Plant Mol Biol (2010) 74:563–571
DOI 10.1007/s11103-010-9696-2

Stability of the MON 810 transgene in maize

Jose Luis La Paz · Maria Pla · Nina Papazova · Pere Puigdomènech · Carlos M. Vicient

Received: 1 June 2010 / Accepted: 21 September 2010 / Published online: 9 October 2010

Abstract We analysed the DNA variability of the transgene insert and its flanking regions in maize MON 810 commercial varieties. Southern analysis demonstrates that breeding, since the initial transformation event more than 10 years ago, has not resulted in any rearrangements. A detailed analysis on the DNA variability at the nucleotide level, using DNA mismatch endonuclease assays, showed the lack of polymorphisms in the transgene insert. We conclude that the mutation rate of the transgene is not significantly different from that observed in the maize endogenous genes. Six SNPs were observed in the 5′flanking region, corresponding to a Zeon1 retrotransposon long terminal repeat. All six SNPs are more than 500 bp upstream of the point of insertion of the transgene and do not affect the reliability of the established PCR-based transgene detection and quantification methods. The mutation rate of the flanking region is similar to that expected for a maize repetitive sequence. We detected low levels of cytosine methylation in leaves of different transgenic varieties, with no significant differences on comparing different transgenic varieties, and minor differences in cytosine methylation when comparing leaves at different developmental stages. There was also a reduction in *cryIAb* mRNA accumulation during leaf development.

Keywords Genetically modified organism · MON 810 maize · Single nucleotide polymorphisms · DNA methylation

Electronic supplementary material The online version of this article (doi:10.1007/s11103-010-9696-2) contains supplementary material, which is available to authorized users.

J. L. L. Paz · P. Puigdomènech · C. M. Vicient (✉)
Molecular Genetics Department, Centre for Research in Agricultural Genomics CRAG (CSIC-IRTA-UAB), Jordi Girona 18-24, 08034 Barcelona, Spain
e-mail: cvsgmp@cid.csic.es

M. Pla
Institute for Agricultural and Food Technology, University of Girona, Campus Montilivi, EPS-1, 17071 Girona, Spain

N. Papazova
Unit Technology and Food, Institute for Agricultural and Fisheries Research, Burg. Van Gansberghelaan 115, 9820 Merelbeke, Belgium

Introduction

Exogenous DNA is introduced into plant genomes for GMO production using *Agrobacterium*-mediated transformation or particle bombardment. After transformation, plant regeneration through in vitro culture can cause rearrangements in the transgene (Register et al. 1994; Kohli et al. 1999), but once transgenic plants have been generated, it is assumed that the transgene is subject to the same mutation rates as the endogenous genes. Stable inheritance of foreign genes has been demonstrated in some transgenic plants based on field trials, the expression of the transgene mRNA or protein, and by Southern analysis (Müller et al. 1987; Delannay et al. 1989; Padgette et al. 1995; Duan et al. 1996; Fearing et al. 1997; Widmer et al. 1997). However, these analyses do not permit the detection of point mutations and small insertions or deletions that could potentially result in the production of altered recombinant proteins or in changes in the regulation of the transgene expression. Moreover, point mutations could also affect the reliability of the established methods for detection and quantification based on real-time quantitative PCR.

Change in primary DNA sequence is not the only mechanism that can produce alterations in the expression

of a transgene. Epigenetic changes, often associated with changes in DNA methylation, may also contribute to phenotypic variability and transgene silencing (Mette et al. 2000). The expression of some maize genes is epigenetically regulated by cytosine methylation and is variable among inbred lines (Makarevitch et al. 2007). Some studies have showed that, in some cases, transgene expression is influenced by the developmental stage of the plant or by environmental factors (Meyer et al. 1992; Dorlhac de Borne et al. 1994). A detailed analysis of transgene methylation patterns in transgenic commercial crops is necessary to be able to assess its possible consequences on expression.

The GM YieldGard® MON 810 maize event contains a transgene cassette carrying the 35S promoter derived from the *Cauliflower Mosaic Virus* (P-35S), the hsp70 intron, and the (truncated) *cryIA(b)* sequence that encodes a toxin giving resistance to *Lepidoptera*, particularly the European corn borer (van Rie et al. 1989). The 3′-end of the *cryIA(b)* coding sequence and the complete NOS terminator were lost during the transformation process (Hernández et al. 2003), and transcription read-through into the host 3′ flanking sequence has been observed (Rosati et al. 2008; La Paz et al. 2010). Determination of the maize genome 5′ sequence flanking the insert sequence has showed that it is homologous to a retrotransposon long terminal repeat (LTR) (Holck et al. 2002), while the 3′- flanking sequence corresponds to a gene putatively coding for a HECT type E3 ubiquitin ligase (Rosati et al. 2008). The lack of the 3′-region of the original transgene construct and the lack of continuity between the two flanking sequences suggest that a genomic rearrangement occurred, involving the original insertion locus, during the integration of the MON 810 transgene.

The activity of CryIA(b) and the fitness and yield of the maize YieldGard® varieties are, however, satisfactory. Maize MON 810 was approved for cultivation and commercialization in the EU in 1998. The transgene has since been introduced into many different local elite varieties by classical breeding methods: the transgenic and elite varieties are crossed, and the progeny is subjected to several cycles of back-crossing with the local elite variety, selecting for the presence of the transgene (Holst-Jensen et al. 2006).

Here we studied, in detail, the genomic variability of the MON 810 transgene cassette and flanking regions in several commercially available transgenic maize lines. The study addressed the analysis of large rearrangements using Southern analysis, point mutations and small indels using the mismatch endonuclease assay, and cytosine methylation using bisulfite sequencing. DNA methylation of the transgene and *cryIA(b)* mRNA levels were also determined throughout the plant development.

Results

MON 810 transgene locus structure and variability

Overall conservation of the genetic structure of the insertion locus was analysed by Southern analysis. Genomic DNA was extracted from three commercial YieldGard® maize varieties obtained by three different breeders (DKC6575, PR33P67 and Aristis Bt; Table 1), digested with restriction enzymes and hybridized with three probes (Fig. 1). The band patterns obtained with the three varieties were identical, indicating that there were no major rearrangements associated with the transgene locus.

Table 1 YieldGard®maize event MON 810 lines

Line	Breeder company	Year[a]
Aristis Bt	Semences coop. de PAU	2003
Asturial Bt	Euralis Genetique	2006
Beles Sur	Holden Foundation Seed	2006
Campero Bt	Advanta Seeds B.V.	2004
Cuartal Bt	Euralis Genetique	2004
DKC6575	Monsanto Technology LLC.	2003
DKC6041-YG	Monsanto Technology LLC.	2005
DKC5784-YG	Monsanto Technology LLC.	2005
DK513[b]	R.a.g.t. Semences	1998
Elgina	Pioneer Hi-Bred International	1998
Foggia	Maisadour Semences	2005
Gambier Bt	Semences coop. de PAU	2004
Helen Bt	Advanta Seeds B.V.	2005
Jaral Bt	Semillas Fitó, S.A.	2004
Kaper-YG	Holden Foundation Seed	2007
Mas-60-YG	Maisadour Semences	2007
Mas-73-YG	Maisadour Semences	2007
PR31N28	Pioneer Hi-Bred International	2006
PR32P76	Pioneer Hi-Bred International	2004
PR32R43	Pioneer Hi-Bred International	2005
PR33P67	Pioneer Hi-Bred Spain S.L.	2003
PR33P67[b]	Pioneer Hi-Bred International	–
PR34N44	Pioneer Hi-Bred International	2005
Protect	Golden Harvest/J.C. Robins	2004
SF1112T	Semillas Fitó, S.A.	2005
Sancia Bt	Holden Foundation Seed	2006
Kardan	KWS SAAT AG	2006
Mas-58-YG	Maisadour Semences	2007

[a] Year of inscription in the European catalogue of varieties of agricultural species

[b] Not validated for commercial use in Europe

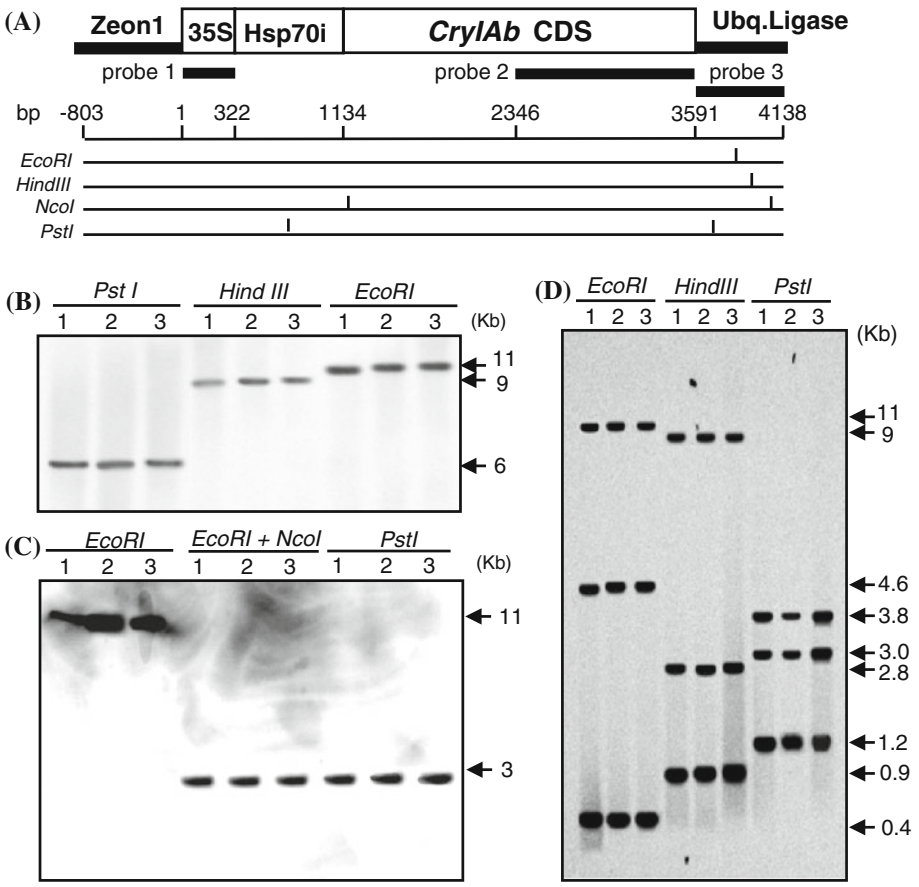
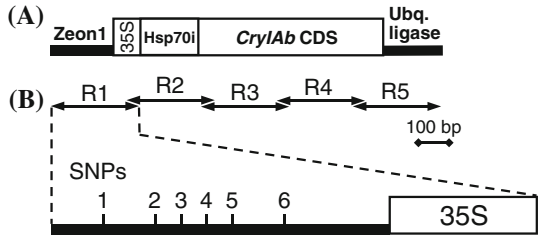


Fig. 1 Southern analysis of three MON 810 maize varieties. **a** Schematic representation and restriction map of the MON 810 transgene locus and host genome flanking regions (Acc. no. AF434709 and AY326434). *Black bars* indicate the position of the probes used in Southern analysis. 35S, 35S promoter; Hsp70i, intron of the maize heat shock protein 70 gene; *CryIAb* CDS, gene encoding the CryIAb toxin (coding region); Ubq.Ligase, exon 1 of the gene coding for HECT ubiquitin ligase 3; Zeon1, Zeon 1 retrotransposon. On the "bp" bar, numbers indicate the positions of the different elements in bp, position 1 corresponds to the first nucleotide of the 35S promoter. Below, restriction enzymes are indicated on the left; *vertical bars* indicate the position of the restriction sites identified in the sequences. **b**, **c**, **d** Southern analysis of genomic DNAs of three maize varieties digested with the indicated enzymes and hybridized with probe 1 (**b**), probe 2 (**c**) and probe 3 (**d**). Probe 3 produced three bands because the MON 810 transgenic lines are hemizygous, so two of the bands correspond to the chromosome carrying the transgene and the other to the chromosome without the transgene. Numbers on the top indicate the variety: 1, DKC6575; 2, PR33P67; 3, Aristis Bt. Fragment size is indicated on the right

Screening for SNPs in the MON 810 insert and flanking regions

A mismatch endonuclease assay was used to detect SNPs and indels within the MON 810 transgene and the flanking sequences in 28 commercial varieties of maize MON 810 (Table 1). These commercial varieties were obtained by different breeders and introduced onto the market in different years, so providing a broad representation of available MON 810 lines. Genomic DNA was extracted from seeds of each variety (three biological replicates) and PCR amplified. To cover all the transgene and flanking sequences (0.7 Kbp per side), five pairs of primers were used which amplify about 1 Kbp each. The amplified fragments partially overlap each other, covering all the nucleotide positions (Fig. 2a, b). The PCR products from five different varieties obtained with the same pair of primers were mixed to form heteroduplex DNA and digested with *CelI* endonuclease. The MON 810 reference variety DK513 was present in all pools as a control.

The size of the endonuclease digested fragments was determined by capillary micro electrophoresis (CμE). A single peak in CμE indicates that all sequences within the pool are identical. The presence of more than one peak indicates that at least one sequence in the pool contains an SNP(s) or an indel(s). All assays were performed in triplicate. Representative examples of the electropherograms are shown in Online Resource 1. A single peak was always observed in the electropherograms corresponding to the regions R2, R3, R4 and R5, which indicates that there are no polymorphisms within the transgene insert or in the 3′ flanking region in any of the 28 varieties. Analysis of the



(C)

SNP n.	SNP type	Position	Variety
1	C→T	-784	Mas-73-YG
2	G→C	-681	DKC6041-YG
3	G→C	-621	SF1112T
4	T→C	-618	Helen
5	A→C	-585	Protect
6	C→G	-531	PR31N28

Fig. 2 SNP detection within the MON 810 transgene and host genome flanking regions. **a** Schematic representation of the MON 810 insert and flanking regions and location of the five fragments used in the SNP/indel detection analyses (R1-R5). 35S, 35S promoter; Hsp70i, intron of the maize heat shock protein 70 gene; *CryIAb* CDS, gene encoding the CryIAb toxin (coding region); Ubq.Ligase, exon 1 of the gene coding for HECT ubiquitin ligase 3; Zeon1, Zeon 1 retrotransposon. **b** Graphic representation of the position of the six SNPs detected in the 5′ flanking region. **c** Type, position and maize variety in which the six SNPs were identified

R1 region revealed multiple digestion products, indicating at least six SNPs or indels in the 5′-flanking sequence. R1 PCR fragments of the 28 varieties were cloned and sequenced. Sequence alignment confirmed the presence of an SNP in each of six different varieties (Fig. 2b, c, and sequence alignment in Online Resource 2). The six SNPs were located more than 500 bp upstream of the transgene.

Cytosine methylation in the MON 810 transgene

Epigenetic changes in DNA methylation can contribute to phenotypic variability among varieties (Makarevitch et al. 2007). We analysed the level of cytosine methylation in the MON 810 transgene using bisulfite sequencing PCR in leaves of seven commercial MON 810 varieties collected at the V7 developmental stage. The levels of cytosine methylation were analysed in two regions of the transgene: the P-35S and a fragment of the *cryIA(b)* coding region delimited by the primers Bscry1F1 and Bscry1R1 (Table 2). We also analysed the methylation status of the 5′- flanking region, corresponding to an LTR of the Zeon1 retrotransposon. The percentage of methylation was determined in symmetric (CpG and CpNpG) and asymmetric sites (CpNpN) (Fig. 3). The average total methylation in the 35S promoter was 0.6 and 1.2% in the coding region (Online Resource 3), without any significant differences between varieties ($P < 0.05$, Student's T test; Online Resource 4). The distribution of the methylated sites along the sequences was not uniform, and certain sites were more frequently methylated than others (Online Resource 5). The average methylation in the flanking 5′LTR sequence was much higher than in the transgene (76.6%), as expected for a moderately repetitive sequence (Online Resource 3). In this case, we found significant differences among varieties, but this was restricted to the asymmetric sites (Fig. 3c; Online Resource 4).

DNA methylation is involved in developmental regulation of gene expression (Yakovlev et al. 2010). We looked for possible changes in the methylation status of the transgene at different stages of development. We analysed the percentage of methylated cytosines in leaves of two MON 810 varieties collected at four different developmental stages (Fig. 4). As previously, the percentage of methylated sites obtained for the transgene was very low, not exceeding 2.5% (Online Resources 6 and 7). In some cases, significant differences were observed between varieties ($P < 0.05$, Student's T test; asterisks in Fig. 4), but these differences were not generalized and only involved specific fragments in a unique developmental stage and type of site (symmetric or asymmetric), never exceeding 1.5%. A slight increase in the methylation level was observed during development, but not in all cases and not significant in most, never exceeding 2.5%. The levels of methylation in the 5′-flanking region were much higher than in the transgene, the percentage of total methylation varying from 66 to 78% (Online Resource 6). Significant differences were observed among varieties (Fig. 4), especially for asymmetric sites during later stages of development.

Accumulation of *cryIA(b)* mRNA during leaf development

The level of *cryIA(b)* mRNA accumulation was analysed during development in leaves of two MON 810 varieties (Fig. 5). A previously validated qPCR assay targeting *cryIA(b)* (Hernández et al. 2003) was used with the α-actin sequence as an in-house validated reference gene (the GeNorm M value was below 0.5 in these samples). There was a significant decrease in *cryIA(b)* mRNA accumulation during leaf development in the two varieties. Similar results were obtained in both analyzed varieties (DKC6575 and PR33P67) ($P < 0.05$, Student's T test).

Discussion

The stability of the transgene over successive generations and after back-crossing has recently received considerable attention. Here we analysed the overall structure of the MON 810 transgene by Southern analysis in three

Table 2 Oligonucleotides used

Name	Sequence	Comments
MONF	5′-CAAGTGTGCCCACCACAGC-3′	QRT–PCR Cry1Ab target
MONR	5′-GCAAGCAAATTCGGAAATGAA-3′	
α-Actin2F	5′-TACCCAACTAAGCGCATGCC-3′	QRT-PCR Housekeeping target
α-Actin2R	5′-GCATCTGAATCACGAAGCAGG-3′	
LTRSNPsF	5′-CTCGTGGTGCGCTCTTATTT-3′	SNPs analysis region R1
35SSNPsR	5′-AGCTTGTCAGCGTGTCCTCT-3′	
35SSNPsF	5′-AAGAAGACGTTCCAACCACG-3′	SNPs analysis region R2
Hsp70iSNPsR	5′-GCCGCTTGGTATCTGCATTA-3′	
Hsp70iSNPsF	5′-GCCTTCTCCCTAGTGTTGACC-3′	SNPs analysis region R3
CrySNPsR1	5′-GATGCTGTTGAGGATGTCCA-3′	
CrySNPsF2	5′-TGGAGAACTTCGACGGTAGC-3′	SNPs analysis region R4
CrySNPsR2	5′-GAGGCAGAACTCATCGGAGA-3′	
CrySNPsF3	5′-ACCACATCGACCAAGTCTCC-3′	SNPs analysis region R5
3FLSNPsR	5′-GGCCCGCGGAAATTCGATT-3′	
35SprobeF	5′CCATTGCCCAGCTATCTGTC3′	Southern probe 35S-promoter
35SprobeR	5′CGTTGATGTTTGGGTTGTTG-3′	
cryprobeF	5′GGCACGGTGGATTCCCTGGACGAGAT-3′	Southern probe Cry1Ab
cryprobeR	5′GCGGCCGCCTACCGAAAGTCCTCGT-3′	
3FLprobeF	5′CTTCTTTCATTTCCGAATTTGC-3′	Southern probe 3′ Flanking
3FLprobeR	5′TGGCACTGTTGGTTCAGAAA-3′	
BsLTRF	5′-AAATGTAGAGGGIATGGGTGTAA-3′	Bisulfite sequencing LTR
BsLTRR	5′-CCTTTTCCACTATCTTCACAATAA-3′	
Bs35SF	5′-TATITGTTATTTTATTGTGAAGATAGTGGA-3′	Bisulfite sequencing P-35S
Bs35SR	5′-CTAAACCACTCTCAICAATCACCACAC-3′	
Bscry1F1	5′-GTTGGTATAATATTGGTTTGGAG -3′	Bisulfite sequencing Cry1Ab
Bscry1R1	5′-AAATATAAACTCCTAATAAAACCCTC-3′	

commercial maize MON 810 varieties and found no evidence of new rearrangements within the inserted sequences or in the flanking regions. We further analysed the genomic DNA of 28 varieties of maize MON 810 looking for point mutations and indels within the insert and in the proximal flanking regions. The mutation rate of maize genes has been estimated as 3×10^{-8} substitutions per site per generation (Clark et al. 2005), and the mutation rate of a maize hypervariable microsatellite as 8×10^{-4} (Vigouroux et al. 2002). According to these rates and considering that, on average, 17 generations have passed since the introduction of the transgene in the maize varieties, we would expect to observe 0.002 SNPs where the transgene behaves as a maize endogenous gene, or 57 SNPs where it behaves as a hypervariable sequence. As we did not detect any SNP in the transgene, we can conclude that the transgene behaves more similarly to an endogenous maize gene than to a hypervariable region in terms of mutation rates. Similar mutation rates have been estimated for the transgene of the Roundup Ready® soybean (Ogasawara et al. 2005). This suggests that, once integrated into the genome, transgenes are not mutation "hot spots" and have similar rates of mutation to endogenous genes.

Six polymorphisms were detected in the 5′flanking sequence corresponding to a Zeon1 retrotansposon LTR, a moderately highly-repetitive sequence (Holck et al. 2002). This number of SNPs represents a mutation rate of about 1.6×10^{-5} substitutions per nucleotide and generation, which is similar to the mutation rate expected for a maize hypervariable region (8×10^{-4}). Nucleotide changes in the flanking regions could potentially have a negative effect on the reliability and accuracy of existing GMO detection and quantification methods. Event specific detection and quantification methods are based on PCR amplification using a primer located within the transgene and a second primer located in the flanking sequence, close to the insertion site. All six mutations detected were located at least 500 bp upstream of the transgene, and therefore do not affect the PCR target sequences or the reliability of the detection and quantification methods.

The genetic background of a particular variety can influence the epigenetic regulation of transgenes (Makarevitch et al., 2007). Consequently, differences in the levels of DNA methylation in the transgene may be possible when it is introduced into different genetic backgrounds through classic breeding. However, our analyses show that

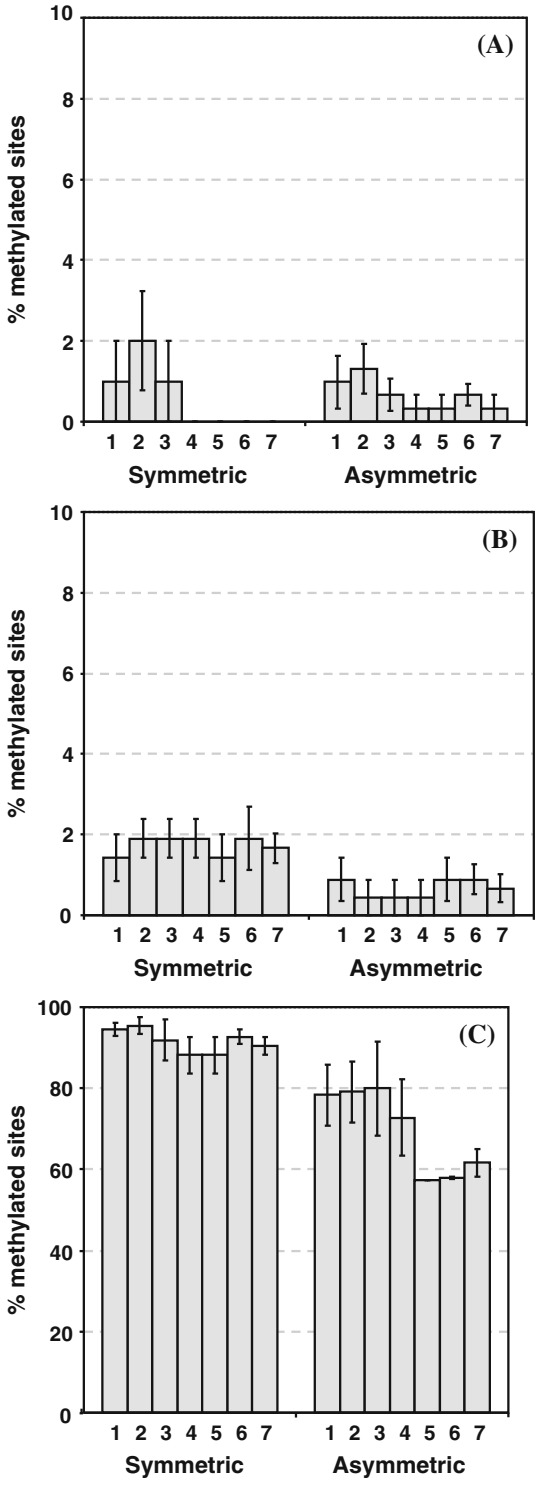


Fig. 3 Cytosine methylation in leaves of maize MON 810 varieties. Average DNA cytosine methylation levels of the symmetric and asymmetric sites in leaves of seven maize MON 810 varieties: 1, PR34N44; 2, HELEN Bt; 3, DKC5784-YG; 4, GAMBIER BT; 5, DKC6041-YG, 6, DKC6575; and 7, PR33P67. Three different regions were analysed: (**a**) 35S promoter; (**b**) CryIAb coding region fragment; (**c**) 5′ flanking LTR. Bars show the average percentages of methylation in at least 5 repeats. Error bars represent the standard errors

there are only minor differences in cytosine methylation when comparing seven MON 810 varieties. Significant differences in DNA methylation among varieties were only observed in the LTR flanking sequence, and restricted to the asymmetric sites. Cytosine methylation has been implicated in the silencing of transposable elements (Rabinowicz 2003) but asymmetrical DNA methylation is much less important than symmetrical CpG methylation in maintaining TE silencing (Lippman et al. 2003). In consequence, the observed differences in the asymmetric methylation levels in the LTR in the 5′flanking region are unlikely to represent a difference in the activity of the retrotransposon.

Previous studies have shown that cytosine methylation varies with the age of the plant, which may explain differences in the expression of some genes at different stages of development (Ruiz-Garcia et al. 2005). The small differences in the levels of methylation of the transgene in MON 810 maize leaves, during plant development, cannot explain the reduction in the *cry1A(b)* mRNA accumulation that we found during leaf development. Although generally considered a constitutive promoter, some reports suggest that CaMV 35S promoter is not similarly active in all cell types or in all stages of development (Sunilkumar et al. 2002). For example, the accumulation of mRNA from a transcriptional fusion of the CaMV 35S promoter and a 19 kD zein cDNA was lower in young than in old tobacco leaves (Williamson et al. 1989). If the 35S promoter behaves similarly in maize, this would explain the observed differences in accumulation of mRNA in the case of MON 810 varieties.

Some studies describe the presence of very different levels of Bt protein in plants growing in different areas or individual plants growing in the same field (Nguyen and Jehle 2007). Since we have shown that the accumulation of *cry1A(b)* mRNA decreases rapidly with leaf age, the differences observed in these analyses based on field samples could be due, at least in part, to deviations regarding the precise developmental stage of the analyzed plants rather than to intrinsic differences in transgene expression. Our experiments based on plants grown under controlled environmental conditions showed no significant differences of mRNA accumulation between samples at the same developmental stage.

In conclusion, our data demonstrate that any tendency towards genetic instability of the sequence introduced in the MON 810 YieldGard® maize is no higher than for endogenous maize genes. There is very little difference in cytosine methylation status of the transgene among leaves of different varieties and among different developmental stages. However, the level of *cry1A(b)* mRNA accumulation is reduced throughout leaf development.

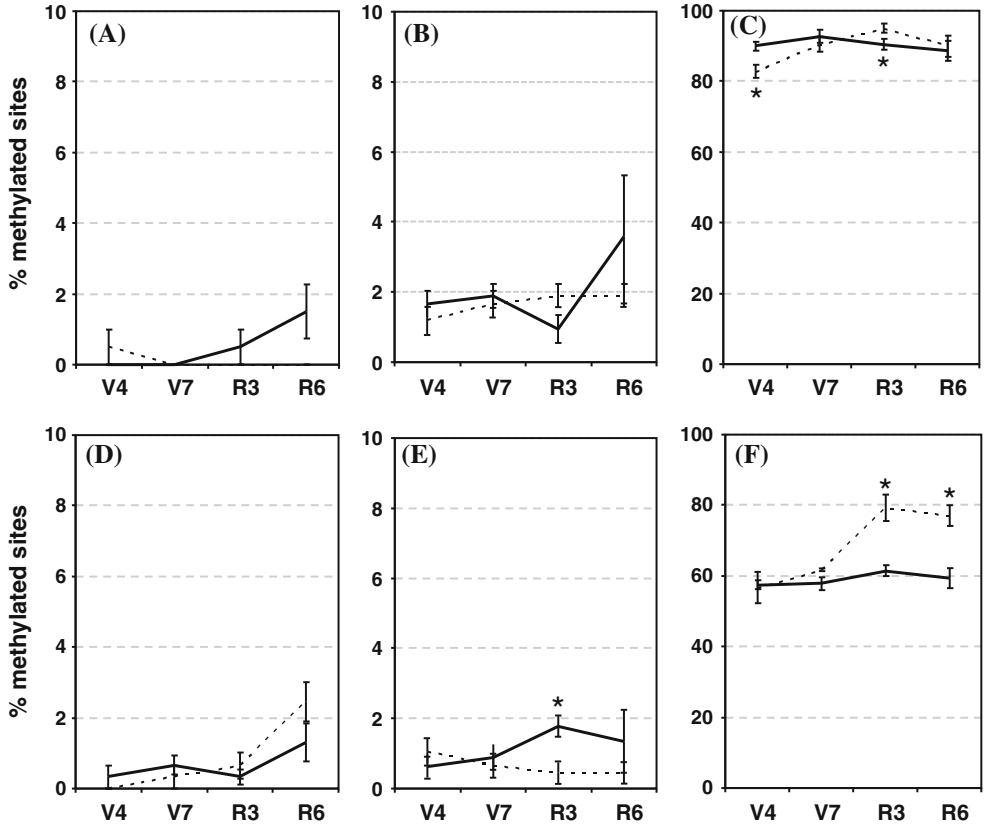


Fig. 4 Cytosine methylation in leaves of maize MON 810 at different developmental stages. DNA cytosine methylation level in leaves of maize MON 810 varieties in symmetric and asymmetric sites at different stages of plant development. **a** and **d** in the 35S promoter; **b** and **e** *CryIA(b)* coding region fragment; **c** and **f** 5′ flanking LTR. **a**, **b** and **c** represent symmetric sites, and **d**, **e** and **f** represent asymmetric sites. Continuous line represents the average percentage of methylation for the DKC6575 variety and discontinuous line for PR33P67. *Error bars* represent the standard errors. V4, V7, R3 and R7 are developmental stages of the plant. Asterisks indicate statistically significant differences (*t*-Student, $P < 0.05$)

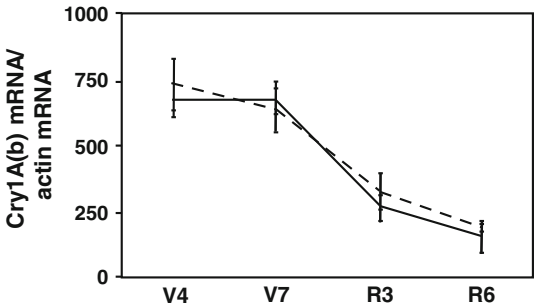


Fig. 5 *Cry1A(b)* mRNA accumulation during maize MON 810 leaf development. mRNA content was determined using quantitative real time RT-PCR, with actin as the normalizing gene. V4, V7, R3 and R7 are developmental stages of the plant. The continuous line corresponds to the DKC6575 variety and discontinuous line to PR33P67. Error bars represent the standard errors

Experimental procedures

Plant material

Seeds of commercial hybrid varieties of the YieldGard® maize event MON 810 included in the Spanish catalogue "Registro de Variedades Vegetales" were obtained on the Spanish market and are listed in Table 1. Certified reference material (CRM) of powdered seeds of YieldGard® maize event MON 810 was purchased from the Institute of Reference Materials and Measurements (IRMM, Belgium).

For cytosine methylation, mRNA and protein analyses, MON 810 plants were grown under controlled conditions (day temperature 28 ± 2°C, night temperature 22 ± 2°C, relative humidity 60 ± 5% and 14/10 h light/dark). Leaf samples were taken at the vegetative developmental stages V4 (4 leaves, 2 weeks) and V7 (7 leaves, 4 weeks), and at the reproductive stages R3 (30 days after pollination, 8 weeks) and R6 (dry kernels; 12 weeks).

Nucleic acid isolation and cDNA first-strand synthesis

Genomic DNA was extracted from 200 mg of leaves or 1 g of seeds using the DNA Plant Kit (Qiagen, Hilden, Germany). Total RNA was extracted using TRIZOL reagent (Invitrogen, Carslbad, USA) and purified with Qiagen RNeasy kit (Qiagen, Hilden, Germany). DNA and RNA were quantified using a NanoDrop ND-1000 spectrophotometer

(NanoDrop Technologies, Wilmington, USA) and their integrity checked by capillary micro-electrophoresis (CμE) in an Agilent-2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). First strand cDNA was synthesized from 1 μg of total RNA using the superscript III kit (Invitrogen, Carslbad, USA).

Southern analysis

20 μg of genomic DNA were digested with restriction enzymes, electrophoresed on 0.8% (w/v) agarose gels and blotted onto positively charged nylon membranes (Hybond N^+, Amersham Biosciences, UK). Blots were hybridised with probes prepared using the PCR DIG probe synthesis kit (Roche, Basel, Switzerland). Labelling, hybridisation, washings and detection procedures were according to the manufacturer's instructions.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed with the MONF and MONR (targeting *cryIA(b)*) or α-Actin2F and α-Actin2R (targeting the *α-actin* gene used for normalization) primers. All oligonucleotides used in this study are listed in Table 2. qPCR assays were in a final volume of 20 μL containing 1 × SYBR Premix EX Taq™ (Takara Bio Inc., Shiga, Japan), 0.2 μM of each primer and 2 μL cDNA as template.

The qPCR reactions were carried out in a Lightcycler LC480 (Roche Applied Science, USA) with the following program: initial denaturation at 95°C for 30 s followed by 45 cycles of 95°C 5 s and 60°C 35 s. The specificity of the assays was tested by melting curve analysis. Relative quantification was performed using a standard curve prepared by serial dilution of a plasmid solution containing a known number of copies of the MON 810 transgene and α-actin. The criteria to validate qPCR assays were R2 above 0.98 (i.e. high linearity) and slope between −3.1 and −3.6 (i.e. high efficiency). All data were analyzed using the geNORM v.3.4 (Center for Medical Genetics, University Hospital Ghent, Belgium; http://medgen.ugent.be/genorm) and NORMfinder statistical algorithms (Molecular Diagnostic Laboratory, Aarhus University Hospital Skejby, Danmark; http://www.mdl.dk/ publicationsnormfinder.htm).

Mismatch endonuclease assay

A Mastercycler S thermal cycler (Eppendorf, Germany) was used for the PCR reactions, in 20 μL final volume containing 1 × Buffer II (100 mM Tris–HCl pH 8.3, 500 mM KCl), 1.5 mM $MgCl_2$, 1.6 mM dNTPs and 0.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, USA), 0.2 μM of each primer and 100 ng of genomic DNA as template. The PCR products obtained from 5 to 6 varieties were pooled in equal amounts and heteroduplex DNA formation was carried out in a Mastercycler S thermal cycler using the program: 95°C 15 s, 85°C 15 s (ramp −0.5°C/s), and 25°C 15 s (ramp −0.1°C/s). Each genomic region was analysed with different primer pairs, i.e. LTRSNPsF and 35SSNPsR (region R1), 35SSNPsF and Hsp70iR (R2), Hsp70iF and CryIAbSNPsR1 (R3), CryIAbSNPsF2 and CryIAbSNPsR2 (R4) and CryIAbSNPsF3 and 3flankSNPsR (R5) (Table 2).

SNPs were detected using the Surveyor® endonuclease assay kit (Transgenomic Inc., Omaha, USA) according to the manufacturer's instructions. Basically, 15 μL of each heteroduplex DNA were mixed with 1 μL Enhancer™ and 1 μL Surveyor™ endonuclease and incubated at 42°C for 20 min. The reaction was stopped by addition of 1 μL Stop solution. Fragments generated by endonuclease digestions were analysed by CμE using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc.; Santa Clara, CA, USA) with the DNA-chip-1000 kit. All electropherograms were analysed with the same fluorescence threshold (20 FU) and baseline. Single nucleotide polymorphisms (SNPs) were confirmed by sequencing the two strands in an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, USA) using the Big Dye terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, USA). Sequences obtained were deposited in GeneBank under the accession numbers FN706511to FN706516.

Bisulfite genomic sequencing PCR

The DNA cytosine methylation was analysed by bisulfite genomic sequencing PCR using 500 ng of genomic DNA. Bisulfite conversion was performed using the EZ DNA Methylation-Gold kit (Zymo research, Orange, USA) according the manufacturer's instructions. After treatment, 1.5 μL of bisulfite-treated DNA was used to PCR amplify different transgene fragments (spanning from the P-35S to the *cry1A(b)* coding region) using the modified primers Bs35SF and Bs35SR; and Bscry1F and Bscry1R. The PCR conditions were 1× Buffer II (Applied Biosystems, Foster City, USA), 0.8 mM dNTPs, 1.5 mM MgCl2, 0.2 μM each primer and 1U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, USA). The PCR running program was 95°C 10 min, followed by 45 cycles of 95°C 15 s, 52°C 30 s, 72°C 30 s and a final extension step of 72°C 3 min. The PCR products were resolved in 1% (w/v) agarose gels and bands were excised and purified using the QIAEXII gel extraction kit (Qiagen, Hilden, Germany), and cloned into pCRII-TOPO (Invitrogen, Carslbad, USA). At least five clones per band were sequenced with M13 primers using an ABI3730 sequencer (Applied Biosystems, Foster City, USA). Bisulfite-generated sequences were

analyzed using the plant specific methylation analysis software Kismeth^{TM} (Gruntman et al. 2008).

Acknowledgments This work was funded by the European collaborative project Co-Extra (Co-Existence and Traceability of Genetically Modified Organisms in the European Supply Chain; http://www.coextra.eu/; VI Framework Programme), the Centre CONSOLIDER on Agrigenomics and the Xarxa de Referencia en Biotecnologia of the Generalitat de Catalunya. We thank Drs. Josep M. Casacuberta and Michael A. Phillips for their comments on the manuscript.

References

Clark RM, Tavaré S, Doebley J (2005) Estimating a nucleotide substitution rate for maize from polymorphism at a major domestication locus. Mol Biol Evol 22:2304–2312

Delannay X, La Vallee BJ, Proksch RK, Fuchs RL, Sims SR, Greenplate JT, Marrone PG, Dodson RB, Augustine JJ, Layton JG, Fischhoff DA (1989) Field performance of transgenic tomato plants expressing the Bacillus thuringiensis var kurstaki insect control protein. Bio/Technology 7:1265–1269

Dorlhac de Borne F, Vincentz M, Chupeau Y, Vaucheret H (1994) Co-suppression of nitrate reductase host genes and transgenes in transgenic tobacco plants. Mol Gen Genom 243:613–621

Duan X, Li X, Xue Q, Abo-el-Saad M, Xu D, Wu R (1996) Transgenic rice plants harboring an introduced potato proteinase inhibitor II gene are insect resistant. Nat Biotech 14:494–498

Fearing PL, Brown D, Vlachos D, Meghji M, Privalle L (1997) Quantitative analysis of Cry1A (b) expression in Bt maize plants, and silage and stability of expression over successive generations. Mol Breed 3:169–176

Gruntman E, Qi Y, Slotkin RK, Roeder T, Martienssen RA, Sachidanandam R (2008) Kismeth: analyzer of plant methylation states through bisulfite sequencing. BMC Bioinf 9:371

Hernández M, Pla M, Esteve T, Prat S, Puigdomenech P, Ferrando A (2003) A specific real-time quantitative PCR detection system for event MON 810 in maize YieldGard based on the 3′-transgene integration sequence. Transg Res 12:179–189

Holck A, Vaïtilingom M, Didierjean L, Rudi K (2002) 5′-Nuclease PCR for quantitative event-specific detection of the genetically modified MON 810 MaisGard maize. Eur Food Res Tech 214: 449–453

Holst-Jensen A, De Loose M, van den Eede G (2006) Coherence between legal requirements and approaches for detection of genetically modified organisms (GMOs) and their derived products. J Agric Food Chem 19:2799–2809

Kohli A, Griffiths S, Palacios N, Twyman RM, Vain P, Laurie DA, Christou P (1999) Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology mediated recombination. Plant J 17:591–601

La Paz JL, Vicient CM, Puigdomènech P, Pla M (2010) Characterization of polyadenylated cryIA(b) transcripts in maize MON 810 commercial varieties. Anal Bioanal Chem 396:2125–2133

Lippman Z, May B, Yordan C, Singer T, Martienssen R (2003) Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification. PLoS Biol 1:e67

Makarevitch I, Stupar RM, Iniguez AL, Haun WJ, Barbazuk WB, Kaeppler SM, Springer NM (2007) Natural variation for alleles under epigenetic control by the maize chromomethylase zmet2. Genetics 77:749–760

Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJ (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J 19:5194–5201

Meyer P, Linn F, Heidmann I, Meyer H, Niedenhof I, Saedler H (1992) Endogenous and environmental factors influence 35S promoter methylation of a maize A1 gene construct in transgenic petunia and its colour phenotype. Mol Gen Genom 231:345–352

Müller AJ, Mendel RR, Schiemann J, Simoens C, Inzé D (1987) High meiotic stability of a foreign gene introduced into tobacco by *Agrobacterium*-mediated transformation. Mol Gen Genom 207: 171–175

Nguyen HT, Jehle JA (2007) Quantitative analysis of the seasonal and tissue specific expression of Cry1Ab in transgenic maize MON 810. J Plant Dis Protec 114:82–87

Ogasawara T, Chikagawa Y, Arakawa F, Nozaki A, Itoh Y, Sasaki K, Umetsu H, Watanabe T, Akiyama H, Maitani T, Toyoda M, Kamada H, Goda Y, Ozeki Y (2005) Frequency of mutations of the transgene, which might result in the loss of the glyphosate-tolerant phenotype, was lowered in roundup ready soybeans. J Health Sci 51:197–201

Padgette SR, Kolacz KH, Delannay X, Re DB, Re DB, LaVallee BJ, Tinius CN, Rhodes WK, Otero YI, Barry GF, Eichholtz DA, Peschke VM, Nida DL, Taylor NB, Kishore GM (1995) Development, identification, and characterization of a glyphosate-tolerant soybean line. Crop Sci 35:1451–1461

Rabinowicz PD (2003) Constructing gene enriched genomic libraries using methylation filtration technology. Methods Mol Biol 236: 21–36

Register JC 3rd, Peterson DJ, Bell PJ, Bullock WP, Evans IJ, Frame B, Greenland AJ, Higgs NS, Jepson I, Jiao S, Lewnau CJ, Sillick JM, Wilson HM (1994) Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. Plant Mol Biol 25:951–961

Rosati A, Bogani P, Santarlasci A, Buiatti M (2008) Characterisation of 3′ transgene insertion site and derived mRNAs in MON 810 YieldGard maize. Plant Mol Biol 67:271–281

Ruiz-Garcia L, Cervera MT, Martínez Zapater JM (2005) DNA methylation increases throughout development. Planta 222: 301–306

Sunilkumar G, Mohr L, Lopata-Finch E, Emani C, Rathore KS (2002) Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP. Plant Mol Biol 50: 463–474

Van Rie J, Jansens S, Höfte H, Degheele D, Van Mellaert H (1989) Specificity of *Bacillus thuringiensis* delta-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects. Eur J Bioch 186:239–247

Vigouroux Y, Jaqueth JS, Matsuoka Y, Smith OS, Beavis WD, Smith JS, Doebley J (2002) Rate and pattern of mutation at microsatellite loci in maize. Mol Biol Evol 19:1251–1260

Widmer F, Seidler RJ, Donegan KK, Reed GL (1997) Quantification of transgenic plant marker gene persistence in the field. Mol Ecol 6:1–7

Williamson JD, Hirsch-Wyncott ME, Larkins BA, Gelvin SB (1989) Differential accumulation of a transcript driven by the CaMV 35S promoter in transgenic tobacco. Plant Physiol 90:1570–1576

Yakovlev A, Khafizova M, Abdullaev Z, Loukinov D, Kondratyev A (2010) Epigenetic regulation of caspase-3 gene expression in rat brain development. Gene 450:103–108