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Proteomic Analysis of Four Brazilian MON810 Maize Varieties and Their Four Non-Genetically-Modified Isogenic Varieties

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Supporting Information

ABSTRACT: Profiling techniques have been suggested as a nontargeted approach to detect unintended effects in genetically modified (GM) plants. Seedlings from eight Brazilian maize varieties, four MON810 GM varieties and four non-GM isogenic varieties, were grown under controlled environmental conditions. Physiological parameters (aerial part weight, main leaf length, and chlorophyll and total protein contents) were compared, and some differences were observed. Nevertheless, these differences were not constant among all GM and non-GM counterparts. Leaf proteomic profiles were analyzed using two-dimensional gel electrophoresis (2DE) coupled to mass spectrometry, using six 2DE gels per variety. The comparison between MON810 and its counterpart was limited to qualitative differences of fully reproducible protein spot patterns. Twelve exclusive proteins were observed in two of four maize variety pairs; all of these leaf proteins were variety specific. In this study, MON810 leaf proteomes of four varieties were similar to non-GM counterpart leaf proteomes.

KEYWORDS: proteome, profiling techniques, GMO, two-dimensional gel electrophoresis, maize

INTRODUCTION

Maize is an important grain in Brazil. The expected Brazilian corn yield for the 2010/2011 crop is 56 million tons, and corn total planted area in Brazil will reach 13 million hectares.[1] Genetically modified (GM) maize has been cultivated in Brazil since 2008; in the first year of commercial cultivation GM maize planted area started at 1.3 million hectares and rose to 5 million hectares in 2009.[2] The first GM maize approved for commercialization in Brazil by the National Biosafety Technical Commission and the National Biosafety Council was event MON810.[3] This GM crop contains recombinant DNA leading to ectopic expression of a synthetic CryIA(b) endotoxin, which confers resistance to lepidopteran insects, especially the European corn borer.[4] A unique recombinant DNA element of about 3.6 kb with a truncated *cry*IA(b) gene is present in the genome of MON810 maize, and the truncated inserted expression cassette lacks a terminator.[4] The *cry*IA(b) gene present in MON810 is under the control of the strong constitutive promoter P35S with duplicated enhancer regions,[5,6] resulting in protein expression levels of 9.35 μg/g fresh weight in leaf and 0.31 μg/g fresh weight in grain.[7] The Cry1Ab protein constitutes <0.001% of total protein content in MON810 maize tissues.[7]

After MON810, other GM maize events have been approved in Brazil in the past years: T25, Bt11, TC1507, GA21, NK603, Roundup Ready 2, MON89034, MON88017, and MIR162 and stacked events TC1507×NK60, MON810×NK603, MON89034×TC1507×NK603, MON89034×NK603, and Bt11×MIR162x-GA21 (http://www.ctnbio.gov.br/index.php/content/view/14785.html). Brazilian Normative Resolution 05 describes the rules for commercial release of genetically modified organisms (GMO) and their derivatives (http://www.ctnbio.gov.br/index.php/content/view/12857.html). The basis for the approval procedure is a comparative safety assessment. The dossier includes information related to the GMO, as molecular characterization, composition analysis, risk assessment to human and animal health, environmental risk assessment, and monitoring plan.

In different international guidelines for the composition analysis of new GM plant varieties, the current approach is the comparison of GM plant with their appropriate counterpart already on the market grown side-by-side in different locations in subsequent growth seasons. It is also suggested that it may be important to investigate the possibility of replacing the current numbers of field experiments supporting regulatory decisions by a limited set of experiments under controlled conditions.[8]

There are concerns that unintended, unexpected, and uncontrolled side effects might occur in GMOs. Profiling techniques (genomics, transcriptomics, proteomics, metabolomics, and glycomics) have been suggested as nontargeted approaches to detect unintended effects in GM plants and other plant-breeding processes.[9−13] These unbiased fingerprinting approaches could provide a more complete insight into any unpredicted changes in the physiology of the plant that might go undetected when single compounds are focused on.[14] However, significant research is required before these techniques produce confirmed and validated information.[15] Until now, 44 studies have been conducted using profiling techniques to evaluate GM crops; these studies were recently reviewed.[16] Among the profiling techniques,

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proteomic analysis of the tissues of interest is a direct method of investigating unpredicted alterations.

Various methods are available for the qualitative and quantitative comparison of plant proteomes.[17] So far, two-dimensional gel electrophoresis (2DE) combined with mass spectrometry (MS) is one of the most widely used approaches to compare plant proteomes to identify differentially expressed proteins.[18−21] The disadvantages of 2DE include its low efficiency in the analysis of hydrophobic proteins; its advantages are its power of good separation of proteins, robustness, parallelism, and unique ability to analyze complete sets of proteins at high resolution.[22]

2DE-based proteomic approach has been used to compare grain protein profiles of MON810 maize varieties grown in agricultural fields[23,24] and also MON810 maize varieties grown in environmentally controlled growth chambers.[19] Previous studies analyzed grain proteomes of MON810 maize varieties and their counterparts.[19,23−25] The MON810 proteomic profiles were analyzed in two subsequent generations with their respective isogenic controls grown in environmentally controlled growth chambers,[19] and the authors concluded that environment has the main influence on proteomic profiles of MON810 seeds and also that the transformation method used to generate MON810 maize, particle bombardment, by itself induces additional genome alterations which cause a different protein expression.

The grain transcriptomic, proteomic, and metabolomic profiles of two transgenic maize lines (MON810 and NK603) were compared, [23] and the plants were grown in agricultural fields in the same location over three growing seasons to evaluate the extent of environmental variation. In the earlier study, MON810 and control maize grown in three different locations were also compared in one growing season to evaluate the effect of growing conditions. These authors concluded that environmental factors caused more variation in the different transcript/protein/metabolite profiles than the different genotypes.

The proteomic profiles of two MON810 varieties and two near-isogenic counterparts grown in agricultural fields in one location (two different microplots) were analyzed using three protein extractions from each sample.[24] These authors concluded that MON810 and non-GM grains had virtually identical proteomic patterns, with only 10 differentially expressed proteins, and these proteins were all variety specific.[24] In the earlier study, 2DE patterns obtained from different non-GM maize varieties were highly different and the differences between two conventionally bred varieties were larger than those between a GM variety and its non-GM counterpart. Leaf transcriptome of MON810 maize was previously analyzed for plants grown in vitro[26] or in the field.[27] The authors reported the differential expression of a few transcripts (1.7 and 0.1%) in leaves of MON810 versus near-isogenic varieties. Although leaf transcriptomes have been compared between MON810 and non-GM maize varieties,[26] there is not a comparison of leaf proteomes yet. Despite the fact that leaf proteome should not be directly implicated in risk assessment to human and animal health, it could be useful for environmental risk assessment and for investigation of unintended physiological effects.

In the present study, 2DE was used to compare leaf protein profiles of four MON 810 maize varieties with the four isogenic varieties grown in an environmentally controlled growth chamber.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Chemicals. Seeds from eight Brazilian maize varieties, four MON810 varieties (DKBYG 240, DKBYG 330, DKBYG 350, AGYG 6018) and four non-GM varieties (DKB 240, DKB 330, DKB 350, AG 6018), were provided by Monsanto Co., St. Louis, MO. Seeds were surface sterilized with 0.5% hypochlorite for 20 min, washed three times with 70% alcohol, and finally rinsed with distilled water. The seeds were germinated on water-saturated filter paper at 25 °C in the dark. Germinated seedlings were transferred to soil. Twenty-four seedlings from each variety were grown side-by-side in two years (2010 and 2011), in a controlled-environmental chamber for 10 days (14 h photoperiod at 100 photon μmol m^{-2} s^{-1}, 25 °C). The same set of seeds was grown in December 2010 and February 2011. Maize seedlings were harvested, and leaves were frozen in liquid nitrogen and stored at −80 °C for further analysis.

Genomic DNA of the eight varieties was isolated from leaves, previously powdered in liquid nitrogen, using a CTAB method.[28] Samples were subject to nested PCR as already described[29] to confirm they were indeed MON810. Certified reference material (5% MON810) from European Reference Materials, Geel, Belgium, was used as a positive control.

Immobiline DryStrip Gels (IPG strips) and ampholyte-containing buffer (IPG buffer) were purchased from GE Healthcare, acrylamide, CHAPS, and Coomassie Brillant Blue G250 were purchased from Bio-Rad, and other chemicals were purchased from Sigma and Promega.

Protein Extraction for Two-Dimensional Gel Electrophoresis. Plants were grown side-by-side in two years (2010 and 2011), and three protein extractions were carried out from each variety in each year, generating six 2DE patterns from each variety. Total protein was extracted from all entire leaves of a pool including five plants, using the protocol previously described.[30] Leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle, and approximately 1 g of each sample was extracted with 10 mL of extraction buffer (50 mM Tris-HCl, pH 8.5, 5 mM EDTA, 30% sucrose, 100 mM KCl, 2% β-mercaptoethanol and 1 mM PMSF) and 10 mL of saturated phenol Tris-HCl buffer for 30 min at 4 °C under agitation. After centrifugation at 15500*g* for 30 min at 4 °C, 10 mL of extraction buffer was added to the phenolic phase under the same conditions.

Proteins were precipitated overnight with 0.1 M ammonium acetate in cold methanol. After centrifugation, dried protein pellets were resuspended in approximately 300 μL of a solution containing 7 M urea, 2 M thiourea, 3% (w/v) CHAPS, 2% (v/v) IPG buffer pH 3−10, and 1.5% (w/v) DTT. Protein concentrations were determined using a 2D Quant kit (GE Healthcare).

Two-Dimensional Gel Electrophoresis. Isoelectric focusing (IEF) of 450 μg of protein in buffer (7 M urea, 2 M thiourea, 3% (w/v) CHAPS, 2% (v/v) IPG buffer pH 3−10, 0.2% (w/v) DTT, and 0.01% (w/v) bromophenol blue) was run using immobilized nonlinear pH gradient 3−10 and 13 cm IPG strips on an IPGphor instrument (GE Healthcare). Focusing was done with the following program: gradient until 500 Vh, 1000, 14500, and 17800 Vh until a total of 33800 V was reached.

After IEF, strips were stored at −80 °C until equilibration. The strips were equilibrated for 15 min in 5 mL of equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.002% (w/v) bromophenol blue) containing 1% (w/v) DTT, followed by 15 min in equilibration buffer containing 2.5% (w/v) iodoacetamide. The strips were then attached to the top of the second-dimension gels by means of 0.5% agarose melted in cathode buffer (1% (w/v) SDS in Tris−glycine, pH 8.3, and traces of bromophenol blue).

Second-dimension SDS-PAGE gels were run in 12% (w/v) acrylamide gels using the Hoefer SE 600 Ruby System (GE Healthcare) at 10 mA/gel for 1 h, 20 mA/gel for 1 h, and 30 mA/gel using Precision Plus Protein Standards 10−250 kDa (Bio-Rad). Temperature was kept at 10 °C using a Multitemp III thermostatic circulator (GE Healthcare). Upon electrophoresis, the gels were rinsed in ultrapure water for 1 min and stained in staining solution containing 0.1% (w/v) Coomassie

Table 1. Physiological Parameters (Aerial Part Weight, Main Leaf Length, Chlorophyll Content, and Total Protein Content) of Four MON810 and Four Non-GM Isogenic Maize Varieties[a]

		2010		2011	
variety	*n*	non-GM	GM MON810	non-GM	GM MON810
Aerial Part Weight (g)					
DKB 240	10	0.45 ± 0.05 a	0.36 ± 0.04 b	0.36 ± 0.03 b	0.27 ± 0.03 c
DKB 330	10	0.30 ± 0.05	0.25 ± 0.05	0.32 ± 0.05	0.30 ± 0.05
DKB 350	10	0.34 ± 0.05	0.32 ± 0.06	0.30 ± 0.06	0.34 ± 0.05
AG 6018	10	0.29 ± 0.06 b	0.37 ± 0.04 a	0.32 ± 0.04 ab	0.35 ± 0.05 ab
Main Leaf Length (cm)					
DKB 240	10	34 ± 3 a	28 ± 2 b	33 ± 2 a	30 ± 2 b
DKB 330	10	21 ± 2 b	20 ± 4 b	26 ± 2 a	28 ± 2 a
DKB 350	10	21 ± 3 b	21 ± 3 b	26 ± 1 a	27 ± 2 a
AG 6018	10	21 ± 4 b	23 ± 1 b	30 ± 1 a	30 ± 1 a
Chlorophyll ($a + b$) Content ($\mu g\ g^{-1}$ FW)					
DKB 240	3	332 ± 18 b	508 ± 29 ab	629 ± 215 ab	697 ± 121 a
DKB 330	3	415 ± 114 b	299 ± 60 b	775 ± 35 a	703 ± 132 a
DKB 350	3	386 ± 18	402 ± 80	504 ± 227	600 ± 94
AG 6018	3	546 ± 62	421 ± 132	568 ± 132	475 ± 130
Total Protein Content (mg g^{-1} FW)					
DKB 240	3	6.74 ± 0.97	7.96 ± 2.84	8.37 ± 1.86	10.63 ± 1.66
DKB 330	3	7.89 ± 0.41	9.97 ± 1.88	9.54 ± 1.57	7.98 ± 1.84
DKB 350	3	8.45 ± 1.10	6.84 ± 1.91	7.56 ± 0.85	8.78 ± 0.93
AG 6018	3	7.30 ± 2.03	8.86 ± 1.45	7.91 ± 2.46	8.16 ± 1.44

[a] Values are the mean ± SD; means followed by different letters in the rows are significantly different at $P < 0.05$ according to the Tukey test. FW, fresh weight.

Brillant Blue G250 (Bio-Rad), 8% (w/v) ammonium sulfate, 2% (w/v) phosphoric acid, and 20% (v/v) methanol for 4 days on an orbital shaker, destained with 100 mM Tris−phosphoric acid, pH 6.5, for 3 min, 25% (v/v) methanol for 30 s, and 20% (w/v) ammonium sulfate for 12 h, and washed for 3 days with ultrapure water.[31] Stained gels were kept in 20% (w/v) ammonium sulfate at 4 °C.

Stained gels were imaged using a Labscan scanner (GE Healthcare). The resulting gel images were exported to and subsequently normalized with ImageMaster Platinum v.7.0 software (GE Healthcare). The following parameters were used for spot detection: saliency ≥ 95; area ≥ 11; and smooth ≥ 5. Reproducible spots in all replicates were considered in the analysis.

Protein Digestion and MALDI-TOF-MS Analysis. Protein spots from stained gels were excised manually and submitted to destaining procedure in 400 μL of a 50% (v/v) acetonitrile, 25 mM NH_4HCO_3 solution for 30 min at room temperature. The procedure was repeated twice. The solution was replaced by 200 μL of pure acetonitrile for 5 min and dried under vacuum. The proteins were then incubated for 30 min on ice in the presence of 10 μL of 20 ng/μL trypsin (Trypsin V5280, Promega), followed by 18 h at 37 °C. The peptides were extracted with 30 μL of a 5% trifluoroacetic acid solution. Then the extract was dried under vacuum and solubilized in 0.1% trifluoroacetic acid. The spots were applied onto a MALDI-TOF sample plate. MALDI-TOF mass spectra were acquired on a Bruker Daltonics mass spectrometer (MALDI-TOF/TOF Autoflex III Smartbean) with a 200 Hz Smartbeam pulsed nitrogen laser emitting at 337 nm. The extraction voltage was 19 kV, and all spectra were recorded under delayed extraction conditions and in the reflector mode. Spectra were also acquired with deflector mode of 400 Da, and each spectrum represents an average of 300 single laser shots.

Protein identification was performed using the Mascot software database (MSDB). Subsequently, the NCBI-nr database was used to look for homologous proteins. The following parameters were used for database searches: taxonomy, Viridiplantae; enzyme, trypsin; fixed modifications, carbamidomethyl cysteine; variable modifications, oxidized methionine; peptide tolerance, 0.3 Da; peptide charge, 1 H^+. Identifications were considered with a Mascot score >40 and sequence coverage of at least 25%.

Table 2. Results of Spot Detection and Matching in the Repeatability Assay[a]

	detected spots						
	Gel 1	Gel 2	Gel 3	av	SD	CV (%)	reproducible spots
extract 1	526	473	444	481	42	8.7	193
extract 2	480	344	439	421	69	16.4	222
extract 3	544	426	378	449	85	18.9	104
extract to extract				450	30	6.4	173 ± 61

[a] av, average; SD, standard deviation; CV, coefficient of variation.

Table 3. Detected Spots and Exclusive Spots of Four MON810 and Four Non-GM Isogenic Maize Varieties

	non-GM		GM (MON810)	
	detected spots[a]	exclusive spots[b]	detected spots[a]	exclusive spots[b]
DKB 240	466 ± 79	6	401 ± 99	1
DKB 330	445 ± 94		523 ± 75	
DKB 350	672 ± 32	4	525 ± 165	1
AG 6018	328 ± 142		330 ± 142	

[a] Values are the mean ± SD ($n = 6$). [b] A spot was considered to be exclusive when it was present in all six maps of a given variety and absent in all six maps of the corresponding isogenic variety.

2DE Repeatability Assay. The repeatability of the 2DE approach was evaluated using a pool of leaves from 10-day-old maize seedlings (DKB240 variety) that was processed identically to the analyzed samples. Three protein extractions from the same pool were conducted in parallel, and three 2DE gels were performed from each protein extract. Coefficients of variation (CV) were calculated as the standard deviation divided by the average value of detected spots for each extract, multiplied by 100. The 2DE patterns of the nine gels were compared, and the correlation coefficients (r^2) among gels were calculated from scatter plots.

Determination of Total Protein and Chlorophyll. Total protein was extracted by homogenizing the main leaf, previously powdered in liquid nitrogen, in 4 volumes of 125 mM Tris-HCl buffer, pH 8.8, 1% (w/v) SDS, 10% (w/v) glycerol, and 1 mM PMSF. The homogenate was centrifuged for 10 min at 13000*g* at 4 °C, and the protein content of the supernatant was determined using Bio-Rad Protein Assays Dye Reagent Concentrate and bovine serum albumin as standard.[32] Chlorophyll was extracted by homogenizing the main leaf, previously powdered in liquid nitrogen, in 4 volumes of 80% (v/v) precooled acetone. The homogenate was centrifuged for 20 min at 13000*g* at 4 °C, and the chlorophyll content of the supernatant was measured at 663 and 647 nm in a spectrophotometer (Hitachi U2910).[33]

Statistical Analysis. The data (aerial part weight, main leaf length, total protein content, and chlorophyll content) were analyzed by one-way analysis of variance (ANOVA) and compared by *F* test; the means were compared using the post hoc test of Tukey's multiple range.

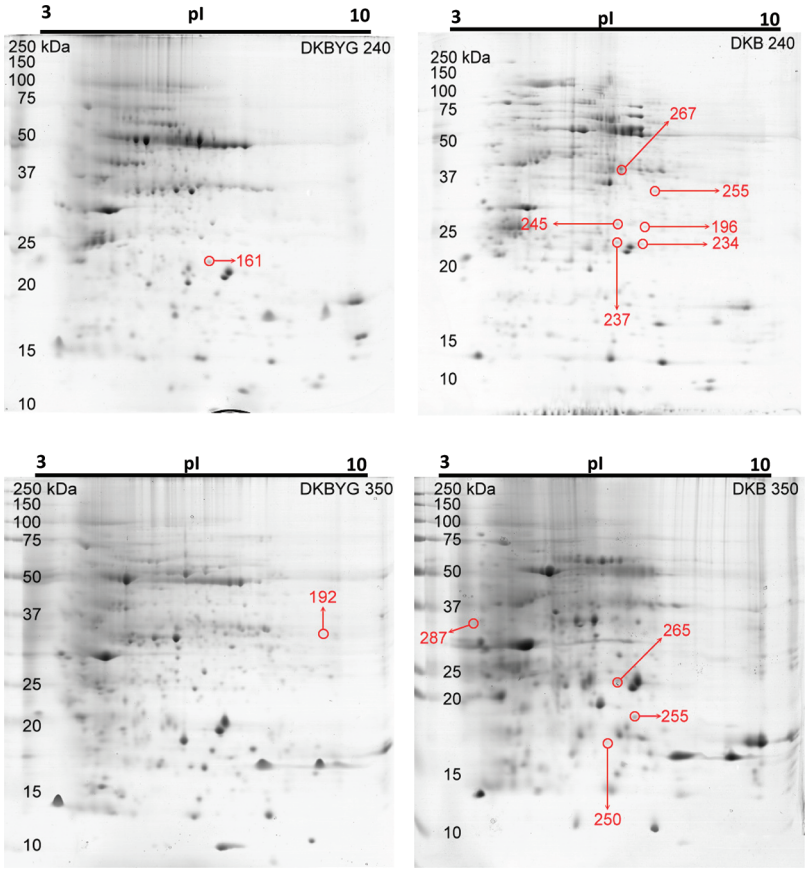

Figure 1. Representative leaf proteome (2DE) patterns of MON 810 (DKBYG 240 and DKBYG 350) and non-GM isogenic (DKB 240 and DKB 350) maize varieties (450 μg of total leaf protein); the exclusive spots are circled.

Data were analyzed using the Statistica 7 (StatSoft Inc., Tulsa, OK) software package. Differences were considered to be significant when $p < 0.05$.

RESULTS

Seedlings from eight maize varieties commonly cultivated in Brazil, four MON810 varieties (DKBYG 240, DKBYG 330, DKBYG 350, AGYG 6018) and four non-GM isogenic varieties (DKB 240, DKB 330, DKB 350, AG 6018), were grown side-by-side. The four MON810 varieties were positive for the MON810 nested PCR, and the four non-GM isogenic varieties were negative (Figure S1 of the Supporting Information).

Physiological parameters were determined for the eight varieties in 2010 and 2011, and they were compared between MON810 and the corresponding non-GM isogenic variety (Table 1). The aerial part weight of seedlings differed between MON810 and non-GM isogenic varieties DKB 240 grown in both years; the highest weights were observed for non-GM DKB 240. It differed between GM and non-GM AG 6018 grown in 2010; the highest weight was observed for MON810 AG 6018. Otherwise, significant differences were not observed for DKB 330 and DKB 350 varieties (Table 1). The main leaf length of MON810 DKB 240 was longer than the corresponding non-GM variety in both years; otherwise, for the other varieties (DKB 330, DKB 350, AG 6018), the main leaf length did not differ between GM and non-GM varieties, but it was longer in 2011 compared to 2010 (Table 1). Chlorophyll content differed from 2010 to 2011 for DKB 240 and DKB 330 varieties; the highest chlorophyll contents were observed in 2011. It did not differ for DKB 350 and AG 6018 varieties. Total protein contents of leaves were similar among all analyzed samples (Table 1). In summary, physiological parameters were compared and some differences were observed between the four MON 810 varieties and their non-GM isogenic counterparts with regard to aerial part weight, main leaf length, and chlorophyll contents. Nevertheless, these differences were not constant among all GM and non-GM counterparts.

2DE coupled to mass spectrometry was used to compare leaf proteomic profiles of MON810 and isogenic maize varieties. To determine the repeatability of the 2DE approach, three protein extractions from the same pool were conducted in parallel and three 2DE gels were run from each protein extract. Averages of 450 $\pm$ 60 distinct spots were detected in each of the nine gels as well as 173 $\pm$ 61 reproducible spots (Table 2). The gel-to-gel standard deviations (42−85) were larger than extract-to-extract standard deviation (30), and the CV were <19%.

Comparing matched spots of three gels from the same extract showed correlation coefficients (r^2) of scatter plots were in the range 0.75−0.89 and the slopes varied from 0.8 to 1.06 (Table S1 of the Supporting Information). Comparing matched spots of three reference gels between the different extracts showed correlation coefficients of scatter plots were 0.68, 0.79, and 0.87 and the slopes were 0.69, 0.77, and 0.89 (Table S1 of the Supporting Information).

The leaf proteome was compared between MON810 and the corresponding non-GM isogenic variety. For every variety, three protein extractions for 2DE were carried out in each year;

Table 4. Identification of Exclusive Spots in Leaves of MON810 (DKBYG 240 and DKBYG 350) and Non-GM Isogenic (DKB 240 and DKB 350) Maize Varieties[a]

							MW (kDa)		pI		
variety	spot ID	NCBI acession no.	Mascot score	sequence coverage (%)	matching peptides	source	theor	exptl	theor	exptl	protein
DKB 240	196	125534318	63	58	7	*O. sativa*	14.16	27	9.22	7.17	putat uncharact prot
	234							24		7.04	
	237	219362787	55	33	7	*Z. mays*	29.36	24	9.08	6.7	putat uncharact prot
	245	212722290	100	39	9	*Z. mays*	32	27	9.11	6.6	NAD-dependent epimerase/ dehydratase (putative)
	255	56784482	45	31	6	*O. sativa*	15.96	30	5.14	6.87	putat uncharact prot
	267	49388308	64	38	5	*O. sativa*	24.4	40	5.15	6.61	putat uncharact prot
DKBYG 240	161	226508790	41	32	5	*Z. mays*	15.48	23	9.05	6.56	putat uncharact prot
DKB 350	255	51535454	48	37	3	*O. sativa*	14.65	18	9.96	6.87	putat uncharact prot
	265	293331611	49	25	5	*Z. mays*	53.7	24	7.98	6.7	putat uncharact prot
	250	226491253	58	58	5	*Z. mays*	37.32	17	6.72	6.2	protein phosphatase 2c
	287	55296384	49	25	3	*O. sativa*	20.12	34	10.79	3.7	putat uncharact prot
DKBYG 350	192	293335549	55	28	9	*Z. mays*	48.38	33	8.41	8.75	putat uncharact prot

[a] Theor MW, theoretical molecular weight; exptl MW, experimental molecular weight; theor p*I*, theoretical isoeletric point; exptl p*I*, experimental isoeletric point; putat uncharact prot, putative uncharacterized protein.

therefore, total soluble proteins were extracted from 48 samples, and values ranged from 0.84 to 2.77 mg g^{-1} of fresh weight. The adopted protocol permitted an extraction yield in the range from 9.48 to 35.11% of soluble proteins extracted using phenol protocol compared to total protein content (Table S2 of the Supporting Information). Six protein extracts were prepared for each variety, generating 48 protein extracts and 48 corresponding proteome maps. The averages of detected spots observed in six replicate maps of each variety are shown in Table 3. DKB 350 presented the highest number of detected spots (703) and AG 6018 the lowest number of detected spots (127). The number of detected spots did not differ significantly between GM and non-GM isogenic varieties. Quantitative protein differences were investigated between each MON810 variety and its non-GM counterpart. Five differentially expressed spots were observed; otherwise, these spots were not differentially expressed in all six gels of one variety in comparison to six gels of its counterpart variety.

Qualitative protein differences between each MON810 variety and its comparable isogenic variety were investigated by comparison of all 12 2DE gels of each pair. Representative examples of 2DE gels are shown in Figure 1. The exclusive spots observed in all six replicate maps of each variety and absent in the counterpart were considered in this analysis. One exclusive spot (spot 161, Figure 1) was observed in all six 2DE maps of MON810 DKB 240, and six exclusive spots were observed in non-GM DKB 240 (spots 196, 234, 237, 245, 255, and 267). One exclusive spot was observed in MON810 DKB 350 (spot 192), and four exclusive spots were observed in non-GM DKB 350 (spots 250, 255, 265, and 287). In a comparison of DKB 330 or AG 6018, exclusive spots were not observed (Table 3).

The 12 exclusive spots were analyzed by mass spectrometry (Table 4). Proteins were identified from their peptide mass fingerprint by searching the MSDB database, and at least one presumed identity could be assigned to 11 of 12 spots (Mascot score > 40). Protein identification was based on homologies to *Zea mays* proteins in six spots; however, in five spots it was not possible to find presumed identities based on homologies to maize proteins; in these cases it was based on homologies to described *Oryza sativa* proteins (Table 4). Nine of 11 identified proteins presented homologies to putative uncharacterized proteins, spot 245 presented homology to a putative NAD-dependent epimerase/dehydratase, and spot 250 presented homology to a protein phosphatase 2C.

DISCUSSION

In our study, four Brazilian representative MON810 varieties and four non-GM isogenic counterparts were used to compare leaf proteomes and physiological parameters. Aerial part weight, leaf length, and chlorophyll content presented variations among the analyzed varieties, but the differences were not constant between all GM and non-GM counterparts. For example, aerial part weight was higher for non-GM DKB 240 than for its GM counterpart, but it was higher for MON810 AG 6018 than for its non-GM counterpart (Table 1). These differences were in agreement with the high natural variability of maize varieties observed in other studies.[34]

Reliable comparative proteomic analysis requires validated methodology. Key parameters in the validation are specificity, accuracy, precision, and linearity.[10] First, the impact of extraction procedure and 2DE on the repeatability of the spot pattern was investigated by comparing the qualitative (spot presence/absence) variability of spot patterns among three extracts and nine replicate gels using the approach described previously.[18]

The observed variability among the detected spots of nine gels carried out from one sample was considered to be acceptable,[35] because the CVs were <19% (Table 2). The CV inherent to the 2DE technique has been defined in the range of 20−40%, a value that is maintained across laboratories and experiments.[35]

The gel-to-gel spot number standard deviation and extract-to-extract spot number standard deviation suggest that the variation in spot number was affected by the 2DE method itself and by the sample preparation. The correlation coefficients (r^2) and the slopes of scatter plots were also considered to be acceptable, extract-to-extract slopes were >0.69, and extract-to-extract r^2 values were >0.68 (Table S1 of the Supporting Information). Slopes around 0.7 were observed when variations from experiment-to-experiment repeats were examined, and slopes around 0.8 were observed when technical variations were investigated.[36]The 2DE pattern of the nine replicate gels displayed some inconsistency between samples, with significant gel-to-gel and extract-to-extract standard deviation (Table 2). Nevertheless, six repetitions of each variety were performed, the variability being in this way diminished.

In this study, the proteome was compared by performing protein extraction from six pools of five plants for each variety. An extraction yield of about 17% of soluble proteins extracted using a phenol protocol compared to total protein content was achieved using the adopted protocol (Table S2 of the Supporting Information), comparable to other authors' data. For example, an extraction yield of about 20% for maize leaf soluble proteins has been obtained by other authors.[37]

Forty-eight 2DE maps were analyzed using the approach proposed;[24] it was based on six 2DE gels per variety and establishment of a filter to limit the analysis to qualitative differences for spots with fully reproducible patterns. Leaf proteomes of four MON810 varieties were compared to their isogenic counterparts. In the present experimental condition, 12 exclusive proteins were observed in two of four MON810 varieties, and these leaf proteins were variety specific (Tables 3 and 4). Nine identified proteins presented homologies to hypothetical proteins due to scarce available data about leaf maize protein characterization.

Previous studies about MON810 grain proteome and MON810 leaf transcriptome concluded that MON810 and non-GM grains had virtually identical proteomic[24] and transcriptomic patterns.[26,27] In our study, MON810 leaf proteomes of four varieties were similar to non-GM counterparts leaf proteomes. The observed leaf proteome profiles seem to support the observed differences not being attributable to the MON810 character.

Our leaf proteomic analysis of MON810 was an exploratory study. In the future, it is crucial to establish normalized validated approaches for the routine assessment of GM plants.[16] Proteomic screening has the potential to reduce the uncertainty of routine assessment by providing more data on plant protein composition than data obtained with targeted analysis.[19]

■ ASSOCIATED CONTENT

Supporting Information. Additional tables and figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

2DE, two-dimensional gel electrophoresis; GM, genetically modified; GMO, genetically modified organism; MS, mass spectrometry; CV, coefficient of variation.

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