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Title: Proteomic analysis of MON810 and comparable non-GM maize varieties grown in agricultural fields

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Keywords: Genetically Modified Organism (GMO); MON810; proteome; two-dimensional gel electrophoresis (2DE); mass spectrometry (MS); unexpected effects; maize.

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Abstract: Worldwide maize is the second major agricultural commodity and around one fourth is currently biotech, with significant application of the insect resistant event MON810 particularly in the European Union. Grains are the major commercialized part of the plant, both harvested at late milky-starchy grains (for forage uses) or after maturity (for food and feed purposes). We assessed possible proteomic unintended effects of the MON810 transgene using two-dimensional gel electrophoresis coupled to mass spectrometry. To keep in a realistic scenario we used plants grown in agricultural fields in a region where ~50% of maize was MON810, and analyzed grains at milky-starchy stage. In maize, differential transcripts and metabolites between GM and comparable non-GM varieties tend to be variety specific. Thus, we analyzed two variety pairs, DKC6575/Tietar and PR33P67/PR33P66 which are considered representative of Food and Agriculture Organization 700 and 600 varieties commercially grown in the region.

MON810 and non-GM milky-starchy grains had virtually identical proteomic patterns, with a very small number of spots showing fold-variations in the 1-1.8 range. They were all variety specific and had divergent identities and functions. Although 2DE allows the analysis of a limited dataset our results support substantial equivalence between MON810 and comparable non-GM varieties.

# Proteomic analysis of MON810 and comparable non-GM maize varieties grown in agricultural fields

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## **ABSTRACT**

Worldwide maize is the second major agricultural commodity and around one fourth is currently biotech, with significant application of the insect resistant event MON810 particularly in the European Union. Grains are the major commercialized part of the plant, both harvested at late milky-starchy grains (for forage uses) or after maturity (for food and feed purposes). We assessed possible proteomic unintended effects of the MON810 transgene using two-dimensional gel electrophoresis coupled to mass spectrometry. To keep in a realistic scenario we used plants grown in agricultural fields in a region where ~50% of maize was MON810, and analyzed grains at milky-starchy stage. In maize, differential transcripts and metabolites between GM and comparable non-GM varieties tend to be variety specific. Thus, we analyzed two variety pairs, DKC6575/Tietar and PR33P67/PR33P66 which are considered representative of Food and Agriculture Organization 700 and 600 varieties commercially grown in the region.

MON810 and non-GM milky-starchy grains had virtually identical proteomic patterns, with a very small number of spots showing fold-variations in the 1-1.8 range. They were all variety specific and had divergent identities and functions. Although 2DE allows the analysis of a limited dataset our results support substantial equivalence between MON810 and comparable non-GM varieties.

**Key words:** Genetically Modified Organism (GMO), MON810, proteome, two-dimensional gel electrophoresis (2DE), mass spectrometry (MS), unexpected effects, maize

## **ABBREVIATIONS**

2DE: two-dimensional gel electrophoresis

CRM: certified reference material

EU: European Union

FAO: Food and Agriculture Organization

GMO: Genetically Modified Organism

MS: mass spectrometry

MSDB: mass spectrometry protein sequence data base

qPCR: real-time polymerase chain reaction

V8: vegetative eight-leaf stage

DAF: days after flowering

## INTRODUCTION

History reveals that high yield crop production has been achieved by different technology developments throughout years. The technology of hybrid maize developed in the 1930s and the green revolution in the 1960s made substantial contributions to crop productivity (James 2010); more recently genetic engineering represented another important step. To ensure consumers' safety strict legislation was established in many countries that regulates marketing of Genetically Modified Organisms (GMOs). The concept of substantial equivalence is the leading principle in safety assessment of GM crops, involving targeted compositional analyses and field and animal nutrition studies (EFSA 2004). However, concerns that unintended effects might remain undetected using targeted analyses encouraged the development of unbiased profiling methods as complementary tools.

Proteomics is a non-targeted approach that has emerged in the last years as a powerful tool to detect possible unintended effects derived from genetic manipulation of various plant species. A two-dimensional gel electrophoresis (2DE) based proteomic approach was used to compare protein profiles of transgenic *Arabidopsis* (Ren et al. 2009; Ruebelt et al. 2006), tomato (Corpillo et al. 2004), potato (Khalf et al. 2010; Lehesranta et al. 2005) and maize (Barros et al. 2010; Zolla et al. 2008) lines and their non-GM counterparts. These studies revealed low percentages of proteins with significantly altered levels in transgenic and non-GM lines, and the differences in spot quantities either were part of the intended effects or fell within the range of natural variability. Transcriptomics (Cheng et al. 2008; Dubouzet et al. 2007; El Ouakfaoui and Miki 2005; Gregersen et al. 2005; Miki et al. 2009) and metabolomics (Baker et al. 2006; Catchpole et al. 2005; Kristensen et al. 2005) approaches also supported the substantial equivalence of transgenic plants and comparable non-GM lines.

With 161 million ha grown in 2008, maize is the second major crop in terms of global production (<http://faostat.fao.org/>). It is used for food and industrial applications, feed being the main usage. Worldwide about 60% coarse grains were used for feed in 2009-2010 ([www.fao.org/giews/english/index.htm](http://www.fao.org/giews/english/index.htm)). Similarly, most maize grown in Europe is commonly used as feed: above 75% total maize is harvested before maturity and the entire plant is employed for forage; while the rest is harvested at maturity and grains are used for feed and food. Maize is the species with the most approved events (53 in 2009) and MON810 is the second event approved in most countries (21 approvals in 2009) and the only biotech crop officially planted on a commercial basis in the European Union (EU) with 94.750 ha cultured in 2009 (James 2010). MON810 maize has single copy genomic insert comprising the cauliflower mosaic virus 35S promoter and maize *hsp70* intron sequences driving the expression of a synthetic *cryIA(b)* gene encoding a delta-endotoxin that acts as potent and highly specific insecticide. The inserted expression cassette has a 3' truncation partially affecting the *cryIA(b)* coding sequence and resulting in the lack of terminator (Hernández et al. 2003). Transcription of the transgene was reported to read-through 3'-past the truncation site (Rosati et al. 2008) and give rise to a variety of polyadenylated transcripts of different sizes that extend to around 1 kbp downstream the truncation site (La Paz et al. 2010). A Stop codon at position +7 downstream the *cryIA(b)* sequence indicates the transgenic protein has two additional amino acids. Several authors have used analytical profiling technologies for safety

assessment of the MON810 commercial GMO. Transcriptomics, proteomics and metabolomics analyzes showed small differential transcripts / proteins / metabolites between MON810 and non-GM samples (Coll et al. 2008; Manetti et al. 2006; Piccioni et al. 2009); and the effects of varying environmental conditions were higher than those of the transgene (Barros et al. 2010; Coll et al. 2010).

Many different elite varieties have been commercialized that contain the MON810 transgene while displaying different agronomic properties. The transgenic insert is introduced into selected local varieties by classical breeding methods (Holst-Jensen et al. 2006): the transgenic line is first crossed with the elite variety and the progeny is subjected to several cycles of backcrossings with the local elite. This implies that the MON810 transgene will be placed in very different genetic backgrounds; and that genetic differences between a transgenic and a conventional "near-isogenic" variety may not solely rely on the presence of the transgene but possibly on other portions of the genomes used for breeding. Thus, comparison of MON810 and conventional maize requires selection of a MON810 variety and careful identification of the best comparable non-GM variety. The differences between GM and non-GM maize ("unexpected effects") may not be the same when different variety pairs are compared. Only a few previous works based on transcriptomics and metabolomics took into account different MON810 and near-isogenic varieties; and remarkably they showed that transcripts and metabolites mostly show altered concentrations in MON810 and non-GM samples in a variety specific manner (Coll et al. 2008; Coll et al. 2010; Levandi et al. 2008). Although this approach has been recommended by several authors (Ruebelt et al. 2006; Van Dijk et al. 2009), a proteomics based research to cover this aspect is lacking. It would be most desirable to know to which extend protein differences found in particular MON810 and non-GM pair are common to different pairs and thus, can be associated to the transgenic character of MON810, in particular in the tissues and developmental stages used for feed and food purposes.

The aim of the present study was to assess possible unintended effects of the MON810 transgene in different maize commercial varieties by means of a 2DE and mass spectrometry (MS) based proteomics approach. We analyzed representative MON810 and comparable non-GM variety pairs at the most relevant grain maturity stage from the economic perspective: late milky-starchy grains (used in forages). Samples were collected from agricultural fields.

## **MATERIAL & METHODS**

### **Plant Material**

Seeds from two MON810 varieties (company, date of authorization in the BOE Spanish official publication): PR33P67 (Pioneer Hi-Bred, 2003) and DKC6575 (DeKalb, Monsanto Agricultura, 2003), and their corresponding near-isogenic varieties (PR33P66 and Tietar) from the same companies were used.

Genomic DNA from 0.2 g of seeds of the two GMO varieties was isolated using the Nucleospin food kit (Macherey-Nagel Int, Easton, PA) and analyzed to confirm they were MON810. To that end they

were subjected to event specific real-time polymerase chain reaction (qPCR) (Hernández et al. 2003) using *hmg* as the endogenous control (Hernández et al. 2005). Powdered certified reference material (CRM, ref#ERM-BF413A,B,D,F), purchased from Fluka (Fluka-Riedel, Geel, Belgium) was used as control.

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. Close to the sea and with a Mediterranean climate, the soil type in this area is Xerofluvent oxisol, coarse-loamy, mixed, calcareous, thermic. The field under study was divided into micro-plots, 4 rows wide (row spacing 0.75 m) and 20 m long. They were sown at a density of 80,000 plants/ha (4 April 2006) and were treated following standard agricultural practices in the region. One hundred kg N/ha, 100 kg P/ha and 100 kg K/ha were applied before sowing and an additional 150 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 (Dow Agrosiences, Indianapolis, IN, USA) (35% acetochlor + 15% atrazine + 5.8% Diclorimid) and with post-emergence application of 1.25 l/ha of Samson (Syngenta, Basel, Switzerland) (4% nicosulfuron). When necessary, the fields under study were irrigated following conventional agricultural practices. Maize grains were harvested at 40 days after flowering (daf) and they were carefully checked for the absence of corn-borer, other infections and lesions. Grains of 3 plants from a single micro-plot were harvested at the same time of the day, immediately frozen in liquid nitrogen and stored at -80°C. Two biological replicates from different micro-plots were sampled per maize variety.

### **Protein Extraction**

Protein extraction was performed using a modified thiourea/urea protein solubilization method described by Natarajan et al. (2005). Frozen maize grains were ground in liquid nitrogen in a mortar. Approximately 100 mg of this fine powder was solubilized at room temperature in 800 µl of lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 0.1% Triton-X and 14 mM DTT) containing protease inhibitors (1 mM PMSF and 10 mM A, P, L, E-64), 1 µl/ml DNase I and 1 µl/ml RNase. Protein extracts were clarified twice at 13000rpm for 10min at 4°C and finally ultracentrifuged at 240,000 rpm for 40 min at 4°C. Obtained supernatants were saved and protein concentration was determined by the Bradford method (Bradford 1976).

### **Two-Dimensional Electrophoresis**

To explore variations in protein levels associated with biological material, two replicate samples from each protein extract were firstly analyzed by using small IPG strips (7 cm, pH 4-7) (GE Healthcare, Uppsala, Sweden) in the first dimension. Protocol was similar to that used for 18cm strips (see below) with some modifications. After loading 50 µg of protein in an IPGphor™ II system (GE Healthcare), rehydration (6h at room temperature) and focusing (30 V for 6.5 h, 500 V for 1 h, 1000 V for 1 h and 5000 V for 7 h) were performed. The second dimension was carried out in a miniprotean apparatus; strips were loaded onto 12% SDS-polyacrylamide gels and were run at 50 V for 5 min and 100 V for 1 h.



Once assessed that variability within samples fall in an optimal range for proteomic analysis, three protein extractions from each sample were analyzed by 2-DE using 18 cm IPG strips and 12% SDS-PAGE gel in an Ettan™ DALT Electrophoresis Bidimensional system (GE Healthcare).

For the first dimension samples containing 400 µg of total protein were diluted in thiourea/urea rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% IPG buffer at the same range of IPG strips and trace of bromophenol blue) containing 1.6% (v/v) DeStreak Reagen (GE Healthcare) and loaded onto IPG strips (18 cm, pH 4-7 linear). By using an Ettan™ IPGphor™ Isoelectric Focusing System (GE Healthcare), strips were firstly allowed to rehydrate for 10h at 50V and immediately they were focused with a linear voltage ramp up to 8000 V in 8 h followed by a constant voltage of 8000 V until reaching 60 kV h. For the second dimension, strips were equilibrated first for 15 min in slow agitation with a buffer containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 10 mg/ml DTT and 0.002% (w/v) bromophenol blue and then for another 15 min in the same solution except for DTT which were replaced with 25 mg/ml iodoacetamide. Strips were then loaded onto 12% SDS-polyacrylamide gels (25 x 20 x 0.1 cm) and were run in an Ettan™ DALTsix Electrophoresis System (GE Healthcare) at 3 W/gel for 30 min followed by 20 W/gel for 4 h.

Gels were stained with colloidal Coomassie Brilliant Blue (CBB) following the method by Neuhoff et al. (1988) with some modifications. After 2 h protein fixation in a 40% methanol and 10% acetic acid solution gels were incubated in a staining solution containing 2% phosphoric acid, 10% (w/v) ammonium sulphate and 0.1% (w/v) Serva Blue G-250 for 24 h at room temperature on an orbital rotator. Imaging of the stained proteins was performed using an ImageScanner™ (GE Healthcare) and images were acquired using the LabScan scanning application, in transmission mode at (16 bits) grey scale level, 300 dpi, zoom factor set at 1:1 (100%) and saved as TIFF (Tag Image File Format) files.

ImageMaster Platinum software v. 2.0 (GE Healthcare) was used to process and analyse scanned images of the 2DE gels. The analysis included spot detection, background subtraction, gel matching, generation of an average gel (master) and relative quantification of each spot. To identify quantitative differences between GM and non-GM maize grains proteome, Student t-test was performed. In the statistical analysis only spots present in at least three replicate gels of each sample that match with its comparator were considered. Spots with Student t p-values < 0.05 and at least three fold increase or decrease in their relative quantities were further analyzed (in the absence of differential spots, the threshold was further placed at one-fold).

## **MS Analyses**

Spots of interest were manually excised from 2DE gels and washed automatically using Multiprobe II robot (Perkin Elmer, Waltham, MA). Protein spots were firstly washed with distilled water for 15 min and incubated in 25 mM ammonium bicarbonate for 30min. They were subsequently dehydrated two times in 50% 25 mM ammonium bicarbonate, 50% acetonitrile for 30 min and two times in 100% acetonitrile for 15 min. The gel pieces were finally dried to completion at 45°C. Digestion was performed incubating each gel spot with 8µl of 12.5 µg/ml trypsin (Promega,

Madison, WI) in 25 mM ammonium carbonate, at 37°C overnight. The resulting tryptic fragments were extracted in 10 µl formic acid 2% with sonication.

Tryptic peptides were analyzed by an ESI-Ion Trap mass spectrometer (Esquire HCT; Bruker Daltonik GmbH, Bremen, Germany), interfaced with an HPLC-Chip system (Agilent Technologies, Palo Alto, CA) at the Proteomic Platform of INRA, Montpellier, France. A sample volume of 2 µl was loaded onto a C-18 enrichment cartridge (40 nL) with a flow rate of 4 µl/min of 0.1% (v/v) formic acid. After preconcentration and cleanup, peptides were separated in the column (HPLC-Chip C18, 5 µm, 75 µm x 43 mm, 40 nL enrichment column; Agilent Technologies, Palo Alto, CA) at a flow rate of 0.3 µl/min using a gradient of 3% to 80% (v/v) acetonitrile in 15 min (0.1% [v/v] formic acid). Peptides were eluted into the High Capacity ion Trap (Esquire HCT; Bruker Daltonik GmbH, Bremen, Germany). Capillary voltage was 1.5-2 kV in the positive ion mode and was used a dry gas flow rate of 4.5 L/min with a temperature of 250°C. The first full-scan mass spectrum was measured for range 310 to 1800 m/z. The second scan was done to measure more exact  $M_r$  of the three major ions with higher resolution, and the third scan was done to measure the collision-induced MS/MS spectrum of the selected ions (range 100 to 2000 m/z).

Identification was performed by searching in the Mass Spectrometry protein sequence DataBase (MSDB) using the MASCOT software (<http://www.matrixscience.com>) (Taxonomy, viridiplantae). The main search parameters were: complete carbamidomethylation of cysteines, peptide mass tolerance +/-0.6 Da, fragment mass tolerance +/-0.8 Da, missed cleavages 1. For positive identification, the score of the result (-10Log(P)) had to be over the significance threshold level ( $P < 0.05$ ). Matches of MS/MS spectra against sequences in the database were also verified manually.

## RESULTS

### Experimental Design

In view of the importance of GM maize in the agricultural sector, the present work aims to compare protein profiles of MON810 and near-isogenic maize varieties by 2DE combined with MS. We used two highly commercialized GM varieties produced by different seed companies (PR33P67 and DKC6575) and their near isogenic counterparts (PR33P33 and Tietar, respectively). Agronomical data collected in the region of Girona, Spain from 2005 to 2008 (López et al. 2009) pointed DKC6575 and PR33P67 as the most representative varieties in the two most commonly cultured in the region Food and Agriculture Organization (FAO) maturity groups (700 and 600 respectively). Although they present some phenotypic differences, both have been recommended for their high yield production and low sensitivity to infections.

To get a realistic picture of proteomic differences among MON810 and non-GM comparable varieties we analyzed maize grains at the harvesting stage for forage applications (~75% maize cultivated): late milky-starchy stage. Grains were sampled from plants grown in field conditions in La Tallada d'Empordà, where 36.6% of cultured maize was transgenic in 2006 (<http://www20.gencat.cat/portal/site/DAR>). Fertilization and irrigation managements were in

accordance with conventional agricultural practices in the region and no phytosanitary treatments were applied. For each variety two replicate biological samples were prepared, each consisting on a pool of seeds harvested from three individual plants. Each sample was independently analyzed in experimental triplicates. Here, proteomics tools were used to demonstrate the similarity among samples.

### **Proteomic comparison of late milky-starchy grains grown under agricultural conditions**

Two-DE protein profiles of late milky-starchy grains of two MON810 and the two corresponding non-GM comparable varieties (PR33P67 and PR33P66; DKC6575 and Tietar) were independently analyzed. For all four varieties, experimental replicates showed correlation values in the 67 to 77 % range, with loading differences below 15% (see Table S1 Supplementary information). Thus, all gels were included in further comparisons. For each variety, a virtual gel was obtained with all reproducible protein spots i.e. those that were present in at least 3 (out of 6) replicate maps of a given sample. The PR33P67 and PR33P66 virtual gels had 737 and 698 spots respectively, from which 601 were the same. DKC6575 and Tietar gels had 535 and 478 spots respectively, with 335 common spots. Common spots in each pair of varieties were around 70-80%.

We investigated quantitative protein differences between each MON810 variety and its comparable non-GM counterpart by comparison of all twelve 2DE gels of each pair (see representative examples of 2DE gels in Figure 1). To err on the safe side and avoid any artefacts to influence our results a filter was applied to specifically identify differential spots that were represented in the two virtual gels. The following thresholds were initially set: fold variation  $\geq 3$  and Student t p-value  $< 0.05$ . No differences were observed in any of the two MON810 and non-GM varieties, indicating strong proteome similarity between comparable variety pairs. We further reduced the fold variation threshold down to 1 and a very small number of differential spots were revealed (Table 1). A total of 4 spots were differentially expressed in DKC6575 and Tietar grains, which represented 1.19% of analyzed spots. They were all induced in GM plants and fold induction rates were between 1 and 1.8. Similarly, 4 spots were differentially accumulated in PR33P67 and PR33P66 (i.e. 3 up-regulated and 1 down-regulated spots). This represented 0.67% of analyzed spots. We further re-analyzed 2DE gels looking for the possible presence of spots that were present in all six gels of a given variety while consistently not detected in gels of the comparator variety. Two additional spots were identified that were present in PR33P66 but below the detection limit in PR33P67. All differential spots were subjected to LC-MS/MS analysis and their presumed identities are listed in Table 1.

Proteins were identified from their peptide mass fingerprint by searching the MSDB database; and at least one presumed identity could be assigned to 9 out of 10 spots (scores  $> 42$ ,  $p < 0.05$  were considered significant). No protein in the green plant taxonomy could be identified in spot 7. Protein identification was mostly based on homologies to known maize proteins; however in some cases it was based on homologies to described *Oryza sativa* proteins. For some putative proteins the GO annotation was available (Supplemental Information Table S2).

## DISCUSSION

Maize is the second major agricultural commodity in terms of global production; according to provisional data from FAO, 819.6 million tonnes of maize were produced in 2008 ([www.fao.org/giews/english/index.htm](http://www.fao.org/giews/english/index.htm)) and 26% of maize production was biotech (James 2010). So statistics reveal a significant application of this genetic engineered crop. The insect resistant maize event MON810 is the second event that has received regulatory approval in most countries and the only biotech crop officially planted on a commercial basis in the EU with over 60,000 ha cultured in 2006, mostly (86%) in Spain. Due to the commercial importance of MON810, we investigated the variability between this event and comparable non-GM varieties by a proteomics approach carried out on the most commercially relevant tissue and developmental stage. We used plants grown in agricultural fields and according to conventional farming practices, in a region where around 50% of maize was MON810. DKC6575 and PR33P67 are the most representative FAO 700 and 600 varieties commercially grown in the region; and grains are as a rule harvested at late milky-starchy grains for forage uses.

Our 2DE coupled to MS-MS identification approach showed that late milky-starchy grains of MON810 and comparable non-GM varieties had essentially the same proteomic patterns. We only observed a very small number of quantitative differential spots between a particular MON810 and non-GM variety pair ( $\leq 1\%$ ), fold variations were minimal and none was differentially accumulated in the two variety pairs tested. In a previous transcriptomics approach (Coll et al. 2008; Coll et al. 2010), leaves of MON810 commercial varieties had similarity levels to their near-isogenic counterparts in the 0.1% to 1.5% range; and (although some sequences were differentially regulated in various variety pairs) most genes were differentially expressed in a variety specific manner. Similarly, seeds of MON810 varieties seem to have unexpected metabolomic variations compared to their corresponding non-GM counterparts (Manetti et al. 2006; Piccioni et al. 2009), and most seem to be depend upon the specific variety. Levandi and co-workers (2008) analyzed three MON810 lines and its corresponding non-GM counterparts: from 27 metabolites identified by a CE-TOF-MS based approach, just two compounds (i.e. L-Carnitine and stachydrine) had different concentrations in all three comparisons. We earlier described the stability of MON810 commercial varieties in terms of transgene expression and CryIA(b) protein accumulation (in similar environmental conditions) (La Paz et al. 2010), thus discounting the differences between GM varieties to be due to different levels of transgenic protein. The variety specific regulation described appears to derive from portions of conventional genomes used to obtain each transgenic commercial variety: the MON810 character was introduced into different commercial varieties through a number of backcrossings with elite conventional varieties.

Remarkably, a 2DE based proteomics approach here demonstrated the lack of consistent differences between MON810 and comparable non-GM varieties. It should be noted that due to the complexity and diversity of proteins in plants, proteomics analyses are limited to a small part of the total proteomic information. According to our experimental conditions, the analysis concerned a defined window in terms of pI and Mw and was restricted to soluble and abundant proteins. Our approach was based on comparison of the levels of protein spots that were consistently detected in the two compared samples. Proteomics 2DE analyses are subjected to large experimental

deviations and our approach is intended to avoid the identification of false differentially regulated proteins. However, we specially looked for the possible presence of spots that were newly produced in large amounts in transgenic plants. No spots were detected fulfilling this criteria but two additional spots were present in all six non-GM PR33P66 gels while consistently absent in PR33P67 gels.

Even though the identities of proteins differentially accumulated in a specific variety pair should not be of great importance in discussing the unintended effects of transgenic maize events, we identified those found in PR33P67 and PR33P66 or those in DKC6575 and Tietar late milk-starchy grains grown in agronomic fields as an example. They did not clearly point towards a specific biological process, molecular function or cellular component affected (which would be especially difficult due to the small numbers of differentially regulated proteins). Up-regulated in DKC6575 and Tietar were Os02g0625500 (a predicted adenosine kinase, producing purines from its derivatives), a putative sorbitol dehydrogenase involved in oxidation reduction processes, a protein matching homocysteine S-methyltransferase-4 (involved in methionine biosynthetic processes) and a heat shock protein involved in the response to stress and protein folding. In contrast, differentially regulated in PR33P67 and PR33P66 were a predicted heat shock protein involved in the response to heat stress (16.9 kDa class I heat shock protein 3, not the same as in DKC6575 vs. Tietar), an enzyme involved in glucose and fatty acid metabolism (triose-phosphate isomerase), a protein most probably belonging to the proteasome complex, a protein predicted to regulate translation initiation (translation initiation factor 4E-1) and a putative WD-40 repeat protein of unknown function. Zolla and co-workers (2008) showed that PR33P67 and PR33P66 mature grains produced under controlled conditions had altered levels of several proteins, among which a few had predicted functions similar to those here reported (e.g. two proteins with desiccation protection function, a triose-phosphate isomerase, a proteasome complex protein and a protein involved in translation initiation factor activity). In contrast, just one 2DE spot had altered levels in MON810 and non-GM plants grown in field conditions along three consecutive seasons (Barros et al, 2010). Environmental and varietal conditions seem to play an important role in MON810 unexpected effects. Transcriptomics, proteomics and metabolomics techniques should be taken as complementary approaches. Among the differentially regulated proteins described here, only homocysteine S-methyltransferase-1 was regulated as well at the mRNA level in the same variety pair (Coll et al., 2008). Similarly, Barros and co-workers (2010) could not establish functional correlations between the genes, proteins and metabolites driving the variation between conventional and GM maize.

Agronomical data obtained from fields in the region under study (López et al. 2009) showed high incidence of corn borer mainly affecting conventional varieties; which was associated with higher levels of fungi in non-GM compared to MON810 mature plants. Additionally, MON810 plants had better stay-green characteristics than their corresponding non-GM varieties along various seasons in the same region when conventional agricultural practices were followed. Stay-green refers to delayed senescence and is considered a positive agronomical property since it is associated to better plant health at the later cultural stages (although also to higher grain humidity at harvest of mature grains for feed or food). Note that these differences mainly appear at the physiological

maturity stage. Our results are in agreement with no clear physiological differences between MON810 and comparable non-GM plants at the stage of harvesting for forage uses.

Previous observations from a number of authors indicated that the unintended effects of transgenes have very little impact, particularly when compared to the large differences observed between lines produced by conventional breeding approaches (Baudo et al. 2006; Catchpole et al. 2005; Ioset et al. 2007; Lehesranta et al. 2005). Moreover, environmental factors cause more variation in the different transcript, protein and metabolite profiles than the different genotypes (Barros et al. 2010; Coll et al. 2010; Zolla et al. 2008). Our 2DE patterns obtained from different conventional maize varieties were highly different (only around 60% matching proteins), thus further statistical analysis was not envisaged. But they seem to support that the differences between two conventionally bred varieties are larger than those between a GM variety and its non-GM counterpart.

In conclusion, protein differences were observed in MON810 and non-GM agronomic field-grown grains harvested for forage purposes. Although the 2DE technology allows the analysis of a limited dataset, differentially accumulated proteins represented less than 1.2% analysed spots. In agreement with previous transcriptomics and metabolomics results, they were all variety specific and thus could not directly be attributed to the MON810 transgenic character.

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## FIGURE LEGENDS

**Figure 1.** Protein profiles of maize grains grown under field conditions. For each sample, the image displays a representative map out of a total of 6 gels. Quantitative comparison of MON810 vs. near-isogenic maize lines [DKC6575 vs. Tietar (upper gels) and PR33P67 vs. PR33P66 (lower gels)] is shown: significantly different spots are indicated with circles and numbers according to protein identification in Table 1. Linear isoelectric focusing pH 4-7 for the first dimension and 12% SDS-PAGE gels in the second dimension were used. Gels were stained with Comassie Brilliant Blue G-250. For each variety pair and differential spot, normalized spot volumes (mean and SD) are indicated in the bar graphs on the right

## TABLES

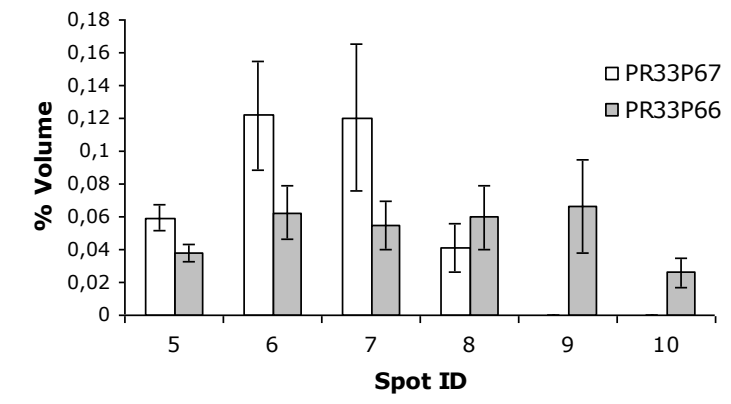
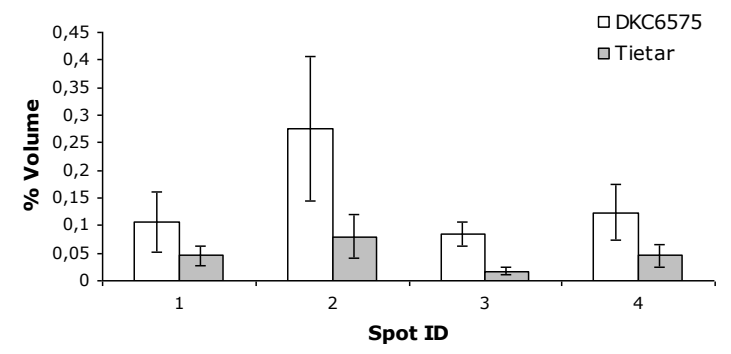
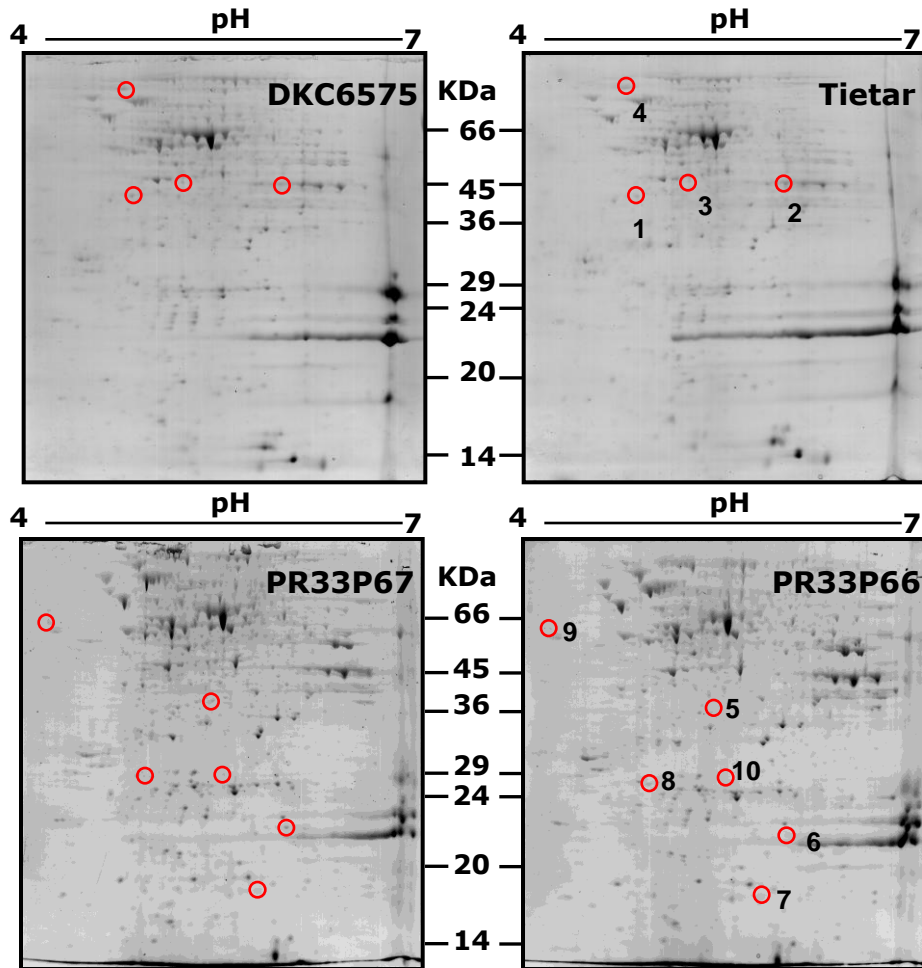
**Table 1.** Identified spots presenting differential accumulation in late milky-starchy grains of MON810 and comparable non-GM varieties

Comparison	I/R*	Spot ID	protein**			Score	Matching peptides	Sequence coverage (%)	Mw (kDa)		pI	
			NCBI accession	UniProt accession	Protein Name				calculated	measured	calculated	measured
DKC6575/Tietar	I	1	gi:115447399	Q0DZE5_ORYSJ	Os02g0625500 ( <i>O. Sativa</i> )	258	4	12	37.4	41.0	5.07	4.91
DKC6575/Tietar	I	2	gi:115477633	Q6ZBH2_ORYSJ	Os08g0545200 ( <i>O. sativa</i> ) (Putative sorbitol dehydrogenase)	222	5	10	40.0	45.0	6.03	6.19
DKC6575/Tietar	I	3	gi:162464417	HMT4_MAIZE	Homocysteine S-methyltransferase4 ( <i>Z. mays</i> )	148	4	13	36.8	46.2	5.39	5.26
DKC6575/Tietar	I	4	gi:158513648	HSP81_ORYSJ	Heat shock protein 81-1 ( <i>O. sativa</i> )	582	12	16	80.4	66.0	5.00	4.87
PR33P67/PR33P66	I	5	gi:21104899	Q69X61_ORYSA	Putative WD-40 repeat protein ( <i>O. sativa</i> )	170	7	17	38.1	37.2	5.43	5.43
PR33P67/PR33P66	I	6	gi:75306027	T04171	Heat shock protein ( <i>O. sativa</i> )	56	3	15	16.8	19.0	6.18	5.79
PR33P67/PR33P66	I	7			unknown					22.1		5.99
PR33P67/PR33P66	R	8	gi:217974	ISZMT	Triose-phosphate isomerase (EC 5.3.1.1) ( <i>Z. mays</i> )	93	3	12	27.2	27.2	5.52	4.94
PR33P67/PR33P66	R	9	gi:75220216	TS1606	Probable 26S proteasome non-ATPase chain 55a ( <i>O.sativa</i> )	289	6	18	42.5	59.4	4.50	4.30
PR33P67/PR33P66	R	10	gi:6016334	T01686	Translation initiation factor eIF-4F chain p26 ( <i>Z. mays</i> )	50	1	5	24.7	29.0	5.52	5.50

\* I: spots over-accumulated in the GM variety, R: spots under-accumulated in the GM variety

\*\* for each spot, only the protein with the highest score is shown

colour figure  
Figure 1



**Supplementary material Table S1.** Two-DE technical parameters: correlation among the three experimental replicates and the two biological replicates analyzed per variety

	<b>Plant variety</b>			
	<b>PR33P67</b>	<b>PR33P66</b>	<b>DKC6575</b>	<b>Tietar</b>
<b>Range of correlation among experimental replicates</b>	77 - 72 %	76 - 67 %	85 - 72 %	96 - 77%
<b>Loading difference among replicates</b>	≤ 12.5 %	≤ 15 %	≤ 14.5 %	≤ 10.5 %
<b># matching spots*</b>	737	698	535	478

\* number of spots represented in at least 3 out of 6 replicates

**Supplementary material Table S2.** Gene Ontology (GO) mapping of identified spots with differential accumulation in late milky-starchy grains of MON810 and comparable non-GM varieties.

Comparison	Spot ID	Protein Name	GO		
			Biological process	Molecular function	Cellular component
DKC6575/Tietar	1	Osl02g0625500 ( <i>O. sativa</i> )	purine ribonucleoside salvage	adenosine kinase activity	
DKC6575/Tietar	2	Osl08g0545200 ( <i>O. sativa</i> ) (Putative sorbitol dehydrogenase)	oxidation reduction	oxidoreductase activity, zinc ion binding	
DKC6575/Tietar	3	Homocysteine S-methyltransferase4 ( <i>Z. mays</i> )	methionine biosynthetic process	homocysteine S-methyltransferase activity, zinc ion binding	
DKC6575/Tietar	4	Heat shock protein 81-1 ( <i>O. sativa</i> )	protein folding, stress response	ATP binding, unfolded protein binding	cytoplasm
PR33P67/PR33P66	5	Putative WD-40 repeat protein ( <i>O. sativa</i> )			
PR33P67/PR33P66	6	Heat shock protein ( <i>O. sativa</i> )	response to heat		cytoplasm
PR33P67/PR33P66	7	unknown			
PR33P67/PR33P66	8	Triose-phosphate isomerase (EC 5.3.1.1) ( <i>Z. mays</i> )	fatty acid biosynthetic process, gluconeogenesis, glycolysis	triose-phosphate isomerase activity	cytoplasm
PR33P67/PR33P66	9	Probable 26S proteasome non-ATPase chain 55a ( <i>O. sativa</i> )			cytosol, proteasome complex
PR33P67/PR33P66	10	Translation initiation factor eIF-4E chain p26 ( <i>Z. mays</i> )	regulation of translation	RNA binding, translation initiation factor activity	cytoplasm