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Characterization of maize allergens — MON810 vs. its non-transgenic counterpart

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ARTICLE INFO

Article history:

Received 17 October 2011

Accepted 4 January 2012

Available online 13 January 2012

Keywords:

Maize allergy

Genetically modified food safety

Allergen identification

2D-gel electrophoresis

Western blot

ABSTRACT

One of the main concerns about genetically modified foods and their potential impacts on human health is that the introduction of a new/ altered gene may putatively alter the expression of others, namely endogenous allergens. We intended to evaluate, and to compare, using quantitative real time RT-PCR technique, the expression of 5 already known maize allergens (Zea m14, Zea m25, Zea m27kD, 50kD Zein and trypsin inhibitor) in MON 810 vs. its non-transgenic counterpart, throughout seed development (10, 16 and 23 days after pollination). We have shown that none of the tested allergen genes presented differential expression, with statistic significance, along all tested seed development stages, in MON810 vs. its conventional counterpart.

We have also used bidimensional gel electrophoresis followed by Western blotting with plasma from two maize allergic subjects to characterize their immunologic responses against MON 810 vs. its non-transgenic control. Immunoreactive spots were characterized by MS.

We have identified fourteen new IgE-binding proteins present in both transgenic and non-transgenic maize.

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1. Introduction

Recombinant DNA technology, also known as genetic engineering, allows the transfer of genes between unrelated species. As a result, a genetically modified organism (GMO) may contain one or more proteins originating from another organism.

The application of genetic engineering, for plant improvement and food production, is becoming a common practice. New and diverse plant varieties have been obtained which are pest and/or disease resistant, more productive and with improved nutritional quality, flavour and shelf life. Accord-

ing to ISAAA report 2010 [1], the accumulated hectareage planted between 1996 and 2010 exceeded, for the first time, 1 billion hectares with maize being the second most widespread GM crop, after soybean (31% and 50% of global biotech area, respectively). In EU, maize event MON810 is the only cultivated GM event for food purposes. This maize was developed through a specific genetic modification to be resistant to attack by European corn borer (*Ostrinia nubilalis*), a major insect pest in maize culture. The inserted truncated cry1Ab gene codes for a delta-endotoxin that acts as a potent insecticide highly specific to Lepidoptera (<http://www.gmo-compass.org>).

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In spite of all the potential benefits, some apprehension persists regarding the putative effects of GMOs on human health and environment. One of the main concerns regards GMOs potential allergenicity, namely the possibility of a new/ altered gene to influence the expression of others, including endogenous allergens with impact on the overall plant allergenicity. The European Food Safety Authority panel on GMOs, recommends searching for known allergens [2] and evaluating the possible effects of the genetic modification in GM plants [3] by comparing compositional data of the GM plant *versus* the non-GM counterpart, using either targeted or non-targeted profiling techniques. There are already several examples that validate the use of proteomics [4–7], transcriptomics [4,8,9] and metabolomics [4,10,11] techniques to perform substantial equivalence analysis of transgenic plants.

Even if the correlation between allergen expression and its clinical relevance is not well established, expression studies and the discovery of new potential allergens, could contribute for the growing knowledge in this area.

In maize, there are already some proteins characterized as allergens (<http://www.allergome.org/>), namely a lipid transport protein (Zea m14) [12], a thioredoxin (Zea m25) [13], two glutelins (Zea m27 [14] and 50kD Zein [15]) and one trypsin inhibitor [12].

In this study, we have used quantitative real time RT-PCR to evaluate the expression of these 5 allergens, during seed development, in MON 810 *vs.* its non-transgenic counterpart.

Proteomics techniques were also used to compare the immunologic response of maize allergic subjects against MON 810 *vs.* its non-transgenic control and to characterize the encountered potential maize allergens.

2. Materials and methods

2.1. Plant growth

In this study, we have used a MON810 maize (*Zea mays*) line (DKC6575) and the respective non-transgenic counterpart. Plants of each line were self-pollinated and grown from May to October, in a mixture of turf and soil (1:1) in a glass house under the same environmental conditions for four generations before the experiment. Ears from the fifth generation plants (1 ear *per* plant) were collected and immediately frozen at -80°C , on the 10th, 16th, 23rd (for RNA extraction) and 50th days after pollination (DAP) (for protein extraction). For RNA extraction, 3 ears collected from three different plants, for each time point and line (18 ears total- 18 plants) were used. For protein extraction 5 ears collected from 5 different plants, for each line (10 ears total- 10 plants) were used.

2.2. Identity of the tested samples (transgenic or not)

Transgenesis was checked along generations by PCR (ISO 21569:2005). ELISA GMO Check Bt maize test kit (SDI Europe) was used to evaluate the presence-absence of Bt CryIA(b) protein in all the tested maize seed samples. Manufacturer's instructions were followed.

2.3. RNA extraction and cDNA production

All the seeds from each tested ear were ground in a mill (IKA A11 basic) with liquid nitrogen, and RNA was isolated using

the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions (≈ 100 mg of starting material was used). Extracted RNAs were used for cDNA production (GeneAmp RNA PCR kit- Applied Biosystems). Random hexamers as reverse transcriptase primers and 500 ng of RNA as template were used and manufacturer's recommendations regarding Master Mix (100 μl total volume) and protocol were followed.

2.4. Quantitative real-time PCR (qPCR) assay

Relative expression of the maize allergens Zea m14, Zea m25, Zea m27kD, 50kD Zein and trypsin inhibitor was achieved using the ABI7000SDS (Applied Biosystems), SYBR Green chemistry and the standard curve method. The PCR reagents consisted of: 1x SYBR Green PCR Master Mix (Applied Biosystems), 400nM of each primer, and 5 μl of sample cDNA, in a final volume of 25 μl . The thermocycling profile was: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C . Three reporter genes: 18S ribosomal, actin 1 and α -tubulin genes were tested and 18S Ribosomal gene was selected since it showed the most stable expression among the diverse samples tested (less than $2C_T$ difference).

Standard curves with cDNA sample masses between 18.75 and 600 ng were used (cDNA from a transgenic sample extracted 16 DAP was used). Only standard curves (run in duplicate in each plate) with higher than 85% efficiency and with correlation coefficients (R^2) higher than 0.99, were considered. Each sample was run in duplicate and 300 ng of cDNA was used as starting material. The amount of each gene and 18SrRNA was determined from the respective standard curve by conversion of the mean threshold cycle values, and normalization was obtained by dividing the quantity of each allergen by the quantity of 18SrRNA. The specificity of the amplified products was verified by analysis of the dissociation curves, generated by ABI 7000 software, based on the specific melting temperature for each amplicon.

qPCR primers for the allergens and tested reporter genes (Supplemental Table 1) were designed using ABI7000SDS-specific software, Primer Express (Applied Biosystems).

2.5. Protein extraction

Proteins were TCA/ acetone extracted from seeds of 5 ears (1 *per* plant, randomly selected from each line) harvested 50 DAP (5 g of ground seeds used as starting material), as previously described [16]. The five protein pellets of each of the tested lines were combined and about 2 g of each of the combined pellets were extracted with 6 ml of Extraction Buffer [100 mM Hepes-KOH pH 7.5; 5% Glycerol; 5 mM EDTA; 0.1% β -Mercaptoethanol (added freshly); 1% Proteinase Inhibitor Cocktail for plant proteins (Sigma, added freshly)] for 10 min at 4°C , followed by vortex and 20 sec of ice incubation. The extract was centrifuged twice at 10,000g, for 10 min at 4°C . The supernatant (with approximately 48 mg of protein *per* g of wet weight starting material) was filtered through a 0.45 μm CME filter, dialyzed against milliQ water and lyophilized.

2.6. Gel electrophoresis of proteins from maize flour

The obtained lyophilized pellets were dissolved in solubilisation buffer [2 M thiourea, 0.4% (v/v) Triton X-100, 7 M urea, 4% (w/v) CHAPS, 1% immobilized pH gradient (IPG) buffer

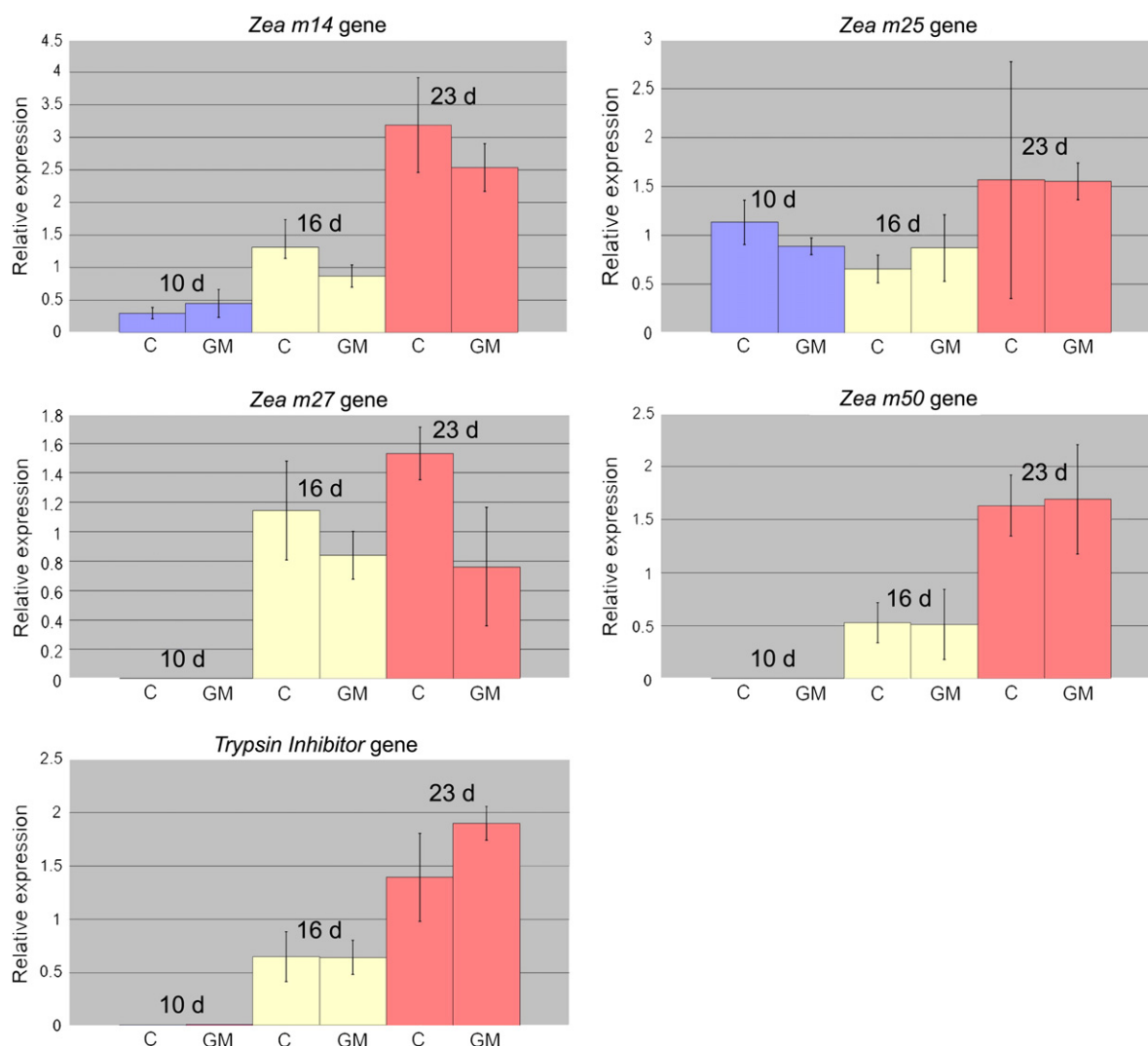


Fig. 1 – Quantitative real time RT-PCR relative expression results for each of the allergens, under test, during seed development. 10 d- 10 days after pollination; 16 d- 16 days after pollination; 23 d- 23 days after pollination; C- control; GM- genetically modified MON 810 maize.

3–11]. The protein was measured according to Ramagli [17], with albumin from chicken egg white (Sigma) as standard.

Isoelectric focusing was done on 13-cm-long IPG strips (GE Healthcare) with a non-linear pH gradient range of 3–11 in an Ettan IPGphor3 instrument, as previously described [18]. SDS-PAGE, on 12.5% T, 1.4% C gels, was performed in a Hoefer SE 600 system. Gels were run, at 15 °C, with constant 30 mA/gel. Bio-line prestained protein molecular weight markers were used.

For one-dimensional SDS-PAGE, the pellets were diluted 1:1 in sample buffer (0.125 M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8) and boiled for 5 min before electrophoresis in 12.5% gels.

2.7. IgE immunoblot reactivity assay of plasma from maize-allergic subjects

Subjects' plasma was purchased from Plasmalab International (Everett, Wash., USA) and obtained from 2 subjects who had a documented history of maize allergy as well as positive

specific UniCAP test values equal or higher than class 3 (Pharmacia Diagnostics) [19]. For negative control, plasma from a non-allergic subject with a class 0 UniCAP test was used.

IgE reactivity in the subjects' plasma, against maize samples, was performed as previously described [18] with the difference that after protein transfer, and before blots blocking, proteins in the nitrocellulose membranes were deep purple stained (GE Healthcare) according to manufacturer's instructions, and the image acquired with a FUJI FLA-5100 scanner. 2D IgE Immunoblot Reactivity Assay was performed in duplicate for each subject.

2.8. Image analysis and spot identification

For image analysis, each chemiluminescence film was overlaid to the correspondent total protein deep purple stained nitrocellulose membrane, enabling the correlation of each spot on the 2-D gel with the immunoreactive protein. For each tested subject, 4 immunoblots were obtained (2 against transgenic and 2 against non- transgenic samples).

Table 1 – MS identification of 2D gel electrophoresis spots.

Spot n°	Protein identification / (Source)	Total ion score	Matches	Theo. Mr (Da)/ pI	Coverage (%)	Blast
1	Prolamin PPROL 17 precursor gi 195658073/ (<i>Zea mays</i>)	154	5	17414/ 9.3	19	
	Globulin-1 S allele precursor gi 195658011/ (<i>Zea mays</i>)	116	16	50275/ 6.2	24	
2	Prolamin PPROL 17 precursor gi 195658073/ (<i>Zea mays</i>)	145	4	17414/ 9.3	19	
3/4	Globulin-1 S allele precursor gi 195658011/ (<i>Zea mays</i>)	328/311	24/24	50275/ 6.2	40/34	
	Prolamin PPROL 17 precursor gi 195658073/ (<i>Zea mays</i>)	194/156	5/4	17414/ 9.3	19/19	
5/7	LEA protein, group 3 gi 226532662/ (<i>Zea mays</i>)	428/307	12/17	18588/ 7.8	47/75	
8	Ethylene-responsive protein gi 226498678/ (<i>Zea mays</i>)	131	9	19513/ 7.8	36	
	Dehydrin DHN1 gi 118484/ (<i>Zea mays</i>)	103	5	17150/ 9.1	31	
	LEA protein, group 3 gi 162463970/ (<i>Zea mays</i>)	144	2	22745/ 8.8	15	
9/10/11/12	Dehydrin gi 18964/ (<i>Zea mays</i>)	115/121/ 119/145	10/8/2/12	16945/ 8.0	51/31/14/51	
13	Hypothetical protein LOC100272498 gi 226500748/ (<i>Zea mays</i>)	105	24	33152/ 5.8	64	gi 108885236 Glucose and ribitol dehydrogenase homolog (I=80%; P=89%; G=3%)
	Vicilin-like embryo storage protein gi 22284/ (<i>Zea mays</i>)	77	16	66635/ 6.2	32	
14	Hypothetical protein LOC100272498 gi 226500748/ (<i>Zea mays</i>)	500	37	33152/ 5.8	68	gi 108885236 Glucose and ribitol dehydrogenase homolog (I=80%; P=89%; G=3%)
15/16/17	Hypothetical protein LOC100382989 gi 293331027/ (<i>Zea mays</i>)	443/346/ 382	33/36/35	57479/ 5.5	58/54/52	gi 15231092 ketol-acid reductoisomerase (I=90%/92%/92%; P=95%/97%/97%; G=0%/0%/0%)
18/29/30	Unknown gi 194690156/ (<i>Zea mays</i>)	384/858/ 837	28/39/37	39022/ 7.5	74/83/74	gi 162462282 fructose-bisphosphate aldolase, cytoplasmic isozyme (I=99%/99%; P=100%/100%; G=0%/0%)
20,21	50kD gamma zein gi 16305113 / (<i>Zea mays</i>)	483/390	15/12	35401/ 6.8	39/30	
22	Hypothetical protein LOC100191846 gi 212275438/ (<i>Zea mays</i>)	564	45	52317/ 5.2	77	gb ACL80329.1 UDP-glucose pyrophosphorylase (I=98%, P=99%, G=0%)
	Hypothetical protein SORBIDRAFT_04g035840 gi 242066822/ (<i>Sorghum bicolor</i>)	103	1	62121/ 7.6	3	sp Q6K669.1 Leucine aminopeptidase 2, chloroplastic (I=92%; P=96%; G=0%)
23	Enolase gi 195619804/ (<i>Zea mays</i>)	122	21	48386 /5.6	59	
	UDP-glucose pyrophosphorylase gi 222142505/ (<i>Saccharum officinarum</i>)	108	12	29652/ 6.0	50	
25	UDP-glucose pyrophosphorylase gi 222142505/ (<i>Saccharum officinarum</i>)	110	16	29652/ 6.0	58	
	Chaperonin CPN60-1, mitochondrial gi 2506275/ (<i>Zea mays</i>)	77	22	61458/ 5.7	43	
26	Chaperonin CPN60-1, mitochondrial precursor gi 162458253 / (<i>Zea mays</i>)	335	42	61438/ 5.7	70	
27	Vicilin-like embryo storage protein gi 22284/ (<i>Zea mays</i>)	28	21	66635/ 6.2	44	
32	Malate dehydrogenase, cytoplasmic gi 162464321/ (<i>Zea mays</i>)	583	33	35909/ 5.8	74	

Table 1 (continued)

Spot n°	Protein identification / (Source)	Total ion score	Matches	Theo. Mr (Da)/ pI	Coverage (%)	Blast
33	Unknown gi 238008704/ (<i>Zea mays</i>)	236	16	60878/ 5.9	39	gi 281312184 Beta-glucosidase 31 (I=70%; 81%; G=3%)
	Succinyl-CoA ligase [GDP-forming] subunit beta gi 15225353/ (<i>Arabidopsis thaliana</i>)	37	1	45602/ 6.3	3	
34	Triacylglycerol lipase gi 226533365/ (<i>Zea mays</i>)	263	27	43017/ 5.2	60	sp B7F7K7.1 Beta-glucosidase 31 (I=70%; P=81%; G=3%)
	Unknown gi 238008704/ (<i>Zea mays</i>)	200	18	60878/ 5.9	39	
	Glutamine synthetase gi 4138351/ (<i>Pinus sylvestris</i>)	78	21	39430/ 5.9	44	
35	Unknown gi 238014324/ (<i>Zea mays</i>)	210	20	59630/ 5.5	31	sp B7F7K7.1 Beta-glucosidase 31 (I=83%; P=92%; G=0%)
36	Adenosine kinase gi 4582787/ (<i>Zea mays</i>)	366	33	36465/ 5.2	64	
	Salt tolerance protein gi 195612984/ (<i>Zea mays</i>)	99	2	35281/ 4.9	8	
37	Desiccation-related protein PCC13-62 gi 226495167/ (<i>Zea mays</i>)	412	10	34238/ 4.8	23	
	LEA protein D-34 gi 226530343/ (<i>Zea mays</i>)	304	22	27275/ 5.4	71	
39/47	Vicilin-like embryo storage protein gi 22284/ (<i>Zea mays</i>)	347 /126	23/31	66635/ 6.2	36/50	
40	Triosephosphate isomerase, cytosolic gi 226529672 (<i>Zea mays</i>)	301	24	27550/ 5.5	64	No significant homology
	Hypothetical protein gi 195626982 (<i>Zea mays</i>)	187	2	26702/ 7.1	7	
	Hypothetical protein OsI_10386 gi 125542768/ (<i>Oryza sativa</i>)	138	2	68602/ 5.7	4	
41	Hypothetical protein SORBIDRAFT_01g043980 gi 242041881/ (<i>Sorghum bicolor</i>)	228	11	75423/ 6.2	21	gi 108706671 Cupin family protein, expressed (I=69%; P=78%; G=4%)
	Soluble inorganic pyrophosphatase gi 226499836/ (<i>Zea mays</i>)	161	12	24366/ 5.4	47	
42	Hypothetical protein LOC100193826 gi 212720920/ (<i>Zea mays</i>)	20	26	29137/ 5.7	85	gi 195619270 hydroxyacylglutathione hydrolase (I=99%; P=99%; G=0%)
	Aspartic proteinase oryzasin-1 gi 226503984/ (<i>Zea mays</i>)	54	17	55263/ 5.4	27	
43	Cysteine proteinase 1 precursor gi 162459555/ (<i>Zea mays</i>)	295	25	40949/ 5.9	35	
44	Chitinase gi 214014554/ (<i>Zea mays</i>)	339	35	29895/ 8.8	79	
	Globulin-1 S allele precursor gi 195658011/ (<i>Zea mays</i>)	330	23	50275/ 6.2	44	
45	Endochitinase A gi 226506778 (<i>Zea mays</i>)	568	43	30490/ 8.4	68	
46	LOC100286330 gi 226529441 (<i>Zea mays</i>)	320	25	23528/ 8.9	61	gb ACV91270.1 LEA group 3 protein (I=44%; P=53%; G=30%) gb ABF95817.1 Cupin family protein, expressed (I=61%; P=69%; G=9%)
	LOC100280650 gi 226509468 (<i>Zea mays</i>)	37	15	56776/ 6.0	27	
50/51/52	27kDa storage protein gi 22550 (<i>Zea mays</i>)	33/21/35	1/1/1	20217/ 8.5	4/4/4	

Identification was not possible for spots 6, 19, 24, 28, 31, 38, 48 and 49; I- Identities; P- Positives; G- Gaps; **Blue** - Significant identification; **Orange** - Several proteins found in the spot; **Red** - already reported maize allergens; LEA- Late embryogenesis abundant. Theo.- theoretical.

The immunoreactive spots were excised from colloidal Coomassie blue [20] or compatible silver [21] stained 2-D gels and MS characterized. These, corresponded to proteins that originated a signal in all four immunoblots obtained for each tested subject.

2.9. Digestion of 2D Gel spots and MS analysis

The silver stained gel plugs have been manually destained in 30 mM $K_3[Fe(CN)_6]$ and 100 mM $Na_2S_2O_3$ and rinsed in milliQ water before the automated digestion and spotting with the Ettan Spot Handling Workstation. After washing and desalting in 50 mM ammonium bicarbonate/50% (v/v) methanol, followed by 75% v/v acetonitrile, spots were digested with Trypsin Gold for 6 h at 37 °C (MS grade, Promega, $5\mu g.mL^{-1}$, in 20 mM ammonium bicarbonate). After extraction, the dried peptides dissolved in a 50% acetonitrile/0.5% trifluoroacetic acid were spotted on disposable target plates (Applied Biosystems,) prior to the deposit of the matrix (α -Cyano-4-hydroxycinnamic acid $7 mg.mL^{-1}$ in 50% v/v acetonitrile, 0.1% v/v trifluoroacetic acid, Sigma Aldrich).

2.10. Database search

Peptide mass determinations were carried out using the 4800 Proteomics Analyzer (Applied Biosystems) in reflectron mode for both peptide mass fingerprint and MS/MS. Calibration was performed with the peptide mass calibration kit for 4700 (Applied Biosystems). Protein identification was done by searching the MS and MS/MS data against NCBI database, downloaded on September 24, 2010, in the viridiplantae taxonomy (1143124 sequences), using MASCOT (<http://www.matrixscience.com>). Two missed trypsin cleavages, four dynamic modifications (methionine and tryptophan oxidation, tryptophan dioxydation and tryptophan to kynurenin), and carboxyamidomethylation of cysteine as fixed modification were allowed. Mass accuracy was set to 150 ppm for parent ions and 0.75 Da for MS/MS fragments. Proteins were identified with a minimum of two MS/MS spectra matching the databank sequence, with a total ion score above 60. All the identifications were manually validated.

3. Results and discussion

3.1. Expression of the five tested allergens during seed development (quantitative real time RT-PCR assay)

The results of relative expression obtained throughout seed development for each allergen tested, are presented in Fig. 1. One of the conclusions we can retrieve from these data is that gene expression varies between replicas, which was particularly evident in samples collected 23 DAP. The fact that at this developmental stage, starch reserves are reaching the maximum [22] may have contributed to the observed variability. Additionally the eventual presence of other non-nucleic acid compounds may have also interfered with RNA purification and qRT-PCR. These conditions together with the natural plant variability may have negatively impacted replicate reproducibility. As previously recommended plant natural variability should be

always considered in any food safety assessment [16]. Another clear observation was that gene expression was not constant during seed development. Three of the tested genes, Zea m27, Zea m50 and trypsin inhibitor were almost not expressed 10 DAP, and their expression increased throughout seed development. Zea m14 although slightly expressed 10 DAP also increased during seed development; on the contrary Zea m25 expression seemed to be relatively stable along all the analysed time points. For all the tested genes no statistical difference ($p < 0.05$ in t-test) was found in expression levels when comparing GM vs. non-GM plants.

3.2. IgE-binding proteins in transgenic vs. non-transgenic maize (immunoblot assay)

Table 1 and Supplemental Table 2 summarize our MS data and, in Table 2 the potential maize allergens reported so far are listed together with the ones identified in our work. Moreover, the reactions of the two maize allergic subjects and the non-allergic control, against total protein extracts of the two maize lines analysed, are shown in Figs. 2 and 3 and Supplemental Fig. 1. These experiments aimed not only to compare the reaction of these subjects against protein samples from transgenic vs. non-transgenic maize, but also to identify maize IgE-binding proteins. We found that the two subjects under test had completely different reactions against the same maize protein sample although with some common immunoreactive proteins (blue shadowed in Table 2). Furthermore, the two subjects reacted very similarly against transgenic vs. non-transgenic samples. While in subject 1 (male, 41 years-old, maize UniCAP class 4), we found one signal present only in the MON 810 maize chemiluminescence film, in subject 2 (male, 27 years-old, maize UniCAP class 3) two signals were exclusively present in the non-transgenic maize chemiluminescence film (green arrows in Fig. 3). It was not possible to correlate any of these signals with spots in the respective gels. This may have happened because of the much higher sensitivity of the IgE immunoblot assay as compared with the protein available dyes. This fact together with the use of only two maize allergic subjects in this assay, didn't allow us to speculate about transgenic vs. non-transgenic maize lines potential allergenicity.

The control subject presented no reaction against both maize protein extracts under test (Supplemental Fig. 1).

3.3. Identified IgE-binding proteins already reported as potential maize allergens

Several already known maize allergens (<http://www.allergome.org/>) were detected in this experiment. Spots 3 and 4 correspond to globulin-1 S allele precursor, a storage protein belonging to cupin superfamily. This protein has 98% identity with globulin-2 precursor (Uniprot Q7M1Z8) already reported as a maize allergen [23]. Spots 20 and 21 regard 50 kDa gamma zein allergen (Zea m50kD zein) [14,23]. Spot 23 corresponds to enolase, a glycolytic enzyme related to drought stress in rice [24] and already in silico identified as a potential allergen (Zea m22). Spots 27, 39 and 47 correspond to potential maize allergen Vicilin-like embryo storage protein (Zea mG1), a nutrient reservoir activity protein belonging to cupin superfamily [23]. Finally, spots 44 and [45 46]

Table 2 – Maize allergens already reported and new potential maize allergens identified in this study.

	Maize allergen	Spots N°	Protein information
	Zea m11		Trypsin inhibitor /2S albumin family/ 16kDa (exp.); 13.6 (theor.)
	Zea m14		Lipid Transfer protein/ Prolamin superfamily/ 9kDa (exp.); 11.3 (theor.)
	Zea m22	23	Enolase/ Glycolytic enzymes family/ 48.3 kDa (theor.)
	Zea m25		Thioredoxin/ 13.9 kDa (theor.)
	Zea m27	50-52	Glutelin / Prolamin superfamily/ 27kDa (exp.); 20.2 (theor.)
	Zea m 50kD zein	20, 21	Glutelin/ Prolamin superfamily/ 35.4kDa (theor.)
	Zea m chitinase	44,45	Chitinase/ 29.0kDa (theor.)
	Zea mG1	27, 39, 47	Vicilin-like embryo storage protein/ Cupin superfamily/ 66.6kDa (theor.)
	Zea mG2	3, 4	Globulin-2 precursor/ Cupin superfamily/ 49.9kDa (theor.)
		1,2	Prolamin PPROL 17 precursor 17.4kDa (theor.)
		5,7	LEA protein, group 3/ 18.6kDa (theor.)
		8	Ethylene-responsive protein/ 19.5kDa (theor.)
		9-12	Dehydrin/ 16.9 kDa (theor.)
		15- 17	Ketol-acid reductoisomerase
		22,25	UDP-glucose pyrophosphorylase/ 29.7kDa (theor.)
		26	Chaperonin CPN60-1, mitochondrial precursor/ 61.4kDa (theor.)
		18, 29, 30	Fructose-bisphosphate aldolase
		32	Malate dehydrogenase/ 35.9kDa (theor.)
		34	Triacylglycerol lipase/ 43.0kDa (theor.)
		36	Adenosine kinase (ADK)/ 36.5kDa (theor.)
		37	Maize desiccation-related protein/ 34.2kDa (theor.)
		40	Triosephosphate isomerase/ 27.5kDa (theor.)
		43	Cysteine proteinase 1 precursor/ 41.0kDa (theor.)

Green: Expression along maize seed development tested by real time quantitative RT-PCR; **Red**: Already reported allergens that were also identified in the IgE Immunoblot reactivity assay of plasma from the maize-allergic patients; **Purple**: New potential maize allergens identified in this study; **Yellow**: Stress related proteins; **Blue**: Allergens detected in the chemiluminescence films of both individuals. Protein identification of spots 27, 50, 51 and 52 (in **bold**) was not significant. exp.- experimental; theo.- theoretical.

matched with chitinase and endochitinase A, respectively. These proteins belong to the group of the “pathogenesis-related proteins” and are involved in plant defence against chitin containing fungal pathogens. Endochitinases had been previously identified as potential allergens in several food plants, namely grape [25], gold kiwifruits [26], raspberry [27] and maize [23,28].

3.4. New potential maize allergens identified

Spots 5 and 7 matched a late embryogenesis abundant (LEA) protein. LEA proteins occur naturally in maturing seeds as they desiccate for dormancy helping to stabilize membranes and other proteins of the dehydrated cells. Also, the expression of LEA proteins under drought stress in plants and animals support their role in desiccation tolerance [29,30]. When studying soybean allergy, we have already indicated the involvement of LEA proteins in plant allergy [18].

In this experiment, several other stress-related proteins were also identified as potential maize allergens. Spots 9–12 corresponded to a Dehydrin, which is a plant protein family known to accumulate in response to low temperatures [31] and drought

stress [32]. Spot 8 has significantly matched three stress-related proteins with the highest score matching a transcription factor induced by ethylene, that may be important in regulating gene expression during the plant defence response [33]. Spot 26 has significantly matched with a Chaperonin CPN60-1, mitochondrial precursor, from maize. This protein belongs to a family involved in correct folding and assembly of polypeptides into oligomeric structures. They are required for normal cell growth and may be stress-induced, acting to stabilize or protect disassembled polypeptides under heat-shock conditions [34]. Spot 36 highest score leads to a maize Adenosine kinase (ADK), an enzyme that catalyzes the phosphorylation of adenosine to adenosine monophosphate. In plants, ADK is believed to be the predominant enzyme acting for the salvage of adenosine which, besides contributing to adenylate levels, can also impact numerous metabolic processes. Wang *et al.* also reported a role of ADK in plant defence against viral infection [35]. The highest score correspondence of spot 37 leads to a maize desiccation-related protein, and spot 43 to a Cysteine proteinase 1 precursor. This protein belongs to peptidase C1 family and may be involved in the response to herbivory [36]. Additionally to all these proteins, we have also identified, as potential maize allergens, several metabolism-related proteins that were also

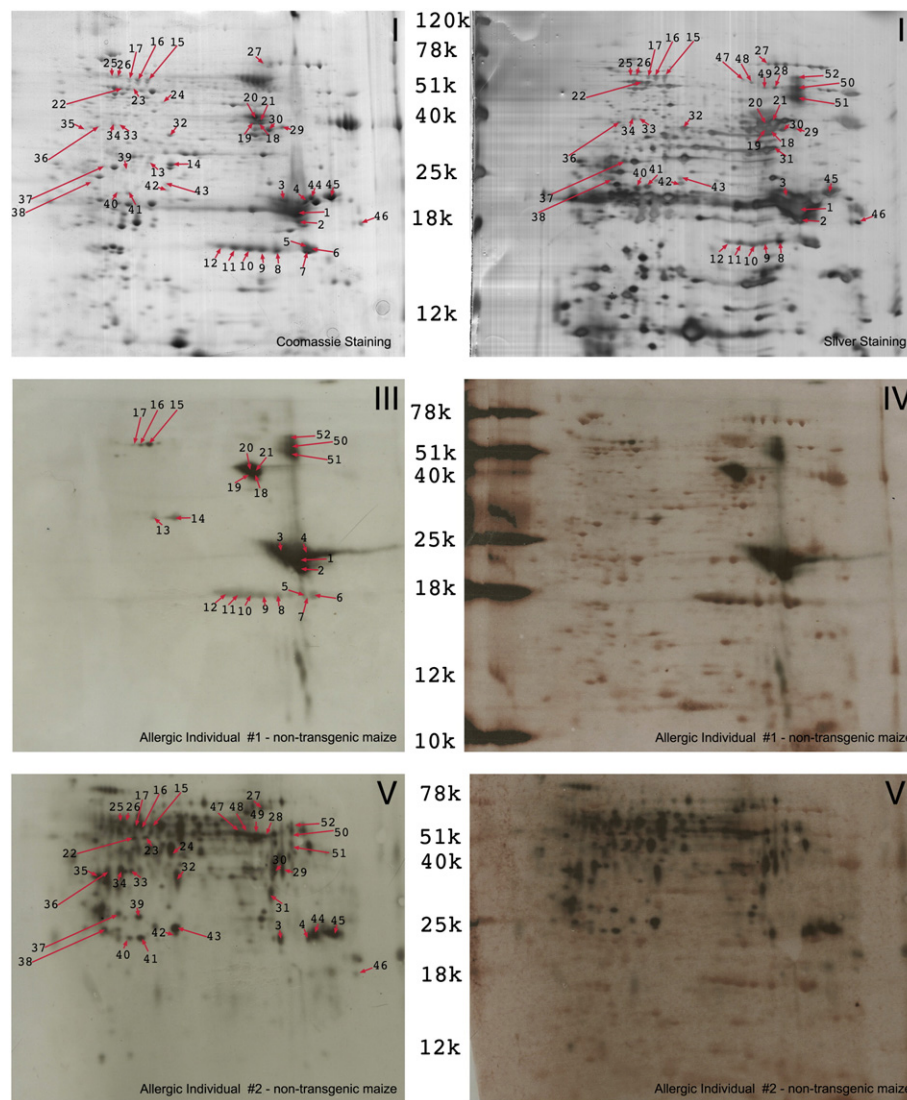


Fig. 2 – IgE Immunoblot Reactivity Assay of plasma from maize-allergic subject 1 and 2 against non-transgenic total protein maize sample: I Example of a Coomassie blue stained maize seed total protein 2D electrophoresis gel; II Example of a silver stained maize seed total protein 2D electrophoresis gel; III and V IgE immunoblots of subject 1 and 2, respectively, against non-transgenic maize protein extracts; IV and VI IgE immunoblots of subject 1 and 2, respectively, against non-transgenic maize protein extracts over respective Deep-purple stained nitrocellulose membrane; Red arrows- immunoreactive protein spots. Two-dimensional gels were run with 400 µg of total protein in 12.5% gels. k=kDa.

associated with drought stress in rice [24]; ketol-acid reductoisomerase (spots 15–17 - involved in the biosynthesis of branched-chain amino acids isoleucine, leucine and valine); UDP-glucose pyrophosphorylase (spots 22 and 25- produces UDP-glucose which is essential for sucrose and polysaccharide synthesis); fructose-bisphosphate aldolase (spots 18, 29 and 30- involved in glycolysis) and malate dehydrogenase (spot 32 - involved in the tricarboxylic cycle). From the 23 potential maize allergens presented in Table 2, 16 may be related with stress (70%). The potential association of stress-related proteins with allergenicity was previously documented for pathogenesis-related proteins [37,38].

Finally, the seed storage protein- prolamin PPROL 17 precursor (spots 1 and 2) and the metabolism related proteins-

triosephosphate isomerase (spot 40 - involved in glycolysis) and triacylglycerol lipase (spot 34 - catalyzing the initial steps of lipid mobilization) were also identified as potential maize allergens.

3.5. Already reported maize allergens that were not identified in the immunoblot assay

Although fourteen new potential maize allergens and several already reported maize allergens were identified in the immunoblot assay, 4 previously reported maize allergens (Zea m11-Trypsin inhibitor; Zea m14-Lipid Transfer protein; Zea m25-Thioredoxin and Zea m27-Glutelin) could not be identified. Interestingly, although the protein identification of

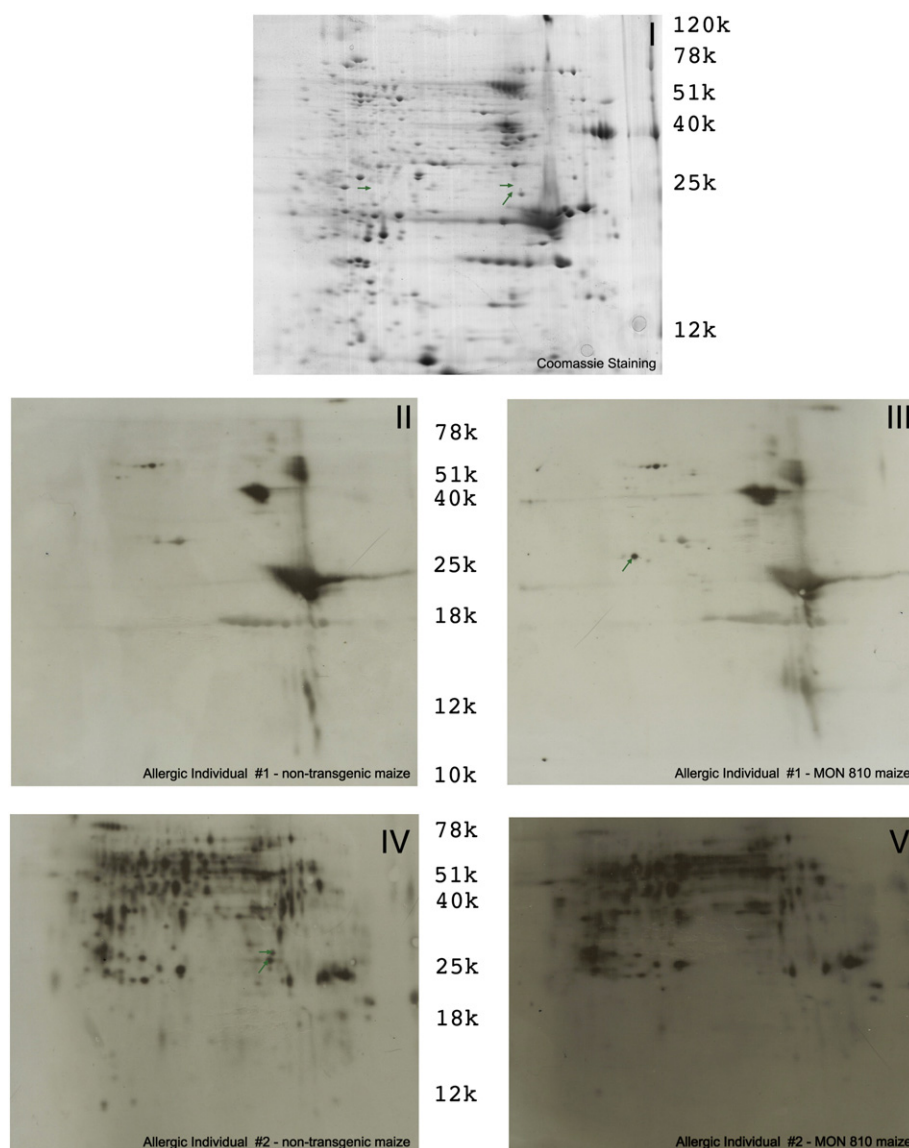


Fig. 3 – Immunoreactivity differences against non-transgenic vs. transgenic maize samples. I Example of a Coomassie blue stained maize seed total protein 2D electrophoresis gel; **II** and **III** IgE immunoblots of subject 1 against non-transgenic and transgenic maize protein extracts, respectively; **IV** and **V** IgE immunoblots of subject 2 against non-transgenic and transgenic maize protein extracts, respectively. Green arrows- Spots that presented different immunoreactivity in transgenic vs. non-transgenic samples. It was not possible to correlate any of these signals with spots in the respective gels (Coomassie or silver stained).

Spots 50–52 was not significant, according to the statistical criteria used, all 3 spots had a peptide match with the Zea m 27 maize storage protein.

As already stated, and pointed out in Table 2, a high percentage of the proteins identified as potential maize allergens may be also related with stress response. Three of these four proteins were also already associated to stress. Maize Trypsin inhibitor was already related to drought stress response [39] and confirmed as involved in resistance to *Fusarium moniliforme* [40]. Lipid transfer proteins are potent inhibitors of bacterial and fungal plant pathogens [41,42] and some LTP genes are known to be induced under drought stress [43]. Finally, maize Thioredoxin was also involved in abiotic stress response [44].

4. Conclusions

We found no statistically significant differences in the expression of the five tested maize allergens (Zea m14, Zea m25, Zea m27kD, 50kD Zein and trypsin inhibitor) throughout seed development of MON 810 transgenic vs. its non-transgenic counterpart.

Considering the small number of allergic subjects tested and the impossibility to correlate the differences found in the Immunoreactivity assays with spots in the corresponding gels, we could not draw conclusions regarding the differential allergenicity of the two maize lines (transgenic vs. non-transgenic). Nevertheless, in this work we could identify 14 new IgE-binding proteins from maize.

Author's contributions

Cátia Fonseca- Contributed to the study conception and design; IgE immunoblot reactivity assay of plasma from maize-allergic subjects data acquisition its analysis and interpretation; MS data interpretation; writing and revision the final version of the paper; final approval of the version to be published.

Jenny Renaut- Contributed to MS data acquisition its analysis and interpretation; writing and revision the final version of the paper; final approval of the version to be published.

Sébastien Planchon- Contributed to MS data acquisition its analysis and interpretation; writing and revision the final version of the paper; final approval of the version to be published.

Maria Margarida Oliveira- Contributed to the study conception and design; writing and revision the final version of the paper; final approval of the version to be published.

Rita Batista- Contributed to the study conception and design; experimental steps of plant growth, RNA and protein extractions and cDNA production; Real time quantitative PCR assay and IgE Immunoblot Reactivity Assay of Plasma from Maize-Allergic Patients data acquisition its analysis and interpretation; MS data interpretation; writing and revision the final version of the paper; final approval of the version to be published.

Conflict of interest statement

The authors warrant that the article is original, does not infringe upon any copyright or other proprietary right of any third part, is not under consideration by another journal, and has not been previously published. The authors confirm that they have reviewed and approved the final version of the manuscript and concur with submission. All authors declare that there are no conflicts of interest.

Supplementary materials related to this article can be found online at [doi:10.1016/j.jprot.2012.01.005](https://doi.org/10.1016/j.jprot.2012.01.005).

Acknowledgements

We thank Prof. Margarida O. Krause for the revision of the manuscript.

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Supplemental table 1- Primers used in the real time quantitative PCR assay after cDNA synthesis

	Gene	NCBI entry	Primers (5'-3')
Target genes	Zeam 14	NM_001111841	Zeam14 (7/8) Forward: CCGCCTGAAGACGATCCAT Reverse: CAAGTAGTGATCATGCGTAGGTAGATATG
	Zeam 25	NM_001112341	Zeam25 (1/2) Forward: CGATGCCGACCTTCCACTT Reverse: CTTGGCACCCACGATGGT
	Zeam 27	NM_001111884	Zeam27 (1/2) Forward: TCACCACCACTGGGTCTTCA Reverse: TGGTGTGATCGGGTTCTTC
	Zeam 50	NM_001111583	Zeam50 (7/8) Forward: TGCTTTCATTGCTTTAGTATCAAGTGT Reverse: CAGCCGCCTGTCTGTGTACA
	Trypsin inhibitor	NM_001112762	Trypsin (7/8) Forward: GTCTGAATAATAAGCACAGCAAGAAGA Reverse: TGAACGATCCGAGAAATGCAT
Reporter genes	18S	AF168884	18S (1/2) Forward: CCTGAGAAACGGCTACCACATC Reverse: CCCGTGTCAGGATTGGGTAA
	Actin 1	J01238	Actin (1/2) Forward: CTTCGAATGCCCAGCAATG Reverse: GTTCGCCCCACTAGCGTACAAC
	α -Tubulin	AF249276	Tubulin (3/4) Forward: TCCAGGCTGATGGTCAGATG Reverse: CATCACCACCCCAATGGT