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A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard® based on the 3′-transgene integration sequence

Marta Hernández, Maria Pla, Teresa Esteve, Salomé Prat, Pere Puigdomènech & Alejandro Ferrando*

Instituto de Biología Molecular de Barcelona (IBMB)-Consejo Superior de Investigaciones Científicas (CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain

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Abstract

The increasing presence of transgenic plant derivatives in a wide range of animal and human consumables has provoked in western Europe a strong demand for appropriate detection methods to evaluate the existence of transgenic elements. Among the different techniques currently used, the real-time quantitative PCR is a powerful technology well adapted to the mandatory labeling requirements in the European Union (EU). The use of transgene flanking genomic sequences has recently been suggested as a means to avoid ambiguous results both in qualitative and quantitative PCR-based technologies. In this study we report the identification of genomic sequences adjacent to the 3′-integration site of event MON810 in transgenic maize. This genetically modified crop contains transgene sequences leading to ectopic expression of a synthetic CryIA(b) endotoxin which confers resistance to lepidopteran insects especially against the European corn borer. The characterization of the genome–transgene junction sequences by means of TAIL-PCR has facilitated the design of a specific, sensitive and accurate quantification method based on TaqMan chemistry. Cloning of event MON810 3′-junction region has also allowed to compare the suitability of plasmid target sequences versus genomic DNA obtained from certified reference materials (CRMs), to prepare standard calibration curves for quantification.

Introduction

In recent years, cultivation and/or commercialization of several genetically modified organisms (GMOs) has been approved worldwide in different countries, and the tendency to increase the cultivated area as well as the number of modified traits is becoming evident (James, 2001). However, the norms that regulate the use and exploitation of GMOs differ from one country to another, and they are probably influenced by consumer perception (Kleter et al., 2001). In particular, the European Union (EU) establishes mandatory rules for labeling GMO foods or food ingredients with a 1% threshold level for adventitious presence of GM material (European Commission, 2000a) or all GM food additives introduced to the market (European Commission, 2000b). These strict European labeling norms have encouraged the development and application of technologies aimed to precisely quantify the presence of GMO in food or additives.

The most accepted techniques for accurate DNA quantification consist of either the PCR co-amplification of target and competitor DNA (Gilliland et al., 1990) or the real-time PCR product quantification initially developed by monitoring fluorescence emission of ethidium bromide at each cycle (Higuchi et al., 1992). Subsequent variations of the real-time quantitative PCR include novel DNA-binding dyes such as the SYBR Green (Morrison et al., 1998), or fluorescent oligonucleotides such as the Scorpion primers (Whitcombe et al., 1999), Molecular Beacons

*Author for correspondence
E-mail: afmgmp@ibmb.csic.es

(Tyagi & Kramer, 1996), fluorescence resonance energy transfer (FRET) probes (Wittwer et al., 1997) and TaqMan probes (Heid et al., 1996). Recent efforts to introduce the use of quantitative PCR for GMO detection and quantification have been reported using either quantitative competitive PCR methods (Studer et al., 1998; Zimmermann et al., 2000) or real-time quantitative PCR TaqMan technologies (Vaïtilingom et al., 1999; Berdal & Holst-Jensen, 2001; Taverniers et al., 2001; Terry & Harris, 2001). A notable advantage of the TaqMan chemistry is the high specificity based on the simultaneous use of three oligonucleotides in the PCR reaction. Two of the primers allow amplification of the product to which a third dual-labeled fluorogenic oligonucleotide, the TaqMan probe, will anneal. Upon polymerase amplification, the 5′–3′ exonuclease activity of the Taq polymerase releases a 5′ fluorescent tag from the annealed TaqMan probe, yielding a real-time measurable fluorescence emission directly proportional to the concentration of the target sequence. The peculiarities of the TaqMan system allow great versatility in the rational design of primers and probes, leading to very specific detection and quantification methods. In this work we have used the TaqMan technology to establish a method for the specific detection and quantification of the MON810 event in transgenic maize.

The *Zea mays* L. line MON810 from Monsanto (YieldGard®) is one of the 18 GMO authorizations granted to date in the EU for placing onto the market. This GMO contains a genome integrated plant expression cassette comprised of the cauliflower mosaic virus 35S promoter and hsp70 maize intron sequences driving expression of a synthetic *cryIA(b)* gene. The *cryIA(b)* gene codes for a delta-endotoxin which binds to specific sites localized on the midgut epithelium of susceptible lepidopteran insect species and interferes with midgut function, thus acting as a potent and highly specific insecticide (Van Rie et al., 1989). We have identified by means of the TAIL-PCR technique (Liu et al., 1995) the maize genomic sequences flanking the 3′-site of the MON810 event integration locus, and developed a specific and precise real-time PCR method to identify and quantify this transformation event. The specificity of the method is based on the design of a TaqMan probe that contains annealing sites for both the 3′-site of the transgene construct and the maize genome sequences immediately adjacent. The amplicon containing the TaqMan probe binding site is amplified using primers specific to the transgene and genomic flanking sequences, thus providing an unambiguous and sensitive quantification method for event MON810. Furthermore we have compared the detection and quantification limits attained with this method using either genomic DNA purified from certified reference materials (CRMs) or cloned target constructs as external calibration standards.

Materials and methods

Plant materials

Powdered CRMs with Bt176, Bt11 and MON810 maize were from the Institute for Reference Materials and Measurements (IRMM) and commercialized by Fluka (Buchs, Switzerland). Leaves of *Z. mays* cultivar W64A, *Zea diploperennis*, *Sorghum bicolor*, *Oryza sativa*, *Hordeum vulgare*, *Brassica napus*, *Brassica oleracea* ssp. *Botrytis*, *Arabidopsis thaliana* ecotype *Columbia*, *Solanum tuberosum* var. *Désirée*, *Lycopersicum esculentum* var. *Ailsa Craig*, *Helianthus annuus* were from plants cultivated in greenhouses at the IBMB-CSIC. Leaves of the isogenic wild-type line of MON810 were kindly provided by INRA. DNA samples of other plant species were provided from the Qpcrgmofood European project.

DNA isolation and quantification

Large-scale genomic DNA was isolated from 30 g of leaves according to Dellaporta et al. (1983), and purified by using cesium chloride density gradient centrifugation. Small-scale genomic DNA was isolated from 0.1 g of plant material using a CTAB-based protocol as described (Meyer & Jaccaud, 1997). DNA concentration was quantified using the spectrophotometer GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). Concentrations were further checked by agarose gel electrophoresis and ethidium bromide staining. UV-fluorescent emission was recorded and quantified with the Quantity One™ software (Bio-Rad Laboratories Inc., Hercules, CA).

PCR reactions

Unless stated all PCR reactions were carried out in a GeneAmp™ PCR System 9600 (Perkin Elmer Cetus Instruments, Emeryville, CA) and primers were purchased from MWG-Biotech AG (Ebensburg, Germany). Primer sequences are shown in Table 1. Standard PCR reactions were performed with the

Table 1. Oligonucleotides

PCR system	Name	Orientation	Sequence
Mapping integrated *cryIA(b)*	CRY2235R	Reverse primer	5′-GATGTAGCCGCGGAGCTGGTAGCGAGTGTAA-3′
	CRY2571R	Reverse primer	5′-GCTTCTCGCGCTTGTCCCTCCACTTCTTCTCA-3′
3′-junction PCR	CRY2388F	Forward primer	5′-GCCCACCACAGCCACCACTTCTCC-3′
	GENOMONR	Reverse primer	5′-TCCCGAGCTCATGGCGAAAAATCAC-3′
TAIL-PCR	CRY1213F	Forward primer	5′-GGCACGGTGGATTCCCTGGACGAGAT-3′
	CRY1825F	Forward primer	5′-GTCACCTTCGAAGCCGAGTACGACCTGGAGAG-3′
	AD2[a]	Arbitrary primer	5′-(AGCT)GTCGA(GC)(AT)GA(AGCT)A(AT)GAA-3′
Long PCR	810F[b]	Forward primer	5′-CGAAGGACTCTAACGTTTAACATCCT-3′
	GENOMONR	Reverse primer	5′-TCCCGAGCTCATGGCGAAAAATCAC-3′
Real-time quantitative PCR	MONF	Forward primer	5′-CAAGTGTGCCCACCACAGC-3′
	MONR	Reverse primer	5′-GCAAGCAAATTCGGAAATGAA-3′
	MONP	Forward probe	FAM-5′-CGACCTGAACGAGGACTTTCGGTAGCC-3′-TAMRA

[a] Liu et al. (1995).
[b] M. Vaïtilingom, Personal communication.

TaqMan PCR core reagents (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, NJ) in 50 µl of PCR mixture including: 1 × buffer II (100 mM Tris–HCl, pH 8.3, 500 mM KCl), 3 mM $MgCl_2$, 250 µM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 2 U of AmpliTaq Gold™ DNA polymerase and DNA template, according to the PCR program: 10 min at 95°C, 40 cycles of 20 s at 95°C, 30 s at 60°C and 30 s at 72°C with a final extension step of 7 min at 72°C.

TAIL-PCR

PCR reactions were performed according to the protocol described (Liu et al., 1995). Slight modifications for maize DNA samples were as follows. The first TAIL-PCR amplification was performed in a total volume of 10 µl containing: 1 × buffer II, 2.5 mM $MgCl_2$, 250 µM dNTPs, 0.3 µM primer *CRY1213F*, 5 µM primer *AD2*, 0.8 U of AmpliTaq Gold™ DNA polymerase and 50 ng of cesium chloride purified *MON810* template DNA. The PCR program for the primary TAIL-PCR consisted of a denaturation/activation step of 10 min at 95°C followed by 6 min at 68°C, and the amplification cycles: five cycles of 30 s at 94°C and 6 min at 68°C, one cycle of 15 s at 94°C, 3 min at 45°C and 6 min at 68°C, five cycles of 15 s at 94°C, 30 s at 45°C and 6 min at 68°C, and a final step of 12 cycles of 15 s at 94°C, 7 min at 68°C, 15 s at 94°C, 7 min at 68°C, 15 s at 94°C, 30 s at 45°C and 7 min at 68°C. Secondary TAIL-PCR amplification was carried out in a total volume of 20 µl containing: 1 × buffer II, 2.5 mM $MgCl_2$, 250 µM dNTPs, 0.2 µM primer *CRY1825F*, 2 µM primer *AD2*, 0.8 U of AmpliTaq Gold™ DNA polymerase and 1 µl of a 50-fold dilution of the primary PCR products. The secondary TAIL-PCR program was 10 min at 95°C followed by 15 cycles of 15 s at 94°C, 7 min at 68°C, 15 s at 94°C, 7 min at 68°C, 15 s at 94°C, 30 s at 45°C and 7 min at 68°C. The secondary PCR amplification product was analyzed by agarose gel electrophoresis and ethidium bromide staining. Parallel amplifications with wild-type isogenic DNA were carried out to identify MON810 specific PCR products. These DNA fragments were purified and subsequently cloned into the pGEM®-T Easy vector (Promega Corp., Madison, WI). DNA inserts in this vector were sequenced in both directions using the Big Dye™ Terminator Cycle Sequencing kit and an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems Division of Perkin Elmer Corp., Foster City, CA).

Long-range PCR

Primer *GENOMONR* matching the 3′-junction genomic sequence was used in combination with primer *810F* annealing at the 5′-flanking genomic sequence to perform long-range PCR amplification using either *MON810* or isogenic wild-type genomic DNA as template. Expand™ High Fidelity Taq polymerase (Boehringer-Mannheim, Germany) was used accordingly to manufacturer instructions. A PCR product

of ca. 4.2 kb was specifically amplified from the *MON810* DNA and cloned into the pGEM®-T Easy vector to yield plasmid pMON3.

Real-time quantitative PCR

TaqMan assay

The Primer Express™ 1.5 software (Applied Biosystems Division of Perkin Elmer Corp., Foster City, CA) was used to design the oligonucleotides shown in Table 1. The TaqMan® probe (Eurogentec, s.a., Seraing, Belgium) was labeled on the 5′-end with the fluorescent 6-carboxyfluorescein (FAM) reporter dye and the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) was attached to its 3′-end. For real-time PCR, primers *MONF* and *MONR* leading to a 106 bp product were used in combination with the *MONP* probe. PCR reactions were performed in a 20 µl reaction volume containing 1 × PCR TaqMan buffer A (includes ROX as a passive reference dye for real-time PCR), 6 mM $MgCl_2$, 400 µM each dATP, dCTP, dGTP, 800 µM dUTP, 500 nM primers, 200 nM probe, 1 U of AmpliTaq Gold™ DNA polymerase, 0.2 U of AmpErase® uracil *N*-glycosylase (UNG), and 2 µl of the DNA solution. Real-time PCR reactions were run on an ABI PRISM® 7700 Sequence Detection System device (Applied Biosystems Division of Perkin Elmer Corp., Foster City, CA) using the following program: 2 min at 50°C, 10 min at 95°C and 50 cycles of 15 s at 95°C and 1 min at 60°C. The real-time PCR products were analyzed using the sequence detection system software 1.7 (Applied Biosystems). Quantification was performed by interpolation in a standard regression curve of Ct values generated from DNA samples of known concentrations. Negative values or lack of amplification was considered for those real-time PCR reactions with Ct value of 50 or above.

Construction of standard curves

Two sets of standards were used for quantification and determination of the detection and quantification limits. Genomic DNA isolated from powdered 5% MON810 maize (Fluka) was serially diluted to final concentrations equivalent to 500, 250, 50, 5, 2, 1, 0.5 target molecules/µl (amounts of DNA per reaction tube ranged from 1000, 500, 100, 10, 4, 2 and 1 target molecules) considering 2504 Mb per haploid genome in the case of maize according to Arumuganathan and Earle (1991). Following these calculations one haploid genome molecule corresponds to 2.6 pg of maize DNA assuming that 965 Mb weigh 1 pg. Standards from plasmid DNA were constructed using the cloned *MON810* transgene in pGEM®-T Easy vector. Plasmid DNA purified using a QIAGEN® Plasmid Midi Kit (QIAGEN GmbH, Hilden, Germany) was serially diluted in a solution containing 50 ng/µl BSA to yield 10^6, 10^5, 10^4, 10^3, 10^2, 10, 4, 2, 1 molecules. According to the plasmid size one plasmid molecule (7264 bp) corresponds to 7.5 ag of plasmid DNA considering that 965 Mb weigh 1 pg. Mean Ct values are the average of a total of 14 replicates of two independent experiments. The statistical analysis was applied to every serial dilution calculating a confidence interval at the given probability. The calculations were performed according to a binomial distribution as reported (Kay & Van den Eede, 2001). The results were also confirmed by Monte-Carlo simulations.

Results

Maize genomic sequences at the 3′-site of MON810 event integration locus

Based on a thorough scrutiny of genome and patent databases, we retrieved and aligned a large number of DNA sequences corresponding to the *cryIA(b)* gene. The consensus sequences derived allowed to design a battery of oligonucleotides covering the whole *cryIA(b)* coding sequence that we used to precisely map the presence of this transgene in MON810 transgenic maize. PCR amplifications were performed with MON810 genomic DNA as a template using the complete set of forward oligonucleotides designed on *cryIA(b)* sequence together with reverse primers *CRY2235R* or *CRY2571R* (Table 1). No amplification was obtained for the combinations that included *CRY2571R* as a reverse primer, whereas expected fragments were amplified when using *CRY2235R* as a reverse primer (data not shown). Initial reports from Monsanto, available through the Agbios web site (http://www.agbios.com/) suggested a truncation of the CryIA(b) protein, based on western studies, thus indicating that during transformation, a partial integration or truncation of the transgene might have occurred. Our data suggest a truncation of the *cryIA(b)* gene between positions 2235 and 2571. This would indicate, as recently updated, that the 3′-region of the *cryIA(b)* gene together with NOS terminator sequences could be absent from the MON810 transgenic maize genome (Figure 1(A)). Upon narrowing down

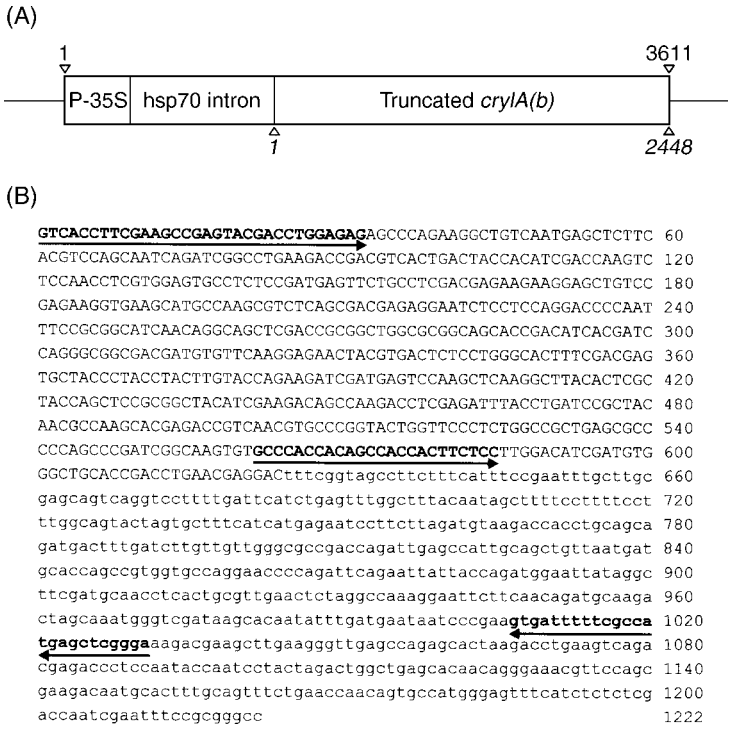

Figure 1. Genome organization of event MON810 in transgenic maize. (A) Schematic representation of the genomic arrangement of event MON810 transgene. Transgene elements are drawn to scale with the top arrow-heads indicating the position of the whole insert and lower arrow-heads showing the numbering of *cryIA(b)* gene coding sequence. Adjacent solid lines represent maize genome. (B) The TAIL-PCR amplified 3′-junction sequence was cloned and sequenced. Capital letters represent the 3′ coding region of *cryIA(b)* gene, and lowercase letters show the flanking genomic sequence. Primers used for PCR amplifications detailed in Table 1 are highlighted in bold and underlined.

the putative truncation site of the *cryIA(b)* gene, we were able to design appropriate specific primers to amplify genomic flanking sequences by means of the TAIL-PCR technique. The combination of the *AD2* arbitrary primer together with the specific primers *CRY1213F* and *CRY1825F* in first and second amplification reactions, respectively, amplified a discrete product of ca. 1.2 kb. No amplification product was observed in parallel experiments with genomic DNA of wild-type isogenic maize as template. The TAIL-PCR product was cloned into the pGEM®-T Easy vector and fully sequenced (GenBank accession number AF490398). Figure 1(B) shows the sequence of the cloned DNA fragment indicating the precise location of the truncation site at position 2448 of the *cryIA(b)* gene coding sequence. To further demonstrate that the PCR-amplified sequences do indeed correspond to maize flanking sequences, we used this genomic sequence to design primer *GENOMONR* and performed standard PCR reactions with the *cryIA(b)* gene primer *CRY2388F*. A discrete amplification product of 470 bp was obtained as expected when using MON810 genomic DNA as a template, but no amplification was observed with the wild-type maize isogenic DNA template (Figure 2(B)). In the course of our investigation, parallel efforts to characterize the genomic organization of the MON810 event in YieldGard® maize led to the identification of the genomic flanking sequences located at the 5′-transgene integration site (Holck et al., 2002). We have used primers flanking both the 5′- and 3′-integration sites to perform long-range PCR amplifications with either MON810 maize or the wild-type isogenic genomic DNA as templates. As shown in Figure 2(A), a unique amplification product of approximate size 4.2 kb was obtained with maize MON810 DNA, whereas no visible amplification was obtained from wild-type DNA (Figure 2(A)). The whole long-range PCR product was cloned into the pGEM®-T vector, yielding plasmid pMON3. These data, together with southern-based information provided by Monsanto (http://www.agbios.com), suggest that a unique transgene element of about 3.6 kb corresponding to a truncated *cryIA(b)* gene is present in the genome of YieldGard® maize.

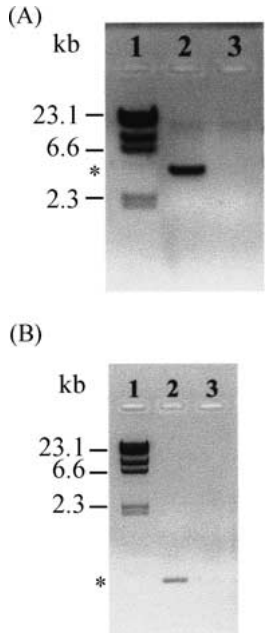

Figure 2. Event MON810 and 3′-junction region amplifications. (A) Long-range PCR reactions were performed with primers *810F* and *GENOMONR*. PCR reactions using either MON810 or isogenic wild-type DNA as templates in lanes 2 and 3, respectively, were separated by agarose electrophoresis and photographed under UV- light after ethidium bromide staining. (B) Standard PCR reactions with *CRY2388/GENOMONR* as primer combinations were performed using MON810 or isogenic wild-type DNA as templates in lanes 2 and 3, respectively, and processed as in (A). Lane 1 shows in both panels the molecular weight markers of *Hin*dIII digested lambda DNA. Asterisks indicate the presence of specific PCR products.

Use of the 3′-integration site sequence to design a TaqMan-based quantification method specific for the MON810 event in transgenic maize

Availability of the 3′-end insertion sequences corresponding to the unique insertion event in MON810 maize offers an excellent opportunity for the development of specific detection and quantification methods based on real-time quantitative PCR. We have exploited this to design a suitable TaqMan assay based on the sequence obtained. As shown in Figure 3, the combination of one forward primer located at the 3′region of the *cryIA(b)* gene together with a reverse primer annealing to the maize flanking genomic sequence, yields a 106 bp amplicon with binding sites for the TaqMan probe. The dual-labeled fluorescent TaqMan probe contains annealing sequences for both the 3′-border of the truncated *cryIA(b)* gene and the maize genomic DNA, thus providing extra specificity to the detected signal. We have tested this new assay to evaluate its specificity in different samples, using the ABI PRISM® 7700 Sequence Detection System from Applied Biosystems. To assay the specificity of the method, we have tested different plant species, and GMO materials containing *cryIA(b)* based constructs different from MON810, such as maize Bt11 and Bt176. As shown in Table 2, only detectable quantitative amplification was observed with the MON810 event.

Comparison of external standards based on plasmid or genomic DNA for the evaluation of detection and quantification limits

Estimation of the accuracy and precision of the TaqMan assay is important regarding labeling norms in the EU that establishes 1% of transgenic material as the threshold limit for compulsory labeling. We have used both CRM and plasmid pMON3 as external standards to build the calibration curves used in quantification. As CRM for the MON810 event, we have used genomic DNA isolated from 5% MON810 maize validated by the IRMM and commercialized by Fluka. Plasmid pMON3, containing the full MON810 transgene integrated in the pGEM-T vector, was obtained in our laboratory as described in the Materials and methods section. Appropriate dilutions of both types of DNA templates were prepared to yield an estimated number of target DNA molecules ranging from 10^6 to 1 for plasmid pMON3, and 10^3 to 1 for IRMM standard genomic DNA. The narrower linear range for the standard curve based on genomic DNA, is due to signal inhibition of this sample in the upper limits (Berdal & Holst-Jensen, 2001). In spite of the wide linear range of plasmid target molecules to build the standard curves, high reproducibility and accuracy can be obtained as shown in Figure 4. A very efficient amplification was obtained as indicated by the slope of the linear regression analysis (−3.498) with a good correlation coefficient (0.999). Two parameters have to be considered in terms of quantification, the absolute detection limit and the absolute quantification limit, the latter being more relevant for legal issues. As shown in Table 3, both type of standards yielded similar results in terms of absolute detection values, with similar threshold cycle values for two target DNA molecules and a practical detection limit of up to one molecule. The estimation of the practical limit of quantification also requires a careful experimental design to

Figure 3. Design of a TaqMan assay for event MON810 in YieldGard® maize. The sequence represents the 3′-junction region of event MON810, with uppercase letters displaying the sequence of transgene *cryIA(b)* gene and in lowercase the genome flanking sequence. Forward and reverse primers *MONF* and *MONR* amplify a 106 bp amplicon with annealing sequences for dual-labeled fluorescent probe *MONP* placed in the transgene–genome border sequence.

Table 2. Specificity of event MON810 TaqMan assay tested with genomic DNA from different plant species or CRM

Plant species	Amplification
Z. mays W64A (maize)	–
Z. diploperennis (teosinte)	–
S. bicolor (sorghum)	–
S. cereale (rye)	–
O. sativa (rice)	–
T. aestivum (wheat)	–
P. miliaceum (millet)	–
H. vulgare (barley)	–
B. napus (rapeseed)	–
B. oleracea ssp. *Botrytis* (cauliflower)	–
A. thaliana	–
S. tuberosum (potato)	–
L. esculentum (tomato)	–
H. annuus (sunflower)	–
G. max (soybean)	–
L. esculenta (lentils)	–
V. faba (white bean)	–
P. aureus (mung bean)	–
L. albus (lupine)	–
GMO (DNA from CRM)	Amplification
Maize event MON810	+
Maize event 176	–
Maize event Bt11	–

minimize the bias associated with serial dilutions of DNA samples, and in this case the probable range of GMO copies that would be sampled needs to be considered. The results obtained according to our experimental design are shown in Table 4. The confidence interval for dilutions containing 10, 4, 2 and 1 copy overlap with each other meaning that a reliable quantification of target molecules is only possible above 10 copies at the described confidence level both for plasmid and genomic standards.

Discussion

The cultivation and commercialization of GMOs in the EU is subjected to exhaustive control measurements that require detailed analysis of the organism to be introduced into the market (Kleter et al., 2001). These studies usually cover a number of issues related to agronomic, nutritional, environmental or consumer safety impacts, and minor importance is usually given to the molecular characterization of the GMOs. In spite of the little attention paid to date to the molecular genomic organization of the transgenes present in GMOs, there are reasons to change this tendency due to (i) the highly sensitized consumer perception of biological safety aspects, and (ii) the potential biotechnological applications derived from these studies. One example of such application is the establishment of precise detection and quantification assays, based on the transgene flanking sequences. As further developments of GMO technology may lead to the introduction of identical transformation events into different crop species, a detailed description of individual genomic arrangements is a valuable tool for the design of event-specific assays.

Current methods for GMO production cause random integration of the transgene DNA into the plant genome independently of the method of choice, either *Agrobacterium*-mediated transformation or direct DNA transfer. The transgene–genome border regions will therefore be unique for each particular transformation event, occurring only once per haploid genome. In our study, transgenic maize containing MON810 event was generated by direct DNA transfer through microprojectile bombardment. The molecular rules governing this type of transformation have been precisely studied in rice, suggesting a two-step mechanism of transgene integration mediated by the establishment of integration hot spots (Kohli et al.,

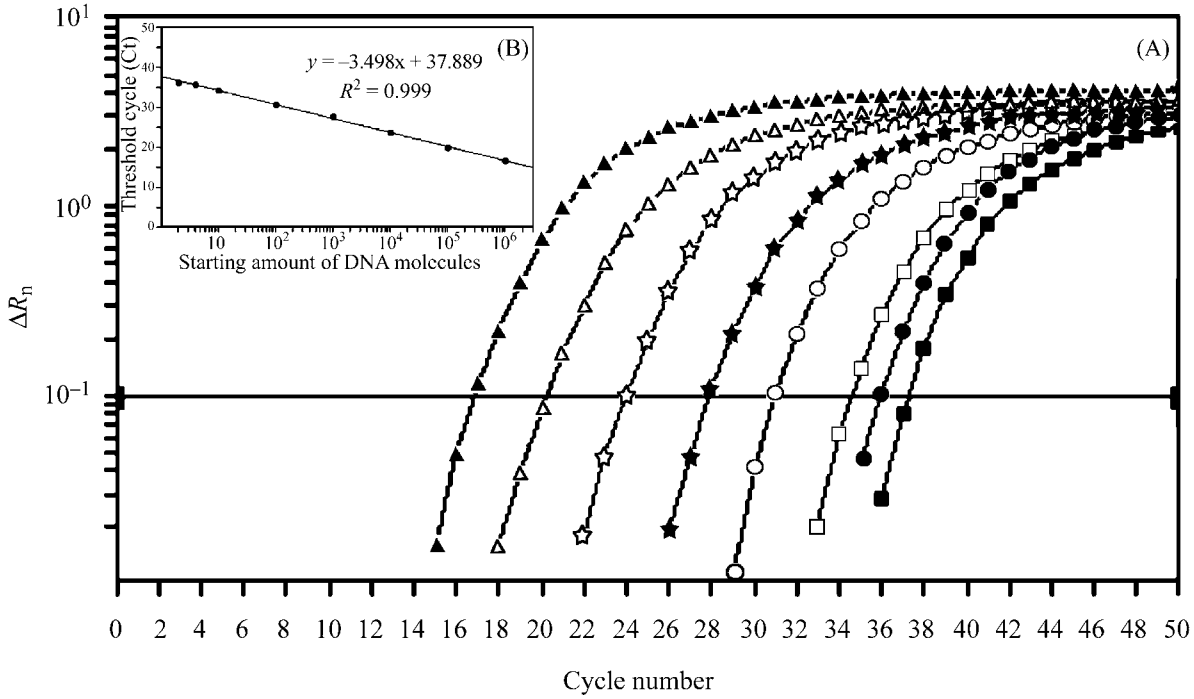

Figure 4. Detection and amplification of event MON810 in real-time PCR. (A) Typical amplification plot generated by using primers *MONF*, *MONR* and probe *MONP* with serial dilutions of plasmid pMON3 to 10^6, 10^5, 10^4, 10^3, 10^2, 10, 4 and 1 target molecules shown in closed triangle, open triangle, open star, closed star, open circle, open square, closed circle and closed square, respectively. (B) Representative standard curve generated from the amplification data given in (A).

Table 3. Detection and quantification limits obtained with the two external standards. Ct refers to threshold cycle value, SD is the standard deviation

Plasmid DNA									
Template molecules (approximate)	10^6	10^5	10^4	1000	100	10	4	2	1
Signal ratio (positive signal/total reactions)	14/14	14/14	14/14	14/14	14/14	14/14	14/14	12/14	10/14
Mean Ct values	16.93	20.15	23.82	28.18	30.64	34.15	35.87	38.03	40.74
SD Ct values	0.08	0.06	0.09	0.09	0.19	0.55	1.03	5.18	6.14
Genomic DNA from certified material									
Template molecules (approximate)	1000	500	100	10	4	2	1		
Signal ratio (positive signal/total reactions)	14/14	14/14	14/14	14/14	14/14	11/14	6/14		
Mean Ct values	27.81	28.88	31.34	34.92	36.12	39.94	45.10		
SD Ct values	0.13	0.14	0.18	0.66	0.49	5.52	6.11		

1998). A first step in the pre-integration process may cause transgenic construct rearrangements due to multimerization, whereas a second phase of chromosomal integration seems to be favored by the presence of hot spots, sometimes leading to tandem transgene repeats with interspersed plant DNA sequences in a single genetic locus. Similar results were observed with different relevant crop species such as oat (Pawlowski & Somers, 1998) and maize (Register et al., 1994). In addition to cellular mechanisms controlling the transgene integration process, subsequent selection procedures for the modified biological material may introduce further genomic reorganizations. This might be the case for event MON810 where the structure of the transgene differs notably from the original plasmid construct as reported in the safety

Table 4. Statistical analysis for the determination of practical quantification limits

Plasmid DNA									
Template molecules (approximate)	10^6	10^5	10^4	1000	100	10	4	2	1
Confidence level	95.0%	95.0%	95.1%	95.2%	95.5%	96.3%	96.1%	98.4%	98.1%
Lower limit confidence interval	998042	99381	9804	938	80	4	1	0	0
Upper limit confidence interval	1001958	100619	10196	1062	120	16	8	5	3
Genomic DNA from certified material									
Template molecules (approximate)	1000	500	100	10	4	2	1		
Confidence level	95.1%	95.2%	95.1%	96.4%	96.2%	98.5%	98.2%		
Lower limit confidence interval	939	457	81	4	1	0	0		
Upper limit confidence interval	1061	543	119	16	8	5	3		

assessment by Monsanto (http://www.agbios.com/). In this work we have characterized the 3′-region of the MON810 event, thus allowing a precise mapping of the *cryIA(b)* gene truncation site. Intriguingly, the missing 3′-region of the original construct and the genomic flanking sequences identified suggest that a genomic rearrangement involving the original insertion locus for the MON810 transgene might have occurred. Several lines of evidence support this possibility. First, the recently published 5′-genomic junction sequence (Holck et al., 2002) shows homology with LTR sequences of the *Z. mays* 22 kDa alpha Zein gene cluster reported in the GenBank Accession Number AF090447, whereas the 3′-genomic sequence identified by us does not match with this annotated genomic DNA fragment. Second, we could not find any significant database homology when using as query the 3′-genomic junction sequence reported here. Moreover, the long-range PCR reactions performed with the 5′- and 3′-flanking genomic sequence primers yielded an amplified product only with the MON810 maize template, but not with the wild-type isogenic genomic DNA template. Finally, the genomic organization of MON810 event in transgenic maize reveals an unusual arrangement with respect to the common genomic structure generated after particle bombardment that usually consists of several multimeric transgene copies with intervening genomic DNA. The presence of a unique truncated transgene could indicate that recombination events leading to a partial loss of the inserted element and adjacent genomic sequences might have happened, with no apparent consequences for either the activity of the partial CryIA(b) endotoxin or vigor and yield of the maize YieldGard® line. Further experiments are needed to precisely characterize the genomic organization at this locus for the maize YieldGard® and to unequivocally demonstrate the possible genomic rearrangement that our reported data suggest.

The elucidation of DNA transgene–genome junction sequences at the 3′-site of the integration event, has paved the way to design and implement a specific detection and quantification method for the presence of the MON810 event transgenic maize. We have chosen the real-time quantitative PCR TaqMan system to design a TaqMan probe which is unique to the 3′-end of the MON810 insertion event. A number of advantages illustrate selection of this type of quantification method as (i) no post-PCR manipulation of the amplified product is required, thus avoiding carry-over contaminations, (ii) simultaneous use of three oligonucleotides in the TaqMan PCR reaction entails a high specificity of the captured fluorescent signal which is directly proportional to the amount of amplification product, and (iii) the small amplicon size required for TaqMan chemistry is well suited for the analysis of processed food samples, in which large amounts of fragmented DNA are to be expected. Altogether, these benefits concede an extraordinary level of specificity, precision and robustness to the TaqMan real-time quantitative PCR based on TaqMan probes annealing to genome–transgene junction sequences particular of a given GMO. We have demonstrated here the validity of our designed TaqMan method for the 3′-transgene integration MON810 event both in terms of specificity and accuracy in accordance to labeling requirements.

Further studies using this assay include a comparison of the estimated accuracy reached by addition of external plasmid or genomic DNA calibration standards. A commonly accepted practice is the use of GMO CRMs as calibration standard for quantification

purposes (Vaïtilingom et al., 1999; Zimmermann et al., 2000; Berdal & Holst-Jensen, 2001; Terry & Harris, 2001). Intrinsic features of these type of validated standards may lead to imprecise quantification of the DNA copy number values, whereas curves built up with cloned target plasmid DNA may be more suitable to quantify GMOs in a wide range of food-derived processed samples (Taverniers et al., 2001). In our hands both types of external standards yielded similar detection and quantification limits in good agreement with recent literature (Berdal & Holst-Jensen, 2001), being difficult to favor either plasmid DNA or genomic DNA as calibration standards. Among the advantages of using plasmid DNA as standard are the simplicity in preparation, low costs, and a wider linear range of target molecules due to lack of inhibition. On the other hand, the use of GMO-derived genomic DNA as standard may be more faithful to the analytical situation, especially in terms of PCR efficiency. Nevertheless the intrinsic properties of a processed food sample such as poor DNA quality and presence of undesired chemical compounds, makes almost impossible to determine what sort of standard better simulates the amplifications required for quantification analysis.

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