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PROTEOMIC ANALYSIS OF A GENETICALLY MODIFIED MAIZE FLOUR CARRYING *CRY1AB* GENE AND COMPARISON TO THE CORRESPONDING *WILD-TYPE*

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ABSTRACT - Protein expression in a maize hybrid flour (hereafter called WT) and its corresponding transgenic version resistant to European corn borer (hereafter called BT and carrying a gene encoding for the *Bacillus thuringiensis* insecticidal protein Cry1Ab) has been studied by means of two-dimensional gel electrophoresis coupled with mass spectrometry. This comparison has been chosen as a model to verify proteomics capability in detecting unexpected differences between near-isogenic lines (differing in the ideal case only for the presence of the transgenic protein). Some unpredictable differences were detected: i) glucose and ribitol dehydrogenase spot was unique of BT maize; ii) endochitinase A spot was unique of WT maize; iii) triosephosphate isomerase 1 and one spot of globulin-1 S were overexpressed while cytosolic 3-phosphoglycerate kinase and one spot of aldose reductase were down-regulated in BT maize with respect to WT. These results outline the potential of the new non-targeted "–omics" technologies (in particular proteomics) in the detection of unexpected, unintended and unwanted variations in Genetically Modified (GM) *versus* non-GM food comparison and suggest the possible employment of these technologies in *substantial equivalence* evaluation. Moreover, a reference map for maize flour was built: forty spots, corresponding to twenty-five different proteins, were successfully identified.

KEYWORDS: Cry1Ab; Genetically modified plants; Maize; Proteomics; Substantial equivalence.

INTRODUCTION

Genetically modified (GM) food crops were commercially introduced in 1994. Since then, adoption of GM food crops has increased continuously achieving a cultivated area of 90 million hectares worldwide (James, 2005). GM crops that are cultivated today carry foreign traits introduced by genetic engineering that are predominantly of agronomic importance. The best-known examples include herbicide-resistant soybeans and insect-resistant maize (www.agbios.com/dbase.php). GM crops have also been designed to have traits that are beneficial in food processing or that might positively influence the nutritional and health status of the crop for consumers (Kleter *et al.*, 2000) and to contrast certain nutritional deficiencies (Ye *et al.*, 2000; Lucca *et al.*, 2001).

Despite the exploitation opportunities given by genetic engineering, food derived from GM crops is often perceived disparagingly by consumers, mostly because of concerns about unintended effects that could be dangerous to human health (Frewer *et al.*, 2004). To address the problem of the safety of GM derived food, guiding principles and regulatory frameworks have been proposed (König *et al.*, 2004), the main tenet of which is "*substantial equivalence*" or "*comparative safety*" (Oecd, 1993; Fao/Who, 2000; Efsa, 2004). In this context, it was proposed that the most practical method to assess the safety of a novel GM food is to compare its composition with that of the nearest existing wild-type counterpart (e.g. a non-GM organism with the same genetic background and with a known history about safety). The new GM food is considered substantially equivalent to the existing one if no sizeable differences are detected in the composition, with the exclusion of those differences that can be directly and functionally ascribed to the in-

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Abbreviations: 2-DE, two-dimensional electrophoresis; BT, genetically modified maize; GM, genetically modified; WT, control maize.

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tended genetic modification (e.g. predictable effects; CELLINI *et al.*, 2004; RISCHER and OKSMAN-CALDENTEY, 2006), which are always accurately considered from a safety viewpoint. In this case, the new food is regarded exactly as its counterpart in terms of safety (KUIPER *et al.*, 2002). If there is a non-complete correspondence between the new GM food and any existing organism, the modified organism is not necessarily considered as dangerous to human health, but further considerations about safety are required.

Currently, only key compounds, such as macro- and micro-nutrients, anti-nutrients and plant specific toxins are included in comparative analyses (KUIPER *et al.*, 2002), and targeted analytical assays are employed for their assessment. This approach requires the *a priori* knowledge of the possible unwanted species, and unpredicted toxins or allergens can not be detected. Moreover, the limits beyond which differences have to be considered as "sizeable" are not well-defined, leading to somewhat subjective judgments (HODGSON, 2006). For these reasons, the concept of "substantial equivalence" as originally proposed was soon criticized as being a pseudo-scientific concept, not properly defined and inadequate to serve as a safety assessment tool (MILLSTONE *et al.*, 1999).

Along with the debate about risk assessment of GM food, new profiling/fingerprinting methods (including DNA microarray technology, mRNA profiling, proteomics and metabolomics) were developed (WILKINS *et al.*, 1997; ABBOTT, 1999; BURKS, 1999; RABILLOUD, 2000) and proposed as innovative tools to address the problem of substantial equivalence (KUIPER *et al.*, 2001). These methods allow for the simultaneous screening of many components without requiring the *a priori* selection of the species to be searched for and analyzed (non-targeted methods). Taken together, targeted and non targeted techniques can be very suitable for a complete, holistic comparative analysis, as they offer a range of complementary approaches to the assessment of "substantial equivalence".

Despite the huge potential of proteomics and the outstanding importance to improve the concept of substantial equivalence, the studies published in this field are scanty. CORPILLO *et al.* (2004), by investigating tomato hybrids genetically modified for resistance to tomato spotted wilt virus, were unable to detect sizeable differences between transgenic and control tomato lines. Similarly, LEHESRANTA *et al.* (2005), by comparing varieties, landraces and GM potato lines, found nine proteins showing statistically significant differences between GM and control lines, while no new protein unique to GM lines was observed. They concluded that there was no evidence for any major changes in protein pattern in GM lines when compared to their control lines. Finally, RUEBELT *et al.* (2006) analyzed seeds of 12 transgenic *Arabidopsis thaliana* lines, which were compared to their parental line as well as to 12 *Arabidopsis* ecotype lines. They found that the genetic modification of the *Arabidopsis* lines, using three different genes and three different promoters, did not cause unintended changes to the analyzed proteome, as differences in spot quantity between transgenic and non-transgenic lines fell in the range of values found in the 12 *Arabidopsis* ecotype lines or were related to the introduced gene.

In this work, we have analyzed protein expression in the flour of a maize hybrid (hereafter called wild-type maize, WT) and its corresponding transgenic version (hereafter called *Bacillus thuringiensis* maize, BT, and obtained by crossing the commercial transgenic maize line MON810 to La73 and La17 non-transgenic backgrounds). BT carries a gene encoding for a truncated version (91 kDa) of the insecticidal protein Cry1Ab, derived from *Bacillus thuringiensis*. Delta-endotoxin Cry1Ab acts by selectively binding to specific sites localized on the brush border midgut epithelium of susceptible insect species (ROMEIS *et al.*, 2006). Following binding, cation-specific pores are formed that disrupt midgut ion flow and thereby cause paralysis and death. Cry1Ab is insecticidal only to lepidopteran insects such as the European corn borer (*Ostrinia nubilalis)*, and its specificity of action is directly attributable to the presence of specific binding sites in the target insects. There are no binding sites for delta-endotoxins of *B. thuringiensis* on the surface of mammalian intestinal cells, therefore, livestock animals and humans are not susceptible to these proteins (detailed information about MON810 can be found at http://www.agbios.com/dbase.php?action=ShowProd&data=MON810&frmat=LONG).

From the proteomics viewpoint, the only expected difference between BT and WT should be the presence of the Cry1Ab protein. We therefore performed a comparison of protein expression in BT and WT maize grains to assess whether or not other unintended or unexpected modifications in the protein expression profile can be unveiled by means of proteomics methods.

MATERIALS AND METHODS

Plant materials

Seeds of MON810 maize line containing the C*ry1Ab* gene from *Bacillus thuringiensis*, which confers resistance to the European corn borer (*Ostrinia nubilalis*), were kindly provided by Monsanto Co. (St. Louis, MO, USA). The MON810 insertion event was introduced into the La73 and La17 backgrounds, respectively, by backcrossing six times to the recurrent parents to minimize mixed genetic background, followed by two self-pollinations to obtain homozygous plants (La73-Bt and La17-Bt, respectively) for each inbred lines. The BT line used in this study was then derived by crossing La73-Bt and La17-Bt. In the same manner, the WT line, used as non-GM control line, was derived by crossing La73 and La17 inbred lines.

The transgenic plants were selected following an immunological assay for Cry1Ab (Flashkit™ from Biofords, Evry, France) and RT-PCR analyses. Plants derived from the seeds of the two set of inbred lines (BT and WT) were grown in contiguous plots under containment, according to the guidelines of the Italian laws for biosafety. At flowering, the plants of the 2 sets of inbred lines were, respectively, crossed by hand pollination; the ears were harvested after physiological maturity, and dried at 30°C. For each set of crosses including reciprocal sample, seeds deriving from the central position of each ear were stored in sealed plastic bag at –4°C. For the analyses, seed samples (100 kernels) were ground using a Cyclotec mill. Flour samples derived from the two grain versions were kept in sealed plastic bags at 0°C until chemical analysis.

Chemicals

Immobilized pH gradient (IPG) strips, IPG buffer and Coomassie brilliant blue R350 were purchased from GE Healthcare (Uppsala, Sweden). Modified trypsin was from Promega (Madison, WI, USA). Flashkits™ for rapid detection of Cry1Ab were purchased from Biofords (Evry, France). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification.

Protein extraction

To minimize effects of internal variability, each sample of both BT and WT flour proteins was extracted from maize grains deriving from an independent plant (all plants were grown and harvested identically). Before proceeding to the protein extraction, a PCR assay on genomic DNA was carried out to confirm the presence of *Cry1Ab* gene in each BT flour and its absence in each WT flour (data not shown).

Maize flour (100 mg) was homogenized in 2 ml of a lysis buffer (0.5 M Tris-HCl pH 7.5 containing 0.7 M sucrose, 5 mM EDTA, 0.1 M KCl, 10 mM thiourea, 2% β-mercaptoethanol and protease inhibitors) and diluted with an equal volume of phenol saturated solution. After centrifugation at 12,000 x g for 10 min, proteins were recovered in the phenolic phase and precipitated by adding 5 volumes of 0.1 M ammonium acetate in methanol and freezing overnight at –20°C. After centrifugation at 12,000 x g for 10 min at 4°C, pellets were washed twice with 80% acetone, then with 70% ethanol and finally resuspended in 100 µl of 7 M urea, 2 M thiourea and 4% CHAPS (Bestel-Corre *et al.*, 2002). Protein content was estimated by the method described by Bradford (1976).

2-DE and data analysis

Proteins were extracted from four samples of BT and four of WT flour. Each sample was analyzed separately and in triplicate. 2-DE was performed according to Jacobs *et al.* (2001), with some modifications. Protein samples (about 1 mg) were diluted to 350 µl with a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer 3-10NL, 2 mM tributylphosphine and traces of bromophenol blue and loaded on 18 cm IPG strips with a 3-10 non-linear pH gradient by in-gel rehydration (1 h at 0 V, 10 h at 30 V). IEF was performed at 16°C on an IPGphor (GE Healthcare, Uppsala, Sweden) according to the following schedule: 1h at 200 V, 30 min of a linear gradient to 3500 V, 3 h at 3500 V, 2.5 h of a linear gradient to 8000 V, 6 h at 8000 V. Prior to SDS-PAGE, the IPG strips were equilibrated for 2 x 45 min in 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and traces of bromophenol blue containing 1% DTT for the first equilibration step and 2.5% iodoacetamide for the second. SDS-PAGE was performed using 12.5% T, 2.6% C separating polyacrylamide gels (1.5 mm thick) according to Laemmli (1970) but without stacking gel, using Hoefer SE 600 system (GE Healthcare, Uppsala, Sweden). The second dimension was carried out at 45 mA/gel at 16°C and was terminated when the bromophenol dye front had migrated to the lower end of the gels. The gels were stained with Coomassie brilliant blue R350. After staining, gels were scanned with ImageMaster Labscan V3.0 (GE Healthcare, Uppsala, Sweden) and images were analyzed with ImageMaster 2D Platinum software package (GE Healthcare, Uppsala, Sweden).

Statistical analysis

Coomassie-stained spots were quantified on the basis of their relative volume (%Vol, i.e. the spot volume normalized to the total volume of gel spots). Data were expressed as means ± SD (standard deviation) and statistically analyzed according to the non parametric Mann-Whitney test by GraphPad-Instant 3.00 (GraphPad software, San Diego, CA, USA). Relationships were considered statistically significant when $P<0.05$.

Immunoassay for Cry1Ab

1g of each flour sample was resuspended in 40 ml of distilled water and vortexed. Then, the end of a Flashkits™ strip was inserted in each flour suspension. After 10 min, the appearance of check (in each sample) and test (only in Cry1Ab containing samples) red lines was finally verified, as specified by the manufacturer.

Tryptic digestion of 2-DE spots

Coomassie-stained spots were manually excised and destained overnight with 25 mM ammonium bicarbonate in 40% ethanol. Gel pieces were washed twice with 25 mM ammonium bicarbonate and desiccated three times with acetonitrile. Each piece was then reswollen in 25 mM ammonium bicarbonate containing 0.6 µg of modified porcine trypsin and digestion proceeded overnight at 37°C. Peptides were extracted by sonication in 25 mM ammonium bicarbonate and analyzed by mass spectrometry.

MALDI-TOF experiments

Each peptide sample was mixed 1:1 with a saturated solution of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.2% TFA and 0.5 µl was spotted directly on a MALDI target plate. MALDI mass spectra were acquired in the positive reflectron ion mode with delayed extraction on a Ultraflex II TOF-TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 200 Hz all-solid-state laser. Ion acceleration voltage was set to 25.00 kV, the reflector voltage was set to 26.30 kV and the first extrac-

tion plate was set to 21.75 kV. Mass spectra were obtained by averaging 1500 laser shots. Calibration of the spectra was performed internally by a two-points linear fit, using the autolysis products of trypsin at *m/z* 842.50 and 2211.10. Peak lists were obtained using the FlexAnalysis software (Bruker Daltonics, Bremen, Germany).

NanoLC-nanospray-ion trap experiments

1 µl of each peptide sample was loaded onto a ZORBAX 300 SB C18 RP column (75 µm x 150 mm, 3.5 µm particles, Agilent Italia, Milan, Italy) and eluted with a gradient of acetonitrile from 5% to 80% (containing 0.1% formic acid) at a flow rate of 0.3 µl/min by a HP 1100 nanoLC system coupled to a XCT-Plus nanospray-ion trap mass spectrometer (Agilent Italia, Milan, Italy). Mass spectrometer parameters were the following: scan range 100-2200 *m/z*, scan speed 8100 $m/z\ s^{-1}$, dry gas flow 5 L/min, dry temperature 300°C, capillary 1.8 kV, skimmer 40 V, ion charge control (ICC) target 125000, maximum accumulation time 300 ms. Positively charged peptides ions were automatically isolated and fragmented, and spectra were deconvoluted by the DataAnalysis software (Bruker Daltonics, Bremen, Germany).

Database search

Database search from MALDI-TOF experiments was performed against the UniProtKB/SwissProt-TrEMBL database using Aldente search algorithm (http://www.expasy.org/tools/aldente/), while for nanoLC-nanospray-ion trap experiments the NCBInr database and Mascot search algorithm (http://www.matrixscience.com/search_form_select.html) or Spectrum Mill software (Agilent Italia, Milan, Italy) were chosen. Mass tolerance was set to 25 ppm for MALDI-TOF experiments and to 1.2 Da (parent ion) and 0.6 Da (fragments) for nanoLC-nanospray-ion trap experiments. For both type of experiments, the maximum number of missed cleavages was set to 1. Allowed modifications were cysteine carbamidomethylation and methionine oxidation. Identification was accepted when validated by the chosen software: Aldente minimum protein score: 0.49; Mascot minimum protein score: 46; Spectrum Mill minimum peptide score: 9.

RESULTS AND DISCUSSION

Sample choice for comparative protein profiling

Our first study about substantial equivalence assessment by proteomics (CORPILLO *et al.*, 2004) was performed on seedlings of a GM tomato resistant to tomato spotted wilt virus. In our opinion, that study had two major drawbacks: i) it was not performed on the edible portion of the plant (that is of course the most interesting, as the concept of substantial equivalence is strictly connected to that of food safety evaluation) and ii) that particular GM plant did not express the transgenic protein (preventing to evaluate the possible effects of an exogenous protein on plant metabolism). To overcome these limitations, in the present work we decide to analyze the edible portion of a GM plant expressing the transgenic protein. To the best of our knowledge, this is the first study satisfying both these conditions, as LEHESRANTA *et al.* (2005) did not investigate the real presence of transgenic proteins in their GM potato lines, while RUEBELT *et al.* (2006) analyzed transgenic lines of a plant that is not addressed to either human or animal feeding (*Arabidopsis thaliana*).

As GM material, we chose seeds of MON810 maize line, containing the C*ry1Ab* gene from *Bacillus thuringiensis*. To perform a detailed proteomic study of a GM species, not only the GM material, but also the exact non-GM counterpart are needed: unfortunately, the corresponding *wild-type* maize was not available. In order to do a comparison between near-isogenic lines (differing in the ideal case only for the presence of the transgene), the MON810 insertion event was introduced into the La73 and La17 backgrounds by backcrossing several times, to finally obtain homozygous BT plants (see Methods), which were then compared to WT plants, used as non-GM control line and obtained by crossing La73 and La17 inbred lines.

The flour of this GM maize and that of the non-GM counterpart can represent a good model for our proteins profiling studies, as our purpose is to assess the usefulness of proteomics in GM food safety evaluation rather than to assess the safety of this particular "pseudo commercial" food.

In principle, BT maize should differ from WT in a single trait, namely the insertion of the *Cry1Ab* gene, that confers resistance to attack by insects (ROMEIS *et al.*, 2006; SHIMADA *et al.*, 2006).

To investigate whether the genetic modification affected the overall expression of proteins, both qualitative and quantitative differences on Coomassie-stained gels were considered.

BT versus *WT: qualitative comparison*

Proteins were extracted from each kind of flour and separated by two-dimensional electrophoresis (2-DE). All BT and WT 2-DE gels were then Coomassie-stained and analyzed by Image Master 2D Platinum software, introducing for each group an average gel, a synthetic image which contains only spots present in all gels of each group (113 spots in total in both average gels). These virtual gels were then superimposed by the software, and unmatched spots were considered as qualitative differences (i.e., such spots were unique to a given group of samples). While 112 out of 113 spots were easily matched in BT *versus* WT comparison, one spot remained unmatched in BT flour gels (spot A

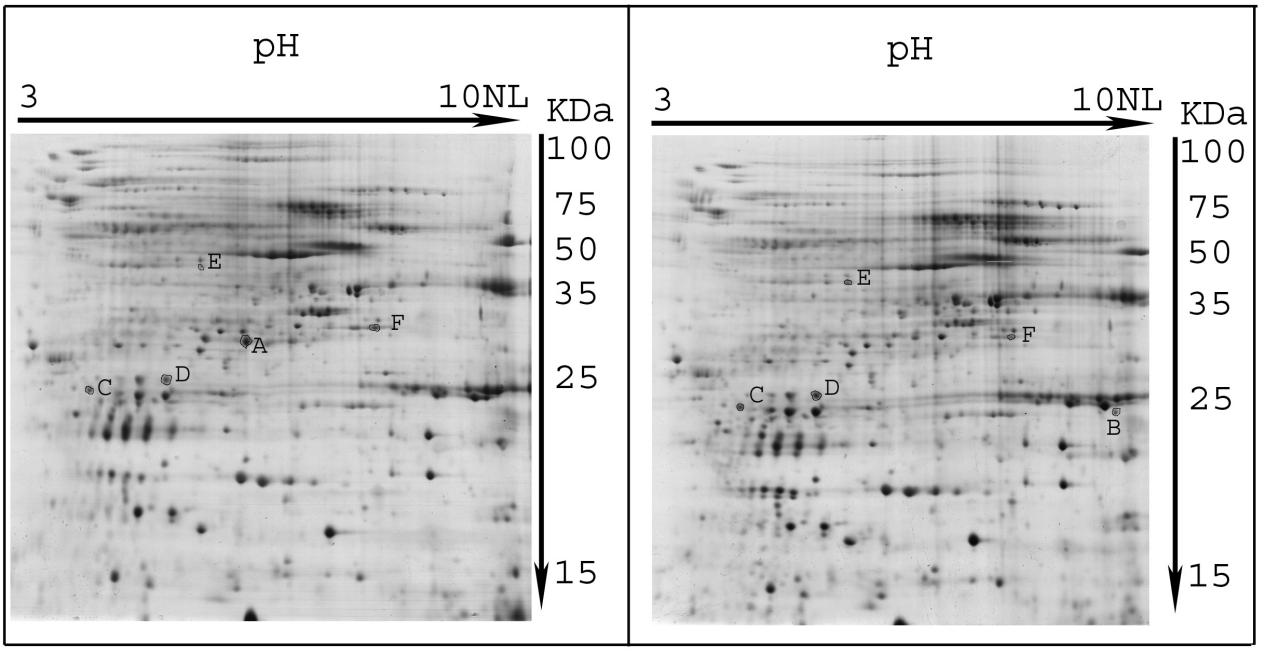

FIGURE 1 - Left panel: Coomassie-stained 2-DE of proteins from BT flour. Right panel: Coomassie-stained 2-DE of proteins from WT flour. Spot A is unique of BT gels, spot B is unique of WT gels, while spots C, D, E and F are differentially expressed between BT and WT flours (see Table 2). Labeled spots were identified as listed in Table 1.

in Fig. 1, left panel) and one in WT gels (spot B in Fig. 1, right panel).

Spot A, which was verified to be present in all BT gels and in none WT gels, resulted to be a glucose and ribitol dehydrogenase homolog (Table 1), an enzyme that may act as a short alcohol-polyol-sugar dehydrogenase, possibly related to carbohydrate metabolism and to the acquisition of desiccation tolerance and possibly involved in signal transduction (as reported at http://expasy.org/uniprot/Q75KH3). The appearance of this spot could be due to *Cry1Ab* insertion event in La73/La17 lines, obtained by crossing them with MON810 maize. We cannot exclude that the same difference in spot A expression is already present between MON810 maize and its original *wild-type*, even if it must be stressed that, being BT and MON810 likely generated from different parental lines (i.e. different genetic background), results obtained on BT can not be directly extended to MON810.

Spot B, which was verified to be present in all WT gels and in none BT gels, was instead assigned by mass spectrometry to endochitinase A (Table 1), a protein induced in response to fungal infection (Bravo *et al.*, 2003). It is known that insecticidal

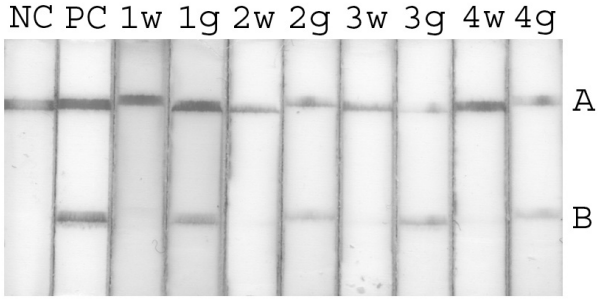

FIGURE 2 - Flashkits™ test for rapid detection of Cry1Ab in flours.
NC: negative control. PC: positive control. 1w, 2w, 3w, 4w: four WT samples. 1g, 2g, 3g, 4g: four BT samples. A: check line. B: test line.

proteins like Cry1Ab, preventing insect damage to plants, reduce in turn infection by mycotoxigenic fungi (Wu *et al.*, 2004), the possible final effect being the downregulation of endochitinase A. However, it is unlikely that its presence in WT gels is due to a higher fungal infection correlated to insect attack, as the plants were grown in controlled conditions. Its disappearance in BT gels could again be correlated to *Cry1Ab* insertion event in La73/La17 lines.

TABLE 1 - *Identification by mass spectrometry of spots (labeled according to Figs. 1 and 3) from transgenic (BT) and/or non transgenic (WT) maize flours gels.*[a]

Spot	Protein (AC, species)	Identified by	Peptides	$MW_{theoretical}$ (kDa)	$pI_{theoretical}$
A	Glucose and ribitol dehydrogenase homolog (108885236, rice)	MS/MS	2	32.3	5.76
B	Endochitinase A (116329, maize)	MS/MS	2	29.1	8.30
C	Triosephosphate isomerase 1 (168647, maize)	MS/MS	2	27.0	5.52
D	Globulin-1 S, 464-499 (121205, maize)	MS/MS	2	65.0 (full length)	6.63 (full length)
E	Cytosolic 3-phosphoglycerate kinase (28172917, maize)	MS/MS	1	31.6	5.01
F	Putative aldose reductase (46200527, maize)	MS/MS	1	65.1	6.35
1	Globulin-1 S, 102-544 (P15590, maize)	MS	10	65.0 (full length)	6.63 (full length)
2	Globulin-1 S, 102-499 (P15590, 121205, maize)	MS, MS/MS	7, 2	65.0 (full length)	6.63 (full length)
3	Globulin-1 S, 102-499 (P15590, 121205, maize)	MS, MS/MS	7, 6	65.0 (full length)	6.63 (full length)
4	Globulin-1 S, 102-499 (P15590, 121205, maize)	MS, MS/MS	9, 4	65.0 (full length)	6.63 (full length)
5	Globulin-1 S, 102-499 (P15590, 121205, maize)	MS, MS/MS	9, 4	65.0 (full length)	6.63 (full length)
6	Globulin-1 S, 102-527 (P15590, maize)	MS	11	65.0 (full length)	6.63 (full length)
7	Enolase 2 (P42895, maize)	MS	7	48.2	5.71
8	Cytosolic 3-phosphoglycerate kinase (Q84JX6, 28172917, maize)	MS, MS/MS	11, 11	31.6	5.01
9	Cytosolic 3-phosphoglycerate kinase (Q84JX6, maize)	MS	8	31.6	5.01
10	Cytosolic 3-phosphoglycerate kinase (Q84JX6, 28172917, maize)	MS, MS/MS	7, 6	31.6	5.01
11	Globulin-1 S, 102-499 (P15590, 121205, maize)	MS, MS/MS	5, 3	65.0 (full length)	6.63 (full length)
12	Hypothetical protein (Q6R9E8, maize)	MS	4	17.0	10.89
13	Globulin-1 S, 102-384 (P15590, maize)	MS	7	65.0 (full length)	6.63 (full length)
14	Osr40c1 (Q40705, 1296955, rice)	MS, MS/MS	8, 3	38.8	6.30
15	Osr40c1 (Q40705, 1296955, rice)	MS, MS/MS	7, 2	38.8	6.30
16	Protein b-32 (Q41777, 22190, maize)	MS, MS/MS	9, 2	33.4	6.02
17	Knolle protein (Q84R43, rice)	MS	6	35.0	6.07
18	Rab28 (Q41850, 22460, maize)	MS, MS/MS	7, 2	27.7	4.90
19	Silencing group B protein (Q94F75, maize)	MS	4	20.3	7.75
20	2310003L22Rik protein (Q2QW87, rice)	MS	6	23.4	8.88
21	Globulin-2, 271-428 (100876, maize)	MS/MS	4	49.9 (full length)	6.16 (full length)
22	Hypothetical protein (Q60ES1, rice)	MS	5	18.7	5.03
23	Peroxiredoxin (1694833, barley)	MS/MS	3	24.0	6.31
24	MADS-box protein 9 (Q9LEH9, barley)	MS	6	28.6	8.74
25	Globulin-2, 41-210 (Q7M1Z8, 100876, maize)	MS, MS/MS	7, 6	49.9 (full length)	6.16 (full length)
26	Glutathione S-transferase (Q9FQA9, maize)	MS	5	25.2	5.33
27	Hypothetical protein (Q5GAR2, maize)	MS	10	38.2	4.67
28	Globulin-2, 56-210 (Q7M1Z8, maize)	MS	7	49.9 (full length)	6.16 (full length)

TABLE 1 - *Continued*

Spot	Protein (AC, species)	Identified by	Peptides	$MW_{theoretical}$ (kDa)	$pI_{theoretical}$
29	Globulin-2, 56-210 (Q7M1Z8, maize)	MS	9	49.9 (full length)	6.16 (full length)
30	Superoxide dismutase (P41980, maize)	MS	4	25.2	6.71
31	CI2F (Q8LLB1, barley)	MS	3	9.5	6.57
32	17.4 kDa class I heat shock protein (P31673, rice)	MS	5	17.4	6.18
33	MAPK (Q6RXW7, maize)	MS	3	16.1	6.65
34	Starch branching enzyme IIB fragment (Q7XZN5, maize)	MS	4	8.3	5.09

[a] MS: MALDI-TOF experiments; MS/MS: nanoLC-nanospray-ion trap experiments; Reported Access Code (AC) refers to either UniProtKB/SwissProt-TrEMBL (for MS experiments) or NCBInr (for MS/MS experiments) databases. For globulin-1 S and globulin 2, present in the 2-DE map as different fragments, the minimal trait of sequence detected for each spot is indicated, while reported theoretical molecular weight (MW) and isoelectric point (pI) refer to full length proteins.

TABLE 2 - *Statistical analysis of the spots showing quantitative differences (P<0.05) in spot % Vol Av. between transgenic (BT) and non transgenic (WT) maize flours (see Fig. 1 and Table 1).*[a]

SPOT	%Vol Av. (±SD) BT	%Vol Av. (±SD) WT	BT/WT (%)	P
C	0.44 (±0.14)	0.32 (±0.09)	138	0.0325
D	0.79 (±0.20)	0.57 (±0.16)	139	0.0052
E	0.15 (±0.05)	0.26 (±0.06)	58	0.0015
F	0.65 (±0.26)	1.04 (±0.44)	62	0.0333

[a] %Vol Av.: average relative volume; SD: standard deviation; P: probability (according to Mann-Whitney test).

Cry1Ab was not found among the unmatched spots. Therefore, BT and WT flours were tested by Flashkits™, an immuno-test for rapid detection of this transgenic protein in flours. This analysis confirmed the presence of the protein in BT flours and its absence in WT ones (Fig. 2).

Cry1Ab was not detected by Coomassie-staining of BT maize gels because of a level of expression below the detection limit of the staining technique. In fact, the Flashkits™ immunoassay has a markedly higher sensitivity (about 5 ppb, i.e. 5 ng/g of flour) than Coomassie-staining. The very low amount of Cry1Ab in BT maize flour is not surprising, since it is known that the levels of transgenic proteins in currently registered biotechnology-derived maize hybrids are very low, ranging from non detectable quantities (less than 0.005 µg/g of plant tissue) to 4 µg/g (Carpenter *et al.*, 2002). Specifically, in the MON810 grain the concentration of expressed toxin is found to range from 0.19 to 0.39 µg/g (**www.agbios.com/dbase.php**). Considering a total protein content of 5-10% in maize flours, it means that we can expect to have only a few ng of Cry1Ab in the protein extract (about 1 mg) loaded onto 2-DE gels, i.e. surely below the limit of detection of Coomassie staining (around 100ng/spot).

BT versus *WT: quantitative comparison*

Quantitative comparison (i.e., increase or decrease in spot relative volume, %Vol) of BT *versus* WT spots was performed on the 112 matched spots. A precise calculation of their %Vol in each gel was obtained in order to do statistical analysis, and data for each group were finally expressed as spot %Vol averages ± SD (standard deviation). Four spots showed a statistically significant variation in their %Vol, according to the non-parametric Mann-Whitney test (P<0.05). Considering the average values of the %Vol of these spots, two of them resulted over-expressed (spots C and D in Fig. 1, both panels) and two down-regulated (spots E and F in Fig. 1, both panels) in BT samples, respectively (see Table 2). Differences ranged from -42% to +39%. Spots C, D, E and F were identified by mass spectrometry as

triosephosphate isomerase 1, globulin-1 S, cytosolic 3-phosphoglycerate kinase and aldose reductase, respectively (Table 1). Triosephosphate isomerase 1 is an enzyme of the glycolysis/gluconeogenesis pathway, while globulin-1 S belongs to the 7S seed storage protein family. Cytosolic 3-phosphoglycerate kinase, instead, is involved in ATP synthesis and, finally, aldose reductase acts as a regulator of K+ ion channels.

The possible contribution of proteomics to "substantial equivalence" assessment

The results presented in this paper show how proteomics can evidence subtle unexpected differences even when near-isogenic lines are compared. Detected differences (in respect to WT) could either be already present in the original Monsanto *wild-type*, or due to *Cry1Ab* gene insertion in MON810 or correlated to La73/La17 lines crossing with MON810 maize. It is not the aim of this work to understand which difference correlates to which event nor to evaluate how the detected differences can influence the safety of the analyzed flour as a new food. It must be stressed, in fact, that BT was produced for research purpose and is not addressed to either human or animal feeding. The scope of this section is to discuss the usefulness of the proteomic analysis in the evaluation of the principle of "substantial equivalence".

According to this principle, the safety of a new GM food is assessed by comparing some of its attributes with those of a "traditional" counterpart having the closest genetic background. For what concerning *Cry1Ab* insertion, several studies report that there are no significant differences between the control and event MON810 for various traits, including protein, fat, ash, neutral and acid detergent fibers and carbohydrates (**www.agbios.com/dbase.php**); additionally, these differences are all within the ranges of published literature values (JUGENHEIMER, 1976; WATSON, 1987). In this respect, maize grains derived from the event MON810 were judged substantially equivalent to other commercial maize varieties.

With the non-targeted approach presented in this work, i.e. the analysis done at the proteome level, an investigation of possible unexpected alterations of the plant metabolism can be performed. In principle, differential protein expression in GM organisms with respect to non-GM counterparts can result from at least two major causes: i) the position where the transgene has been introduced, which could influence expression of close genes, and/or ii) the metabolic response of the plant to the presence of the transgene and/or the transgenic protein. This view is consistent with a recent metabolomic study of transgenic maize grain carrying the MON810 trait (MANETTI *et al.*, 2004, 2006), in which it is shown that this genetic modification induces metabolic variations in osmolytes and in branched-chain amino acid concentrations. Whatever is the mechanism linking the genetic modification with the protein expression profile, the proteomic analysis can evidence differences that are undetectable by the traditional targeted approach. In the present work, the detected changes (appearance of spot A, disappearance of spot B and quantitative modification of the expression of spots C, D, E and F), even if unexpected, concern either enzymatic or storage proteins, naturally synthesized by the maize kernels. No change in proteins, known to be toxic or allergenic, was detected in this study. In addition, a number of articles have been published indicating minimal risk for maize varieties containing the Cry1Ab protein (PILCHER *et al.*, 1997; AULRICH *et al.*, 1998; DAENICKE *et al.*, 1999).

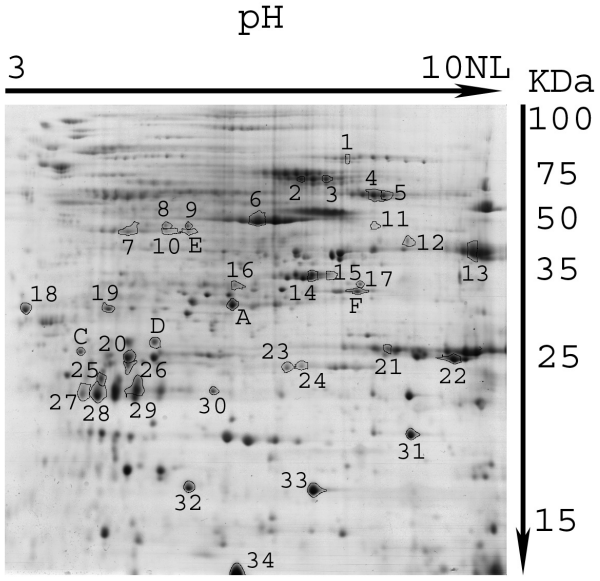

FIGURE 3 - Coomassie-stained 2-DE of proteins from BT maize flour. Labeled spots were identified as listed in Table 1.

Protein characterization in maize flour

Finally, to build a reference map of the proteins extracted from maize flour, most intense spots from BT and WT gels were analyzed by MALDI-TOF (peptide mass fingerprinting experiments) or nanoLC-nanospray-ion trap (fragmentation experi-

ments) mass spectrometry or both. Forty of them, corresponding to twenty-five different proteins, were successfully identified (Figs. 1 and 3, and Table 1) as maize (*Zea mays*) proteins or as homologous of proteins from two other cereals, namely rice (*Oryza sativa*) and barley (*Hordeum vulgare*). Among the identified spots, there are storage proteins (globulin-1 S and globulin-2), proteins involved in carbohydrate metabolism (glucose and ribitol dehydrogenase, triosephosphate isomerase 1 and enolase 2), stress response (endochitinase A (BRAVO *et al.*, 2003), osr40c1 (MOONS *et al.*, 1997), peroxiredoxin, glutathione S-transferase, superoxide dismutase, C12F (WEI *et al.*, 2002), MAPK (ZHANG *et al.*, 2006) and 17.4 kDa class I heat shock protein), ATP synthesis (cytosolic 3-phosphoglycerate kinase), K+ ion channel regulation (aldose reductase), zein expression (protein b-32 (HARTINGS *et al.*, 1990)), cytokinesis (Knolle protein (VÖLKER *et al.*, 2001)), embryogenesis (Rab28 (NIOGRET *et al.*, 1996)), transcription regulation (MADS-box protein 9) or kernel starch biosynthesis (starch branching enzyme IIB fragment (WILSON *et al.*, 2004)) and proteins of unknown function (silencing group B protein, 2310003L22Rik protein and 3 hypothetical proteins). Interestingly, globulin-1 S and globulin 2 resulted represented in the 2-DE map by various fragments.

To the best of our knowledge, this is the first 2-DE map of maize flour from entire kernels. Instead, maps from maize embryo (KOLLIPARA *et al.*, 2002; CAMPO *et al.*, 2004) and endosperm (MECHIN *et al.*, 2004, 2007) are available. These maps share 11 out of the 25 proteins identified in the present paper (triosephosphate isomerase 1, cytosolic 3-phosphoglycerate kinase, enolase 2, glutathione S-transferase, superoxide dismutase, 17.4 kDa class I heat shock protein, starch branching enzyme IIB, osr40c1, aldose reductase, globulin-1 S and globulin-2, the latter two again represented by various fragments), while the remaining 14 proteins were not identified in the reported references.

CONCLUDING REMARKS

Results presented in this paper concern flours of plants arising from successive crossbreeding of maize carrying or not the Cry1Ab insertion event (to achieve an acceptable degree of isogenicity between BT and WT plants). This protocol for generating GM maize is not suited for production of novel crops for commercial purposes (that is not the scope of this work). However, the maize flours we have analyzed constitute a good model to test the concept of comparative proteomics for the assessment of substantial equivalence between two populations of crops. Analysis on WT *versus* BT maize flour evidenced differences that can be classified as unintended/unpredictable, on the basis of the criteria proposed by CELLINI *et al.* (2004) and RISCHER and OKSMAN-CALDENTEY (2006). On these grounds, the results of the proteome analysis should constitute the starting point for the safety assessment of the components that have been found to have a different expression pattern in BT *versus* WT line.

These results concern only the subproteome we were able to extract, separate by 2-DE and detect by Coomassie staining. Of course, each step could be optimized. However, even in these suboptimal conditions, the proteomic approach was found to be a useful tool for the detection of unintended and/or unpredictable variations in GM *versus* non-GM plants comparison, which could lead, together with traditional targeted techniques, to a more complete evaluation of their substantial equivalence. The key feature of the proteomic approach to the substantial equivalence is, in fact, that it is a non-targeted method, allowing for the detection of unpredictable variations (METZDORFF *et al.*, 2006) that could otherwise go unnoticed. However, prior to the application of this method on a routine basis for the screening of unintended effects in transgenic plants of real commercial interest (KUIPER *et al.*, 2001), some items should be addressed. First, the variability of the protein expression in conventional crops should be assessed, to ensure that any difference eventually found with respect to a GM version is truly due to the genetic manipulation rather than to natural variations within conventional varieties or to crossing events among different lines (RUEBELT *et al.*, 2006; SHEPERD *et al.*, 2006). Then, well-described guidelines for proteome analysis, comparative statistics and evaluation of the results must be validated and agreed. Together with other non-targeted profiling methods, such as transcriptomics and metabolomics (FIEHN, 2002; WECKWERTH, 2003; METZDORFF *et al.*, 2006), comparative proteomics should improve the knowledge of single GMOs, making them more easily acceptable by public opinion and providing crucial information for a scientific evaluation of the problem of food safety.

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Appendix

TABLE S1 - *Spot identification by MS experiments.*

Spot	Aldente score	Sequence coverage (%)	Spot	Aldente score	Sequence coverage (%)
1	1.82	22	17	1.37	31
2	1.22	17	18	2.83	41
3	1.07	17	19	0.70	30
4	2.16	24	20	1.09	29
5	2.14	24	22	1.45	36
6	2.77	32	24	0.81	25
7	1.40	21	25	1.06	16
8	5.64	60	26	1.08	22
9	2.87	37	27	1.12	29
10	2.18	34	28	1.47	22
11	0.71	14	29	1.95	22
12	0.66	32	30	1.32	34
13	1.25	20	31	1.03	37
14	1.70	23	32	0.96	24
15	1.36	22	33	0.70	29
16	2.44	31	34	1.77	83

TABLE S2 - *Spot identification by MS/MS experiments.*

Spot	Mascot (M)/Spectrum Mill (S) score	Peptides (charge state)
A	148 (M)	VAIVTGGDSGIGR (+2) TLLDYTATK (+2)
B	106 (M) 26.11 (S)	DIGFNGLADPNR (+2) VAQDAVIAFK (+2)
C	96 (M)	ALLGESNEFVGDK (+2) EAGSTMDVVAAQTK (+2)
D	100 (M) 37.51 (S)	VFLAGADNVLQK (+2) AEEVDEVLGSR (+2)
E	48 (M)	LAAALPEGGVLLLENVR (+3)
F	14.45 (S)	HGIHVTAYSPLGSSEK (+3)
2	163 (M) 36.71 (S)	VFLAGADNVLQK (+2) AEEVDEVLGSR (+2)
3	400 (M) 70.86 (S)	VLRPFDEVSR (+2) NPESFLSSFSK (+2) GYAEIVCPHR + carbamidomethyl(C) (+2) SEEEEESSEEQEEVGQGYHTIR (+3) VFLAGADNVLQK (+2) AEEVDEVLGSR (+2)
4	309 (M) 49.84 (S)	VLRPFDEVSR (+2) SEEEEESSEEQEEVGQGYHTIR (+3) VFLAGADNVLQK (+2) AEEVDEVLGSR (+2)
5	280 (M) 40.34 (S)	VLRPFDEVSR (+2) SEEEEESSEEQEEVGQGYHTIR (+3) VFLAGADNVLQK (+2) AEEVDEVLGSR (+2)

TABLE S2 - *Continued.*

Spot	Mascot (M)/Spectrum Mill (S) score	Peptides (charge state)
8	716 (M) 120.98 (S)	LSELLGVEVVMANDCIGEEVEK + carbamidomethyl(C) (+3) LAAALPEGGVLLLENVR (+2) AHASTEGVTK (+2) ELDYLVGAVANPK (+2) IGVIESLLAK (+2) SLVEEDKLELATSLIEK (+2) GVSLLLPTDIVVADK (+2) IVPATAIPDDWMGLDVGPDATK + oxidation (M) (+2) TFNEALDTTK (+2) TVIWNGPMGVFEFEK + oxidation (M) (+2) GVTTIIGGGDSVAAVEK (+2)
10	394 (M)	LAAALPEGGVLLLENVR (+3, +2) LASVADLYVNDAFGTAHR (+3) ELDYLVGAVANPK (+2) IGVIESLLAK (+2) TVIWNGPMGVFEFEK + oxidation (M) (+2) GVTTIIGGGDSVAAVEK (+2)
11	184 (M)	VLRPFDEVSR (+2) VFLAGADNVLQK (+2) AEEVDEVLGSR (+2)
14	255 (M)	DEEGNPAFALVNK (+2) DEEGYPAFALVNR (+2) LVPYNPGYQDESVLWTESR (+2)
15	153 (M)	DEEGNPAFALVNK (+2) LVPYNPGYQDESVLWTESR (+2, +3)
16	30.86 (S)	TPGGVWWEFGK (+2) TVDAGFNSQHGVTLTVTQGK (+3)
18	155 (M)	MGAVGHDQATDATAVQGVTVSETR + oxidation (M) (+3) VTIGEALEATALAAGDAPVER (+2)
21	298 (M)	LLDMDVGLANIAR + oxidation (M) (+2) EGSVIVIPAGHPTALVAGEDK (+3) VFLAGTNSALQK (+2) LLAFGADEEQQVDR (+2)
23	155 (M)	PGLTIGDTVPNLELDSTHGK (+2, +3) QLNMVDPDEK + oxidation (M) (+2) MFPQGFETADLPSK + oxidation (M) (+2)
25	434 (M)	FTHELLEDAVGNYR (+2, +3) AFLQPSHYDADEVMFVK + oxidation (M) (+2) VVMLLSPVVSTSGR (+2) VVMLLSPVVSTSGR + oxidation (M) (+2) FEEFFPIGGESPESFLSVFSDDVIQASFNTR (+3) GEITTASEEQIR (+2)