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Assessment of the allergenicity of soluble fractions from GM and commercial genotypes of wheats

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ABSTRACT

Breeding has enabled the development of wheat genotypes with different quality characteristics and end uses. Despite its numerous positive properties that make it one of the most cultivated crops, wheat is known to induce allergic reactions in predisposed consumers. Genetic modification (GM) technology for crop improvement has recently emerged and its impact on allergenicity must be evaluated, as recommended by the Codex Alimentarius.

Our aim was to determine whether the variation in the amount of allergenic polypeptides in five GM wheats, along with their untransformed genotypes, was equivalent to the variation observed among twenty commercial cultivars, either durum or bread wheats. Since the most important factor involved is the amount of allergenic polypeptides, we performed Enzyme-Linked Immunoabsorbent Assays with IgE from twenty-two patients suffering from food or respiratory allergy to wheat on two well characterised soluble protein fractions. Statistical analyses showed a significant effect of the genotypes and sera, both by considering GM lines and untransformed genotypes. This study leads us to conclude that a wide variation exists in the amount of allergenic polypeptides among durum and bread wheat cultivars, and that the differences observed between GM wheats and their parents are within the range of cultivated wheats.

1. Introduction

Cereal breeding has been particularly prolific in the last century, resulting in spectacular improvements in suitable traits such as increased yields, biotic and abiotic resistance and technological performances. Safety has never been an issue of traditional plant breeding because it was assumed that crop varieties were safe based on their long history of use, even if no derived food is safe for all consumers (Goodman and Tetteh, 2011). Typical standard seed certification therefore only focuses on seed authenticity, genetic purity and stability over time.

The two most important cultivated wheat species are *Triticum aestivum* L. (bread wheat) and *Triticum durum* Desf. (durum wheat), which are mainly processed into a range of breads and other baked products, pasta and other foods.

Wheat is the most commonly consumed staple food, although it is known to present health risks for a fraction of the population in which it triggers reactions, some of which are IgE-mediated. The estimated prevalence of food allergy to wheat varies from 0.3 to 0.6% of the population, and 4–10% of bakers are affected by a respiratory allergy due to wheat (baker's asthma) (Salcedo et al., 2011).

Wheat proteins are typically classified into four fractions: albumins, which are water soluble; globulins, which are soluble in salt solutions; gliadins, which are soluble in concentrated alcohol solutions; and glutenins, which are soluble in diluted acid or alkaline solutions. Many proteins of the salt-soluble fraction have already been described as allergens in baker's asthma (Salcedo et al., 2011; Tatham and Shewry, 2008), and some of them are also frequently associated with food allergy, mainly in children (Battais et al., 2005; Larré et al., 2011; Pastorello et al., 2007; Sotkovsky et al., 2008). Although gliadins and, to a lesser extent, glutenins, are implicated in food allergy (Matsuo et al., 2004), some gliadins may also bind with IgE from patients with baker's asthma. Most allergen studies have been performed on *T. aestivum*, but little

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Table 1
Wheat genotypes included in the study.

Genotype	Bread wheat *T Aestivum*		Durum wheat *T Durum*	
wt	Bobwhite *wt*		Svevo *wt*	
or		Bobwhite		Ofanto
Null-segregant lines	x	Null-segregant of GM PGIP-GS	x	Null-segregant
GM lines	Bobwhite (over-expressing LMW-GS)	Bobwhite (over-expressing PGIP-GS)	Svevo (over-expressing *Wx-B1*) Svevo (RNAi silencing SBEIIa)	Ofanto (SBEIIa silencing with *Agrobacterium*)
Commercial varieties	Antille Apache Avorio Colledoro Exotic Feria Genesi Lilliput Masaccio Valbona		Casanova Claudio Creso Dorato Minosse Neodur Pitagora Simeto Tripudio Vinci	

is known about the content of allergens, and even less about their variability between and within species and cultivars. Only a few studies suggest that certain endogenous allergen concentrations are highly variable across crop cultivars, including LTP in soybean (Houston et al., 2011) and maize (Kuppannan et al., 2011), but that they also vary depending on environmental and growing conditions. Moreover, it is important to consider that the protein thresholds for eliciting an allergic reaction vary per individual.

The introduction of transgenesis as an alternative breeding procedure has caused a general concern in consumers and new regulations have been implemented as a result. Even if transgenesis offers immense opportunities, there has been, and continues to be, considerable public resistance to the use of genetically modified (GM) plants, particularly in Western Europe and in relation to staple food crops such as wheat. The Codex Alimentarius (http://www.codexalimentarius.net) has introduced the need to assess the safety of GM plants that are used for human consumption. The recommended approach is a comparison between the transgenic plant and its conventional counterpart at different levels. Few comparative studies have been reported in which transcriptomic, proteomic or metabolomic comparisons have been performed. However, most of them reveal only a small impact of transgenesis (Baudo et al., 2006; Laino et al., 2010; Lovegrove et al., 2009; Scossa et al., 2008) and infer that adverse effects or health risks remain unlikely (Domingo and Gine Bordonaba, 2011).

The Codex Alimentarius now requires a list of the contents of allergenic polypeptides of GM plants in order to assess their safety when the recipient is known to be an allergen.

This assessment is performed by comparing the GM with its direct parent line for which the "history of safe use" is generally not or poorly documented (Kok et al., 2008). When focussing on allergy, it is necessary to determine whether or not the genetic modifications introduced have potentially increased the intrinsic allergenicity as an unintended effect. In a previous study (Lupi et al., 2013), few differences at the molecular and quantitative levels were revealed when comparing two wheat GM lines with their corresponding untransformed lines. However, the EFSA suggests extending this comparison with appropriate comparator(s) in order to take the natural variability in allergens into account (EFSA Panel on Genetically Modified Organisms (GMO Panel, 2010; http://www.efsa.europa.eu/en/efsajournal/pub/1700.htm). In the present study, we focused on soluble wheat seed proteins in which many allergens are found, especially those correlated with respiratory allergies, including baker's asthma. Classical varieties were included in this study in order to compare the allergenic variation induced by the transgene presence to that present in genotypes obtained by classical breeding.

Five GM lines were included in this study, all of them were developed and maintained for research purposes only. They were selected based on different criteria: they relate to the two most cultivated species (T. *aestivum* and T. *durum*), several types of transformations were used (Agrobacterium, biolistic and silencing) and finally the selected genes target either proteins with metabolic activity or storage protein of the gluten. They were compared with their untransformed counterparts and with other varieties (ten durum wheats and ten bread wheats).

In order to obtain enough sensitivity in the IgE response, soluble seed proteins were divided into their subfractions: metabolic and chloroform-methanol (CM-like) soluble proteins. Their composition was examined by electrophoresis and mass spectrometry, as was their ability to bind with IgE. Subfractions were then prepared for each of the 29 varieties and tested by ELISA with the IgE of 23 patients with different clinical profiles of wheat allergies (baker's asthma and food allergy to wheat).

2. Materials and methods

2.1. Wheat samples

Ten varieties of *T. aestivum* and ten of *T. durum* were used in this study; they are listed in Table 1. All these cultivars were kindly provided by APSOVSEMENTI S.p.A. (Voghera, Pavia, Italy). Bread wheats were grown in Voghera (Italy) and durum wheats in Grosseto (Italy) in 2010 and certified for their homogeneity. Two GM lines of the bread wheat *cv.* Bobwhite and three GM genotypes of durum wheat (two of *cv.* Svevo and one of *cv.* Ofanto) along with their corresponding *wt* and null-segregant genotypes (when available) grown in a growth chamber at the University of Tuscia (Italy) were also included in this study (Table 1).

One of the transgenic bread wheat *cv.* Bobwhite genotypes was transformed with a LMW-GS as described in Masci et al. (2003). This GM bread wheat line showed a strong overexpression of the transgenic polypeptide due to the high number of transformation events and a drastic decrease in the amount of all other endogenous proteins, including CM-like proteins, with respect to the *wt* genotype (Scossa et al., 2008).

The second transgenic bread wheat *cv.* Bobwhite genotype was transformed with a bean PolyGalacturonase Inhibitor Protein (PvPGIP2 gene) in order to investigate whether the PGIP protects wheat tissue from PG degradation (Janni et al., 2008). The authors tested the activity of the transgene protein (PvPGIP2) against *Fusarium moniliforme* endopolygalacturonase (FmPG). Transformation resulted in a GM line capable of inhibiting FmPG, whereas the null segregant and *wt* genotypes did not affect the

activity of this enzyme. The null-segregant line was included in our study.

As regards the transgenic durum wheat lines, two of them are described in Sestili et al. (2010), and correspond to *cvs.* Svevo and Ofanto, in which the genes encoding starch branching enzymes of class II (SBEIIa) have been silenced using the RNA interference (RNAi) approach, in order to increase amylose content. The Svevo GM line was transformed by the biolistic method, whereas the Ofanto GM line was transformed with *Agrobacterium*. The silencing of *SBEIIa* genes in the two GM wheat lines caused alterations in granule morphology and starch composition with increased amylose content, regardless of the transformation method used (Sestili et al., 2010).

The third transgenic durum wheat line corresponded to *cv.* Svevo transformed with the Wx-B1 gene in order to investigate the effect on starch composition, which turned out not to be substantially altered with respect to the *wt* genotype (Sestili et al., 2012).

2.2. Human sera

Sera of 22 patients with wheat allergy (11 food allergies: FA, and 11 baker's asthma: BA) and a control serum (non-atopic) were used. Clinical data of the patients (symptoms, age, and wheat-specific IgE reactivity against the albumin/globulin fraction of the reference cultivar Récital) are summarized in Table S1. Sera were obtained from the Clinical Immunology and Allergy service of the Hospitals of Epinal (France) and Udine (Italy) with the informed consent of the patients. Control serum was obtained from healthy volunteers.

2.3. Extraction of the salt soluble protein fraction

2.3.1. Extraction of the albumin/globulin fraction (A/G)

The albumin/globulin (A/G) fraction was extracted from Bobwhite flour and from the reference bread wheat cultivar, Récital, as described in Larré et al. (2011). The final supernatant was collected, dialysed against water and freeze-dried.

2.3.2. Extraction of metabolic (Met) and chloroform-methanol (CM-like) soluble protein fractions

Metabolic (Met) and chloroform-methanol (CM-like) protein fractions were extracted from milled wheat seeds with 2% PVPP (polyvinylpolypyrrolidone) for 30 min and centrifuged at 8500 rpm for 1 h. The pellet was suspended in cold (4 °C) KCl buffer (50 mM Tris–HCl, 100 mM KCl, 5 mM EDTA, pH 7.8), as described by Hurkman and Tanaka (2007). The protein concentration was determined by Kejdahl's method or by the BC Assay Protein Quantification Kit (*Uptima*), as described in Larré et al. (2011).

2.4. SDS-PAGE, western and immunoblotting

The A/G, Met and CM-like fractions of the bread wheat *cv.* Bobwhite were solubilised in Tris–HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 4% β-mercaptoethanol and bromophenol blue; 35 μg were loaded and separated by 1D SDS-PAGE on 15% or 10–15% gradient acrylamide gels (18 × 16 cm gel with SE600X-Hoefer). Gels were either stained with Coomassie Brilliant Blue G250 (Sigma–Aldrich) according to the procedure of Devouge et al. (2007) or used for blotting. In the latter case, semidry transfer was achieved at 300 mA for 40 min to nitrocellulose membranes (0.2 μm, Sartorius, Germany) in 25 mM Tris, 192 mM glycine, 0.1% SDS and 20% ethanol. After washings with PBS-0.05%/Tween 20, the membrane was successively blocked overnight with PBS-0.05%/Tween 20, incubated for 1 h with anti-LTP IgG antibody diluted at 1/500, and then for 1 h with alkaline phosphatase conjugate anti-rabbit (REF, diluted at 1/3000). After further washing with PBS and 0.1 M Tris (pH 9.5), the membrane was incubated for 10 min with 0.1 M Tris (pH 9.5)/Alkaline Phosphatase Conjugate Substrate Kit (BIO RAD), and stopped with 0.01 M acetic acid and washed with water. The colorimetric reaction was detected using the Luminescent ImageAnalyzer LAS 3000 (Fujifilm).

In the case of immunoblotting with patients' sera, nitrocellulose sheets were revealed according to the procedure described in Lupi et al. (2013). Sera were prepared at the appropriate dilution (ranging from 1/20 to 1/50) in the washing buffer.

2.5. Protein identification by mass spectrometry

The fractions A/G, Met and CM-like as well as protein electrophoretic bands were prepared for mass spectrometry using trypsin hydrolysis following the protocol described by Larré et al. (2010).

2.5.1. Liquid chromatography and mass spectrometry

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), or using a Switchos-Ultimate II capillary LC system (LC Packings/Dionex, Amsterdam, the Netherlands) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Global, Micromass/Waters, Manchester, UK). Chromatographic separation and mass data acquisition performed using the Q-TOF instrument were performed as described in Larré et al. (2010).

In the case of the LTQ-Orbitrap instrument, chromatographic separation was conducted on a reverse-phase capillary column (Acclaim Pepmap C18 2 μm 100A, 75-μm i.d. × 15-cm length, Dionex) at a flow rate of 200 or 300 nL.min-1. Mobile phases were composed as indicated: (A) 99.9% water, 0.1% formic acid; (B) 90% acetonitrile, 0.08% formic acid. The gradient consisted of a linear increase from 4% to 45% of B in 30 min, followed by a rapid increase to 70% within 1 min. The composition was maintained at 70% B for 5 min and then decreased to 4% B for re-equilibration of the column. Mass data acquisitions were performed using Xcalibur 2.1 software. Full MS scans were acquired at high resolution (FWMH 30,000) in the Orbitrap analyser (mass-to-charge ratio (m/z): 400–2000), whereas collision-induced dissociation (CID) spectra were recorded on the five most intense ions in the linear LTQ traps.

2.5.1.1. Databank searches and interpretation. Raw data collected during LC-MS/MS analyses were processed into mgf (mascot generic format) files and further searched against databanks using the XTandem! software 2008.02.01 (http://www.thegpm.org/TANDEM/), with a user-interface designed by B. Valot (B. Valot, 2010) and available at http://pappso.inra.fr/bioinfo/xtandempipeline/. Protein identification was achieved by confronting mass data (MS and MS/MS spectra) against the UniProt databank restricted to Poaceae (release 2013_02, February 2013). Another databank search was performed against the Wheat TIGR Gene Indices databank (http://compbio.dfci.harvard.edu/tgi/, release 12, April 2010). Fixed modification of cysteine residues by iodoacetamide was considered, as well as oxidised 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 their E-value was below 10^{-4} and when they were identified with a minimum of two different peptides that matched their sequence with an E-value below 0.05. Protein identifications were compared in the two databanks (Uniprot and Wheat TIGR): best matches were validated. When the results were identical in the two databanks, the identification in UniProt was chosen. Proteins were

sorted into groups and subgroups: groups consisted of proteins that shared at least one peptide, and subgroups consisted of proteins that could not be distinguished on the basis of their peptides. Moreover, 56% of the groups contained more than one protein, reflecting the presence of numerous protein isoforms.

2.6. Enzyme-linked ImmunoSorbent assay (ELISA)

ELISA was performed using the Biomek® NXP Laboratory Automation Workstation. The wells on microtiter plates (Nunc MaxiSorp 384-well plates) were coated with 20 μL of each antigen diluted at 5 μg/mL in 100 mM carbonate buffer (pH 9.6) for 2 h at room temperature. After washing (PBS-0.1% Tween 20), plates were blocked with PBS-0.1% Tween 20 and 0.5% porcine gelatin (G2500 SIGMA) for 1 h at 37 °C and washed, and patients' sera diluted at 1:10 with 0.5% gelatin/PBS/Tween were added for 15 h at 37 °C. Goat anti-human IgE antibody (A3525-SIGMA) diluted at 1:500 in 0.5% G–PBST was incubated for 2 h at 37 °C. Finally, the fluorescent substrate (4-Methylumbelliferyl phosphate M3168-SIGMA) diluted at 1:5 in 1 M Tris/HCl (pH 9.8) was added for 90 min at room temperature and in the dark. The fluorescence was measured at 440 nm (excitation: 360 nm). Measurements were run in triplicate and the fluorescence for each antigen was corrected by subtracting the fluorescence of the serum with no antigen. The concentration of specific IgE binding to the antigen was calculated by reference to a standard curve run on the same plate (serial dilutions from 160 ng/mL to 0.07 ng/mL of standard human IgE, 2nd WHO international reference NIBSC 75/502, caught by a rabbit anti-human IgE antibody (A0094, DAKO). The standard curve ensured that the quantification and the saturation limits ranged between 0.1 and 32 ng IgE/mL.

2.7. Statistical analysis

IgE reactivity of the 22 sera was measured by ELISA on the Met and CM fractions extracted from the 29 wheat genotypes. Measurable responses were obtained for only 18 sera, which led to a set of 1566 data values for each extract (CM or Met). Using Statgraphics PLUS (version 5.1), two-way analyses of variance (ANOVA) were used to determine the influence of genotype and serum on IgE-binding ($P < 0.05$). An LSD multiple range comparison test was also used to determine differences among genotypes ($P < 0.05$). In the case of cultivars, the effects of species and type of allergies were also examined.

3. Results

3.1. Characterisation of the three fractions obtained from the wild type bread wheat cv. Bobwhite

Two procedures can be considered to obtain salt-soluble allergens: the first provides the allergens in a single fraction referred to as A/G (including albumins and globulins), while the second leads to two fractions referred to as CM-like and Met, in which allergens are present, especially in the CM-like fraction. In order to characterise the allergen composition of A/G, Met and CM-like fractions, we first characterised their proteins by mass spectrometry and, in the specific case of LTP, by immunoblotting. We then compared their IgE reactivity with patient sera.

3.1.1. Protein identification

The three fractions prepared from Bobwhite *wt* seeds were analysed by mass spectrometry using a shotgun approach following protein hydrolysis. Eighty-nine proteins were identified in A/G and Met fractions and 69 in the CM-like fraction. The comparison of the proteins identified revealed that 95% of the proteins identified in the A/G fraction were present either in the Met or CM-like fraction. The proteins identified with at least three peptides are reported in Table S2. Only five proteins of the A/G fraction were not recovered in the two others, and none of these proteins has been described as an allergen. In contrast, in the additional proteins identified in the Met and CM-like sub-fractions, many have been described as allergens (Fig. 1). LTP, a well-known allergen, was identified in the CM-like fraction only. However, an immunoblot performed with an anti-LTP antibody also revealed its presence in the A/G fraction with a specific band of molecular weight of around 10 KDa. Its intensity compared to that observed for the CM-like fraction is weaker, thus indicating a smaller enrichment of LTP in the A/G fraction. (Fig. 2A). LTP detection in the blot strengthened its identification in MS, which can be accepted with only two peptides. In the case of Alpha Amylase Inhibitors (AAI), the two sub-fractions (CM-like and Met) made it possible to obtain a larger coverage of this family, ranging from eight to 14 members with 11 AAIS identified in CM-like and three in Met. Such an enrichment was also obtained for purothionin, whose allergenicity was recently shown by Pahr et al. (2013).

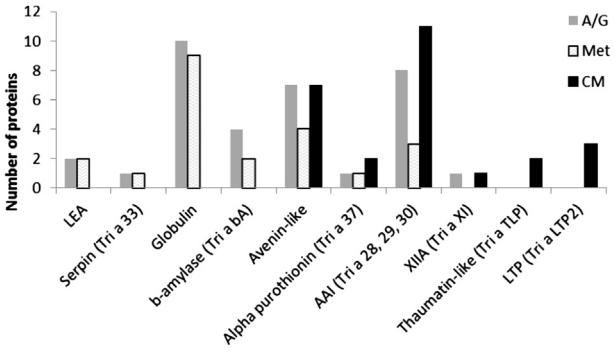

Fig. 1. Allergenic or potential allergenic proteins identified by MS with a shotgun approach following protein hydrolysis in the A/G, Metabolic and CM-Like fractions of Bobwhite *wt*.

3.1.2. IgE-binding capacity

The IgE-binding potential of the three protein fractions was investigated by immunoblotting (Fig. 2B) with three patients' sera (nr 68 affected by FA, nr 458 and nr 858 affected by BA). Several IgE-binding components were detected by serum nr 68 in the Met as well as in the A/G fraction. Two bands (g, h) specific to the Met fraction were analysed by MS (Table 2), revealing the presence of β-amylase, globulins, triosephosphate isomerase and/or serpin, which are known allergens either for FA or BA. A similar profile was observed for serum nr 458 on the Met fraction but with a weak intensity and, surprisingly, no low MW band was detected despite the fact that this serum was obtained from a patient suffering from BA. Serum nr 858 revealed IgE binding proteins of low MW (between 10 and 15 KDa) in the CM-like extract and with lower intensity in the A/G extract. The mass spectrometry analysis of their corresponding SDS-PAGE bands revealed numerous AAIs (bands a, b, c, d, e, f; Table 2).

These results led us to choose Hurkman and Tanaka's procedure (2007) to prepare Met and CM-like fractions from each of the 29 genotypes.

3.2. IgE binding capacity of the Met and CM fractions from wheat genotypes

Both Met and CM fractions were analysed by ELISA to measure their IgE-binding capacity using the sera of 22 patients selected for

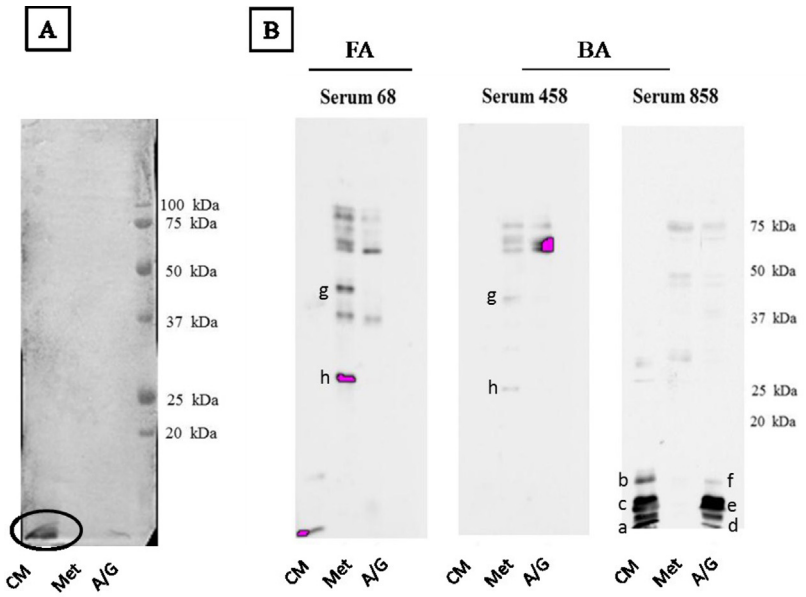

Fig. 2. Separation by SDS-PAGE of proteins from albumin/globulin (A/G), metabolic (Met) and CM-like fractions extracted from bread wheat *cv.* Bobwhite and immunoblotting with anti-LTP antibody (A) or with sera from patients allergic to wheat (sera 458 and 858 from patients affected by baker's asthma and serum 68 from patients suffering from food allergy).

their allergy to wheat. Only 18 sera gave measurable responses and were therefore used in our study (indicated by an asterisk in Table S1).

3.2.1. Analysis of GM versus their natural counterparts

The IgE binding capacity of the CM-like and Met fractions was measured for five GM wheat lines and their corresponding *wt*. The results obtained for the two sub-fractions were analysed separately.

Two-way analyses of variance were conducted to investigate the effect of the nine genotypes and 18 sera on the level of IgE-binding to proteins either in Met or CM-like fractions extracted from the genotypes. Mean IgE-binding values and associated SD are plotted in Fig. 3. Regardless of the fraction, the two main effects, genotype and serum were significant ($p < 0.005$), together with a significant interaction (Table S3). A subsequent comparison of means allocated the genotypes into five and six different groups for Met and CM-like fractions, respectively. Numerous genotypes were different from each other at a confidence level of 95%. The pairs including a GM and its wild-type relative are reported in Table 3. Three significant differences were observed for the Met fraction: GM Bobwhite-over-expressing PGIP-GS and Svevo-SBEII, which showed a lower reactivity in comparison to their *wt* genotypes, whereas Ofanto-SBEIIa silenced with Agrobacterium presented higher reactivity than its null-segregant line. In the case of the CM-like fraction, four of the GMs out of the five tested were different from their controlled lines, and as reported for the Met fraction, they can be either positive or negative (Table 3). Only Svevo-Wx-B1, overexpressing GBSS, was similar to the parent line, regardless of the fraction tested.

3.2.2. Analysis of the natural variability

3.2.2.1. IgE-binding capacity of the metabolic fraction. Among the cultivated wheats, IgE reactivity towards the Met fraction ranged from 29 to 48 ng/mL, with the durum wheat *cv.* Pitagora showing the lowest reactivity, whereas the highest was found for the durum wheat *cv.* Vinci. A two-way ANOVA performed on the responses obtained with the Met fraction of 20 genotypes revealed a significant effect for the genotype, the serum and their interaction as well (Table S3). In a subsequent analysis, the effects of species (T. *durum* versus T. *aestivum*) and of the type of allergy (FA versus BA) were analysed. Only the effect of allergy type was significant, with mean responses of 58.4 ± 2.5 ng and 18.3 ± 3.2 ng for FA and BA, respectively.

3.2.2.2. IgE-binding capacity of the CM fraction. In the case of the CM-like fraction, the IgE reactivity ranged from 42 to 71 ng/mL with the durum wheat *cv.* Minosse showing the lowest reactivity, whereas the highest reactivity was found for the durum wheat *cv.* Claudio. A two-way ANOVA performed on the responses obtained with the CM-like fraction of 20 genotypes revealed a significant effect for the genotype, the serum and their interaction as well (Table S3). In a subsequent analysis, as in the case of the Met fraction, a significant effect was found for the type of allergy and no effect for the species. The mean responses of the two allergy groups were 70.5 ± 3.2 ng and 45.1 ± 4.0 ng for FA and BA, respectively.

4. Discussion

Patients presenting allergic responses specific to the salt-soluble proteins of wheat grain were chosen for this study in order to compare allergenic potential between GM lines, their *wt* and other varieties. They belonged to two groups that differed in their pathology: those suffering from food allergy and those suffering from respiratory allergy. Since only small amounts of each serum were available, an ELISA test was first performed in order to detect the most suitable sera to carry out our experiment since we preferred to use and test individual sera rather than pooled sera. This technical choice requires the preparation of antigen extracts in which the allergens searched for are well represented and in a concentration capable of being detected by IgE and, moreover, quantified by ELISA.

We therefore compared two procedures for isolating salt-soluble seed proteins and obtained them in amounts compatible with subsequent functional analysis and mass spectrometry identification. Using Hurkman and Tanaka's procedure (2007) on Bobwhite genotypes, the salt-soluble proteins (A/G) were spread over two sub-fractions (CM-like or Met) in which IgE-binding

Table 2
List of proteins identified from 1D gel of Bobwhite *wt* fractions: albumins/globulins (A/G), metabolic (Met) and CM (Fig. 1). The columns correspond to: band:assigned protein number corresponding to those indicated in Fig. 1; Prot Id: the protein identity as referred to in Uniprot or the Wheat TIGR databank; Uniprot the best homologue protein name: its corresponding protein or the Uniprot best homologue protein name; log (E-value): Protein E-value expressed in log; %Cov: the per cent of protein coverage; MW: Molecular weight of the protein expressed in KDa; Total Unique Peptides: number of unique peptides for the protein.

N. band	Sub-group	Prot Id TC or Uniprot	Best homologue protein name	log (E-value)	Coverage	MW	Total unique peptides
a (CM)	1.01	A4ZIW9	Monomeric alpha-amylase inhibitor OS = Triticum aestivum PE = 4 SV = 1	−20.63	40	13.10	4
b (CM)	1.01	TC402211	WHEAT Alpha-amylase/trypsin inhibitor CM3 OS = Triticum aestivum PE = 1 SV = 1	−36.72	50	18.10	5
c (CM)	1.01	A4ZIW9	WHEAT Monomeric alpha-amylase inhibitor OS = Triticum aestivum PE = 4 SV = 1	−21.59	40	13.10	4
d (A/G)	1.01	A4ZIW9	WHEAT Monomeric alpha-amylase inhibitor OS = Triticum aestivum PE = 4 SV = 1	−13.29	28	13.10	3
e (A/G)	1.01	A4GFN8	Dimeric alpha-amylase inhibitor OS = Triticum turgidum subsp. dicoccoides PE = 4	−38.64	70	13.10	6
f (A/G)	1.01	TC402211	WHEAT Alpha-amylase/trypsin inhibitor CM3 OS = Triticum aestivum PE = 1 SV = 1	−39.97	50	18.10	5
g (Met)	1.01	TC377918	Cupin family protein, expressed [Oryza sativa Japonica Group].	−17.32	21	32.79	4
	2.01	CA613733	Triosephosphate isomerase OS = Triticum aestivum GN = tpis PE = 2 SV = 1	−11.61	16	26.70	3
	6.01	TC450362	Cupin family protein, expressed; *n* = 2; Oryza sativa Japonica	−12.09	12	28.39	3
h (Met)	1.01	TC388221	Beta amylase [Triticum aestivum]	−42.50	37	37.90	8
	2.02	TC425413	Globulin-2 precursor; *n* = 1; Zea mays	−22.52	18	49.29	5
	4.01	A7UME2	Xylanase inhibitor 725ACCN OS = Triticum aestivum PE = 4 SV = 1	−18.93	17	41.09	4
	5.01	P93693	Serpin-Z1B OS = Triticum aestivum PE = 1 SV = 1	−20.11	20	42.90	5
	6.01	TC383884	Enolase; *n* = 2; Oryza sativa Japonica Group	−25.06	17	52.00	5

proteins were detected. All bands revealed in the A/G fraction by immunoblot with three sera were found in the CM-like or Met fractions, and interestingly, some specific bands were only revealed using the Met fraction, reflecting an enrichment in some allergens. The proteins identified by mass spectrometry in the three fractions confirmed that all proteins of the A/G fraction were found either in the CM-like or Met fractions and sometimes in both. Based on the solubility along with the mobility of the proteins enriched in the CM-like fraction, Hurkman and Tanaka (2007) indicated that they were potentially AAI. We confirmed that this fraction is clearly enriched in AAIs among which there are many known allergens responsible for baker's asthma such as Tri a 15, Tri a 28, Tri a 29, Tri a 30 and Tri a CM16 which are referred in IUIS allergen nomenclature (www.allergen.org) (Tatham and Shewry, 2008). Moreover, two additional allergens were identified in this fraction: Tri a LTP2 (LTP) (Letho et al., 2010) and Tri a 37 (purothionin) (Denery-Papini et al., 2011; Palacin et al., 2007). These results confirmed the interest of this additional fractionation procedure to provide enriched allergen fractions (Hurkman and Tanaka, 2007). Therefore, in order to increase sensitivity, CM-like and Met fractions were extracted from each genotype, both commercial varieties and GMs, and used to measure their IgE-binding capacity.

The safety assessment for food based on transgenic plants requires the evaluation of the allergy potential of the GM plant in comparison with its non-transgenic counterpart. Two of the GM lines included in this study have already been compared to their parents using a classical allergonomic approach and revealed slight differences in their allergen content (Lupi et al., 2013). Others, analysed by transcriptomics, also revealed few differences in their allergen contents (Sestili et al., 2010, 2012, Sestili et al., 2013). Such analyses provide in-depth detail about transcripts, protein quantification and allergen recognition, but since there are no standards of acceptable differences, it is difficult to give an appropriate interpretation of data, especially in terms of risk. In order to obtain information about the acceptable increase in allergen concentration, we explored the natural variability of different genotypes using a large number of sera, taking account of the well-known individual variation for eliciting an allergic reaction. From the clinical and immunological point of view, the sera used in this study reflect the well-known variations in IgE concentration frequently described in IgE-mediated disorders. Interestingly, the profile of responses observed for most of the sera follow the same trend among the genotypes. The higher reactivity of the sera obtained from patients with respiratory allergy, especially on the CM fraction, is consistent with the enrichment of this fraction in AAIs, which are the major agents responsible for bakers' asthma (Salcedo et al., 2011). From the genetic point of view, we revealed that the IgE binding capacity of one genotype shows a difference with nearly half of the other genotypes tested, and that the IgE binding capacity may vary by up to two-fold between genotypes. Regarding the reactivity observed between the GM and their parents, the maximum increase or decrease observed was approximately 20%. These slight variations in allergenicity between the GM and their parents corroborate the results previously reported by Lupi et al. (2013) on the same GMs, but using a different approach.

In this study, we described, for the first time, the natural range of variation in the IgE-binding capacity of a dataset of 20 wheats cultivated at the same location. Our analysis revealed that the IgE binding capacity measurement of the two protein fractions explored was impacted by the type of serum, FA versus BA, but we did not find an effect related to the type of genome, AB or ABD. This latter result suggests that the proteins contributed by the D genome did not have an impact on the allergens involved in the elicitation of allergy. However, a slight tendency can be noted, with the four most highly responsive varieties belonging to bread wheat and the four lowest to durum wheat.

The differences observed between the GM and their parents were always within the variability measured for all genotypes. The varieties used in our study are tetra (AB) or hexaploides (ABD) and belong to wheat cultivated at this time. Exploration of diploid genotypes would be of interest. Environmental conditions could also be interesting to examine, especially because the salt-soluble proteins in wheat are primarily involved in metabolism or defence and, as such, they can be affected by biotic or abiotic stress. This study is comparable with analytical profiling methods such as transcriptomic, proteomic and metabolomic analyses that have been used for safety assessment of GMs. They generally revealed slight differences between the GM and its natural counterpart, and when environmental conditions were explored, they induced variations higher than those induced by the transgene (Baudo et al., 2006; Barros et al., 2010). The strategy developed in this study for wheat grains allows the quantification of the IgE-binding to a well-defined allergic source material. It may therefore be useful to determine the level and/or the nature of the endogenous allergic proteins that may have been altered as a result of biotechnology as well as breeding. Such an *in vitro* test is very sensitive, and when it

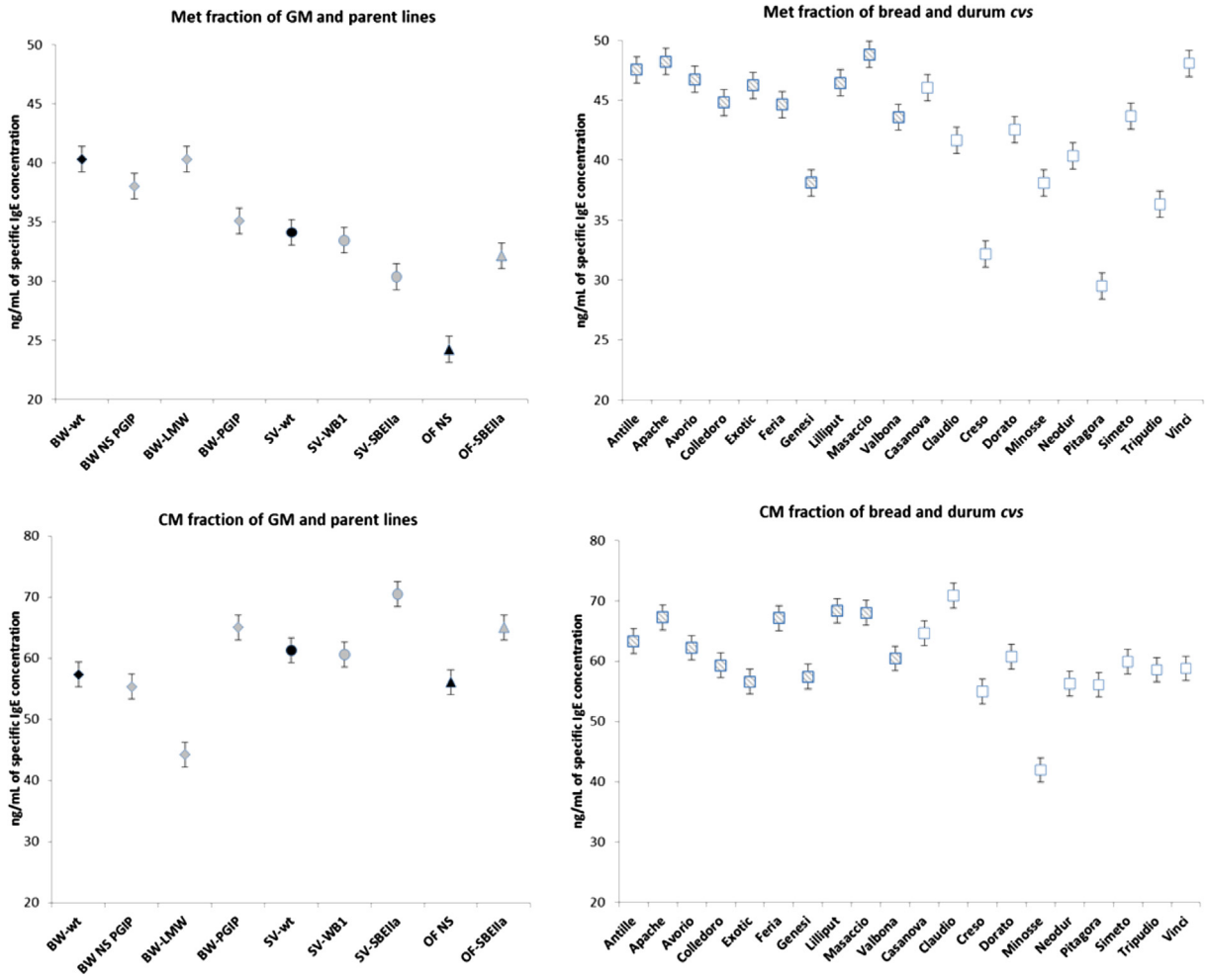

Fig. 3. Mean of specific IgE concentrations calculated for 18 sera from patients affected by food and respiratory allergy to wheat for Met and CM fractions of GM wheat lines (●) and their corresponding parental genotypes (●), and 10 wheat *T. aestivum cvs* (▨) and 10 *T. durum* cvs (□).

is performed with many sera, it is potentially highly predictive regarding the variation in the content of allergens in wheat grains.

Consequently, it has to be assumed that transgenesis may affect the allergenicity of wheat in positive as well as in negative ways. In the case of the GM wheats studied here, the differences observed with their natural counterparts are encompassed in the natural variability of conventional wheat cultivars. This confirms that the assessment of food safety in GM plants, at least in terms of wheat allergenicity, should be evaluated case by case, and that it is not possible to leave out the comparison with the natural variation of parameters under evaluation.

Table 3
Results of pair-wise comparisons of three genotypes and the GM lines was performed by using Fisher's Least Significant Difference (LSD) test. * indicates significant differences between GM line and its *wt* or null-segregant genotype. Differences and mean were expressed in ng/mL.

Contrast	Mean of parental line	Difference
Met fraction		
Bobwhite *wt* vs. Bobwhite null-segregant	40.31	2.28
Bobwhite *wt* vs. Bobwhite-LMW-GS	40.31	0.01
Bobwhite *wt* vs. Bobwhite-PGIP-GS	40.31	5.20*
Bobwhite null-segregant vs. Bobwhite-PGIP-GS	38.02	0.66
Svevo *wt* vs. Svevo-Wx-B1	34.11	0.66
Svevo *wt* vs. Svevo-SBEIIa	34.11	3.75*
Ofanto null-segregant vs. Ofanto-SBEIIa	24.22	−7.93*
CM fraction		
Bobwhite *wt* vs. Bobwhite null-segregant	57.34	2.00
Bobwhite *wt* vs. Bobwhite-LMW-GS	57.34	13.07*
Bobwhite *wt* vs. Bobwhite-PGIP-GS	57.34	−7.74*
Bobwhite null-segregant vs. Bobwhite-PGIP-GS	55.34	−9.74*
Svevo *wt* vs. Svevo-Wx-B1	61.32	0.71
Svevo *wt* vs. Svevo-SBEIIa	61.32	−9.19*
Ofanto null-segregant vs. Ofanto-SBEIIa	56.13	−8.95*

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcs.2014.02.009.

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