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BRIEF COMMUNICATION

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, and can be harvested after maturity (for food and feed purposes) or at late milky-starchy stage (for forage uses, with the whole plant). 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.

Keywords 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
PCR	Polymerase chain reaction
V8	Vegetative eight-leaf stage
DAF	Days after flowering

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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. Transciptomics (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). 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 event with the second largest number of approvals (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). Nevertheless, an in-frame termination codon at bp +7 to +9 downstream the *cryIA(b)* truncated gene 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 analysis 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 will not only rely on the

presence or absence of the transgene but also 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, 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 two highly commercialized GM varieties produced by different seed companies (PR33P67 and DKC6575) and their near isogenic counterparts (PR33P33 and Tietar, respectively); at the most relevant grain maturity stage from the economic perspective, late milky-starchy grains used in forages. Samples were collected from agricultural field.

Materials and 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 (PCR) (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 oxiaqüic, 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 Agrosciences, Indianapolis, IN, USA) (35% acetochlor + 15% atrazine + 5.8% Diclormid) 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. Cross-pollination between plants of different micro-plots was prevented by physical distance among micro-plots and by only analysing plants in the inner rows of each plot. 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 13,000 rpm for 10 min 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

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 18 cm strips (see below) with some modifications. After loading 50 µg of protein in an IPGphor™ II system (GE Healthcare), rehydration (6 h at room temperature) and focusing (30 V for 6.5 h, 500 V for 1 h, 1,000 V for 1 h and 5,000 V for 7 h) were performed. The second dimension was carried out in a miniprotean apparatus; strips were loaded onto 12% (v/v) 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% (v/v) 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% (w/v) CHAPS, 1% (v/v) 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 10 h at 50 V and immediately they were focused with a linear voltage ramp up to 8,000 V in 8 h followed by a constant voltage of 8,000 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% (v/v) SDS–polyacrylamide gels (25 × 20 × 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 Comassie Brilliant Blue (CBB) following the method by Neuhoff et al. (1988) with some modifications. After 2 h protein fixation in a 40% (v/v) methanol and 10% (v/v) acetic acid solution gels were incubated in a staining solution containing 2% (v/v) 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 bips) 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 threefold relative change in their quantities were further analyzed. In the absence of differential spots, the threshold was further placed at a relative change as small as 1.1-fold. Note that all differential spots were detected in at least one experimental replicate from each biological sample, which guaranteed they were not due to technical or possible biological variation. Furthermore, absolute differences between GM and non-GM maize grains proteome were identified as spots present in all six replicate gels of a sample and undetected in all six gels of the comparator.

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 30 min. They were subsequently dehydrated two times in 50% (v/v) 25 mM ammonium bicarbonate, 50% (v/v) acetonitrile for 30 min and two times in 100% (v/v) acetonitrile for 15 min. The gel pieces were finally dried to completion at 45°C. Digestion was performed incubating each gel spot with 8 µl 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% (v/v) 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 Plataform 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 × 43 mm, 40 nL enrichment column; Agilent Technologies) at a flow rate of 0.3 µl/min using a gradient of 3–80% (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). 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 310–1,800 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 100–2,000 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

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 minimum correlation values in the 67–77% 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 including all reproducible protein spots (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 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 Fig. 1). To err on the safe side and avoid differences among technical replicates to influence our results a filter was initially applied to specifically identify quantitative differential spots, i.e. those detected in at least 3 out of 6 replicate gels per variety. The following thresholds were set: fold variation ≥3 and Student *t* *P*-value < 0.05. No quantitative differences were observed in any of the two MON810 and non-GM varieties, indicating strong similarity of analyzed proteins between comparable variety pairs. We further reduced the fold variation threshold down to 1.1 and a very small number of differential spots were revealed, with relative change rates between 1.1- and 1.8-fold (Fig. 1). A total of 4 spots were accumulated at higher levels in DKC6575 than Tietar (1.2% analyzed spots); and 4 spots (0.7% analyzed ones) were differentially accumulated in PR33P67 and PR33P66: 3 up-regulated and 1 down-regulated in the transgenic variety. Most differential spots were detected in all 6 gels of a given variety; and only

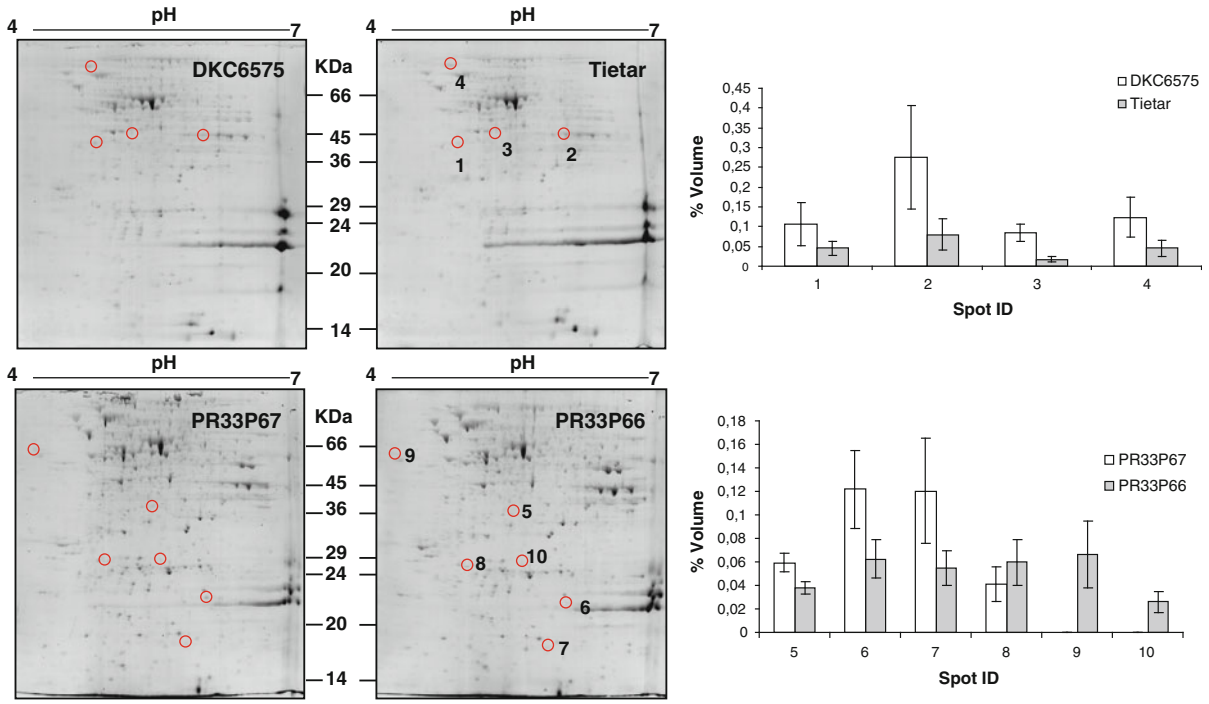

Fig. 1 Protein profiles of maize grains grown under field conditions. For each sample, the image displays a representative map out of a total of 6 gels. Quantitative comparison of MON810 vs. near-isogenic maize lines [DKC6575 vs. Tietar (*upper gels*) and PR33P67 vs. PR33P66 (*lower gels*)] is shown: 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 Brillant 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

differential spots 3 and 4 (see Fig. 1) were detected in 3 out of 6 gels in Tietar. Remarkably, they were detected in the two biological replicates and the lack of consistency was attributed to technical variability.

We further re-analyzed 2DE gels looking for the possible presence of absolute differences, i.e. 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 below the detection limit in PR33P67 while present in conventional PR33P66 samples. Quantitative and absolute 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

Due to its commercial importance, we investigated the variability between MON810 and comparable non-GM varieties by a proteomics approach carried out on a commercially relevant tissue and developmental stage. We used plants grown in agricultural fields in a region where 37% of maize was MON810 (http://www20.gencat.cat/portal/site/DAR) and is often harvested before maturity for forage uses (when grains are at the milky-starchy stage). 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

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	Uniport accession	Protein Name				Calculated	Measured	Calculated	Measured
DKC6575/ Tietar	I	1	gi:115447399	Q0DZE5_ORYSJ	Os02g0625500 (*O. sativa*)	258	4	12	37.4	41.0	5.07	4.91
DKC6575/ Tietar	I	2	gi:115477633	Q6ZBH2_ORYSJ	Os08g0545200 (*O. sativa*) (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 (*Z. mays*)	148	4	13	36.8	46.2	5.39	5.26
DKC6575/ Tietar	I	4	gi:158513648	HSP81 ORYSI	Heat shock protein 81-1 (*O. sativa*)	582	12	16	80.4	66.0	5.00	4.87
PR33P67/ PR33P66	I	5	gi:21104899	Q69X61 ORYSA	Putative WD-40 repeat protein (*O. sativa*)	170	7	17	38.1	37.2	5.43	5.43
PR33P67/ PR33P66	I	6	gi:75306027	T04171	Heat shock protein (*O. sativa*)	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) (*Z. mays*)	93	3	12	27.2	27.2	5.52	4.94
PR33P67/ PR33P66	R	9	gi:75220216	T51606	Probable 26S proteasome non-ATPase chain S5a (*O. sativa*)	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 (*Z. mays*)	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

Agriculture Organization (FAO) maturity groups (700 and 600 respectively).

For comparative proteomics, 2DE is a platform that allows analysis of protein maps with high protein resolution. However, the intrinsic characteristics of the technique (electrophoretic systems, sample preparation strategies, possible identification of several proteins from one spot) and natural variations could influence the comparisons (Brandao et al. 2010). Thus, our approach was based on six 2DE gels per variety (two biological and three technical replicates); and establishment of a filter to limit our analysis to either quantitative differences among the volumes of common spots, or to qualitative differences for spots with fully reproducible patterns (either detected or no detected in all six replicates of a given variety). Considering the analysis of this subset of proteins, our 2DE coupled to MS–MS identification approach showed that late milky-starchy grains of MON810 and comparable non-GM varieties had very similar patterns. We observed a very small number of quantitative differential spots between a particular MON810 and non-GM variety pair ($\leq$1.2% analyzed proteins). However, none was differentially accumulated in the two variety pairs tested and fold variations were minimal. In a previous transcriptomics approach (Coll et al. 2008, 2010), leaves of MON810 commercial varieties had similarity levels to their near-isogenic counterparts in the 0.1–1.5% 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 initially 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 et al. (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 et al. (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. In agreement with our results, no clear physiological differences have been described between MON810 and comparable non-GM plants at the stage of harvesting for forage uses.

Previous observations from a number of authors indicated that 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) or in response to varying environmental factors (Barros et al. 2010; Coll et al. 2010; Zolla et al. 2008). Our 2DE patterns obtained from different non-GM 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% analyzed 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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References

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

Barros E, Lezar S, Anttonen MJ, Van Dijk JP, Rohlig RM, Kok EJ, Engel KH (2010) Comparison of two GM maize varieties with a near-isogenic non-GM variety using transcriptomics, proteomics and metabolomics. Plant Biotechnol J. doi: 10.1111/j.1467-7652.2009.00487.x

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

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Brandao AR, Barbosa HS, Arruda MAZ (2010) Image analysis of two dimensional gel electrophoresis for comparative

proteomics of transgenic and non-transgenic soybean seeds. J Proteomics 73:1433–1440

Catchpole GS, Beckmann M, Enot DP, Mondhe M, Zywicki B, Taylor J, Hardy N, Smith A, King RD, Kell DB, Fiehn O, Draper J (2005) Hierarchical metabolomics demonstrates substantial compositional similarity between genetically modified and conventional potato crops. Proc Natl Acad Sci USA 102:14458–14462

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

Coll A, Nadal A, Palaudelmàs M, Messeguer J, Melé E, Puigdomènech P, Pla M (2008) Lack of repeatable differential expression patterns between MON810 and comparable commercial varieties of maize. Plant Mol Biol 68:105–117

Coll A, Nadal A, Collado R, Capellades G, Kubista M, Messeguer J, Pla M (2010) Natural variation explains most transcriptomic changes among maize plants of MON810 and comparable non-GM varieties subjected to two N-fertilization farming practices. Plant Mol Biol. doi: 10.1007/s11103-010-9624-5

Corpillo D, Gardini G, Vaira AM, Basso M, Aime S, Accotto GP, Fasano M (2004) Proteomics as a tool to improve investigation of substantial equivalence in Genetically Modified Organisms: the case of a virus-resistant tomato. Proteomics 4:193–200

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

EFSA (2004) Guidance document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed. EFSA Journal 99:1–94

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

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

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

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

Holst-Jensen A, De Loose M, Van den Eede G (2006) Coherence between legal requirements and approaches for detection of Genetically Modified Organisms (GMOs) and their derived products. J Agric Food Chem 54:2799–2809

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

James C (2010) Global Status of Commercialized Biotech/GM Crops: 2009. ISAAA Briefs 41. ISAAA, Ithaca, NY

Khalf M, Goulet C, Vorster J, Brunelle F, Anguenot R, Fliss I, Michaud D (2010) Tubers from potato lines expressing a tomato Kunitz protease inhibitor are substantially equivalent to parental and transgenic controls. Plant Biotechnol J 8:155–169

Kristensen C, Morant M, Olsen CE, Ekstrom CT, Galbraith DW, Moller BL, Bak S (2005) Metabolic engineering of dhurrin in transgenic Arabidopsis plants with marginal inadvertent effects on the metabolome and transcriptome. Proc Natl Acad Sci U S A 102:1779–1784

La Paz JL, Vicient CM, Puigdomènech P, Pla M (2010) Characterization of polyadenylated *cryIA(b)* transcripts in maize MON810 commercial varieties. Anal Bioanal Chem 396:2125–2133

Lehesranta SJ, Davies HV, Shepherd LV, Nunan N, McNicol JW, Auriola S, Koistinen KM, Suomalainen S, Kokko HI, Karenlampi SO (2005) Comparison of tuber proteomes of potato varieties, landraces, and genetically modified lines. Plant Physiol 138:1690–1699

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

López A, Serra J, Capellades G, Betbesé JA, Salvia J (2009) Experimetnació de noves varietats de blat de moro per a gra. Indicacions per a la Campanya 2009. Dossier Tècnic 35:3–17

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

Miki B, Abdeen A, Manabe Y, MacDonald P (2009) Selectable marker genes and unintended changes to the plant transcriptome. Plant Biotechnol J 7:211–218

Natarajan S, Xu C, Caperna TJ, Garrett WM (2005) Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins. Anal Biochem 342:214–220

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9:255–262

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

Ren Y, Lv J, Wang H, Li L, Peng Y, Qu LJ (2009) A comparative proteomics approach to detect unintended effects in transgenic Arabidopsis. J Genet Genomics 36: 629–639

Rosati A, Bogani P, Santarlasci A, Buiatti M (2008) Characterisation of 3' transgene insertion site and derived mRNAs in MON810 YieldGard maize. Plant Mol Biol 67:271–281

Ruebelt MC, Lipp M, Reynolds TL, Schmuke JJ, Astwood JD, DellaPenna D, Engel KH, Jany KD (2006) Application of two-dimensional gel electrophoresis to interrogate alterations in the proteome of gentically modified crops. 3.

Assessing unintended effects. J Agric Food Chem 54: 2169–2177

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

Zolla L, Rinalducci S, Antonioli P, Righetti PG (2008) Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications. J Proteome Res 7:1850–1861