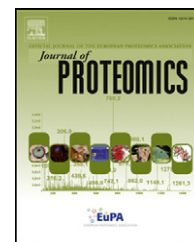


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How much does transgenesis affect wheat allergenicity? Assessment in two GM lines over-expressing endogenous genes

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ABSTRACT

Wheat kernel albumins/globulins (A/G) and gluten proteins are responsible for baker's asthma and food allergy in atopic subjects. Although no commercial genetically modified wheats are currently being grown, they are under study and the allergenicity of GM products is a major concern.

In order to establish the expected and unexpected effects of genetic transformation on allergenicity and also to carry out a safety assessment of genetic transformation, two GM wheat lines (bread and pasta wheat) transformed with endogenous genes were compared to their untransformed counterparts (wt), first by an allergenomic approach, and second, using ELISA with sera from patients suffering from food allergy to wheat and baker's asthma. The 2D immunoblots performed on sera from patients suffering from food allergy and baker's asthma on the A/G fraction of the four lines (two GM and two wt) revealed comparable IgE-binding profiles. A total of 109 IgE-binding spots were analyzed by mass spectrometry, and most of the proteins identified had already been described as allergens or potential allergens. Only two IgE-binding proteins were specific to one GM line. The concentration of specific IgE against the A/G fractions of GM wheat lines and their wt genotypes differed for some sera.

Biological significance: The originality of our paper is to relate the transformation of wheat lines with their potential allergenicity using patient sera, such focus has never been done before in wheat and should be of interest to the researches working in this field.

Another interesting point of this paper is the study of two types of allergies (respiratory and food) on two wheat genotypes and their GM which reveals that some allergens already known in respiratory allergy could be involved in children suffering from wheat food allergy. In this paper we used a classical 2D proteomic analysis and the protein identifications were performed by mass spectrometry after spot picking and in gel trypsin hydrolysis. Concerning the LC-MS/MS analyses classical software and parameters were used as described in [Material and methods](#). We worked on wheat which is actually not fully

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sequenced that was a difficulty; we therefore searched against two databanks (proteins and ESTs) in order to compare the results. Moreover all proteins reported in our paper were identified with at least three unique peptides.

The identified proteins were checked for their potential allergenicity. In order to have a best interpretation of protein identified in terms of potential allergens, BLAST alignments were performed by using an allergen databank (SDAP). This allows the determination of the cross-reactivity of these identified proteins with known allergens of other species and also the prediction of a potential allergenicity.

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1. Introduction

The use of genetic engineering for plant improvement and food production is becoming a common practice with a record increase in the area designated for biotech crops between 1996 and 2010, making GM crops the fastest adopted crop technology in the history of modern agriculture [1]. However, concerns about the unintended effects of gene insertion (such as increased toxicity or allergenicity) necessitate a safety assessment of foods derived from GM plants, which is currently regulated by the Codex Alimentarius (ftp://ftp.fao.org/es/esn/food/guide_plants_en.pdf). This document includes a specific paragraph addressing the question of allergenicity, the modified content of known allergens and the appearance of new and unknown allergens for GM plants. Moreover, the European Food Safety Authority (EFSA) recommends comparing GM food with its appropriate counterpart, when the recipient of the introduced gene is known to be allergenic [2]. Bioinformatic tools can be used to predict the potential allergenicity of proteins, based on known IgE binding sites of allergens [3–6], but are ineffective for a safety assessment of genotypes containing potential allergenic proteins that are not well characterized.

Wheat is an important part of the daily diet of millions of people, with a total production of about 600 million tons per year, 75% of which is used for food [7]. About 95% of the wheat cultivated worldwide is bread wheat, *Triticum aestivum*, with an AABBDD genome, whereas 5% is durum wheat, *Triticum durum*, whose genome is AABB [8]. The distinctive feature that makes wheat unique compared to other cereals is the viscoelastic properties of its dough, which determine its capacity to give a specific end-product [9]. However, this staple food is one of the six major food allergens [10,11]. Wheat grain proteins are classically subdivided into the water/salt-soluble fraction (including albumins and globulins, A/G), which represents about 20% of the total amount of proteins, and the water/salt-insoluble gluten, which contains gliadins (Gli) and glutenins (Glu) [12]. Gliadins are monomeric proteins that can be broken down into three categories: α/β gliadins, γ -gliadins and ω -gliadins. Glutenins are subdivided into high- and low-molecular weight glutenin subunits (HMW-GS and LMW-GS, respectively) [13]. Most gluten proteins, as well as some of the A/G fraction, are responsible for triggering food allergic reactions [13–15]. Moreover, the latter are also responsible for one of the most common types of occupational allergy, known as baker's asthma [16,17]. The officially recognized allergens are listed in the WHO/IUIS Allergen Nomenclature (www.allergome.org). Even if some gliadins have also been

identified as respiratory allergens [18], they are less relevant than those found in the A/G fraction.

Although no commercial GM wheat is currently grown anywhere in the world, there is an increasing interest in this alternative procedure to classic plant breeding. Whatever the target of the transformation, assessment of the safety of such new GM wheat genotypes is essential as it may induce a change in the expression of endogenous allergens. Such studies have been reported for other GM crops, such as rice, soybean and maize [19–22].

In this study, we evaluated the expected and unexpected effects of two different genetic transformation lines on wheat food and respiratory allergenicity: first a GM bread wheat line (based on the cultivar Bobwhite) obtained through the over-expression of an endogenous wheat gene coding for a LMW-GS [23], a class of proteins known to be allergenic [24,25], and second, a durum wheat line (based on the cultivar Svevo) over-expressing the endogenous Wx-B1 gene encoding a granule-bound starch synthase [26]. In both cases, the transgenes are only expressed in the endosperm. These lines are referred to in the text as: BW-wt for the untransformed Bobwhite genotype, BW-GM for the transgenic line, SV-wt for the untransformed Svevo genotype and SV-GM for the transgenic line. The determination of the composition of the three main protein classes (A/G, Gli and Glu) revealed large variations that could potentially induce differences in IgE reactivity. In the case of BW-GM, the reactivity of the Glu class was specifically examined because LMW-GS proteins have already been defined as allergens (Tri a 36, <http://www.allergen.org>). We used an allergenomic approach to identify IgE-binding polypeptides in the A/G fraction of both the transgenic lines and compared them to their wild-type parents. Finally, for both genotypes Svevo and Bobwhite, we also examined the IgE-binding potential against each A/G fraction as it was particularly affected by the two genetic transformation events.

2. Material and methods

2.1. Patient sera

Sera were obtained from 21 patients with clinically documented baker's asthma (BA) or a food allergy (FA). The patients' clinical data (symptoms, age, wheat cv Réctal-specific IgE) are summarized in Table S1. Sera were obtained from the Service of Clinical Immunology and Allergology at Epinal Hospital, France, and the University Hospital of Udine, Italy, with the informed

consent of the patients. Control sera were obtained from healthy volunteers.

2.2. Wheat samples

Wheat flours were prepared from mature kernels of two transformed lines obtained using the biolistic method and their wt parents as controls. The first line was *T. aestivum* L. cv Bobwhite, which strongly over-expresses a transgenic *lmw-gs* gene coding for a storage protein (GenBank ID: AAO53265) as described by Masci et al. [23]. The transgenic genotype used corresponds to the T₅ generation. The second line was *T. durum* cv Svevo (line MJ 11–12), which over-expresses the Wx-B1 gene involved in amylose synthesis and expresses the granule-bound starch synthase (GBSSI, GenBank ID: AAB26860.1) [26]. In both cases, the transgenes were under the control of endosperm-specific promoters. The transgenic genotype used corresponds to the T₄ generation. Both GM lines are produced for research purposes only. The two GM wheat lines and their control wt genotypes were cultivated in the greenhouse under the same conditions.

2.3. Wheat protein extraction (A/G, gliadin and glutenin subunits)

Salt-soluble proteins (A/G fraction) were extracted from 3 g of wheat flour mixed with 80 mL of extraction buffer containing 0.05 M phosphate buffer/0.1 M NaCl, pH 7.8, for 2 h at 4 °C. After centrifugation at 8000 *g* for 15 min at 4 °C, the supernatant corresponding to the A/G was collected and dialyzed at 4 °C against water for 3 days. The remaining pellet corresponding to the salt-insoluble fraction was further fractionated into gliadins and glutenins according to Battais et al. [27].

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA tests were performed to determine the concentrations of specific IgE against the A/G fractions and, for BW-wt and BW-GM only, against various gliadins (α/β -, γ -, ω 2- and ω 5-gliadins) and LMW-GS.

ELISA tests were performed with patient sera in microtiter plates (Nunc Immobilizer Amino) as described by Larré et al. [28]. The concentration of specific IgE binding to the antigen (A/G) was calculated using a standard curve, as described by Bodinier et al. [29]. Four replicates and four controls with 10 mM ethanolamine in carbonate were performed for each serum. Data are presented as mean values with their standard errors. The t-test was used to compare the two GM lines and their wt genotypes, and differences were considered significant when the *p*-value was <0.01.

To analyze the gluten protein fractions, antigen-coated plate ELISA was performed. The wells of microtiter plates (Nunc MaxiSorp) were coated with 5 μ g/mL in 100 mM carbonate buffer (pH 9.6) of antigen for 1 h at room temperature. After three washes with PBS-0.5% Tween 20, the plates were blocked with 2% PBS-milk for 1 h at 37 °C. The plates were washed three times with PBS-0.5% Tween 20, and incubated for 1 h at 37 °C with anti-peptide antibodies (specific to α/β -, γ -, ω 2-, ω 5-, LMW-GS or HMW-GS) [30,31]. The curves were obtained with a serial dilution of antibodies

from 1:500 to 1:8000 in PBS. Goat anti-rabbit IgG anti-body (H+L) Horseradish Peroxidase Conjugate Human IgG Adsorbed (BioRad), diluted to 1:3000 in PBS, was incubated for 1 h at 37 °C after three washes with PBS-0.5% Tween 20. The colorimetric substrate (OPD, o-phenylenediamine/H₂O₂) in 0.05 M citrate buffer, pH 5.5, was added for 30 min at room temperature. The OPD reaction was stopped with 4 N H₂SO₄ and read at 490–630 nm. Three to five replicates were performed for each antibody dilution, and the average was calculated.

2.5. Gliadin and glutenin subunits separation by SDS-PAGE and immunoblotting

This procedure was performed on the two bread wheat genotypes, BW-wt and the BW-GM line. The gliadin and glutenin subunit extracts were solubilized in Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, bromophenol blue (and 4% β -mercaptoethanol for the glutenin subunits), adjusted to a concentration of 1 mg/mL, and 10 μ L was loaded onto the 1D SDS-PAGE on 15% polyacrylamide mini-gels (BioRad). Polypeptides were either stained with Coomassie Brilliant Blue G-250 [32] or electroblotted to nitrocellulose membranes (0.2 μ m, Sartorius, Germany) in 25 mM Tris, 192 mM glycine, 0.1% SDS with a semidry transfer for 45 min at 300 mA. The membranes were blocked with PBS containing 0.1% Tween 20 (PBST) and 4% PVP (SIGMA) for 4 h at room temperature. After washing with PBST/2% PVP (buffer 1) three times, the membranes were incubated overnight with patient serum diluted from 1:20 to 1:50, depending on the level of wheat-specific IgE in buffer 1. After three washes with buffer 1, the membrane was incubated for 1 h with peroxidase-labeled rabbit anti-human IgE (P0295, Dako, Denmark) diluted to 1:100,000. Finally, after three further washes with buffer 1 and two with PBS, membranes were incubated with 4 mL of chemiluminescent substrate (Super Signal West Dura Extended Duration Substrate, 34076, Pierce, USA) according to the manufacturer's recommendations. The bound IgE-antibodies were detected with the Luminescent Image Analyzer LAS 3000 (Fujifilm) [28].

2.6. A/G fraction separation by 2D gel electrophoresis (IEF vs. SDS-PAGE)

Before solubilization of proteins, the freeze-dried A/G extracts were desalted by precipitation with 2 mL of acetone at –20 °C. Two-dimensional gel electrophoresis was performed according to the protocol described by Larré et al. [28]. Polypeptides were either stained with Coomassie Brilliant Blue G-250 [32] or electroblotted to nitrocellulose membranes (0.2 μ m, Sartorius, Germany) in 25 mM Tris, 192 mM glycine, 0.1% SDS with a semidry transfer for 45 min at 300 mA. The stained gels were scanned with an Imaging Densitometer (GE Healthcare) at 300 dpi.

2.7. Detection of IgE-binding polypeptides by 2D immunoblotting

The non-stained 2D gels with the A/G fractions were transferred to a nitrocellulose membrane for 1 h. Due to an insufficient amount of sera, only the portion of the membrane

between pI 4 and 9 (10 cm×10 cm), in which the majority of polypeptides were present, was immunoblotted with patient sera, and it was not possible to perform replicates. The membranes were blocked with PBS containing 0.1% Tween 20 (PBST) and 4% PVP (SIGMA) for 4 h at room temperature. After washing with PBST/2% PVP (buffer 1) three times, the membranes were incubated overnight with patient serum diluted in buffer 1 from 1:20 to 1:80, depending on the level of wheat-specific IgE. After three washes with buffer 1, the membrane was incubated for 1 h with peroxidase-labeled rabbit anti-human IgE (P0295, Dako, Denmark) diluted 1:100,000. Finally, after three further washes with buffer 1 and two with PBS, membranes were incubated with 4 mL of chemiluminescent substrate (Super Signal West Dura Extended Duration Substrate, 34076, Pierce, USA) according to the manufacturer's protocol. The bound IgE-antibodies were detected with the Luminescent Image Analyzer LAS 3000 (Fujifilm) [28]. The serum from a non-allergic subject was used as a negative control [33].

2.8. Protein identification by LC-MS/MS

Protein spots were picked up manually and prepared for mass spectrometry. In-gel digestion was performed using trypsin hydrolysis following the protocol described in Larré et al. [34].

Nanoscale capillary liquid chromatography–tandem mass spectrometry (LC-MS/MS) analyses of the digested proteins were performed using an Ultimate U3000 RSLC system (Dionex) coupled with an LTQ-Orbitrap VELOS mass spectrometer (Thermo Fisher). Chromatographic separation and mass data acquisitions were performed as described in Larré et al. [28].

Raw data collected during LC-MS/MS analyses were processed into *mgf* (mascot generic format) files and further searched against databanks using XTandem! software 2008.02.01 (<http://www.thegpm.org/TANDEM/>), with a user-interface designed by B. Valot and available at <http://pappso.inra.fr/bioinfo/xtandempipeline/>. Protein identification was achieved by interrogating the UniProt databank restricted to Viridiplantae (release 2011_012, December 2011) with mass data (MS and MS/MS spectra). Another search was performed against the Wheat TIGR Gene Indices databank (<http://compbio.dfci.harvard.edu/tgi/>, release 12 from April 2010). Fixed modification of cysteine residues by iodoacetamide was considered, as well as oxidized methionine as potential modifications. Precursor mass and fragment mass tolerance were set at 5.0 ppm and

0.8 Da, respectively. One missed trypsin cleavage was allowed. Proteins were considered as valid when they had an E-value below 10^{-4} and when they were identified with a minimum of three unique peptides matching their sequence with an E-value below 0.001. Best matches were validated, and when the results were identical in the two databanks, the identity determined in UniProt was chosen.

2.9. Molecular allergenicity prediction

A FASTA alignment was performed between new IgE-binding proteins (accession number described in Table S2) and the nr databank and the Structural Database of Allergenic Proteins (SDAP; update August 20, 2012; <http://fermi.utmb.edu/SDAP/>). A threshold of amino acid identity above 35% through a window of 80 amino acids was fixed in order to determine potential cross-reactivity with known allergens [6].

3. Results

3.1. Characterization of the three main protein classes of wheat grain (A/G, Gli and Glu)

In order to investigate a possible change in protein accumulation caused by genetic transformation, the amounts of the major classes of proteins, A/G, gliadins and glutenins, were compared between the two GM lines and their corresponding wt genotypes, Svevo and Bobwhite (Fig. 1). As expected on the basis of the over-expressed gene introduced into this line, the BW-GM line showed a marked increase in the glutenin class (45%), accompanied by a decrease of 49% and 30% for the gliadin and A/G classes, respectively. The genetic transformation of the SV-GM line did not directly affect protein accumulation; nevertheless we observed an unexpected decrease in the Gli and Glu classes (10% and 27%, respectively) and an increase (37%) in the salt-soluble A/G class.

3.1.1. Focus on the Gli and Glu fractions of Bobwhite genotypes
The large increase in the gluten protein in BW-GM due to the over-expression of a LMW-GS drove us to examine the composition of its Glu and Gli fractions using specific antibodies. The amounts of different gliadins and glutenins

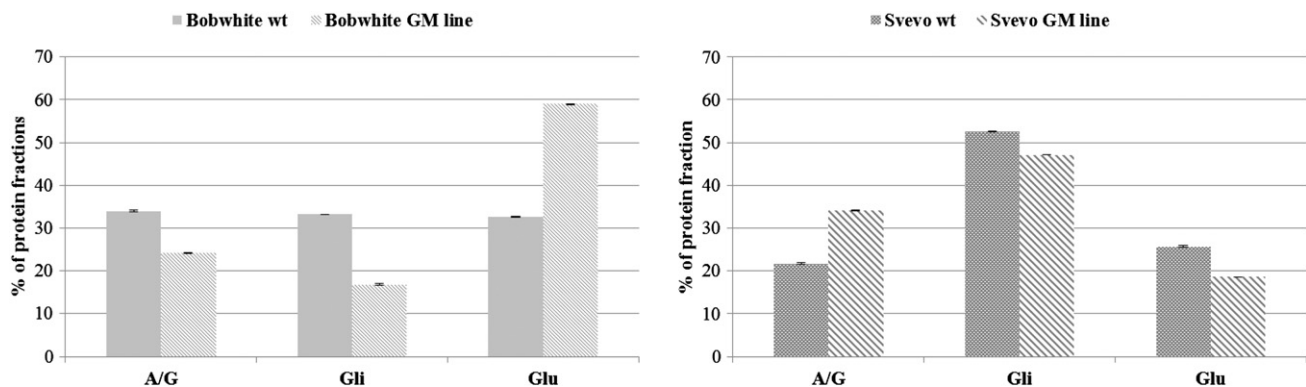


Fig. 1 – Percentage of major protein classes after sequential extraction for the Bobwhite GM line and the wild type genotype (left), and the Svevo-GM line and the wild type genotype (right).

were compared using an ELISA with anti-peptide antibodies specific for α/β -, γ -, ω -2-, ω -5-gliadins, LMW-GS and HMW-GS. The results obtained showed differences between the BW-GM and BW-wt only for the LMW-GS content (Fig. 2). The anti C-terminal LMW-GS antibodies (Fig. 2a) with a broad reactivity to all LMW-GS types indicated an overall increase in this class of proteins in the BW-GM line compared to its wt line. The other antibody, anti N-terminal (Mab 6 $\times$ 1), with a reactivity restricted to a few LMW-GS mostly encoded by the Glu-B3 locus [31], showed a reduction in the amount of these LMW-GS in the GM line (Fig. 2b). In addition, the IgE-binding capacity of these Gli and Glu fractions was assayed by immunoblotting with sera from four patients with food allergy to wheat. The IgE reactivity did not differ between the Gli fractions of GM and wt bread wheat lines, while that against the Glu fractions differed between the four sera (Fig. 3). Among the glutenin subunits of BW-wt, IgE reacted with different polypeptides in the molecular weight range 20–80 kDa while in the case of BW-GM the reactivity profile was clearly dominated by the over-expressed transgenic polypeptide (Fig. 3).

3.1.2. Focus of A/G fraction: identification of IgE-bound polypeptides in the A/G fraction of both GM lines

In order to identify the allergens in the SV-GM, BW-GM and relative wt lines, immunoblots were performed after separation of the flour A/G proteins by 2D electrophoresis and by using sera from three patients suffering from food allergy (FA) (S68, S781, S38) and one from baker's asthma (BA) (S458). Sera were chosen on the basis of their reactivity in 1D electrophoresis or ELISA and the amount available. However, given the difficulties in obtaining sufficient volumes of sera, we chose to carry out a cross-comparison between the two GM lines and their untransformed genotypes with the same sera rather than performing replicates. For each line, a control was performed with the serum of a non-atopic subject: in all cases, no spots were detected (data not shown). These analyses resulted in a total of 606 IgE-binding polypeptides being detected, of which 109 were spotted and further identified by mass spectrometry (Table S1).

3.1.2.1. Comparison between SV-wt and SV-GM. As shown in Fig. 4, many IgE-binding polypeptides were revealed in the A/G fraction of SV-wt and SV-GM, using the three sera 68, 781 and 458. As shown in Table 1, more than 82% of the IgE-binding polypeptides were common to the GM and its parent line. With reference to the same serum, only a few spots were differentially detected between the two lines, either specific to the wt line or to the GM line.

In the case of serum 68, seven polypeptides were specifically detected in SV-wt, but absent from the equivalent blot on its transgenic line. In the case of sera 781 and 458, three and two specific polypeptides, respectively, were detected in SV-wt, whereas six and three specific polypeptides were found in the SV-GM line. Twenty-seven spots in the SV-GM line and 31 in SV-wt were identified by mass spectrometry, 17 of which revealed a common identity between the two lines (Table 1).

3.1.2.2. Comparison between BW-wt and BW-GM line. As shown in Fig. 5, a large number of IgE-binding polypeptides were revealed in the A/G fraction of BW-wt and the BW-GM line using the three sera: 68, 38 and 458. More than 90% of the IgE-binding polypeptides were common to BW-wt and the BW-GM line (Table 1). Some polypeptides were specific to one genotype. In the case of sera 68 and 38, six and seven specific polypeptides were found in the BW-GM line, and two and five in the wt genotype, respectively. In the case of serum 458, four specific polypeptides were detected in BW-wt and one in the BW-GM line. Twenty-seven IgE-binding polypeptides were identified in BW-wt and 24 in the BW-GM line by mass spectrometry, 17 of which revealed a common identity between the two lines (Table 1).

3.2. Immunological characterization of the salt-soluble A/G fractions of wt and GM lines

Sera from 18 allergic patients, eight suffering from BA and ten from FA, were chosen to compare the IgE-binding capacity of the A/G fractions of the two GM wheat genotypes and their two untransformed counterparts. All these sera contained IgE

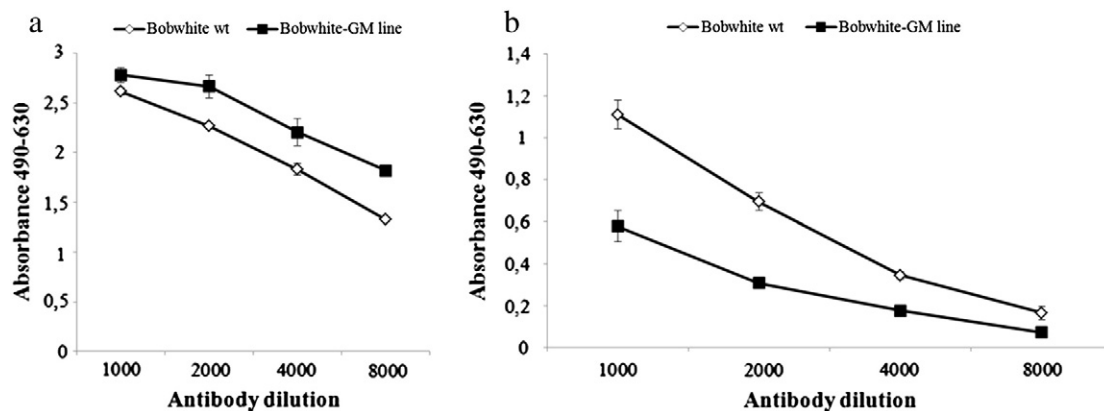


Fig. 2 – Detection by ELISA of LMW-GS by two specific antibodies (a: against the C-terminal and b: against the N-terminal of LMW-GS).

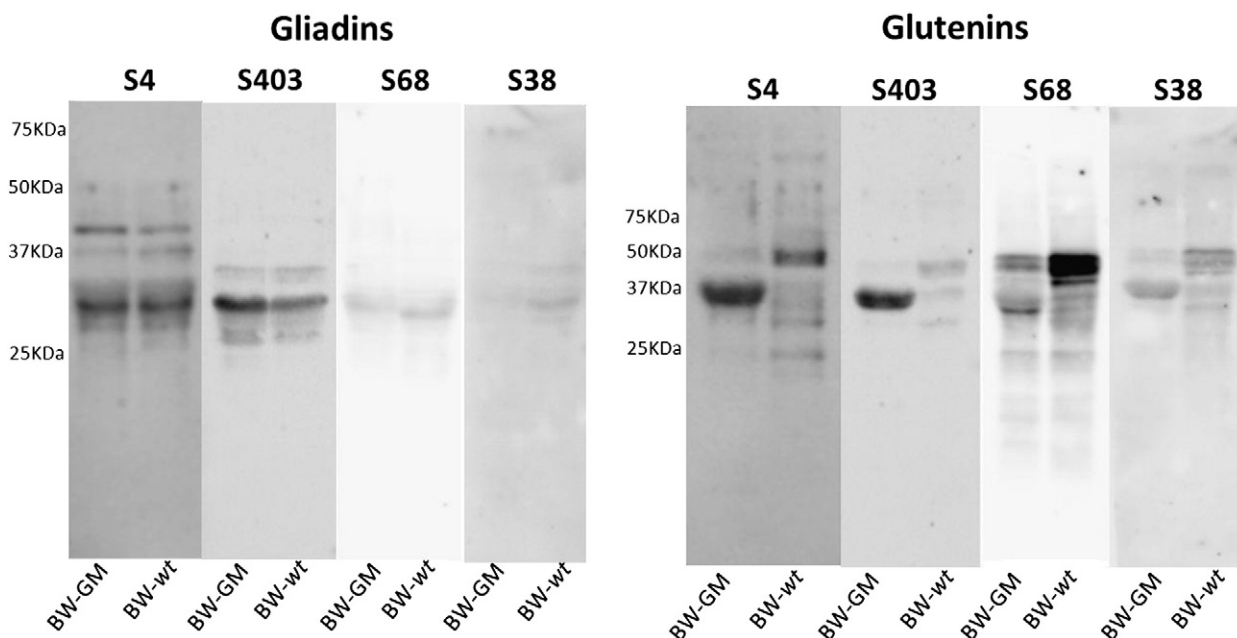


Fig. 3 – 1D immunoblotting on the gliadin and glutenin fractions of the Bobwhite GM line and Bobwhite wt performed with sera from patients suffering from food allergy to wheat (S4, S403, S68, S38).

specific to the A/G fraction of the wheat cv Réclital, with a large range of variability among patients, from 2 to 127 ng/mL (Table S2a, and b). The comparison of IgE binding levels between GM genotypes and their untransformed counterparts revealed significant differences for only two patients suffering from BA (sera 858 and 1021) and for six patients suffering from FA (sera 44, 68, 326, 403, 646 and 781) (these sera are marked with a star in Fig. 6). Of these, five sera (68, 646, 781, 858 and

1021) displayed increased reactivity (of between 14 and 45%) towards the A/G fraction in the SV-GM line, in comparison with SV-wt. Concerning BW-GM versus the wt line, small differences in specific IgE concentrations for the A/G fraction were observed for only three sera, all from patients suffering from food allergy: sera 44 and 326 showed a decrease of 5% and 9%, respectively, whereas serum 403 showed an increase in IgE concentration of 19%.

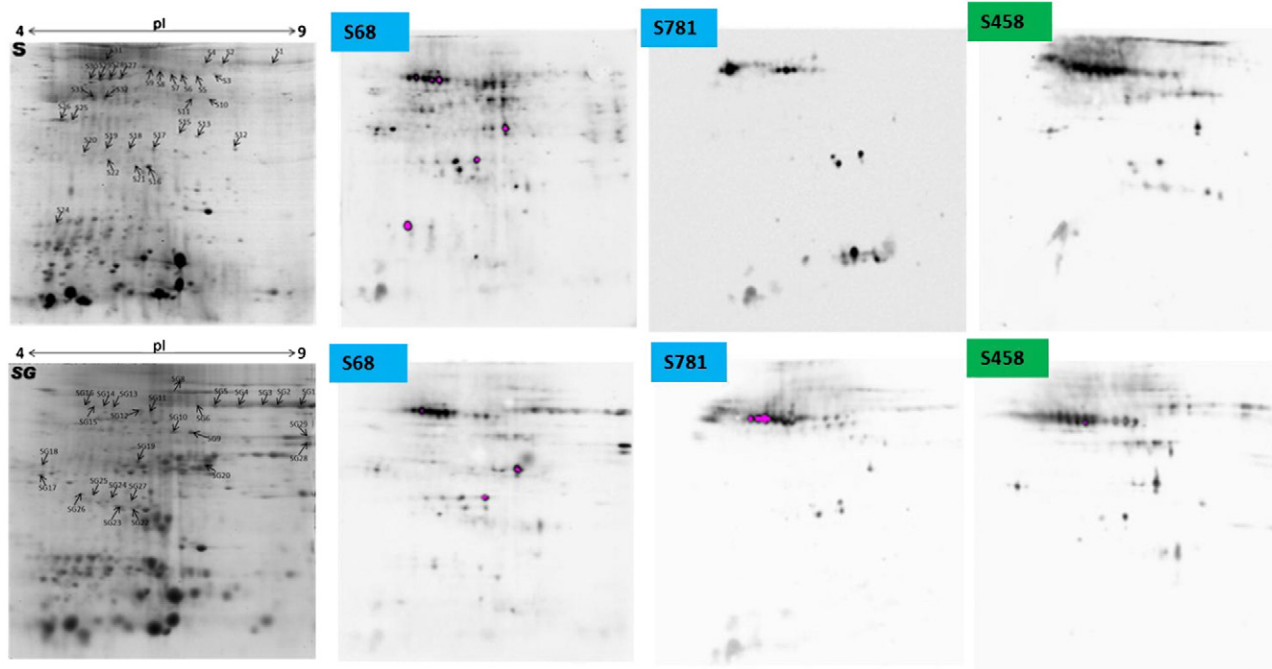


Fig. 4 – 2D immunoblotting of the A/G fraction from the Svevo lines with sera from patients allergic to wheat. S: 2D-SDS-PAGE of the Svevo wt line's A/G fraction, SG: 2D-SDS-PAGE of the Svevo-GM line's A/G fraction. Immunoblotting with sera 68, 781 and 458. The sera from patients suffering from food allergy are shown in blue and the serum from the patient suffering from respiratory allergy is shown in green.

Table 1 – Numbers of detected and identified IgE-binding polypeptides in Svevo and Bobwhite lines for each serum (S38, S68 and S781 obtained from FA patients, S458 obtained from BA patient).

	Svevo lines			
	# of IgE-binding polypeptides		# of common polypeptides	# of common proteins identified
	SV-wt	SV-GM	Between SV-wt and SV-GM	
S68	47	40	40	17
S781	17	20	14	
S458	29	30	27	
	Bobwhite lines			
	# of IgE-binding polypeptides		# of common polypeptides	# of common proteins identified
	BW-wt	BW-GM	Between BW-wt and BW-GM	
S68	62	66	60	17
S38	95	97	90	
S458	53	50	49	

4. Discussion

Allergens in wheat kernels are present in the salt soluble fraction (A/G) and in the insoluble gluten fraction (Gli and Glu). Gluten proteins are generally involved in food allergy, whereas A/G are involved in both food and respiratory allergies. In the present study, we compared two GM lines and their corresponding untransformed cultivars: the bread wheat cultivar Bobwhite [23] and the durum wheat cultivar Svevo [26].

The Bobwhite transformant over-expresses a LMW-GS already described as an allergen [25]. In this specific case, the changes in the composition and immunoreactivity of its gluten

fraction were controlled. In both genotypes, a detailed comparative allergenomic analysis focusing on the A/G fraction was performed to check and identify food or respiratory allergens in the transformed lines.

The bread wheat cultivar Bobwhite was transformed with an endogenous *lmw-gs* transgene in order to increase the amount of this polypeptide, which is known to influence the technological properties of wheat dough [23]. The Bobwhite transformation resulted in a huge increase in the glutenin class and a simultaneous decrease in the gliadin and A/G classes, as already reported by Scossa et al. [35]. Variation in the composition of the glutenin class was observed: besides an increase in the transgenic protein LMW-GS, the other LMW-GS

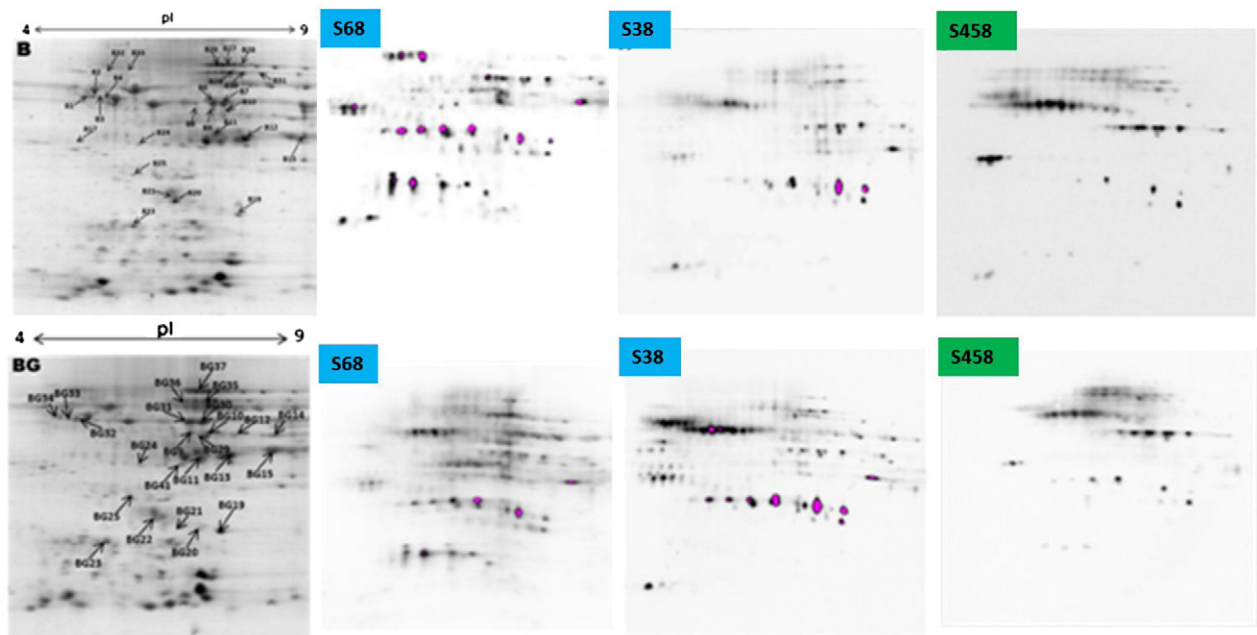


Fig. 5 – 2D immunoblotting of the A/G fraction from the Bobwhite lines with sera from patients allergic to wheat. B: 2D-SDS-PAGE of the Bobwhite wt line's A/G fraction, BG: 2D-SDS-PAGE of the Bobwhite-GM line's A/G fraction. Immunoblotting with sera 68, 38 and 458. The sera from patients suffering from food allergy are shown in blue and the serum from the patient suffering from respiratory allergy is shown in green.

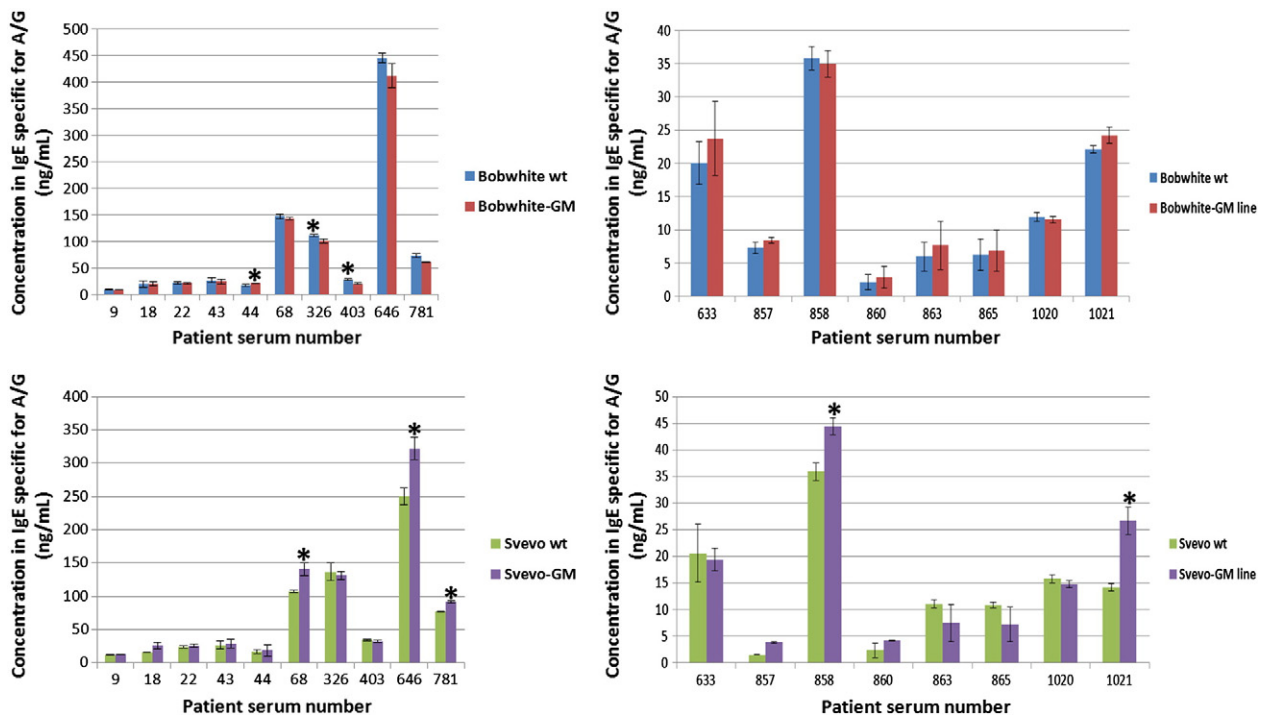


Fig. 6 – Concentrations (ng/mL) of IgE specific to the A/G fraction extracted from the four wheat genotypes. Sera obtained from patients suffering from food allergy (left). Sera obtained from patients suffering from baker's asthma (right). Asterisks indicate patient sera showing significant differences between the GM line and its untransformed counterpart.

protein types decreased, probably as a compensatory effect. Moreover, the amount of all other endogenous proteins of the BW-GM line decreased, although its total seed protein content increased from 15.7% to 17.3% for BW-wt and BW-GM, respectively [23]. The transgenic protein was recognized by the four patient sera used in this work, although with different intensities. The endogenous LMW-GS were detected far more weakly in GM compared to wt, since they were strongly under-expressed. In contrast, the IgE-binding profiles obtained for the gliadin fraction did not differ between the two lines, transgenic or wild type.

The durum GM line was obtained by the over-expression of an endogenous GBSS protein involved in amylose synthesis [26]. This protein has never been described as an allergen. Our results showed that the proportions of the three main classes of wheat proteins (Gli, Glu, and A/G) were affected in SV-GM, with a decrease in gliadins and glutenins balanced by an increase in the A/G class.

Unintended effects were obtained in both transformation events: an overall decrease in the amount of proteins including A/G in the bread wheat line, with the exception of the transgenic protein, and a change in the proportion of gluten and A/G classes in the durum wheat line. As a consequence, these two opposite effects on the amount of the A/G fractions of both transgenic lines and their wild type counterparts led us to examine them in detail with regard to their potential allergenicity.

4.1. Allergen identification

In order to identify the allergens at a molecular level and make comparisons between GM lines and their wt counterparts, we

carried out a comparative allergenomic analysis using sera obtained from patients suffering from FA and BA.

The total number of IgE-binding spots revealed a higher reactivity towards Bobwhite lines with respect to Svevo lines for sera 68 (patient with FA) and 458 (patient with BA). The effect of the wheat genotype on the number of IgE-binding spots has already been reported and might be related to the different levels of ploidy [28], which may induce large variations in the expressed A/G proteins, although this might also be due to intervarietal variation.

Several proteins identified in this study have previously been reported as allergens in wheat (www.allergome.org), or as potential allergens [13,17,36], and are listed in Table 2.

4.1.1. Known wheat allergens found in GM and wt lines

Alpha-amylase inhibitors (WTAI) are known to be among the major allergens in BA [25], and are represented by heterogeneous and numerous polypeptides [37]. Three forms of WTAI were identified in this study: endogenous α -amylase/subtilisin inhibitor and WTAI-0.19 were found in Bobwhite lines, whereas WTAI-CM3 was identified in Svevo lines. Although these polypeptides have mostly been associated with respiratory allergies, they have also been found in sera from patients suffering from FA [28,38]. Many serine protease inhibitors (serpins) were detected as IgE-binding proteins in the four lines studied. Serpin-Z2A (Q9ST57) is a known respiratory allergen (Tri a 33), while other members of this family have already been identified using sera from patients suffering from BA [39–42] and from FA [25,28,38]. The involvement of another serpin isoform (Serp Z2B) in FA has recently been established using a recombinant protein [43]. Triosephosphate-isomerase

Table 2 – Known and potential allergens identified in Bobwhite and Svevo lines. The spots in which the allergen was identified are listed in the first row; the last two rows indicate the sera with which they were detected.

Spot		Bobwhite lines	Svevo lines	Food allergens	Respiratory allergens
<i>Known allergens identified in GM and wt genotypes</i>					
B19,BG19,BG20	Endogenous α -amylase/subtilisin inhibitor	X		38, 68	458
B20, B21, BG22	WTAI-0.19	X		68	458
S11, S15–S17, SG10,SG22,SG27	WTAI-CM3		X	68,781	458
S27–S30, SG13–SG16	Serpin-Z2A	X	X	68, 781	458
B1–B4, BG32–BG34					
S17–S22, S24	Triosephosphate-isomerase		X	68, 781	458
SG24–SG27					
<i>Potential allergens identified in GM and wt genotypes</i>					
S13, S32, S33, SG20	β -amylase	X	X	68	458
B1, B33, BG34					
B19, BG19	Thaumatococcus-like protein	X		68	458
S1–S13, S15, S24–S26,SG1–SG6, SG8–SG11, SG19,SG20	Globulins	X	X	68, 781, 38	458
B7, B9, B10, B12, B15, B23–B31					
BG10, BG15, BG24, BG25,BG29, BG30, BG35–BG37					
S27–S33	Enolase		X	68, 781	458
SG13–SG16					
B32, B33					

(Tri a 31), previously described as an allergen in BA [44], was identified in our study, but this protein was also found in sera from patients suffering from FA.

4.1.2. Potential allergens found in GM and wt lines

β -Amylase was identified in all four genotypes and detected with the two types of sera, suggesting its involvement in both food and respiratory allergies [28,38]. It is noticeable that this protein shares 88% identity with its barley homologue, which has been described as an important allergen in BA [45].

Thaumatococcus-like protein (TLP) was only found in the Bobwhite genotypes. This protein is homologous to the TLP PWIR2, which is responsible for BA as demonstrated by skin prick tests performed with purified extracts [46]. Finally, the protein identified in this study could be a potential allergen for food and respiratory allergies, as it was detected by both types of sera.

Numerous IgE-binding spots were identified as globulin3 or globulin2, which are related to the predominant seed storage 7S globulin class from dicotyledonous plants [28,36,47]. Most of them were common to the wt lines (Svevo and Bobwhite) and their respective GM lines. They were detected by sera from food and respiratory allergic patients but until now there has been no clinical evidence for their allergenicity in wheat. It is noteworthy that the well-known peanut allergen Ara h 1 is a 7S globulin [48].

Enolase was identified in many IgE-binding spots common to SV-wt and its GM line and recognized by the two types of sera. It belongs to a highly conserved family in which many members have been described as fungal and latex allergens [49,50]. This particular wheat enolase, homologous to ten allergens of enolase type, as retrieved from the SDAP database, may therefore be allergenic. It is noticeable that this putative allergen was present in BW-wt and absent in the BW-GM line, which is also an unintended effect of the transformation.

4.1.3. Specific IgE-binding proteins identified in the SV-GM line
Only two spots specific to the SV-GM genotype were detected by IgE and the proteins were identified as 1-cys peroxiredoxin (PER1) and seed maturation protein. The former (PER1) is an allergen already described by the WHO/IUIS as Tri a 32. Phar et al. reported it as a relevant allergen for BA [51]. In our study, the serum from a patient suffering from BA did not recognize this allergen; nonetheless it was recognized by the two sera from patients suffering from FA, suggesting the potential allergenicity of 1-cys-peroxiredoxin regarding food allergy.

The seed maturation protein identified by sera from one patient suffering from BA and another from FA showed no close homology with known allergens by in silico analysis. For this reason and as it was identified only twice, it is important to examine experimentally whether this protein is allergenic.

4.2. IgE patient reactivity against the A/G fractions

The IgE-binding capacity of the A/G fractions measured using a larger set of sera revealed significant differences between the wt genotypes and their GM lines for few sera. In the case of sera from patients with BA, differences were only observed for SV-GM with respect to the untransformed genotype, with two (out of eight) sera showing a significant increase in reactivity. With regard to the sera obtained from FA patients, three (out of ten) of them exhibited higher reactivity against SV-GM compared to SV-wt, whereas a different situation was observed for BW lines, with three sera presenting higher or lower reactivity against the BW-GM compared to BW-wt. These variations in the sera response reflect modifications in the composition of the A/G fraction of the GM-lines. In the case of SV-GM, some allergens recognized specifically by five sera are probably expressed in higher amounts in the A/G fraction; the effect on BW-GM was not as clear cut, but the differences, although significant, were smaller (maximum 8 ng/mL).

In conclusion, at the molecular level, a few differences were revealed between the GM lines and their counterparts: two new IgE-binding proteins were detected for SV-GM, one already known to be allergenic and another that has never been described as an allergen. At the quantitative level, a few differences in IgE reactivity between the GM lines and their natural counterparts were highlighted, involving five patient responses among 18. This exploratory study suggests that the transformation resulted in minor unexpected effects in relation to the potential allergenicity of GM lines but we cannot make any conclusions about their equivalence. In order to establish whether these differences are important, they need to be compared to data on the overall variation in allergenicity within conventional crops.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.01.028>.

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Table S2

Patient serum n°	Age	Symptoms	IgE specific for A/G	IgE specific for gliadins	IgE specific for LMW-GS
Patients suffering from BA					
458**	55	R+AT	+	neg	neg
633	66	AT	49	26	neg
857	20	R	6	neg	neg
858	47	AT	75	+	neg
860	35	AT	2	neg	neg
863	41	AT	12	neg	neg
865	23	AT	12	4	2
1020	49	R+AT	18	neg	neg
1021	30	R+AT	19	+	neg
Patients suffering from FA					
4	A	Urt	neg	35	+
9	1,5	AEDS	9	13	8
18	14	AEDS	18	nd	nd
22	8	AEDS	28	29	25
38**	2	AEDS	10	20	14
43	6	AEDS	33	11	9
44	37	AEDS	20	+	neg
68**	6	AEDS+GI	127	90	115
326	5	AS	110	10	+
403	3	AEDS	29	3	neg
646	5	AEDS	121	200	139
781**	6	AEDS	84	55	neg