used to determine the molecular weight of the amplicons. After ethidium bromide staining, gels were observed on a transilluminator 302 nm UV light and were recorded digitally with a Kodak Digital Science DC120 Zoom Digital Camera. The intensities of the bands were determined by image processing software (QuantityOne Software, Biorad).

2.9 CGE

Analyses were carried out in P/ACE 5500 (Beckman Instruments, Fullerton, CA, USA) CE apparatus, equipped with a diode array UV-Vis detector working at 254 nm. Bare fused-silica capillaries (75 μm id, 40 cm long) were employed for the separations. Injections (60 s) were made at the cathodic end using an N2 pressure of 0.5 psi (1 psi = 6894.76 Pa). The P/ACE 5500 CE instrument was controlled by a PC running the System Gold software (Beckman Instruments). Before use, the uncoated capillary was preconditioned by rinsing with 0.1 M hydrochloric acid (HCl) for 30 min. Between injections, capillary was rinsed for 4 min using 0.1 mM HCl and separation buffer. At the end of the day, capillary was rinsed with deionized water for 5 min and stored overnight with water inside. After the setting up of CGE method, the following conditions were chosen for QC-PCR product separation: temperature of separation 25–27°C, applied voltage 214 kV, electrolyte buffer 20 mM Tris, 10 mM orthophosphoric acid, 2 mM EDTA, and 2.5% hydroxyethylcellulose (HEC) (pH 7.3).

The optimized separation method was evaluated on the basis of LOD and LOQ. The calculation of the LOD of RR-soybean and maize amplicons were based on an S/N ratio of 3, whereas the definition of the lower LOQ was taken to be the signal equal to ten times of mean background signal [17].

3 Results and discussion

The protocol used for DNA isolation gave a DNA yield ranging from 300 to 500 μg/g of leaf material. The spectrophotometer readings gave an A260/A280 ratio of 1.8:1.9 confirming the high levels of purity of DNA samples. Upon electrophoresis only one sharp high molecular weight band was obtained proving the high quality of extracted DNA.

3.1 Construction of RR maize competitor for QC-PCR

The aim of internal control DNA construction is to produce a template with the same priming sites as the target molecule, but whose PCR products can be differentiated from those of the target DNA. A number of techniques are available for constructing internal controls [18–20]. In this study, the internal DNA standard for QC-PCR of RR maize was constructed with the Competitive DNA Construction Kit (Takara), following the manufacturer’s instructions. The construction strategy of competitor is depicted in Fig. 1. On the

Figure 1. Scheme for the construction of RR maize competitor DNA for QC-PCR. Target DNA was amplified with primers A and B (a). On the basis of required size of the competitor, the region of λDNA was determined and primers C and D were designed (b). Primers E and F, respectively, formed by primer A-complementer plus primer C and primer B-complementer plus primer D, were synthesized (c). Finally competitor DNA was amplified by using primers E and F (d).
basis of the sequence of the competitor DNA construction template (∼DNA), the sequences of two primers (C and D, Fig. 1b) were designed. The desired length of the DNA competitor (370 bp) was obtained by adding the length of the fragment amplified with C and D primers to the size of primers amplifying the target region, namely A and B. The final sequences of primers E and F (Fig. 1c) for DNA competitor preparation (Fig. 1d) were calculated as follows: sense primer for DNA competitor preparation (E) = 5’-(A)_complementer + (C)-3’ and antisense primer for DNA competitor preparation (F) = 5’-(B)_complementer + (D)-3’. The relevant parts of the competitor were sequenced and shown to contain the selected portion of ∼DNA (data not shown). In particular, competitor carried intact binding sites of the corresponding primer pair.

3.2 Setting up of CGE separation conditions

Adequate CGE-UV conditions for the fast and reproducible separations of DNA targets and competitors were first investigated. Considering that the expected sizes of DNA competitor and target for both RR maize and soybean were between 169 and 370 bp, the optimization was devoted to DNA fragments up to 400 bp. Different approaches for the CGE efficient separations of DNA fragments have been proposed [21–28]. On the basis of the available literature, the use of HEC in a Tris/orthophosphoric acid/EDTA buffer as originally proposed by Kleemiss et al. [24] and subsequently implemented by García-Cañas et al. [8, 9] seems to offer the best performance for the UV detection of DNA fragments. As a consequence, the CGE separation conditions proposed by García-Cañas et al. [8] were tuned for the specific purposes of the present research. A number of different parameters were evaluated including temperature (15, 20, 25, 30, 35°C), field strength (−10, −12, −14, −16, −18 kV), and polymer concentration (1, 2, 2.5, 3, 4% of HEC in 20 mM Tris, 10 mM orthophosphoric acid, and 2 mM EDTA (pH 7.3) buffer). Arbitrarily, 45 different separation conditions were selected (Fig. 2). Experiments were performed to determine how various changes in analysis parameters affect analysis duration, precision (expressed as percent deviation of the size estimation of DNA target and competitor of both RR maize and soybean with respect to the theoretical value), and resolution (expressed as peak resolution in base units). Relationships between measured parameters were examined through factor analysis. The results analyzed by principal component analysis (PCA) followed by varimax rotation to a set of orthogonal axes for the 45 separations are plotted in Fig. 2. Approximately in the middle of the Cartesian plan, seven separation conditions assuring the best resolutions (between 13 and 15 bp) were observed. In addition, the seven conditions allow for the separation of DNA fragments up to 400 bp within 20 min with good precision (mean deviation of size estimates of RR maize and soybean DNA target and competitor <2.5%). Since no relevant differences for duration time and precision among the seven conditions were observed, for all the subsequent analyses the separation condition assuring the best resolution (13 bp) was chosen (20 mM Tris, 10 mM orthophosphoric acid, 2 mM EDTA, 2.5% HEC (pH 7.3), −14 kV, 25°C temperature) (Fig. 3). The reproducibility of this procedure within the same day (ten injections) and between different days (5 d, 20 injections) for the DNA fragments of 100, 200, 300, and 400 bp (corresponding to the expected migration zone of competitor and target DNA for RR maize and soybean) was tested. A suitable reproducibility was obtained with an intraday mean RSD of migration times (MTs) equal to 1.1% and an interday mean RSD of MTs equal to 2.3%. The peak areas also exhibited good reproducibility with mean RSD values equal to 1.3 and 1.9% within intraday and interday replicates, respectively.

3.3 Slab agarose gel and CGE detection of amplicons

Migration distance (MD) and MT of six DNA fragments (100, 200, 300, 400, 500, 600 bp) were used for the preparation of molecular weight calibration curve in slab gel and CGE separations, respectively. The log (bp) was plotted against 1/MD or MT, and the following regression curves were obtained for slab gel and CGE detection systems, respectively:

\[
\log(\text{bp}) = -23.77(1/\text{MD}) + 3.20 \quad (R^2 = 0.993, \ n = 6) \quad (1)
\]

\[
\log(\text{bp}) = -35.23(1/\text{MT}) + 4.47 \quad (R^2 = 0.997, \ n = 6) \quad (2)
\]

Equations (1), (2) were used to determine the number of base pairs of soybean and maize targets. In agarose gel system the calculated values were 175 and 280 bp for soybean and maize targets, respectively, while competitor amplicon sizes were 230 bp for soybean and 380 bp for maize. For the four amplicons (targets and internal standards), the mean deviation from expected molecular weight was 4.0%. In CGE analysis, the calculated values were 165 and 219 bp for soybean target and internal competitor, while for maize target and internal competitor were 275 and 364 bp. The calculated values were in good agreement with theoretical values. For the four amplicons, the mean deviation from expected molecular weight was 1.7%.

To determine the approximate number of molecules that each specific primer pair was able to amplify, decreasing amounts of target template (in the presence and in the absence of conventional DNA) were amplified in single reactions for 40 cycles. In agarose gel separations, the
Figure 2. Principal component analysis (PCA) of different parameters (polymer concentration, temperature, and applied voltage) affecting analysis duration, precision, and resolution of CGE separations. For each separation condition (circles), the resolution (expressed as base units) and the separation parameters (polymer concentration, temperature, and applied voltage in brackets) are reported. The seven separation conditions (white circles) assuring the best resolutions (between 13 and 15 bp) are evidenced by a dotted line.

Figure 3. CGE separation of a 100-bp DNA ladder (500 ng/μL of DNA injected) using an uncoated fused-silica capillary (75 μm id, 40 cm effective length). Electrolyte buffer: 20 mM Tris, 10 mM orthophosphoric acid, 2 mM EDTA, 2.5% HEC (pH 7.3). Separation conditions: −14 kV at the temperature of 25°C. Detection at 254 nm. Injection for 60 s (0.5 psi). 1 = 100 bp, 2 = 200 bp, 3 = 300 bp, 4 = 400 bp, 5 = 500 bp, 6 = 600 bp, 7 = 700 bp, 8 = 800 bp, 9 = 900 bp, 10 = 1031 bp, 11 = 1200 bp, 12 = 1500 bp, 13 = 2000 bp, 14 = 3000 bp. The smallest number of molecules that produced a visible band was considered to be the lowest LOD for each primer set. GM-soybean mass fractions as low as 0.01% w/w, corresponding to 30 calculated genome copies per single reaction, were clearly detectable in ethidium bromide-stained agarose gels. As for RR maize, the recognition of the 270-bp amplicon was clearly possible up to a GM mass fraction of 0.05%. This dilution step corresponded to calculated 46 copies of the GM-maize genome per single PCR reaction. The analogous setup in CGE resulted in a lower LOD for RR-soybean amplicons with respect to agarose gel separations. In particular, as few as 15 copies of RR-soybean genome (corresponding to a mass fraction GMO equal to 0.005%) were necessary to produce a detectable peak in CE electropherograms, whereas for RR maize the LOD achieved (46 genome copies of GM-maize, corresponding to a mass fraction GMO equal to 0.05%) was the same as agarose gel separations. No interference of background DNA was observed as the LOD with/without conventional DNA was the same for both conventional and CGE separation systems.
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CE and CEC

Figure 4. Calibration of QC-PCR for the detection of RR-soybean (a) and maize (b) in slab gel electrophoresis. The sizes of the target and competitor PCR products are 169 and 220 bp (a), 270 and 370 bp (b), respectively. L = ladder. N = negative sample (0% GMO DNA). P = positive sample (100% GMO DNA). Linear regressions were calculated as described in Section 2. The numbering of the data points is according to the lane numbering of the corresponding agarose gel. For each data point, the SD of three replicates is reported (three gels for each amplification).

3.4 Slab gel electrophoresis quantification of RR-soybean and maize

As the first step to RR-soybean and maize quantification, it was necessary to establish the amount of competitor that should be added to the PCR for each transgenic event. For this purpose, 500-ng samples of genomic DNA from 1% RR-soybean and 5% RR maize were coamplified with different dilutions of respective internal standards (in the range 100 pg to 1 ng). The equivalence point (i.e., the concentration at which the relative areas of target and standard product are equal) was exactly calculated by linear regression between the logarithm of the initial amount of competitor and the logarithm of the ratio of the amounts of competitor and GMO target sequence [6]. The resulting regression equations were solved for the equivalence point as follows:

\[ \log\left(\frac{T}{C}\right) = \log(1) = 0 \]  

(3)

where \( T \) and \( C \) are the peak area of target and competitor DNAs, respectively.

The equivalence points were reached when using 200 pg of CSY and 1 ng of CMZ. Since peak areas are proportional to the amount of PCR products and are not related to the size of the amplified fragment, size correction was unnecessary.

In a second step, QC-PCR was performed with a constant amount of DNA competitor, determined previously, and varying percentages of GMO DNA (10, 5, 2.5, 1, 0.5, and 0%) for both transgenic events (Fig. 4). The slope values of the calibration curves were calculated and found to be 1.19 for RR-soybean and 1.22 for RR maize. These results would suggest different amplification efficiencies (theoretically it should be equal to 1, if the same amplification efficiency applies for target and standard fragments) for the original targets and the corresponding internal standards, precluding any accurate quantification. In spite of this limitation, the regression equations were solved for Equation (3) and the equivalence points were found to be located at 1.19 and 4.50% for RR-soybean and maize, respectively. However, it is to note that separation of amplicons in agarose gel electrophoresis and subsequent evaluation of signal intensities by densitometric analysis could be prone to bias. Peak areas of amplified bands and thus DNA quantification are strictly linked to gel-staining procedures. This characteristic, together with the low reproducibility and the limited resolution of slab gel electrophoresis systems suggest the employment of alternative methods in DNA quantification analysis. CGE technique brings about the independent quantification of QC-PCR products and has proven to be a good alternative to obtain accurate, precise, and sensitive results in the quantity of PCR-amplified DNA fragments [10].
Figure 5. CGE separations of a QC-PCR reaction mixture without DNA template for RR maize (a) and soybean (b). Arrows indicate the expected MTs for target and competitor DNAs. Separation conditions are as reported in Fig. 4.

3.5 CGE quantification of RR-soybean and maize

No interferences from the PCR reaction mixture were observed. In the absence of template DNA, no peak was found in the electropherogram regions where the peaks of amplicons (target and internal competitor) for RR-soybean and maize DNA were expected (Fig. 5).

For the calibration of QC-PCR with CGE detection, 500 ng template DNA mixtures containing 10, 5, 2.5, 1, 0.5, and 0% RR-soybean and maize were coamplified with a constant amount of competitor determined in the previous step in agarose gel analyses. Results of four out of the six GM DNA percentage mixtures employed for CGE calibration are shown in Figs. 6, 7. CGE electropherograms of coamplified targets and internal standards from conventional maize and soybean (0% GMO) showed only the expected internal competitor peaks (Figs. 6a, 7a). CGE separations of amplicons from defined mixtures of DNA for transgenic RR-soybean (0.5, 1, 2.5%) and maize (1, 2.5, 5%) exhibited the peaks corresponding to both targets and internal competitors (Figs. 6b–d, 7b–d).

For the chosen experimental conditions, the exact equivalence points were calculated (Fig. 8). To compensate for the differences in molecular weight of target and standard (namely, 169/220 bp for soybean and 270/370 bp), corrected areas of competitor amplicons were normalized using a factor of 0.77 and of 0.73, respectively.

For RR-soybean, the regression curve was

\[
\log(T/C) = 1.01 \log(T\%) - 0.03 (R^2 = 0.997, n = 5)
\]  (4)

while for RR maize, the following equation was obtained:

\[
\log(T/C) = 1.01 \log(T\%) - 0.62 (R^2 = 0.997, n = 4)
\]  (5)

For both transgenic events slope value was 1.01 indicating thus that the amplification efficiencies of the target and standard DNAs were essentially equal.

The regression equations were solved for the equivalence point which was located at 0.93 and 4.13% for RR-soybean and maize, respectively. These values were similar to those obtained with agarose gel detection under the
**Figure 7.** CGE separations of QC-PCR amplicons for RR maize: (a) 0% GMO; (b) 1% GMO; (c) 2.5% GMO; (d) 5% GMO. *T* = peak of target DNA. *C* = peak of competitor DNA. Separation conditions are as reported in Fig. 4.

**Figure 8.** Calibration of QC-PCR for the detection of RR-soybean (white circles) and maize (black squares) in CGE. Linear regression was calculated as described in Section 2. For each data point, the SD of three replicates is reported (three CGE analyses for each amplification).

The transgene proportions were calculated on the basis of calibration curves reported in Fig. 8. a) Not quantified because it was below the LOQ of CGE.

**Table 2.** Quantification of the proportion of RR-soybean and maize in flour reference mixtures based on CGE separations of QC-PCR amplicons (data refer to CGE results obtained by analyzing six times the same QC-PCR mixture)

<table>
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<th>GMO (%)</th>
<th>Mean amount (%) ± SD</th>
<th>Range (%)</th>
<th>CV (%)</th>
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<tr>
<td>Soybean</td>
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The transgene proportions were calculated on the basis of calibration curves reported in Fig. 8. a) Not quantified because it was below the LOQ of CGE.

In order to demonstrate the suitability of QC-PCR-CGE system not only for the quantification of the glyphosate resistance traits in DNA matrices, but also in practically relevant samples, commercially available flours containing defined amounts of RR-soybean (0, 0.5, 1, 2, 5%) and RR maize (0, 0.5, 1, 1.7, 4.3%) were analyzed. The transgene proportions calculated on the basis of previous calibration curves are reported in Table 2. All maize samples containing less than 1% of GMO could not be quantified, while for soybean the definition of transgenic DNA percentage was possible also for samples containing a minimum of 0.5% GMO. Except for maize CRM containing 0.5% GM DNA, the experimentally determined data for all the other samples were in good agreement with the actual GMO amounts. For soybean and maize samples containing more than 1% of GMO, the determination was more precise (CV between 6 and 12.5% for soybean and between 4.9 and 15.6% for maize) than that for samples containing 0.5–1% of GMO (CV higher than 20%).

**4 Concluding remarks**

Single-competitive PCR was developed for transgenic RR maize detection and quantitation, while for transgenic RR-soybean a commercially available system was tested.
For both systems, using the same experimental conditions, the conventional detection of amplicons by gel electrophoresis was compared with that by CGE. The quantification results confirmed the suitability of single-competitive PCR with CGE detection for the determination of RR traits in homogeneous and low-processed plant samples if reference material analogous to the sample is available. In particular, CGE permits, within the range of QC-PCR systems investigated in this work, the precise quantification of transgenic events independently from the equivalence points, while in slab gel electrophoresis due to the low sensitivity the quantification of GMO DNA is allowed only at the equivalence point. On the other hand, by using CGE it is possible to obtain an accurate quantification of the actual input DNA concentration based on appropriate calibration curves without testing various competitor amounts. This feature is useful for speeding up the analysis procedures permitting thus a high-throughput screening of several samples.

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5 References


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