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Natural variation explains most transcriptomic changes among maize plants of MON810 and comparable non-GM varieties subjected to two N-fertilization farming practices

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Abstract The introduction of genetically modified organisms (GMO) in many countries follows strict regulations to ensure that only safety-tested products are marketed. Over the last few years, targeted approaches have been complemented by profiling methods to assess possible unintended effects of transformation. Here we used a commercial (Affymertix) microarray platform (i.e. allowing assessing the expression of ~1/3 of the genes of maize) to evaluate transcriptional differences between commercial MON810 GM maize and non-transgenic crops in real agricultural conditions, in a region where about 70% of the maize grown was MON810. To consider natural variation in gene expression in relation to biotech plants we took two common MON810/non-GM variety pairs as examples, and two farming practices (conventional and low-nitrogen fertilization). MON810 and comparable non-GM varieties grown in the field have very low numbers of sequences with differential expression, and their identity differs among varieties. Furthermore, we show that the differences between a given MON810 variety and the non-GM counterpart do not appear to depend to any major extent on the assayed cultural conditions, even though these differences may slightly vary between the conditions. In our study, natural variation explained most of the variability in gene expression among the samples. Up to 37.4% was dependent upon the variety (obtained by conventional breeding) and 31.9% a result of the fertilization treatment. In contrast, the MON810 GM character had a very minor effect (9.7%) on gene expression in the analyzed varieties and conditions, even though similar *cryIA(b)* expression levels were detected in the two MON810 varieties and nitrogen treatments. This indicates that transcriptional differences of conventionally-bred varieties and under different environmental conditions should be taken into account in safety assessment studies of GM plants.

Keywords GMO (Genetically Modified Organism) · MON810 · Maize · Nitrogen stress · Transcriptome · Unintended effects · Agricultural field · Natural variation

Abbreviations

cDNA	Complementary DNA
CRM	Certified reference material

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E	Efficiency
EBI	European Bioinformatics Institute
EU	European Union
GM	Genetically Modified
GMO	Genetically Modified Organism
GS2	Glutamine Synthase 2
ISAAA	International Service for the Acquisition of Agri-biotech Applications
mRNA	Messenger RNA
N	Nitrogen
OECD	Organisation for Economic Co-operation and Development
PCA	Principal Component Analysis
RT-qPCR	Reverse transcription—real-time polymerase chain reaction
RMA	Robust Multichip Average
rRNA	Ribosomal RNA
SOM	Self-organizing Map
V6	Vegetative six-leaf stage
V8	Vegetative eight-leaf stage
VT	Vegetative Tasseling

Introduction

Genetically modified (GM) crops are subjected to different legislation worldwide to cover aspects of consumer safety and protection. Authorized GM events have been shown to be equivalent to non-GM lines of the same species by means of targeted analysis of specific compounds that are relevant to the crop species under consideration [(OECD 1993), see reviews in (Cellini et al. 2004; König et al. 2004; Shewry et al. 2007)]. Additionally, more unbiased profiling technologies such as metabolomics, proteomics and transcriptomics, if properly validated and coupled to adequate statistical tools, are considered capable of extending the span of comparative analyses, reducing uncertainty and identifying the need for further risk assessment (Cellini et al. 2004; Hoekenga 2008; Kuiper et al. 2003; Millstone et al. 1999). In various plant species, including *Arabidopsis thaliana*, potato, rice, wheat and maize, it has been shown that non-targeted effects of transgenes do not have a major effect on altering overall gene expression (Baudo et al. 2006, 2009; Cheng et al. 2008; Coll et al. 2008; Dubouzet et al. 2007; El Ouakfaoui and Miki 2005; Gregersen et al. 2005; Metzdorff et al. 2006), proteome or metabolome (Baker et al. 2006; Beale et al. 2009; Lovegrove et al. 2009; Catchpole et al. 2005; Di Carli et al. 2009; Ioset et al. 2007; Ruebelt et al. 2006; Shepherd et al. 2006), in plants grown in optimal controlled environments.

Plant varieties have a wide range of genetic diversity, with large differences observed between commercial varieties. Conventional breeding approaches include the use of techniques that cause genome alteration (e.g. interspecies crosses, tissue culture and mutagenesis) for selecting plants with interesting traits, while discarding those with undesired phenotypes. Many significant differences between the conventional cultivars, caused by genome alteration, were never sought as desired traits in traditional breeding programs and have not given cause for public safety concerns. Recipient varieties can be largely divergent and in consequence, a number of commercial GM varieties with different agronomic properties can be obtained from a single event. A number of studies report the very limited differences between GMO and comparable non-GM plants, in contrast to the extensive variation between conventional varieties [see reviews in (Bradford et al. 2005; Chassy et al. 2008; Kok et al. 2008)].

According to the ISAAA report (James 2008), there is an increase in cultivation and commercialization of GM crops worldwide, with maize being the second most widespread GM crop, after soybean, in particular the corn-borer resistant maize event MON810. This is the only cultivated GM event in the EU, with 108,000 ha grown in 2008, with more than 79,000 ha cultivated in Spain (http://www.gmo-compass.org/eng/home/). The MON810 transgene was introduced into different commercial varieties through breeding programs to produce commercial GM plants containing the new trait resulting from transformation. Previous studies have shown differences between particular pairs of MON810/near-isogenic varieties. These include the lignin contents (Saxena and Stotzky 2001) and composition (Poerschmann et al. 2005); and the enantiomeric composition of certain amino acids in Aristis Bt/Aristis and PR33P67/PR33P66 (Herrero et al. 2007); the amount of L-carnitine and stachydrine in Aristis Bt/Aristis, DK6575/Tietar and PR33P67/PR33P66 (Levandi et al. 2008); and metabolic variations related to the primary nitrogen pathway in La73-Bt/La73 (Manetti et al. 2006). In a transcriptomics approach comparing leaves of in vitro cultured plants (Coll et al. 2008), we showed that 1.7 and 0.1% transcripts in Aristis Bt/Aristis and PR33P67/PR33P66, respectively, had differential expression and that the identity of regulated genes varied between GM and near-isogenic variety pairs. Recently, Piccioni et al. (2009) found several metabolomic differences between PR33P67 and PR33P66 cultured in growth chambers. From these studies it was concluded that different variety pairs had different levels of divergence that were consistently lower than the divergence found among conventional varieties, and most probably associated to the different genomic backgrounds of the compared varieties.

Environmental factors are known to cause considerable changes in plants. MON810 maize is of major agricultural interest, and field cultivation is inevitably subject to diverse environmental conditions and agricultural practices. Comparison of GM and comparable varieties under a variety of environmental and cultural conditions is highly desirable (Kok et al. 2008). A preliminary approach carried out with a limited number of sequences suggested that the differences between MON810 and comparable non-GM plants grown in vitro varied to some extent when they were grown under agricultural field conditions (Coll et al. 2009). To achieve high production yields, maize has major hydrological and nutritional requirements during the vegetative cycle. Nitrogen, normally applied as fertilizers, is the non-carbon mineral nutrient required in greatest abundance. It is particularly relevant due to its economic and environmental effects, and a number of studies focus on the reduction of N supply (Frink et al. 1999; Sylvester-Bradley and Kindred 2009).

The aim of the present study was to assess the relative contribution of (1) the GMO character (using MON810 as example); (2) the variety (referring to closely related MON810/near-isogenic variety pairs obtained through conventional breeding strategies); and (3) the N treatment (as an example of environmental and cultural conditions), on the transcriptional patterns of maize plants grown in agricultural fields.

As a realistic scenario, the present study was carried out in the coastal region of Catalonia, where MON810 maize represents around 70% of the total maize produced (http://www.gencat.cat/darp/). A number of MON810 maize varieties are grown in this area, obtained by different seed companies, particularly Helen Bt (Advanta) and Beles Sur (Limagrain Ibérica). Their corresponding near-isogenic, non GM varieties Helen and Sancia are also widely grown. In the studied region, N fertilization is a main agricultural factor. Usually, around 1/3rd of the nitrogen (N) is supplied before maize sowing, and the remainder is provided at the vegetative 6 (V6) to 8 leaf (V8) stage (J. Serra, personal communication). Based on these variety pairs, and on the conventional N fertilization and the lack of additional N supply during this season as an alternative environmental condition, we used a transcriptomics approach to study the similarity between commercially relevant MON810 and comparable non-GM varieties grown in a real agricultural environment.

Materials and methods

Plant material

Two MON810 varieties (company, date of authorization in the BOE Spanish official publication) were used: Helen Bt (Advanta, 11/08/2005, now commercialized by Limagrain Ibérica) and Beles Sur (Limagrain Ibérica, 07/09/2006), and their corresponding near-isogenic varieties (Helen and Sancia) from the same companies.

Seeds of the two GMO varieties were initially analyzed to confirm they were MON810, using powdered certified reference material (CRM, ref#ERM-BF413A,B,D,F), purchased from Fluka (Fluka-Riedel, Geel, Belgium), as control. Genomic DNAs were isolated from 0.2 g of plant material using the Nucleospin food kit (Macherey–Nagel Int, Easton, PA) and subjected to event specific real-time polymerase chain reaction (qPCR) (Hernández et al. 2003) using *hmg* as the endogenous control (Hernández et al. 2005).

The seeds were grown in La Tallada d'Empordà (Girona), Catalonia, Spain (42°05′N, 3°E), where transgenic insect resistant (MON810) and conventional maize are commercially grown. This area is placed close to the sea and has a Mediterranean climate. The soil type is Xerofluvent oxiaqüic, coarse-loamy, mixed, calcareous, and thermic. The field under study was divided into 24 m^2 micro-plots (4 rows wide, 8 m long, row spacing 0.75 m), sown and treated in a split-plot design. Micro-plots were sown at a density of 80,000 plants/ha (7 April 2007) and treated following standard agricultural practices in the region. One hundred kg P/ha and 100 kg K/ha were applied before sowing. Nitrogen-fertilized micro-plots were also treated with 100 kg N/ha (calcium ammonium nitrate, 27%) before sowing, and an additional 200 kg N/ha were side-dressed at the V8 (vegetative eight-leaf) stage. Weeds were controlled with pre-emergence application of 5 l/ha of Trophy Super (35% acetochlor + 15% atrazine + 5.8% Diclormid, Dow Agrosciences, Indianapolis, IN, USA) and with post-emergence application of 1.25 l/ha of Samson (4% nicosulfuron, Syngenta, Basel, Switzerland). Meteorological conditions were recorded in the region (Mas Badia agro-meteorological station) from sowing to sampling dates. Mean temperatures were 13.1, 16.4 and 20.2°C in April, May and June, respectively, similar to the temperatures recorded between 1984 and 2008 (13.0, 16.9 and 20.5°C, respectively). The recorded rainfall values were 47.6, 76.6 and 76.6 l/m^2 in April, May and June, with mean rainfall values for the same months in 1984–2008 of 61.8, 58.5 and 45.1 l/m^2. Rainfall is more variable between seasons in regions with Mediterranean climate: when necessary, the fields under study were irrigated following conventional agricultural practices.

The relative nitrogen contents of plants in control and low-N micro-plots were indirectly assessed using the N-tester (Yara-Agro) to measure transmittance at 650 and 940 nm (Hawkins et al. 2009). Thirty plants were analyzed per micro-plot at the vegetative tasseling (VT) stage. Maize plants were harvested at the VT stage at the same time of

day, immediately frozen in liquid nitrogen and stored at −80°C. Each sample consisted of 5 cm-long leaf portions of the second fully developed leaf of each of 10 plantlets from a single micro-plot, discarding the 5-cm apical portion and removing the central vein. Plants were carefully checked for the absence of corn-borer (the incidence in 2008 season was considered very low, below 0.1 larvae per conventional plant, J. Serra, personal communication), other infections and other lesions. Three biological replicates were sampled per maize variety and nitrogen condition, each grown in a different micro-plot. Nitrogen content was determined at the Technical Services at the Universitat de Girona by elemental analysis. Two to three mg of dried samples were sealed in tin boats, weighed on a microbalance (Sartorius 2MP, Goettingen, Germany) and immediately transferred to the AE2400 series II Elemental Analyzer (Perkin Elmer, Massachusetts, USA).

Total RNA extraction

Total RNA was extracted using a protocol based on the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and purified with the Qiagen RNeasy MiniElute Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. It was quantified by UV absorption at 260 nm in a NanoDrop ND1000 spectrophotometer (Nanodrop technologies, Wilmington, DE, USA). Agarose gel electrophoresis analysis confirmed the integrity of the RNA samples; and OD 260/280 nm absorption ratios (mean and standard deviation [SD] = 2.05 ± 0.03) confirmed their purity. Thus, all RNA samples were appropriate for use.

Microarray hybridization and analyses

The GeneChip® Maize Genome Array (Affymetrix, Santa Clara, CA, USA) was used to search for transcriptome differences between MON810 and near-isogenic maize varieties and nitrogen conditions. The array has 17,555 probe sets to analyze approximately 14,850 transcripts, which represent 13,339 maize genes, which represent around 1/3rd of the genes of maize (Shnable et al. 2009). It provides comprehensive coverage of over 100 cultivars present in the NCBI UniGene data set (http://www.affymetrix.com/products/arrays/specific/maize.affx).

Three GeneChips were used to analyze three independent replicates per variety and nitrogen level. Hybridization and statistical analysis were performed at the Unidad de Genómica, Parque Científico de Madrid as previously described (Coll et al. 2008). Briefly, the integrity of total RNA samples was assessed by capillary electrophoresis, complementary DNA (cDNA) was synthesized and in vitro transcribed yielding biotin labeled cRNA. The biotinylated cRNA was cleaned, spectrophotometrically quantified, and 15 µg fragmented into sequences of around 100 nt for hybridization to the GeneChip Maize Genome Array (Affymetrix). Chips were subsequently washed and fluorescently labeled with phycoerythrin using antibody amplification, and fluorescence was quantified. The data was extracted by the Robust Multichip Average (RMA) software (Irizarry et al. 2003), which includes background adjustment, quantile normalization and summarization. MultiExperiment Viewer software v.4.2 was used to calculate changes in gene expression as the ratio of normalized fluorescent data between two compared samples. *T*-tests on normalized log2 transformed intensity values and Bonferroni multiple testing correction (Sidak 1971) were also performed with the same software. Sequences showing expression changes greater than two-fold (i.e. above 2- and below 0.5-fold ratios) and *P*-values below 0.05 were defined as differentially expressed. The MapMan tool (Thimm et al. 2004) was used to perform gene ontology analysis of differentially expressed sequences.

Reverse transcription and qPCR amplifications

The expression of 37 selected genes, three maize reference genes and the *cryIA(b)* transgene was assayed by reverse transcription—qPCR (RT-qPCR). Reverse transcription was performed on 500 ng total RNA, previously treated with Turbo DNase (Ambion, Austin, TX, USA) using 50 U of MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) and random hexamer primers (Applied Biosystems) according to the manufacturer's protocol. For each sample, cDNA was prepared at least in duplicate and the 37 sequences were analyzed with all cDNA preparations. The absence of remaining DNA targets was confirmed by qPCR analyses of DNase-treated RNA samples.

The qPCR assays targeting the 37 sequences selected from the microarrays were developed based on SYBR Green technology. PCR primers were designed using the Beacon Designer 7.0 software (Premier Biosoft International, Palo Alto, CA, USA) targeting the sequences used for generation of the GeneChip® Maize Genome Array. They were specific as in silico assessed with the BLAST tool. Amplicon lengths were between 60 and 140 bp. QPCR assays were performed in a 20 µl volume containing 1× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), the optimal concentration of primers [see Supplementary material, Table 1] and 1 µl cDNA (i.e. 1/20th of the RT reaction volume). The reaction conditions were: (1) initial denaturation (10 min at 95°C); (2) amplification and quantification (45 repeats of 15 s at 95°C and 1 min at 60°C); and (3) melting curve program

(60–95°C with a heating rate of 0.5°C/s). Melting curve analysis produced single peaks (no primer-dimer peaks or artefacts), indicating the reactions were specific. The no-template controls (NTC) included in all PCR runs produced negative results. qPCR assays targeting *cryIA(b)* and the reference genes 18S ribosomal RNA, β-actin and α-tubulin were performed as previously reported (Coll et al. 2008). All oligonucleotides were purchased from MWG Biotech AG (Germany).

All reactions were run on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and performed in duplicate or triplicate. Linearity (R^2) and efficiency ($E = 10^{[-1/\text{slope}]}$) of each reaction were within the accepted limits. The suitability of the reference genes as internal standards was confirmed in our samples through the geNORM v3.4 statistical algorithm, with M values below 0.5 in all cases.

Bioinformatics expression analysis

Normalization of RT-qPCR data and statistical analyses (t-test and multiway analysis) were performed using the Genex software v.4.3.1 (MultiD Analyses). The Benjamini and Hochberg False Discovery Rate multiple testing correction was applied (Benjamini and Hochberg 1995). After normalization, principal components analysis (PCA) was used to visualize different sources of variation in the data set (Stahlberg et al. 2008).

Results

The experimental design

Our aim was to assess the importance of transcriptional differences between MON810 and non-GM near-isogenic varieties in plants grown in agricultural environments. We grew two highly commercialized GM varieties (Helen Bt and Beles Sur) and their near-isogenic counterparts (Helen and Sancia) in accordance with conventional agricultural practices in a region where transgenic insect resistant (MON810) and conventional maize are commercially cultured. We included two levels of nitrogen fertilization as an example of different environmental and cultural conditions. Conventional N fertilization was used as control whereas low-N micro-plots were not N fertilized during the season. We analyzed the D leaf of VT stage plants (10 plants per replicate, three replicates per variety and condition). Plants of all varieties and N treatments simultaneously reached the VT stage and were harvested on the same day.

For control purposes, all our GM samples were also analyzed to compare the levels of expression of the transgene through an RT-qPCR assay targeting the *cryIA(b)* coding region (Table 1). Statistical analyses of the results normalized with β-actin messenger RNA (mRNA) levels (ANOVA and Tukey test, $P < 0.05$) indicated that there was no difference in the levels of transgenic mRNA in Helen Bt and Beles Sur plants grown in either control or low-N conditions (significance level, 0.289). This discounted any differential expression pattern among varieties and conditions being attributable to different *cryIA(b)* mRNA levels.

The initial N content in the experimental field was estimated to be 74 kg/ha by the Fenimar software (Domingo et al. 2006), which takes into account soil, climate and previous culture data. Control micro-plots were supplemented with 300 kg N/ha to reach the amount of N required to produce 13 t/ha maize (as expected in the experimental field). For all four varieties, plants grown with and without N fertilization had visible agronomic differences at the VT stage. Most notably, the lower leaves had precocious senescence symptoms that demonstrated N availability differences among the plants. N-tester measurements of chlorophyll content, known to significantly correlate to plant N levels, provided further evidence of the different nutritional status of control and low-N micro-plots [low-N vs. control readings were below 95% (Blackmer and Schepers 1995)]. Additionally, nitrogen was determined in all eight samples by the elemental analysis method. Low-N samples had lower total N content than control samples ($P < 0.05$) (Table 1) with two exceptions, Helen low-N replicate 3, and Beles Sur low-N replicate 3, which may be due to the inherent variability of environmental and cultural conditions in agricultural fields. These two samples were excluded from further analyses.

Our approach was based on transcriptome analysis of the Helen Bt/Helen variety pair (both under low-N and control conditions) by microarrays, and subsequent extension to the Beles Sur/Sancia pair using RT-qPCR on a number of selected transcripts. Globally, the study is a 3-way design: (1) transgenic character (MON810 or non-GM); (2) variety pair (Helen Bt/Helen or Beles Sur/Sancia); and (3) N level (low-N or control). The expression data was analyzed by augmented principal component (PCA) analysis and verified by clustering using the Kohonen self-organizing map (SOM) to evaluate the relative weight of the three defined factors.

Analysis of microarray data

Microarray data are available at the European Bioinformatics Institute (EMBL-EBI) ArrayExpress repository database under accessions E-MEXP-2364, E-MEXP-2366, E-MEXP-2367, E-MEXP-2368. Four independent analyses were performed: Helen Bt was compared to Helen under low-N and control conditions, and low-N and control

Table 1 *cryIA(b)* mRNA levels and percentage of organic N contents of maize plants grown under low-N and control conditions

	Maize variety*	Low-N conditions**	Control conditions**	P-value
cryIA(b) expression***	Helen Bt	7.12e + 07 ± 1.80e07	6.06e + 07 ± 4.59e06	0.289
	Beles Sur	5.27e + 07 ± 6.96e06	5.46e + 07 ± 1.28e07	
N content****	Helen Bt	2.71 ± 0.35	3.78 ± 0.22	8.33e-05
	Helen	2.92 ± 0.18	3.45 ± 0.39	3.79e-02
	Beles Sur	1.63 ± 0.18	2.70 ± 0.30	2.10e-04
	Sancia	2.60 ± 0.15	4.01 ± 0.22	1.43e-07

The ANOVA statistical test was applied to compare *cryIA(b)* mRNA levels among MON810 varieties and N conditions; and the Student *t*-test was used to compare the organic N contents of a given variety between low-N and control conditions (*P*-values are indicated)

* Samples were taken from the D-leaf

** Means and standard deviations (SD) of duplicates of each of the replicate samples

*** *cryIA(b)* mRNA levels are expressed in copy numbers relative to *β*-actin

**** N contents were obtained by elemental analysis

conditions were compared for each variety (Helen Bt and Helen). For each comparison, the data obtained in the three replicates were collectively analyzed using the RMA software for gene expression summary values. The data was subsequently filtered by considering only probes with *P*-values < 0.05 and at least a twofold increase or decrease in the level of a given transcript (Table 2).

Microarray comparisons resulted in a total of 60 sequences with differential transcription patterns between varieties and/or N conditions. Probes with signal intensity differences below 200 fluorescence units were filtered out. Two of the 41 remaining sequences corresponded to the same gene and were both coded as *Zm11* for further analysis. A total of 40 sequences (*Zm01–Zm40*, Supplementary material, Table 1) were used to design qPCR assays to validate the microarray data and further investigate their transcription patterns in other varieties. Sequences *Zm09*, *Zm11* and *Zm14* (array probes belonging to genes with Acc. # BU499844, BM736430, and 11990232-26, respectively) were not suitable to design a qPCR assay in the Beacon Designer software default settings and were discarded. The remaining 37 qPCR assays, optimized with maize genomic DNA samples, all produced unique amplicons, demonstrated by a single sharp peak in the first derivative plot of dissociation curve analysis. Linearity values were above 0.9; mean $R^2 = 0.998 \pm 0.002$ and efficiency values, above 0.9; mean $E = 0.943 \pm 0.047$.

Messenger RNA levels of the 37 selected sequences were assessed in biological triplicates of Helen Bt and Helen leaf samples, both in low-N and control conditions, by qPCR coupled to reverse transcription with random hexamers. Three reference genes (*18S rRNA*, *β-actin* and *α-tubulin*) were used to normalize the data. Application of the geNORM v3.4 statistical algorithm showed that all three reference genes displayed stability measures (*M*) below 0.5, making them suitable internal standards for gene expression under our experimental conditions.

Comparison of the expression profiles in the microarray experiments and qPCR results was performed for all four possible comparisons with the regulated sequences in each case. For each sequence and comparison, the qPCR results

Table 2 Numbers (No. diff. seq.) and percentages (% diff. seq.) of statistically significant differentially expressed sequences in pair-wise comparisons of Helen Bt and Helen maize varieties grown under low N and control conditions

Comparison	No. diff. seq.*	% diff. seq.	No. qPCR*
Helen Bt and Helen (low-N conditions)	23 (8/15)	0.13	14 (5/9)
Helen Bt and Helen (control conditions)	13 (5/8)	0.07	8 (4/4)
Helen Bt and Helen**	24 (8/16)	0.14	14 (5/9)
Control and low-N conditions (Helen Bt)	31 (2/29)	0.17	20 (1/19)
Control and low-N conditions (Helen)	7 (2/5)	0.04	3 (0/3)
Control and low-N conditions***	36 (3/33)	0.20	23 (1/22)

The number of sequences further analyzed by qPCR assays is also indicated (No. qPCR)

* The numbers of sequences that are induced and repressed in the first versus the last compared varieties or conditions are shown in brackets

** Note some sequences may be regulated in control and low-N conditions (see also Fig. 1, Table 3)

*** Note some sequences may be regulated in the two varieties

obtained were statistically analyzed by *t*-test coupled to the Benjamini and Hochberg False Discovery Rate multiple testing correction, with $P < 0.05$ (Supplementary material, Table 2). The degree of coincidence between the microarrays and the RT-qPCR was 71.1%, which is within the expected range (Dallas et al. 2005) and thus verified the microarray results.

According to the microarrays results, the number and percentage of genes showing differential expression among MON810 and non-GM near-isogenic varieties were very low both under control and low N levels (Table 2), overall reaching 0.14% analyzed sequences. As shown in Table 3 and illustrated in Fig. 1 12 genes were regulated in Helen Bt versus Helen in control and low-N conditions (i.e. 50% regulated sequences), with differential expression ratios ranging from 2- to 8-fold. Additionally, *Zm01* was ~30-fold over-expressed in Helen Bt compared to Helen specifically under control conditions. Eleven other sequences were differentially expressed (2- to 6-fold) specifically under low N conditions. The MapMan tool allowed mapping nine differentially expressed sequences (Table 4), 1 in the "not assigned" bin, a glutathione S-transferase (*Zm15*) in the "miscellaneous" bin and seven were assigned to a functional category. They had postulated functions in the minor CHO and lipid metabolism, mitochondrial electron transport/ATP synthesis, signaling, transport and cell wall. There was no significant enrichment of genes in any of the functional categories.

For each variety, the comparison between low-N and control conditions gave very low numbers of differentially expressed sequences (Table 2), reaching 0.03% in Helen. Note that for each variety the N contents of plants grown in low-N and control conditions were statistically different (Table 1); however these differences were 1.07% organic N in Helen Bt and only 0.53 in Helen. The sequences with differential expression were mostly repressed at low N conditions in Helen Bt (see Supplementary material, Table 2). All were repressed less than threefold except for *Zm40* and *Zm39* that were repressed 19- and 7-fold, respectively. Only sequences with GenBank Acc. # CO531 267 (not annotated) and CO532209 (CBL-interacting protein kinase, involved in protein posttranslational modification), were differentially expressed in low-N and control conditions in the two varieties. The 36 regulated sequences were analyzed using MapMan (Supplementary material, Table 3). Up to 27 sequences could be mapped, 6 in the "not assigned" bin and 21 assigned to a MapMan functional category. Among these 21 sequences, 12 had a postulated function in metabolism: amino acids, nitrogen, TCA, polyamine, lipid, cell wall modification, and protein biosynthesis. Four sequences had a presumed regulatory function, two transcription factors and two posttranslational modification proteins. Two sequences mapped in the transport functional category, one in the biotic stress bin, and three corresponded to a gluco-, galacto- and mannosidase, a cytochrome P450 and a phosphatase (miscellaneous bin).

Evaluation of the relative impact of the transgenic character, variety and N availability on maize gene expression

The MON810 transgenic modification has been introduced into many different varieties. The relevance of the differential expression patterns observed between transgenic varieties and the corresponding non-GM near-isogenic lines, and also between two different N availability levels, was assessed by analyzing the expression of the 37 selected sequences in a different MON810 and non-GM near-isogenic pair, using RT-qPCR. Beles Sur (MON810) and Sancia (non-GM) were selected as they are widely cultivated in the region and produced by different companies through independent breeding programs. Beles Sur and Sancia seeds were grown in parallel to Helen Bt and Helen in the same field. Three biological replicates were sampled per variety and nitrogen condition, each grown in a different micro-plot. The 24 samples were analyzed by qPCR using the same three internal controls (validated in these samples, $M < 0.5$) and the results analyzed using GenEX software (Supplementary material, Table 2).

The 37 selected genes analyzed in two pairs of closely related maize varieties, each consisting of one MON810 and one non-GM line, with all four varieties cultured in control or low-N conditions, gave a total of 296 data points [37 (genes) × 2 (pairs of varieties) × 2 (GMO and non-GM character) × 2 (N conditions)]. Our main interest was to compare the transcriptional profiles of all 37 sequences in our eight samples in order to evaluate the grouping of the samples as a function of gene expression, taking the three variables into consideration: variety pair (referring to closely related variety pairs, obtained through different conventional breeding strategies); GMO character (MON810 or non-GM); and the N treatment (conventional fertilization or lack of N-fertilization).

PCA was used to picture different sources of variation in the data set. The first component (PC1) accounted for 37.4% of the information. The second most significant component, PC2, accounts for most of the variability that is not explained by PC1, and reached (in combination with PC1) 69.3%. All eight samples were represented 2-dimensionally using their PC1 and PC2 scores, revealing groups of samples based on around 70% of all variability (Fig. 2). This showed a complete separation of Helen Bt/Helen and Beles Sur/Sancia samples (PC1 values above +2.0 and below −2.0, respectively), indicating that variety pair had the highest impact on gene expression patterns. Moreover, each region showed two

Table 3 Sequences with significant differential expression in pair-wise comparisons of Helen Bt and Helen maize varieties grown under low N and control conditions: Differential expression folds

Internal code*	Accession number	HelenBt versus Helen	
		Low-N conditions (x-fold)**	Control conditions (x-fold)**
Zm01	AY108221.1	22.35	35.56
Zm02	11990232-44	7.77	7.09
	40794996-84	8.19	8.45
Zm03	CK368940	4.49	3.17
Zm04	AY104588.1	2.82	2.70
Zm13	AW566101	0.43	0.39
	BM266792	0.45	0.37
Zm05	BM378498	0.46	0.37
Zm06	CK369628	0.44	0.36
Zm07	40794996-44	0.28	0.26
Zm08	AI855032	0.21	0.22
Zm09	BU499844	0.12	0.17
	BQ578253		0.45
Zm10	11990232-46	2.97	
Zm11	BM736430	2.24	
Zm11***	BM736430	2.16	
Zm12	BM080363	0.50	
	40794996-37	0.49	
Zm14	11990232-26	0.47	
Zm15	AF244689.2	0.42	
Zm16	40794996-39	0.33	
Zm17	CN844137	0.32	
	BM337131	0.30	
	AY105529.1	0.16	

* Only sequences further analyzed by RT-qPCR were assigned an internal code

** Differential expression folds in the first versus the last compared varieties or conditions. Only sequences with differential expression folds >2 or <0.5 are recorded

*** Note that two probes in the microarray corresponded to the same gene Accession number and where both coded as Zm11

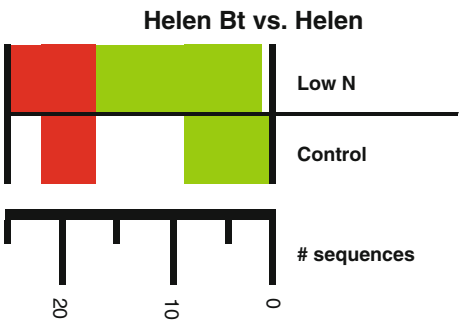

Fig. 1 Schematic representation of differential gene expression in Helen Bt and Helen plants grown under control and low-N conditions. Differentially expressed sequences ($P < 0.05$ and at least twofold difference) are shown in *green* (down-regulated in the MON810 variety) and *red* (up-regulated in the MON810 variety). Sequences with similar expression values in GM and non-GM varieties (about 99.9% of all sequences analyzed) are not included

distinct groups according to the N treatment (negative or positive PC2 values for control and low-N samples, respectively); suggesting that N availability was the second major factor involved in the expression patterns of the selected genes. The inclusion of PC3 in PCA explains 79% of the variability. The samples were then represented 3-dimensionally using their PC1, PC2 and PC3 scores (for clarity, they are represented in two 2-D graphs, Fig. 2). As can be observed, PC3 gave complete separation of Helen Bt and Helen samples (negative and positive PC3 scores, respectively). The MON810 or non-GM character has the lowest impact on gene expression patterns in the studied tissues, varieties and environmental conditions, and the sequences selected to be regulated in a given variety pair (here Helen Bt and Helen) do not allow a clear separation between other MON810 and comparable non-GM varieties.

Table 4 Sequences with significant differential expression in pair-wise comparisons of Helen Bt and Helen maize varieties grown under low N and control conditions: MapMan based functional assignment

Bin code	Bin name	No. seq.	Affymetrix ID	Accession number	Internal code
3	**Minor CHO metabolism**	**1**			
3.5	Minor CHO metabolism, others	1	Zm.13820.1.a1_at	AY104588.1	Zm04
9	**Mitochondrial electron transport/ATP synthesis**	**1**			
9.1.2	Mitochondrial electron transport/ATP synthesis, NADH-DH, localisation not clear	1	Zm.12693.1.a1_at		
10	**Cell wall**	**2**			
10.2	Cell wall, cellulose synthesis	1	Zm.1004.9.1.a1_at	BM378498	Zm05
10.5.1	Cell wall, cell wall proteins, AGPs	1	Zm.8927.1.a1_at		
11	**Lipid metabolism**	**1**			
11.10.3	Lipid metabolism, glycolipid synthesis, UDP-sulfoquinovose synthase	1	Zm.7904.1.a1_at	BM080363	Zm12
26	**Miscellaneous**	**1**			
26.9	Miscellaneous, glutathione S transferases	1	Zm.627.1.a1_at	AF244689.2	Zm15
30	**Signalling**	**1**			
30.3	Signalling, calcium	1	Zm.17082.1.a1_at	AY108221.1	Zm01
31	**Cell**	**1**			
31.4	Cell, vesicle transport	1	Zm.1128.1.a1_at		
34	**Transport**	**1**			
34.9	Transport, metabolite transporters at the mitochondrial membrane	1	Zm.17267.1.a1_at	CK368940	Zm03
35	**Not assigned**	**1**			
35.2	Not assigned, unknown	1	Zm.16709.1.a1_at	CN844137	Zm17

PCA is considered a very robust approach to classify samples based on multiway measurements. Other techniques for unsupervised clustering are available and can be used to confirm the PCA results. Figure 3 shows the graphical output of the clustering by the SOM to confirm the relative weight of the defined factors. A map with two cells was first used and subsequently a map with four cells, to force classification into two and four groups, respectively.

The two-cell map reveals two main groups of samples: I, Beles Sur and Sancia, and II, Helen Bt and Helen. This is in agreement with the two regions clearly separated in the PC1 versus PC2 scatter plot of the PCA (Fig. 2a), giving support to the variety pair being the most significant factor on gene expression patterns with the varieties and conditions used here. The map with four cells assembles the samples as follows: group I, composed of Helen and Helen Bt low-N; group II, composed of Sancia and Beles Sur low-N; group III, with Sancia and Beles Sur control samples; and group IV, with Helen and Helen Bt control samples. Again, these results agree with the regions separated in the PC1 versus PC2 scatter plot of the PCA (Fig. 2a), giving further support to the variety pair and the N conditions being the main factors affecting gene expression in our experiment.

None of the sequences gave the same pattern on comparison of the samples HelenBt/Helen versus Beles Sur/Sancia; low-N versus control; and MON810 versus non-GM, based on the PC1, PC2 and PC3 loadings obtained for each analyzed sequence in the eight different samples. Therefore, none of these sequences could be used as a varietal, N-condition or GM character marker in the conditions assayed. Furthermore, we classified the 37 sequences using PCA to evaluate grouping of the genes as a function of regulation in the different samples analyzed. Analysis of the PC1 and PC2 scores (representing around 70% of the data) did not reveal clear groups of sequences, confirming the lack of clear marker genes for the studied factors among the analyzed sequences. Conversely, the combination of different sequences seems to be the basis for sample classification.

Discussion

Over the last few years, a number of reports have been published focusing on the study of possible unintended effects of the introduction and expression of transgenes in plants, many of them based on general -omics technologies (Baudo et al. 2009; Beale et al. 2009; Kok et al. 2008). These studies have been mainly performed with plants grown under optimal and controlled conditions, i.e.

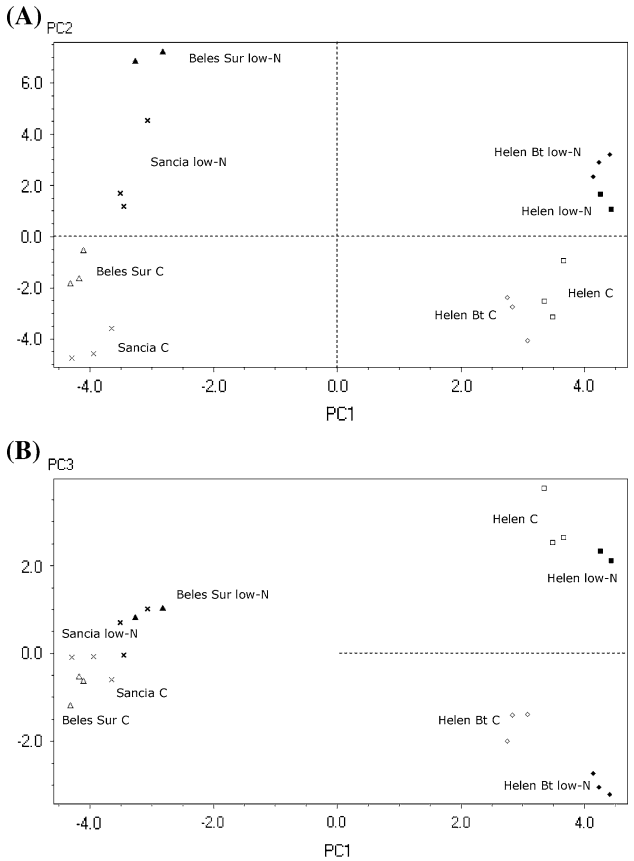

Fig. 2 Principal component analysis (PCA) of the sequence expression data. Classification of samples using PC1 versus PC2 (**a**) and PC1 versus PC3 (**b**). *Rhombus* correspond to Helen Bt samples; *squares*, to Helen samples; *triangles* to Beles Sur and *crosses*, to Sancia samples. *Open figures* represent control (C) and *filled figures*, low-N conditions (low-N). Autoscaled logarithmic expression levels are plotted

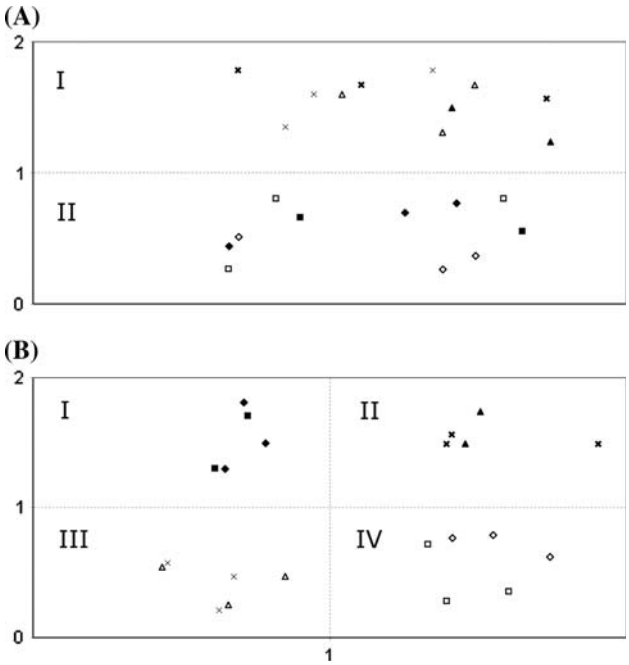

Fig. 3 Kohonen self-organizing maps (SOM) for samples of Helen Bt (*rhombus*), Helen (*squares*), Beles Sur (*triangles*) and Sancia (*crosses*) plants either grown under control (*open figures*) or low-N (*filled figures*) conditions. Maps with two (**a**) and four (**b**) cells are shown for classification into two and four groups, respectively. Autoscaled logarithmic expression levels are plotted

cultured in vitro, in growth chambers or in greenhouses. For commercial GMOs, it is important to assess the unintended effects of transgenes in agricultural conditions, particularly covering different environmental circumstances. Our aim was to broaden the present state of knowledge on transcriptional differences between authorized GM events and non-transgenic crops, taking into account agricultural conditions, using the maize event MON810 as an example, in a region in Catalonia (Spain) where about 70% of the maize grown in the season under study was MON810.

Plants grown in the field were compared at the transcriptome level by microarray hybridization. Field cultivation of plants is inevitably subject to environmental conditions and agricultural practices that can affect different parts of the field in a non-uniform manner, resulting in lower sample homogeneity as compared to greenhouse and, in particular, in vitro culture. The expected variability among individual plants was overcome by sampling up to 10 plants per biological replicate: residual variances of our microarray experiments were between 8 and 13%, values similar to those previously obtained in microarray experiments with MON810 and comparable varieties grown in vitro, (Coll et al. 2008). With this experimental design, possible differences between MON810 and comparable non-GM varieties that could be the result of normal variability of maize plants grown in similar (but not identical) environments are not considered. Natural variation in gene expression has emerged as an important issue in relation to the safety assessment of biotech plants (Van Dijk et al. 2009); and this refers to different varieties, locations and farming practices. We chose the two GM varieties predominantly cultured in the region (Helen Bt and Beles Sur, obtained by different seed companies) and their near-isogenic counterparts (Helen and Sancia, also highly cultivated). We used two levels of nitrogen fertilization to gain insight into the potential impact of environmental and cultivation factors on the degree of similitude between GMO and non-GM comparable varieties. Nitrogen is one of the most relevant nutrients in maize cultivation, with major fertilization treatments being a significant portion of the producers' costs. As such, we considered conventional N fertilization as the control compared to nitrogen stress, with no N fertilization during this season.

According to the microarrays results, only a few genes were regulated between Helen Bt and Helen, and this

occurred in the two cultural conditions assayed. Such numbers are similar to those previously reported for other MON810/near-isogenic variety pairs (Coll et al. 2008): as few as ~1.7 and ~0.14% probes were differentially expressed in leaves of Aristis Bt/Aristis and PR33P67/PR33P66 plants cultured in vitro, respectively. Recent metabolomics comparisons of GM and non-GM maize varieties grown under control conditions also revealed low-percentage differences (Piccioni et al. 2009). MON810 and comparable non-GM varieties appear to have residual and similar levels of regulated sequences in variety pairs, in terms of number of regulated genes, under various environmental conditions. The identity of the sequences regulated between Helen Bt and Helen was, in most cases, the same under low-N and control conditions. Therefore, for a given variety pair, the similarity does not seem to depend substantially upon the assayed environmental conditions. Our compared samples were at the same developmental stage (VT) and, except for the N fertilization, were grown under the same conditions in the field. According to a recent publication by Kok et al. (2008) it is crucial to grow plants under the same conditions when making a comparison, in order to avoid differences that are unrelated to the differences in genotype under investigation. The equivalence of the global pattern of transcription in transgenic and non-transgenic plants has also been demonstrated in transgenic *Arabidopsis* grown in vitro and subjected to temperature or drought stress (El Ouakfaoui and Miki 2005).

In this study, major differences in gene expression were observed between the Helen Bt/Helen and Beles Sur/Sancia variety pairs. In PCA analyses, 37.4% of the variation within the data set was attributed to varietal differences (referring to GM and near-isogenic non-GM varieties bred through conventional programs by different seed companies), with N treatment the second most significant factor. The differential gene expression of VT maize leaves was mainly dependent upon the variety (obtained by conventional breeding), with the nutritional status having a comparatively minor effect. Similarly, in a transcriptome analysis of potato tubers, van Dijk et al. (2009) observed more pronounced differences between two conventional varieties than between different types of organic fertilizer and plant protection regimens applied.

Nitrogen fertilization as a factor with a greater effect on gene expression than the transgene (see below) is especially significant in view of the small differences observed in Helen plants subjected to control and N deficiency treatments (Table 2). From the 36 sequences that were differentially expressed between the fertilizer treatments, most were regulated in Helen Bt (83%), Beles Sur (83%) and Sancia (61%) whereas just 13% were regulated in Helen. Compatible with these results, the variation in total N content in control and low-N samples was over 1% in Helen Bt, Beles Sur and Sancia but, although still significant, was only 0.53% in Helen (Table 1). This was not directly attributable to the transgene since the behavior of the MON810 variety Beles Sur was the same as the two non-GM varieties. In a separate field where Helen and Sancia were sown under the same control and N deficient conditions, the total nitrogen content of the D-leaf of control and low-N plants differed by 1.32 and 0.81%, respectively. This indicated that Helen does not have a better capacity to assimilate or mobilize N under low N conditions but rather the difference in our samples was due to the expected deviations in experiments performed in agricultural fields.

There have been various studies of plant N-responses based on microarray gene expression profiling, most focusing on short-term plant responses to N-induction or N-deprivation (Lian et al. 2006; Price et al. 2004; Scheible et al. 2004; Wang et al. 2000, 2003) (see also Prinsi et al. 2009 for a proteomics approach). In vitro grown plants temporary subjected to low and high N conditions have been shown to exhibit regulation of several hundreds of transcripts. However, plants seem to have a very different regulatory system to cope with N starvation versus chronic N stress. Chronic N stress has been studied in *Arabidopsis* plants cultured in vitro by a transcriptomics approach (Bi et al. 2007). Only a small set of genes (~0.21% analyzed sequences) were differentially expressed by mild chronic stress (1/3rd of control fertilization), whereas about tenfold larger changes were observed as a result of severe chronic stress conditions (1/10th of control fertilization). In our study, 0.17% sequences were regulated in Helen Bt plants grown in the field as a function of chronic N stress (1/5th of control fertilization), which seems to fall within the expected results despite the different plant species and cultural conditions. More than 95% of these sequences were repressed under low N conditions and most of those that could be assigned to a functional category were associated to metabolism, including TCA generation of energy, nitrogen, amino acids, lipid, protein synthesis, and transcription regulation. This overlaps with the biological processes shown to be down-regulated in *Arabidopsis* plants subjected to chronic N stress (Bi et al. 2007).

The N-metabolism related glutamine synthase 2 (GS2) gene was repressed in low N conditions. GS catalyzes the conversion of glutamate into Gln by incorporating a molecule of ammonia. The primary assimilation of N, as well as the re-assimilation of photorespiratory ammonia, consists of incorporation of ammonium into organic molecules by different isozymes of the GS and GOGAT pathway (Coruzzi 2003). The reduced expression of the GS2 gene (known to be induced by NO^{3-} and NH^{4+}) is a result of nitrogen assimilation, photosynthesis and photorespiration

being repressed under N stress. Applying chronic N stress to *Arabidopsis* resulted in increased expression of GS1, probably involved in re-assimilation of ammonia from protein degradation, which usually occurs under N deficiency (Bi et al. 2007). N fertilizer application is directly linked to grain yield and quality: during plant growth, N is accumulated in the vegetative tissues and distributed to the developing seeds concurrently with vegetative tissue senescence. Amino acids (principally Gln) are the major form of N transported from leaf to grain (Lalonde et al. 2004), and lower concentrations of total free amino acids, as well as different amino acids pools, have been measured in the leaves of N-deficient wheat plants (Howarth et al. 2008). We observed that VT maize plants grown under low fertilization conditions under-expressed 3 genes with a possible function in amino acid metabolism, including synthesis (Ala and Pro) and degradation (Arg, Leu and Ile) (Supplementary material, Table 2). Similarly, various genes involved in amino acid metabolism had lower levels of expression in wheat plants transiently subjected to low N stress (Howarth et al. 2008). Most amino acid pools were regulated in *A. thaliana* subjected to abiotic stress by the transcription of catabolic enzymes (Less and Galili 2008). We also found that three genes involved in protein synthesis and posttranslational modification were repressed in maize leaves grown under low N conditions, compatible with N accumulation in the form of amino acids for further mobilization to grains.

In contrast to the variety and nitrogen conditions, the effect of the MON810 transgenic character was only detectable in the Helen Bt and Helen comparison, accounting for less than 10% of the variability of the data set (PC3). This indicates the MON810 GM character has a very minor effect on gene expression in the analyzed varieties and conditions. Similarly, recent publications comparing transcriptome patterns of GM and conventional varieties have shown greater natural variation between conventionally bred varieties than between GM and comparable lines in wheat (Baudo et al. 2006), rice (Batista et al. 2008) and soybean (Cheng et al. 2008). Less than 40% sequences that were regulated in Helen Bt and Helen could be assigned to a functional category; and they were widespread along 8 different bins (Table 4). We could not identify any function that was predominantly altered by the MON810 transgene. Moreover, only around half the sequences that were differentially expressed in Helen Bt and Helen were also regulated in Beles Sur and Sancia in control (50%) and low-N (43%) conditions, although other sets of sequences may be differentially regulated in Beles Sur and Sancia. Only two sequences were regulated in the two variety pairs and N conditions, with signaling related to calcium (*Zm01*) and metabolite transport at the mitochondrial membrane (*Zm03*) functions. The biological relevance of regulated sequences seems to be limited to one particular transgenic variety. Helen Bt and Sancia had similar levels of *cryIA(b)* expression under the two nitrogen conditions, indicating that different transgene mRNA levels were not the cause of the different patterns observed in these varieties. This further supports our previous observations pointing towards a varietal dependence of transcriptional differences between MON810 and comparable varieties (Coll et al. 2008). Statistical differences have also been reported in enantiomeric amino acid composition of particular pairs of MON810/comparable non-GM varieties, such as Aristis Bt and Aristis (% D content of Arg, Ser, and Asp) and PR33P67 and PR33P66 (% D content of Arg, Ser, and Ala) but not of Tietar Bt and Tietar (Herrero et al. 2007). In other plant species, differences between controls and specific GM lines have often been observed but they appear to be random and not associated with any specific insert (Baudo et al. 2006; El Ouakfaoui and Miki 2005; Metzdorff et al. 2006).

The data of the present study indicates that the extent of natural variation of gene expression is, in the varieties and conditions analyzed, larger than the variation due to the insertion and expression of the MON810 transgene. This emphasizes that such natural variation should be taken into account to assess the biological and/or toxicological relevance of observed differences between a GM and a comparable non-GM plant.

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