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

Anna Coll[1], Anna Nadal[2], Rosa Collado[1], Gemma Capellades[3], Mikael Kubista[4], Joaquima Messeguer[5], and Maria Pla[1*]

[1] INTEA, Universitat de Girona. Campus Montilivi, EPS-I; 17071 Girona, Spain

[2] Departament Genètica Molecular, Centre de Recerca en Agrigenòmica. CSIC-IRTA-UAB. Jordi Girona, 18; 08034 Barcelona, Spain

[3] Fundació Mas Badia, ctra. La Tallada s/n, 17134 La Tallada d'Empordà, Girona, Spain

[4] Institute of Biotechnology, Academy of Sciences of the Czech Republic, Videnska 1083, Prague 4, 142, 20 Czech Republic / TATAA Biocenter AB, Odinsgatan 28, 411 03 Goteborg, Sweden

[5] Departament Genètica Vegetal, Centre de Recerca en Agrigenòmica. CSIC-IRTA-UAB. Ctr. Cabrils; 08348 Barcelona, Spain

[*]Corresponding author: Maria Pla. Institut de Tecnologia Agroalimentària (INTEA), Universitat de Girona. Campus Montilivi, EPS-I; 17071 Girona, Spain. Tel. +34 972 419852; Fax. +34 972 418399; e-mail. maria.pla@udg.edu

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.

Key words: GMO (Genetically Modified Organism); MON810; maize; nitrogen stress; transcriptome; unintended effects; agricultural field; natural variation

ABBREVIATIONS

cDNA: complementary DNA

CRM: certified reference material

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; Baudo et al. 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 hectares 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 and co-workers (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 (i) the GMO character (using MON810 as example); (ii) the variety (referring to closely related MON810/near-isogenic variety pairs obtained through conventional breeding strategies); and (iii) 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 & 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 0.2g 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 ºC in April, May and June, respectively, similar to the temperatures recorded between 1984 and 2008 (13.0, 16.9 and 20.5 ºC, 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 -80ºC. 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 260nm 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 (Schnable 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 3 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 15μg 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 twofold (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, 3 maize reference genes and the *cryIA(b)* transgene was assayed by reverse transcription – qPCR (RT-qPCR). Reverse transcription was performed on 500ng total RNA, previously treated with Turbo DNase (Ambion, Austin, TX, USA) using 50U 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 20μl volume containing 1X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), the optimal concentration of primers [see Supplemental material Table 1] and 1μl cDNA (i.e. $1/20^{th}$ of the RT reaction volume). The reaction conditions were: (1) initial denaturation (10min at 95ºC); (2) amplification and quantification (45 repeats of 15s at 95ºC and 1min at 60ºC); and (3) melting curve program (60-95ºC with a heating rate of 0.5ºC/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 ($E= 10^{[-1/slope]}$) 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, 3 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. 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 8 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: (i) transgenic character (MON810 or non-GM); (ii) variety pair (Helen Bt/Helen or Beles Sur/Sancia); and (iii) 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 3 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 conditions were compared for each variety (Helen Bt and Helen). For each comparison, the data obtained in the 3 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 ± 0.002 and efficiency values, above 0.9; mean E = 0.943 ± 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 3 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 4 possible comparisons with the regulated sequences in each case. For each

sequence and comparison, the qPCR results obtained were statistically analyzed by t-test coupled to the Benjamini and Hochberg False Discovery Rate multiple testing correction, with $p<0.05$ (Supplemental 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 3A and illustrated in Figure 1 twelve genes were regulated in Helen Bt vs. 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 9 differentially expressed sequences (Table 3B), 1 in the "not assigned" bin, a glutathione S-transferase (*Zm15*) in the "miscellaneous" bin and 7 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 Supplemental Table 2). All were repressed less than 3-fold except for *Zm40* and *Zm39* that were repressed 19- and 7-fold, respectively. Only sequences with GenBank Acc. # CO531267 (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 (Supplemental 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, 2 transcription factors and 2 posttranslational modification proteins. Two sequences mapped in the transport functional category, one in the biotic stress bin, and 3 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 3 internal controls (validated in these samples, $M < 0.5$) and the results analyzed using GenEX software (Supplemental 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 8 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 (Figure 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 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, Figure 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.

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 vs. PC2 scatter plot of the PCA (Figure 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 vs. PC2 scatter plot of the PCA (Figure 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 vs. Beles Sur/Sancia; low-N vs. control; and MON810 vs. non-GM, based on the PC1, PC2 and PC3 loadings obtained for each analyzed sequence in the 8 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. 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

447 event MON810 as an example, in a region in Catalonia (Spain) where about 70% of the maize
448 grown in the season under study was MON810.

449 Plants grown in the field were compared at the transcriptome level by microarray hybridization.
450 Field cultivation of plants is inevitably subject to environmental conditions and agricultural
451 practices that can affect different parts of the field in a non-uniform manner, resulting in lower
452 sample homogeneity as compared to greenhouse and, in particular, in vitro culture. The
453 expected variability among individual plants was overcome by sampling up to 10 plants per
454 biological replicate: residual variances of our microarray experiments were between 8% and
455 13%, values similar to those previously obtained in microarray experiments with MON810 and
456 comparable varieties grown in vitro, (Coll et al. 2008). With this experimental design, possible
457 differences between MON810 and comparable non-GM varieties that could be the result of
458 normal variability of maize plants grown in similar (but not identical) environments are not
459 considered. Natural variation in gene expression has emerged as an important issue in relation
460 to the safety assessment of biotech plants (Van Dijk et al. 2009); and this refers to different
461 varieties, locations and farming practices. We chose the two GM varieties predominantly
462 cultured in the region (Helen Bt and Beles Sur, obtained by different seed companies) and
463 their near-isogenic counterparts (Helen and Sancia, also highly cultivated). We used two levels
464 of nitrogen fertilization to gain insight into the potential impact of environmental and cultivation
465 factors on the degree of similitude between GMO and non-GM comparable varieties. Nitrogen
466 is one of the most relevant nutrients in maize cultivation, with major fertilization treatments
467 being a significant portion of the producers' costs. As such, we considered conventional N
468 fertilization as the control compared to nitrogen stress, with no N fertilization during this
469 season.

470 According to the microarrays results, only a few genes were regulated between Helen Bt and
471 Helen, and this occurred in the two cultural conditions assayed. Such numbers are similar to
472 those previously reported for other MON810/near-isogenic variety pairs (Coll et al. 2008): as
473 few as ~1.7% and ~0.14% probes were differentially expressed in leaves of Aristis Bt/Aristis
474 and PR33P67/PR33P66 plants cultured in vitro, respectively. Recent metabolomics
475 comparisons of GM and non-GM maize varieties grown under control conditions also revealed
476 low-percentage differences (Piccioni et al. 2009). MON810 and comparable non-GM varieties
477 appear to have residual and similar levels of regulated sequences in variety pairs, in terms of
478 number of regulated genes, under various environmental conditions. The identity of the
479 sequences regulated between Helen Bt and Helen was, in most cases, the same under low-N
480 and control conditions. Therefore, for a given variety pair, the similarity does not seem to
481 depend substantially upon the assayed environmental conditions. Our compared samples were
482 at the same developmental stage (VT) and, except for the N fertilization, were grown under
483 the same conditions in the field. According to a recent publication by Kok and coworkers (Kok
484 et al. 2008) it is crucial to grow plants under the same conditions when making a comparison,
485 in order to avoid differences that are unrelated to the differences in genotype under
486 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; Wang et al. 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) (Supplemental 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 3 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 3B). 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.

ACKNOWLEDGEMENTS

We thank J.M. García-Cantalejo (Parque Científico de Madrid) for technical assistance; J. Serra (E.E.A. Mas Badia), E. Melé and M. Palaudelmàs (CRAG), and Ales Tichopad (Prague) for valuable suggestions; and Prof. P. Puigdomènech (CRAG) for critical reading of the manuscript. This work was financially supported by the Spanish MEC project with ref. AGL2007-65903/AGR. AC received a studentship from the Generalitat de Catalunya (2005FI 00144).

FIGURE LEGENDS

Figure 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 2-fold 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.

Figure 2. Principal component analysis (PCA) of the sequence expression data. Classification of samples using PC1 vs. PC2 (A) and PC1 vs. PC3 (B). Squares correspond to Helen Bt samples; rhombus, to Helen samples; triangles to Beles Sur and circles, to Sancia samples. Open figures represent control (C) and filled figures, low-N conditions (low-N). Autoscaled logarithmic expression levels are plotted.

Figure 3. Kohonen self-organizing maps (SOM) for samples of Helen Bt (squares), Helen (rhombus), Beles Sur (triangles) and Sancia (circles) plants either grown under control (open

606 figures) or low-N (filled figures) conditions. Maps with two (A) and four (B) cells are shown for
607 classification into two and four groups, respectively. Autoscaled logarithmic expression levels
608 are plotted.

609

610 **REFERENCES**
611
612 Baker JM, Hawkins ND, Ward JL, Lovegrove A, Napier JA, Shewry PR, Beale MH (2006) A
613 metabolomic study of substantial equivalence of field-grown genetically modified wheat.
614 Plant Biotechnol J 4:381-392

615 Batista R, Saibo N, Lourenco T, Oliveira MM (2008) Microarray analyses reveal that plant
616 mutagenesis may induce more transcriptomic changes than transgene insertion. Proc Natl
617 Acad Sci USA 105:3640-3645

618 Baudo MM, Lyons R, Powers S, Pastori GM, Edwards KJ, Holdsworth MJ, Shewry PR (2006)
619 Transgenesis has less impact on the transcriptome of wheat grain than conventional
620 breeding. Plant Biotechnol J 4:369-380

621 Baudo MM, Powers SJ, Mitchell RA, Shewry PR (2009) Establishing substantial equivalence:
622 transcriptomics. Methods Mol Biol 478:247-272

623 Beale MH, Ward JL, Baker JM (2009) Establishing substantial equivalence: metabolomics.
624 Methods Mol Biol 478:289-303

625 Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and poweful
626 approach to multiple testing. J Royal Stat Soc B 57:289-300

627 Bi YM, Wang RL, Zhu T, Rothstein SJ (2007) Global transcription profiling reveals differential
628 responses to chronic nitrogen stress and putative nitrogen regulatory components in
629 Arabidopsis. BMC Genomics 8:281

630 Blackmer TM, Schepers JS (1995) Use of chlorophyll meter to monitor nitrogen status and
631 schedule fertigation for corn. J Prod Agric 8:56-60

632 Bradford KJ, Van Deynze A, Gutterson N, Parrott W, Strauss SH (2005) Regulating transgenic
633 crops sensibly: lessons from plant breeding, biotechnology and genomics. Nat Biotechnol
634 23:439-444

635 Catchpole GS, Beckmann M, Enot DP, Mondhe M, Zywicki B, Taylor J et al (2005) Hierarchical
636 metabolomics demonstrates substantial compositional similarity between genetically
637 modified and conventional potato crops. Proc Natl Acad Sci USA 102:14458-14462

638 Cellini F, Chesson A, Colquhoun I, Constable A, Davies HV, Engel KH et al (2004) Unintended
639 effects and their detection in genetically modified crops. Food Chem Toxicol 42:1089-
640 1125

641 Chassy B, Egnin M, Gao Y, Glenn K, Kleter GA, Nestel P, Newell-McGloughlin M, Shillito R
642 (2008) Nutritional and safety assessments of foods and feeds nutritionally improved
643 through biotechnology: case studies. Comp Rev Food Sci Food Safety 7:65-74

644 Cheng KC, Beaulieu J, Iquira E, Belzile FJ, Fortin MG, Stromvik MV (2008) Effect of transgenes
645 on global gene expression in soybean is within the natural range of variation of
646 conventional cultivars. J Agric Food Chem 56:3057-3067

647 Coll A, Nadal A, Collado R, Capellades G, Messeguer J, Mele E, Palaudelmas M, Pla M (2009)
648 Gene expression profiles of MON810 and comparable non-GM maize varieties cultured in
649 the field are more similar than are those of conventional lines. Transgenic Res 18:801-
650 808

651 Coll A, Nadal A, Palaudelmas M, Messeguer J, Mele E, Puigdomenech P, Pla M (2008) Lack of
652 repeatable differential expression patterns between MON810 and comparable commercial
653 varieties of maize. Plant Mol Biol 68:105-117

654 Coruzzi G (2003) Primary N-assimilation into amino acids in Arabidopsis. In: Meyerowitz EM,
655 Rockville MD (eds) The Arabidopsis Book. American Society of Plant Biologists. doi:
656 10.1199/tab.0010, http://www.aspb.org/publications/arabidopsis/

657 Dallas PB, Gottardo NG, Firth MJ, Beesley AH, Hoffmann K, Terry PA, Freitas JR, Boag JM,
658 Cummings AJ, Kees UR (2005) Gene expression levels assessed by oligonucleotide
659 microarray analysis and quantitative real-time RT-PCR -- how well do they correlate? BMC
660 Genomics 6:59

661 Di Carli M, Villani ME, Renzone G, Nardi L, Pasquo A, Franconi R, Scaloni A, Benvenuto E,
662 Desiderio A (2009) Leaf proteome analysis of transgenic plants expressing antiviral
663 antibodies. J Proteome Res 8:838-848

664 Domingo F, Díaz-Pereira E, Mayol F, Lasa B, Lópied H, Irañeta I, Maturano M, Roselló-Martínez
665 A (2006) FENIMAR, a tool for nitrogen recomendation at field scale in irrigated maize.
666 Biblioteca Fragmenta Agronomica 11:383-385

667 Dubouzet JG, Ishihara A, Matsuda F, Miyagawa H, Iwata H, Wakasa K (2007) Integrated
668 metabolomic and transcriptomic analyses of high-tryptophan rice expressing a mutant
669 anthranilate synthase alpha subunit. J Exp Bot 58:3309-3321

670 El Ouakfaoui S, Miki B (2005) The stability of the Arabidopsis transcriptome in transgenic plants
671 expressing the marker genes nptII and uidA. Plant J 41:791-800

672 Frink CR, Waggoner PE, Ausubel JH (1999) Nitrogen fertilizer: retrospect and prospect. Proc
673 Natl Acad Sci USA 96:1175-1180

674 Gregersen PL, Brinch-Pedersen H, Holm PB (2005) A microarray-based comparative analysis
675 of gene expression profiles during grain development in transgenic and wild type wheat.
676 Transgenic Res 14:887-905

677 Hawkins JA, Sawyer JE, Barker DW, Lundva JP (2009) Using realitve chlorophyll meter values
678 to determine nitrogen application rates for corn. Agron J 99:1034-1040

679 Hernández M, Esteve T, Pla M (2005) Real-time PCR based methods for quantitative detection
680 of barley, rice, sunflower and wheat. J Agric Food Chem 53:7003-7009

681 Hernández M, Pla M, Esteve T, Prat S, Puigdomènech P, Ferrando A (2003) A specific real-time
682 quantitative PCR detection system for event MON810 in maize YieldGard based on the 3'-
683 transgene integration sequence. Transgenic Res 12:179-189

684 Herrero M, Ibáñez E, Martín-Álvarez PJ, Cifuentes A (2007) Analysis of chiral amino acids in
685 conventional and transgenic maize. Anal Chem 79:5071-5077

686 Hoekenga OA (2008) Using metabolomics to estimate unintended effects in transgenic crop
687 plants: problems, promises, and opportunities. J Biomol Tech 19:159-166

688 Howarth JR, Parmar S, Jones J, Shepherd CE, Corol DI, Galster AM et al (2008) Co-ordinated
689 expression of amino acid metabolism in response to N and S deficiency during wheat grain
690 filling. J Exp Bot 59:3675-3689

691 Ioset JR, Urbaniak B, Ndjoko-Ioset K, Wirth J, Martin F, Gruissem W, Hostettmann K, Sautter
692 C (2007) Flavonoid profiling among wild type and related GM wheat varieties. Plant Mol
693 Biol 65:645-654

694 Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003)
695 Exploration, normalization, and summaries of high density oligonucleotide array probe
696 level data. Biostatistics 4:249-264

697 James C (2008) Global Status of Commercialized Biotech/GM Crops: 2008. ISAAA Briefs 39.
698 ISAAA, Ithaca, NY

699 Kok EJ, Keijer J, Kleter GA, Kuiper HA (2008) Comparative safety assessment of plant-derived
700 foods. Regul Toxicol Pharmacol 50:98-113

701 König A, Cockburn A, Crevel RW, Debruyne E, Grafstroem R, Hammerling U et al (2004)
702 Assessment of the safety of foods derived from genetically modified (GM) crops. Food
703 Chem Toxicol 42:1047-1088

704 Kuiper HA, Kok EJ, Engel KH (2003) Exploitation of molecular profiling techniques for GM food
705 safety assessment. Curr Opin Biotechnol 14:238-243

706 Lalonde S, Wipf D, Frommer WB (2004) Transport mechanisms for organic forms of carbon
707 and nitrogen between source and sink. Annu Rev Plant Biol 55:341-372

708 Less H, Galili G (2008) Principal transcriptional programs regulating plant amino acid
709 metabolism in response to abiotic stresses. Plant Physiol 147:316-330

Levandi T, Leon C, Kaljurand M, Garcia-Canas V, Cifuentes A (2008) Capillary electrophoresis time-of-flight mass spectrometry for comparative metabolomics of transgenic versus conventional maize. Anal Chem 80:6329-6335

Lian X, Wang S, Zhang J, Feng Q, Zhang L, Fan D, Li X, Yuan D, Han B, Zhang Q (2006) Expression profiles of 10,422 genes at early stage of low nitrogen stress in rice assayed using a cDNA microarray. Plant Mol Biol 60:617-631

Lovegrove A, Salt L, Shewry PR (2009) Establishing substantial equivalence: proteomics. Methods Mol Biol 478:273-288

Manetti C, Bianchetti C, Casciani L, Castro C, Di Cocco ME, Miccheli A, Motto M, Conti F (2006) A metabonomic study of transgenic maize (*Zea mays*) seeds revealed variations in osmolytes and branched amino acids. J Exp Bot 57:2613-2625

Metzdorff SB, Kok EJ, Knuthsen P, Pedersen J (2006) Evaluation of a non-targeted "omic" approach in the safety assessment of genetically modified plants. Plant Biol (Stuttg) 8:662-672

Millstone E, Brunner E, Mayer S (1999) Beyond 'substantial equivalence'. Nature 401:525-526

OECD (1993) Safety Evaluation of Foods Derived by Modern Biotechnology. Available via OECD http://www oecd org/dataoecd/37/18/41036698 pdf Accessed 22 Sep 2009

Piccioni F, Capitani D, Zolla L, Mannina L (2009) NMR metabolic profiling of transgenic maize with the Cry1Ab gene. J Agric Food Chem 57:6041-6049

Poerschmann J, Gathmann A, Augustin J, Langer U, Gorecki T (2005) Molecular composition of leaves and stems of genetically modified bt and near-isogenic non-bt maize - Characterization of lignin patterns. J Environ Qual 34:1508-1518

Price J, Laxmi A, St Martin SK, Jang JC (2004) Global transcription profiling reveals multiple sugar signal transduction mechanisms in Arabidopsis. Plant Cell 16:2128-2150

734 Prinsi B, Negri AS, Pesaresi P, Cocucci M, Espen L (2009) Evaluation of protein pattern changes
735 in roots and leaves of *Zea mays* plants in response to nitrate availability by two-
736 dimensional gel electrophoresis analysis. BMC Plant Biol 9:113

737 Ruebelt MC, Lipp M, Reynolds TL, Schmuke JJ, Astwood JD, DellaPenna D, Engel KH, Jany KD
738 (2006) Application of two-dimensional gel electrophoresis to interrogate alterations in the
739 proteome of gentically modified crops. 3. Assessing unintended effects. J Agric Food Chem
740 54:2169-2177

741 Saxena D, Stotzky G (2001) Bt corn has a higher lignin content than non-Bt corn. Am J Bot
742 88:1704-1706

743 Scheible WR, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios-Rojas N, Schindelasch D,
744 Thimm O, Udvardi MK, Stitt M (2004) Genome-wide reprogramming of primary and
745 secondary metabolism, protein synthesis, cellular growth processes, and the regulatory
746 infrastructure of Arabidopsis in response to nitrogen. Plant Physiol 136:2483-2499

747 Shepherd LV, McNicol JW, Razzo R, Taylor MA, Davies HV (2006) Assessing the potential for
748 unintended effects in genetically modified potatoes perturbed in metabolic and
749 developmental processes. Targeted analysis of key nutrients and anti-nutrients.
750 Transgenic Res 15:409-425

751 Shewry PR, Baudo M, Lovegrove A, Powers S, Napier JA, Ward JL, Baker JM, Beale MH (2007)
752 Are GM and conventionally bred cereals really different? Trends Food Sci Technol 18:201-
753 209

754 Shnable PS, Ware D, Fulton RS, Stein CJ, Wei F, Pasternak S, Liang C et al. (2009) The B73
755 maize genome: complexity, diversity, and dynamics. Science 326:1112-1115

756 Sidak Z (1971) On probabilities of rectangles in multivariate normal Student distributions: their
757 dependence on correlations. Ann Math Statist 41:169-175

758 Stahlberg A, Elbing K, Andrade-Garda JM, Sjogreen B, Forootan A, Kubista M (2008) Multiway
759 real-time PCR gene expression profiling in yeast Saccharomyces cerevisiae reveals altered
760 transcriptional response of ADH-genes to glucose stimuli. BMC Genomics 9:170

761 Sylvester-Bradley R, Kindred DR (2009) Analysing nitrogen responses of cereals to prioritize
762 routes to the improvement of nitrogen use efficiency. J Exp Bot 60:1939-1951

763 Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, Selbig J, Muller LA, Rhee SY, Stitt
764 M (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of
765 metabolic pathways and other biological processes. Plant J 37:914-939

766 van Dijk JP, Cankar K, Scheffer SJ, Beenen HG, Shepherd LV, Stewart D, Davies HV,
767 Wilkockson SJ, Leifert C, Gruden K, Kok EJ (2009) Transcriptome analysis of potato
768 tubers--effects of different agricultural practices. J Agric Food Chem 57:1612-1623

769 Wang R, Guegler K, LaBrie ST, Crawford NM (2000) Genomic analysis of a nutrient response
770 in Arabidopsis reveals diverse expression patterns and novel metabolic and potential
771 regulatory genes induced by nitrate. Plant Cell 12:1491-1509

772 Wang R, Okamoto M, Xing X, Crawford NM (2003) Microarray analysis of the nitrate response
773 in Arabidopsis roots and shoots reveals over 1,000 rapidly responding genes and new
774 linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol
775 132:556-567
776

Table 1. *cryIA(b)* mRNA levels and percentage of organic N contents of maize plants grown under low-N and control conditions. The differences between low-N and control conditions were statistically significant ($p<0.05$) for all four varieties tested (p-values are indicated).

	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	3.79e-02
	Helen	2.92 ± 0.18	3.45 ± 0.39	8.33e-05
	Beles Sur	1.63 ± 0.18	2.70 ± 0.30	1.43e-07
	Sancia	2.60 ± 0.15	4.01 ± 0.22	2.10e-04

**cryIA(b)* mRNA levels are expressed in copy numbers relative to β-actin
***N contents were obtained by elemental analysis
****Helen Bt and Beles Sur had similar transgene mRNA levels under both low-N and control conditions ($p>0.05$). Samples were taken from the D-leaf.
*****Means and standard deviations (SD) of duplicates of each of the 3 replicate samples corresponding to each treatment and variety are shown.

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

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 numbers of sequences that are induced and repressed in the first vs. the last compared varieties or conditions are shown in brackets.
** Note some sequences may be regulated in control and low-N conditions (see also Figure 1 and Table 3).
*** Note some sequences may be regulated in the two varieties (see also Table 3).

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. (A) Differential expression folds; and (B) MapMan based functional assignment.

A

		HelenBt vs Helen	
Internal code*	**Accession number**	**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	

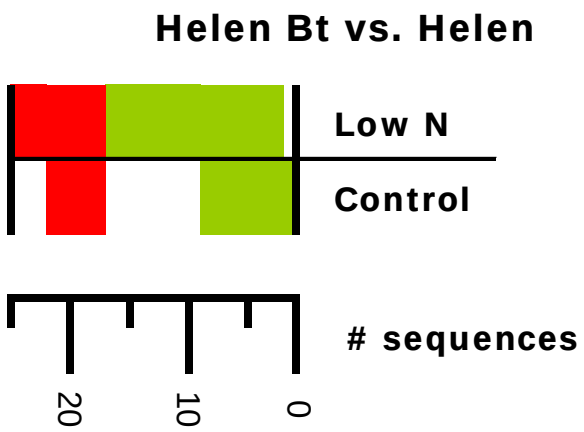
* Only sequences further analyzed by RT-qPCR were assigned an internal code.
** Differential expression folds in the first vs. the last compared varieties or conditions. Only sequences with differential expression folds >2 or <0.5 are recorded.
*** Note that two probes in the microarray corresponded to the same gene Accession number and where both coded as Zm11.

B

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.10049.1.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	misc.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

814

815 **Figure 1.**

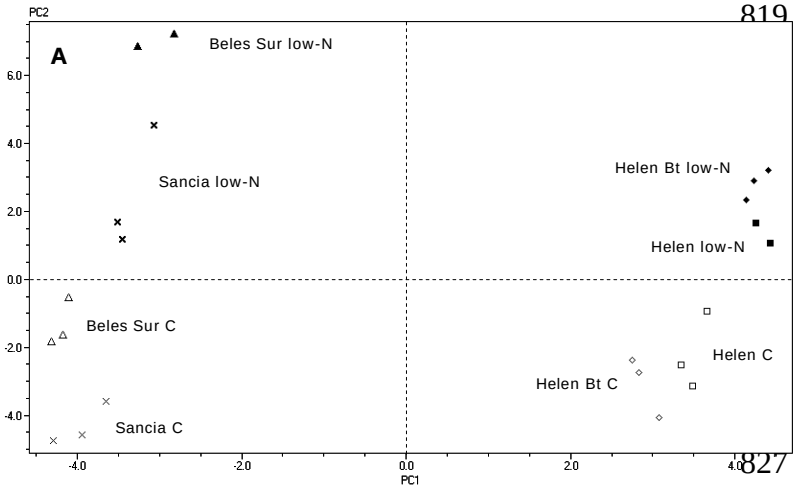


816

817 **Figure 2.**

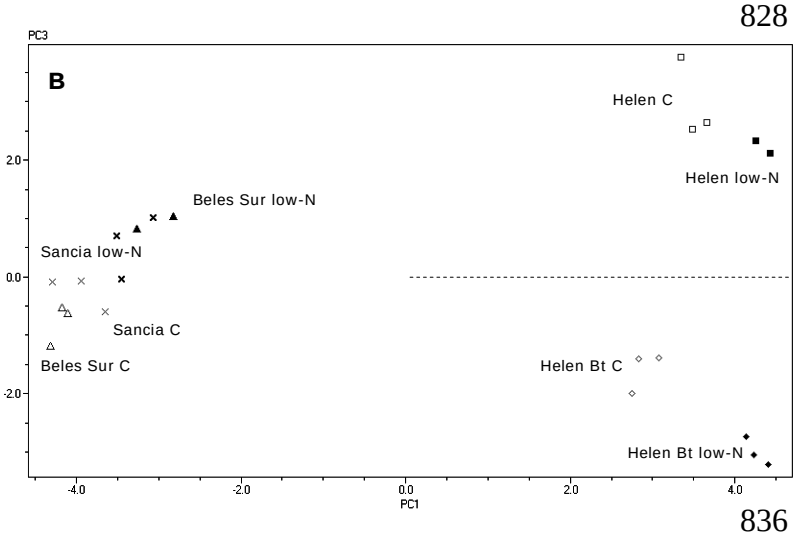
818

819



827

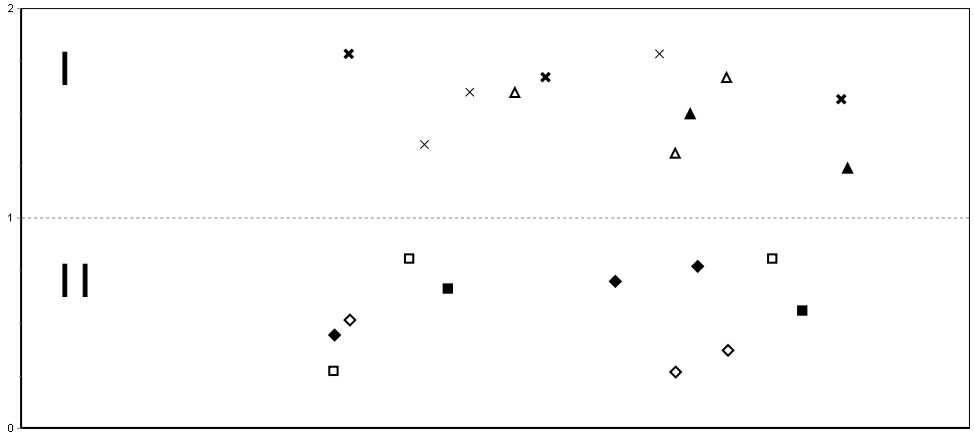
828



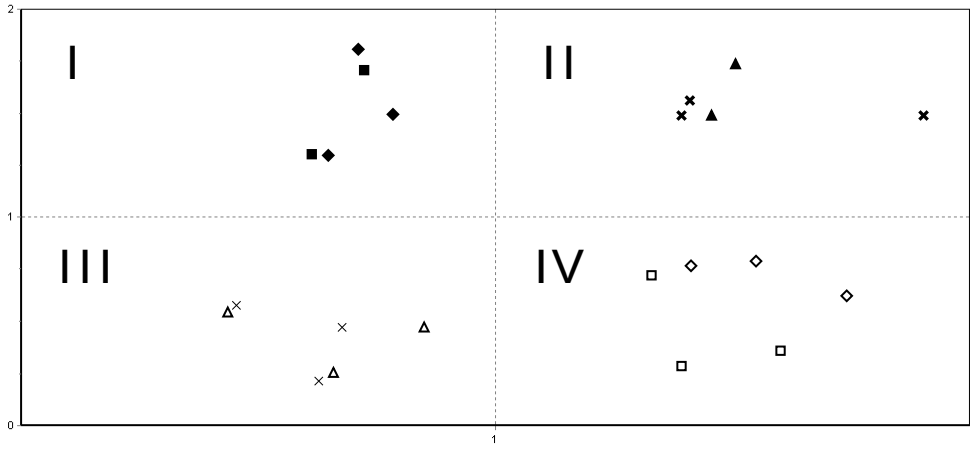
836

Figure 3.

A



B



844 **Supplementary material, Table 1.**

845 Selected sequences and oligonucleotides used for RT-qPCR

HelenBt vs Helen in control and low-N conditions

Accession number	Internal code	Forward Primer	Reverse Primer	Primer concentration (nM)
AY108221.1	Zm01	5'-CGCATGTTTTAAGGCTCATCTCC-3'	5'-CCAGGCTGAATCCCACTTTCG-3'	300
11990232-44	Zm02	5'-ATCGAGATAGAGGCAGGCAA-3'	5'-TGTTGCTGCGTTTATCCGAGTG-3'	300
CK368940	Zm03	5'-TGTCGCGACTTGTACTGAGTTGT-3'	5'-AATTGGGAACGAAGAAGAAACG-3'	300
AY104588.1	Zm04	5'-CCCCATTGTGGTCTCTGTTGC-3'	5'-GCCTGAGGGTGAACTACAAACTC-3'	300
BM378498	Zm05	5'-CGATGGGTTTGTCACTACTCG-3'	5'-TAACAACACACATCCACCCG-3'	300
CK369628	Zm06	5'-CGCACTTCATCACCCATATGATAG-3'	5'-CTCCAGCATGAACACTCTCAGG-3'	300
40794996-44	Zm07	5'-ACCAGACAGCGACGGATAATG-3'	5'-GTAGGTAGATAGAGGCAACGACAC-3'	100
AI855032	Zm08	5'-AGAACAGACTATCGGGCGTTAG-3'	5'-ATTGGGCTACCCTGAAATTCTCC-3'	300

HelenBt vs Helen in low-N conditions

11990232-46	Zm10	5'-TCTATGGTGGCGAACTTAATGGAG-3'	5'-AAAGAAGAGCAAAGCAAAGGTAGC-3'	300
BM080363	Zm12	5'-ACTACAACGCCAAGCACACG-3'	5'-GAGCAGGGAGTCCGAGAGC-3'	300
AW566101	Zm13	5'-GTCTAATGCGTTGCTGCCTC-3'	5'-CAACACATCCTGTTCATCGG-3'	100
AF244689.2	Zm15	5'-TAGGCGTGCTGGAGGAAGTG-3'	5'-CCTTGCTGGCGGTGAAGAAG-3'	100
40794996-39	Zm16	5'-GGGGAAGGGTAAGGAAAACGC-3'	5'-CCCGAAGGAAGGCTGCTATC-3'	300
CN844137	Zm17	5'-TTTGTAATCTCTGCTGCTTCCCA-3'	5'-CGGGTGAACATAGGCTCAAACA-3'	100

Low-N vs control conditions in Helen

Accession number	Internal code	Forward Primer	Reverse Primer	Primer concentration (nM)
11990232-51	Zm18	5'-TGGGCGTTCTCTTAATGGTTTCTG-3'	5'-GCTACTGGGCGACGAAATGG-3'	300
11990232-115	Zm20	5'-GCGTAGAGGAACCACACCAATC-3'	5'-ACAGTATCGTCACCGCAGTAGAG-3'	300
11990232-29	Zm21	5'-GGTATTTATCCCGCAGTGGA-3'	5'-CTTCTGCCACAAAGAACGGT-3'	100

Low-N vs control conditions in HelenBt

AY106317.1	Zm19	5'-CGCGCGCACCTTCAA-3'	5'-CGTTGGCATCGCTGCTT-3'	300
CO531267	Zm22	5'-GTGTTTCGCCTGTACCAGTGC-3'	5'-ACTGGAAACCTGCAACCAGC-3'	100
AY105839.1	Zm23	5'-CGACCTCAAGAGAGTGTTCCC-3'	5'-CGCCTTCCATTTCAAACGCTTC-3'	300
CO528780	Zm24	5'-CGACCTCAAGAGAGTGTTCCC-3'	5'-CGCCTTCCATTTCAAACGCTTC-3'	300
AY107888.1	Zm25	5'-ACACCAGCGAGCACTACAATG-3'	5'-GTTGATGAGAATTTCAGCGGAGTC-3'	50
AW681229	Zm26	5'-CTGCCAAGTCCCCTTCTCTTATAC-3'	5'-GTTGACACCACGGAAGATGCC-3'	100
CF623514	Zm27	5'-CGACGAGGAAGAGGCAGACG-3'	5'-CATCACACGCACGGGTTAAGC-3'	300
CF060279	Zm28	5'-GTCCTAATTGTGGTGCTTGTTTGG-3'	5'-CGCAGACAGTAGGTTCATTCATCC-3'	100
AY104534.1	Zm29	5'-TTGGGCCTGGCTGGTTAG-3'	5'-CCGTGATGGTCATGAATGCTA-3'	100
CD219268	Zm30	5'-TCTCGCAATTCAGTACCGTCAAG-3'	5'-TCTCAGCAGCCTCGTGTATGG-3'	300
AI691771	Zm31	5'-TGTTCATTGCCACCAAGTGATTTG-3'	5'-GCCACATCCATTCTTATTCCGAAG-3'	300
CK369019	Zm32	5'-GTGGAGGCTTATGGACAGATGTTG-3'	5'-GGGTGAAGTCAGACAGGAAATCG-3'	300
AY104270.1	Zm33	5'-AGCCCAGCCACTCCAACG-3'	5'-GTCAGTCCCATTTGCCATCTACG-3'	300
BG316738	Zm34	5'-GCCTCCGCACACCGTCATC-3'	5'-TCGCCGTTGATCTTCCTCTCG-3'	100
BM338021	Zm35	5'-GGCGGAGGTCACGGAGTC-3'	5'-CGAGGCGATGGACATGAAGC-3'	300
BM351351	Zm36	5'-AAACAATGGCACCGAGGCTAG-3'	5'-TCCTGCGGCGAGTTCTGG-3'	300
BM382341	Zm37	5'-GGGTGTGTGTTGGTGTGTGTC-3'	5'-TGACCGTGGGCGGGAGAG-3'	100
AW231741	Zm38	5'-GCGGACCTGTTGGAGTTGATG-3'	5'-CCCTTGTCACCATTCACCACTTG-3'	300
CO526660	Zm39	5'-ATGCCCTACCTCAACGAATACAAG-3'	5'-CGCAGTCGCAGCCAAATCC-3'	300
BM337131	Zm40	5'-CACGCACAGCGGTTACCA-3'	5'-GCCCCTCCGTAGAATGGAAT-3'	300

846

847

Supplementary material, Table 2

Sequences with more than 2-fold differential expression between GM and near-isogenic maize varieties (A) and between low N and control conditions (B), as assessed by microarray hybridizations. The regulation of 37 sequences was verified by RT-qPCR. Pair-wise comparisons giving T-test values with statistical significance ($p<0.05$) are highlighted (red: up-regulated sequences; green: down-regulated sequences).

A

		Low-N conditions				*Control conditions*			
		Helen Bt vs Helen			*Beles Sur vs. Sancia*	*HelenBt vs Helen*			*Beles Sur vs. Sancia*
Sequence internal code	Accession number	Microarray		RT-QPCR		Microarray		RT-QPCR	
		x-fold*	T-test p-value	T-test p-value		x-fold*	T-test p-value	T-test p-value	
Zm01	AY108221.1	22.35	0.00	0.00	0.01	35.56	0.00	0.00	0.00
Zm02	11990232-44	7.77	0.00	0.26	0.62	7.09	0.00	0.19	0.03
Zm03	CK368940	4.49	0.00	0.00	0.00	3.17	0.01	0.00	0.03
Zm04	AY104588.1	2.82	0.00	0.00	0.16	2.70	0.00	0.01	0.02
Zm05	BM378498	0.46	0.03	0.09	0.01	0.37	0.00	0.01	0.24
Zm06	CK369628	0.44	0.00	0.89	0.59	0.36	0.00	0.17	0.62
Zm07	40794996-44	0.28	0.01	0.02	0.18	0.26	0.01	0.02	0.18
Zm08	AI855032	0.21	0.01	0.01	0.02	0.22	0.04	0.05	0.24
Zm10	11990232-46	2.97	0.01	0.29	0.24				
Zm12	BM080363	0.50	0.00	0.09	0.00				
Zm15	AF244689.2	0.42	0.01	0.07	0.82				
Zm16	40794996-39	0.33	0.02	0.06	0.10				
Zm17	CN844137	0.32	0.02	0.07	0.43				
Zm13	AW566101	0.43	0.00	0.01	0.00				
	40794996-84	8.19	0.00			8.45	0.01		
	BM266792	0.45	0.00			0.37	0.01		
	BU499844	0.12	0.02			0.17	0.00		
	BQ578253					0.45	0.01		
	BM736430	2.24	0.04						
	BM736430	2.16	0.01						
	40794996-37	0.49	0.01						
	11990232-26	0.47	0.04						
	BM337131	0.30	0.01						
	AY105529.1	0.16	0.00						

B

		Low-N vs control conditions							
		Helen Bt			*Beles Sur*	*Helen*			*Sancia*
Sequence internal code	Accession number	Microarray		RT-QPCR		Microarray		RT-QPCR	
		x-fold*	T-test p-value	T-test p-value		x-fold*	T-test p-value	T-test p-value	
Zm18	11990232-51				0.67	0.45	0.01	0.26	0.53
Zm20	11990232-115				0.53	0.46	0.03	0.02	0.14
Zm21	11990232-29				0.22	0.48	0.00	0.49	0.53
Zm19	AY106317.1	0.34	0.01	0.00	0.00				0.00
Zm22	CO531267	3.63	0.04	0.02	0.00				0.00
Zm23	AY105839.1	0.49	0.01	0.01	0.00				0.02
Zm24	CO528780	0.48	0.02	0.00	0.00				0.01
Zm25	AY107888.1	0.47	0.01	0.04	0.00				0.00
Zm26	AW681229	0.47	0.02	0.04	0.01				0.01
Zm27	CF623514	0.47	0.03	0.01	0.07				0.04
Zm28	CF060279	0.46	0.02	0.02	0.02				0.06
Zm29	AY104534.1	0.46	0.05	0.05	0.04				0.09
Zm30	CD219268	0.46	0.03	0.03	0.04				0.00
Zm31	AI691771	0.42	0.01	0.04	0.47				0.81
Zm32	CK369019	0.39	0.02	0.01	0.02				0.10
Zm33	AY104270.1	0.39	0.02	0.00	0.05				0.05
Zm34	BG316738	0.39	0.01	0.01	0.00				0.00
Zm35	BM338021	0.39	0.03	0.01	0.00				0.00
Zm36	BM351351	0.35	0.03	0.01	0.00				0.00
Zm37	BM382341	0.33	0.04	0.05	0.02				0.11
Zm38	AW231741	0.29	0.04	0.04	0.04				0.04
Zm39	CO526660	0.14	0.01	0.04	0.00				0.00
Zm40	BM337131	0.06	0.01	0.00	0.01				0.07
	CO532209	0.39	0.00			0.36	0.01		
	11990232-71					2.14	0.04		
	CO522709					0.41	0.01		
	BM334482	2.68	0.03						
	BM381059	0.49	0.01						
	CD978285	0.48	0.03						
	CF349219	0.47	0.01						
	BQ485400	0.45	0.03						
	L00973.1	0.44	0.00						
	BG842207	0.42	0.03						
	CO523535	0.37	0.03						
	AI770584	0.36	0.04						
	AY111675.1	0.28	0.01						

* Differential expression folds in the first vs. the last compared varieties or conditions.

Supplementary material, Table 3

Mapping of sequences with differential expression in low N and control conditions using the MapMan tool.

Bin code	Bin name	No. seq.	Affymetrix ID	Accession number	Internal code
8	**TCA / org. transformation**	**2**			
8.1.3	TCA / org. transformation.TCA.aconitase	1	*Zm.12697.1.S1_at	CF060279	Zm28
8.2.3	TCA / org. transformation.other organic acid transformaitons.aconitase	1	*Zm.12697.1.S1_at	CF060279	Zm28
8.2.9	TCA / org. transformation.other organic acid transformaitons.cyt MDH	1	Zm.5727.2.A1_at	BG842207	
10	**cell wall**	**1**			
10.7	cell wall.modification	1	Zm.7370.1.A1_at	BM382341	Zm37
11	**lipid metabolism**	**2**			
11.3	lipid metabolism.Phospholipid synthesis	2	Zm.16907.1.S1_at	AI691771	Zm31
			Zm.17997.1.A1_at	CK369019	Zm32
12	**N-metabolism**	**1**			
12.2.2	N-metabolism.ammonia metabolism.glutamine synthase	1	Zm.107.1.S1_at	AY104270.1	Zm33
13	**amino acid metabolism**	**3**			
13.1.1.3	amino acid metabolism.synthesis.central amino acid metabolism.alanine	1	**Zm.2321.4.A1_at	CD978285	
13.1.2.2	amino acid metabolism.synthesis.glutamate family.proline	1	***Zm.4019.1.S1_at	AW231741	Zm38
13.2.2.3	amino acid metabolism.degradation.glutamate family.arginine	2	****Zm.15155.1.A1_at	CO526660	
			***Zm.4019.1.S1_at	AW231741	
13.2.4.4	amino acid metabolism.degradation.branched-chain group.leucine	1	**Zm.2321.4.A1_at	CD978285	
13.2.4.5	amino acid metabolism.degradation.branched chain group.isoleucine	1	**Zm.2321.4.A1_at	CD978285	
20	**stress**	**1**			
20.1	stress.biotic	1	Zm.847.1.S1_at	L00973.1	
22	**polyamine metabolism**	**2**			
22.1.3	polyamine metabolism.synthesis.arginine decarboxylase	1	****Zm.15155.1.A1_at	CO526660	
22.2.1	polyamine metabolism.degradation.polyamin oxidase	1	Zm.300.1.A1_at	AY107888.1	Zm25
26	**misc**	**3**			
26.10	misc.cytochrome P450	1	Zm.5605.1.S1_at	CO528780	Zm24
26.13	misc.acid and other phosphatases	1	Zm.1007.1.S1_at	AY106317.1	Zm19
26.3	misc.gluco-, galacto- and mannosidases	1	Zm.14442.1.S1_at	AY105839.1	Zm23
27	**RNA**	**2**			
27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	1	Zm.19076.1.A1_at	CO522709	
27.3.99	RNA.regulation of transcription.unclassified	1	Zm.5794.1.S1_at	BM334482	
29	**protein**	**3**			
29.2.3	protein.synthesis.initiation	1	Zm.16289.1.A1_at	BM337131	Zm40
29.4	protein.postranslational modification	2	Zm.17516.1.S1_at	CF623514	Zm27
			Zm.7031.1.A1_at	CO532209	
34	**transport**	**2**			
34.12	transport.metal	1	Zm.18511.1.S1_at	CO523535	
34.99	transport misc	1	Zm.1659.1.A1_at	BG316738	Zm34
35	**not assigned**	**6**			
35.1	not assigned.no ontology	3	Zm.10451.1.S1_at	BQ485400	
			Zm.12285.1.A1_at	AY104534.1	Zm29
			Zm.2227.1.A1_at	BM381059	
35.2	not assigned.unknown	3	Zm.11185.1.S1_at	CF349219	
			Zm.12065.1.S1_at	CD219268	Zm30
			Zm.17106.1.A1_at	CO531267	Zm22
	not mapped	**9**			
			Zm.15280.1.A1_s_at	BM351351	Zm36
			Zm.694.1.A1_a_at	AW681229	Zm26
			Zm.791.1.S1_s_at	AY111675.1	Zm39
			Zm.8634.2.A1_a_at	BM338021	Zm35
			ZmAffx.1224.1.S1_at	11990232-115	Zm20
			ZmAffx.1303.1.S1_s_at	11990232-71	
			ZmAffx.1462.1.S1_at	11990232-29	Zm21
			ZmAffx.1509.1.S1_at	11990232-51	Zm18
			ZmAffx.837.1.S1_at	AI770584	

* Data points that have been mapped multiple times to different bins