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Assessment of allergenicity of diploid and hexaploid wheat genotypes: Identification of allergens in the albumin/globulin fraction

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ABSTRACT

Wheat is an important part of the daily diet of millions of people. However, this staple food is also responsible for food allergies. Ancient cultivars of wheat are gaining interest today but nothing is known about their allergenicity. Many wheat proteins have been reported as causative food allergens, including some prolamin-type gluten proteins, and salt soluble proteins of the albumin/globulin (A/G) type.

The objective of this work is to obtain information about the allergenicity of the salt soluble A/G fraction of an ancient diploid cultivar compared with a standard hexaploid bread wheat cultivar using 20 sera from patients with wheat allergy. Differences in the IgE reactivity of sera towards the two genotypes were quantified by ELISA. Qualitative differences in IgE-binding proteins were searched after 1D or 2D electrophoresis.

For most of the sera, the concentration in A/G specific IgE was higher for the hexaploid T. aestivum (cv Récital) than for the diploid T. monococcum (cv Engrain). The analysis of 2D spots revealed by immunoblotting leads to the identification by mass spectrometry of 39 IgE-binding proteins, some of them unknown until now as wheat allergens. Numerous allergens were identified, differences observed between Engrain and Récital will be discussed.

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1. Introduction

Wheat is an important part of the daily diet of millions of people. Wheat products are consumed in various forms that include many kinds and types of breads, cakes, pastas, pizza and confectionary items. However, this staple food is also responsible for food allergies. Population-based studies indicate a prevalence as high as 0.5% in children on the basis of positive wheat challenge tests, and a high prevalence of

sensitization to wheat in adults (assessed by IgE) [1]. Hypersensitivity reactions to wheat flour occur both by inhalation (baker's asthma) and ingestion (food allergy and celiac disease), but may also develop by contact in some cases.

Wheat proteins are classically divided into two main groups: the salt soluble fraction mainly composed of albumins/globulins, and the gluten fraction made up of gliadins and glutenins. In the early 1970s, the gluten fraction was reported to be responsible for celiac disease and later on for food allergy. Since then, numerous

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studies have led to the identification of food allergens from this fraction and also to the characterization of epitopes involved in the IgE response [2–6]. Regarding wheat allergies, the flour soluble fraction consisting of albumins/globulins (A/G) has also been reported to contain IgE-binding proteins [7–9]. Proteins of the α -amylase/trypsin inhibitor family [10,11], β -amylase [12], peroxidases [12,13], LTP1 [12,14,15] and serpins [12,16,17] were reported to be allergens in food or respiratory allergies. However, it is noteworthy that the reactivity of the proteins within each family is dependent on their isoforms and that IgE obtained from patients recognize the allergens with variable sensitivity and specificity. Pastorello [12], using two-dimensional electrophoresis followed by immunoblotting, identified new allergens in this A/G fraction, including granule-bound starch synthases, glyceraldehyde-3P-deshydrogenase and puroindolines. Until now, members of 14 families of allergens belonging to the A/G fraction and involved in wheat allergies have been evidenced [18–20].

Wheat genus presents species with different ploidy levels, among which: diploid species with genome A (*T. monococcum*, einkorn); tetraploid with genomes A and B (durum wheat, Emmer); and hexaploid with genomes A, B and D (spelt, bread wheat). The bread wheat currently cultivated (*T. aestivum*) is hexaploid (genome ABD). It originated through spontaneous hybridizations between A, B and D genome of ancestral species and has been widely bred. In the case of prolamins, there is experimental evidence for a natural variation in the degree of biological activity between cereal genus (oat, rye, barley and wheat) [21,22] as well as in the genus *Triticum*. Interestingly, variations in prolamin peptides active in celiac disease have been identified within diploid, tetraploid and hexaploid species of the genus *Triticum* [23,24]. Similarly, the content of a major wheat allergen (ω 5-gliadin) was shown to vary among (hexaploid) bread wheat genotypes [25]. Differences in allergen recognition among seven hexaploid wheat cultivars have already been observed for patients with baker's asthma [26]. Finally, a large study that screened 321 wheat cultivars also revealed wide variations in their recognition by a unique patient serum possessing IgE binding to gliadins, glutenins and α -amylase inhibitors [27].

Until now, the salt soluble fraction has only been studied in hexaploid wheat. As observed for prolamins, it can be hypothesized that the constitutive gene families corresponding to the salt soluble fraction have also been modified through the evolution of modern bread wheat and could therefore induce variations in their allergenicity. The diploid wheat *T. monococcum* is an older species which has good breadmaking performances, although less commonly bred than hexaploid wheat. The A genome, which is currently part of the genome of hexaploid wheat, has probably undergone changes during amphiploidy events resulting in gene evolution between the A genome of diploid and hexaploid wheats. The aim of this work was first to compare the IgE-binding potential of the salt soluble fractions of hexaploid bread wheat (*Triticum aestivum* cv Récital) and its primitive diploid subgroup *Triticum monococcum* cv Engrain Pays de Sault, which only contains the A genome. Secondly, we aimed at characterizing allergen polypeptides at the molecular level in relation to wheat food allergy in both species with emphasis on the ancient diploid wheat.

2. Material and methods

2.1. Material

Wheat flour (type 65) from *Triticum aestivum* cultivar Récital (genomes ABD) and *Triticum monococcum* cultivar Engrain Pays de Sault (genome A) was obtained from INRA Clermont-Ferrand.

2.2. Sequential extraction of wheat proteins

Albumins/globulins (A/G) were extracted from 8 g of flour (cultivar Récital, Engrain Pays de Sault) according to the sequential procedure of Nicolas et al. [28]. A/G were extracted in 240 ml of 0.05 M phosphate buffer, pH 7.8, 0.1 M NaCl for 2 h at 4 °C with constant stirring. After centrifugation (8000 g for 15 min at 4 °C), the supernatant was collected, dialyzed against water and freeze-dried. The protein content was measured according to the Kjeldahl method. Gliadins and glutenins were subsequently extracted according to the method of Nicolas et al. [28].

2.3. Human sera

Sera were obtained from 20 patients (17 children from one to eight years old, and three adults >13 years old) with food allergy to wheat. Two sera were added as controls, one obtained from a non atopic subject who had 200 ng/ml of total IgE and another obtained from a wheat allergenic patient characterized by no specific IgE against A/G fraction but 20 ng/ml of specific IgE against total gliadins. Most of the patients included in the study were suffering from atopic eczema dermatitis syndrome (AEDS), one patient experienced urticaria, another an anaphylactic shock, and two presented gastrointestinal symptoms (Table 1). Sera were obtained from the Service of Clinical Immunology and Allergology, at the University Hospital (CHU) of Nancy, with the informed consent of the patients or their parents, and after ethical committee approval (authorization number DGS2007-0066). Food allergy to wheat was established by evidence of IgE-dependent sensitization by positive prick-in-prick tests to natural wheat flour and by standardized double-blind placebo-controlled food challenges (DBPCFC) with wheat flour, gluten and bread, or by an evident positive effect of avoidance diet when DBPCFC could not be performed for ethical reasons [5].

2.4. ELISA (enzyme-linked immunosorbent assay)

The determination of IgE specific for albumin/globulin extracts from Récital and Engrain Pays de Sault was performed by a fluorimetric ELISA as described by Bodinier et al. [29]. Briefly, an antigen-coated plate ELISA was used for the quantification of specific IgE: the antigens (A/G) were coated at 5 μ g/ml in 100 mM carbonate buffer, pH 9.6, and after washes and saturation, sera were diluted (1:10) and incubated for 15 h at 37 °C. Goat anti-human IgE alkaline phosphatase conjugate (ϵ -chain specific, Sigma A-3525) was added for 2 h at 37 °C (100 μ l/well, 1:500) before addition of fluorescent substrate, 4-MUP (4-methylumbelliferyl phosphate, Sigma M-3168) diluted 1/5 in 1 M Tris/HCl buffer pH9.8 (150 μ l/well) and

Table 1 – Clinical characteristics of patients and their IgE reactivity against albumins/globulins in ELISA or immunoblotting. AEDS: atopic eczema dermatitis syndrome, Urt: chronic urticaria, GI: gastro-intestinal symptoms, AS: anaphylactic shock, and nd: not done.

Patient serum no.	Age (year)	Symptoms	IgE specific for AG in ELISA	IgE specific for AG in immunoblotting
9	1.5	AEDS	+	nd
14	2	AEDS	+	+
18	2	AEDS	+	+
22	8	AEDS	+	nd
32	1	AEDS	+	+
35	35	Urt	+	+
38	2	AEDS	+	+
43	6	AEDS	+	neg
44	37	AEDS	+	+
47	3	AEDS	neg	+
55	8	AEDS	neg	+
265	7	AEDS GI	+	+
269	2	AEDS	+	+
326	5	AS	+	+
342	Child	AEDS	+	nd
397	2	AEDS	+	+
443	5	AEDS	+	nd
450	17	GI	+	nd
451	4	AEDS	+	+
479	6	AEDS	+	+

incubated for 90 min at room temperature in the dark. Fluorescence was measured at 440 nm (excitation 360 nm). The concentration of specific IgE binding to the A/G for a given serum was calculated from the adjusted standard human IgE curves provided that the fluorescence exceeded the quantification limit [29]. The mean quantification limit calculated from 10 experiments was 5 ± 2 ng/ml. Two control wells with 10 mM ethanolamine in carbonate buffer instead of antigen were included for each serum. For seven sera, four replicates were performed permitting the report of the mean and standard deviation of IgE specific concentrations in Table 3. The others were only done in duplicates because only few amounts of sera were available, in this case the value reported in Table 3 is the mean value of the duplicates.

2.5. 1D and 2D electrophoresis separation

Albumins/globulins were separated by SDS-PAGE on 12% acrylamide gels. Proteins were solubilized in Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol and bromophenol blue, adjusted at a concentration of 1 mg/ml and 10 μ l were loaded on the 1D gels. After separation, proteins were either stained with Coomassie Brilliant Blue G250 (Sigma-Aldrich) according to Devouge et al. [30] or transferred for immunoblotting.

Prior to 2D electrophoresis, albumins/globulins were solubilized in 8 M urea, 2% CHAPS and 2 M thiourea. Proteins (600 μ g) were then loaded on immobilized pH gradient gel strips (13 cm Immobiline Dry Strip pH 3–10, GE Healthcare) and isoelectrofocalization was performed at 20 °C. Prior to the second dimension, each gel strip was incubated with equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% SDS, 1% IPG buffer). Proteins were then separated

in the second dimension on 12% polyacrylamide gels. The gels were either stained with CBB as described for 1D gels or cut into pieces of 10 $\times$ 10 cm corresponding to a pH range of 4–9 and a MW range of 10–100 kDa and transferred for immunoblotting. Previous experiments performed on 7 $\times$ 7 cm 2D gels immunoblotted with some patient sera revealed a large amount of IgE-binding spots weakly resolved in the pI range of 4–9 and no IgE-binding spots at extreme pIs ranging between 3 and 4 and 9 and 10. Therefore we choose to perform the first dimension on strips of 13 cm in order to increase the resolution within the range 4–9 and cut the gels into pieces of 10 $\times$ 10 cm corresponding to a pH range of 4–9. For the vertical migration corresponding to the molecular weight, the migration was stopped in order to keep the small proteins (MW around 10 kDa) in the first 10 cm of the gels. The size of the gel and transfer sheet was also chosen because of the low availability of some patient sera, for this reason we were not able to duplicate the immunoblottings. Image acquisition was performed using an Imaging Densitometer (GE Healthcare) with a resolution of 300 μ m pixel⁻¹. The normalization step and spot detection were performed using ImageMaster 2D Platinum software (Amersham Biosciences). Each 2D blot was matched to its corresponding 2D gel, and all 2D gels of each genotype were matched together.

2.6. Analysis of serum reactivity by immunoblotting

After 1D or 2D electrophoresis, A/G gels were electroblotted to nitrocellulose membranes (0.2 μ m, Sartorius, Germany) in 25 mM Tris, 192 mM glycine, 0.1% SDS and 20% ethanol. Semidry transfer was achieved at 300 mA for 45 min for a 10 cm $\times$ 10 cm $\times$ 1 mm gel size.

After transfer of the 2D gels, nitrocellulose sheets were stained with 20 ml of Ponceau S Solution (Sigma) for 15 min, rapidly washed in water in order to reveal protein spots which are detected using white incident light source with a Fujifilm camera. These sheets were then washed four times in water prior immunoblotting. These discoloured nitrocellulose sheets were further washed in PBS solution and blocked for 4 h at room temperature in 4% polyvinylpyrrolidone (PVP-40, Sigma P-0930, Sigma, St Louis, MO, USA) in PBS-0.1% Tween-20 (PBS/T). After three washes with the PBS/T buffer containing 2% PVP, nitrocellulose membranes were incubated overnight with patient or control sera at the appropriate dilution (ranging from 1/10 to 1/80) in the washing buffer. Membranes were then washed three times and incubated for 1 h with peroxidase-labelled rabbit anti-human IgE (P0295, Dako, Denmark) diluted 1/100,000 in the washing buffer. After further washing, membranes were incubated in a chemiluminescent substrate (Super-Signal West Dura Extended Duration Substrate, 34076, Pierce, USA) for 5 min and dried between two paper sheets. Luminescence was then detected using a camera (Luminescent Image Analyzer LAS 3000; Fujifilm).

2.7. Protein identification by mass spectrometry

Protein spots were picked up manually and prepared for mass spectrometry. Each spot underwent the following steps: destaining, reduction, alkylation by iodoacetamide before in-gel digestion using trypsin hydrolysis according to Larré et al. [31].

2.7.1. 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 3000 RSLC system (Dionex) coupled with an LTQ-Orbitrap VELOS mass spectrometer (Thermo Fisher). Chromatographic separation was conducted on a reverse-phase capillary column (Acclaim Pepmap C18 2 μ m 100A, 75- μ m i.d. $\times$ 15-cm length, Dionex) at a flow rate of 300 nL min⁻¹. Mobile phases were composed as indicated: A (99.9% water and 0.1% formic acid), B (90% acetonitrile and 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. 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 (FWHM 30,000) in the Orbitrap analyzer (mass-to-charge ratio (m/z): 400 to 2000), while collision-induced dissociation (CID) spectra were recorded on the five most intense ions in the linear LTQ traps.

2.7.2. Databank searches

Raw data collected during LC-MS/MS analyses were processed into mgf format files and further searched against databanks using MASCOT Server 2.2 (Matrix Science). Protein identification was achieved by confronting mass data (MS and MS/MS spectra) against the UniProt databank (release 2010_029, August 2010). Another databank search was performed against the Wheat TIGR Gene Indices databank (<http://www.compbio.dfci.harvard.edu/tgi/>, release 12 from April 2010). Fixed modification of cysteine residues by iodoacetamide was considered, as well as dynamic modifications (propionamide cysteines and oxidized methionins). One missed trypsin cleavage was set for databank searches and a mass tolerance of 0.0005%. Protein identifications were compared in the two databanks and best matches were validated. Proteins were considered when a minimum of four unique peptides were matched in their sequence, with a MASCOT individual ion score of above 33 for both databases (this threshold corresponds to $p < 0.05$ in the MASCOT software). The concordance between experimental and theoretical molecular weight and/or pI was also used to validate the identifications.

3. Results

3.1. Composition of sequential extracts and purity of the A/G fractions

The sequential fractionation technique based on differential solubility provides an effective way to isolate and quantify the major classes of seed proteins: albumins/globulins (A/G), also referred to as the soluble fraction, gliadins (Gli) and glutenins (Glu). Their proportions are reported in Table 2 for each genotype. Anti-peptide antibodies specific for $\alpha\beta$ - and γ -gliadins [32] were used in immunoblotting to control the potential contamination with gliadins of the A/G fraction. No α -, β -gliadins were detected and only very low amounts of γ -gliadins were detected (results not shown).

Table 2 – Percentage of protein in each fraction after sequential extraction.

	A/G	Gli	Glu
Récital	27	36	37
Engrain	19	63	18

3.2. Reactivity of IgE antibodies with albumin/globulin fractions

Sera were obtained from three adults and 17 children with clinically-documented food allergy to wheat. They were checked for their reactivity to A/G from Récital or Engrain Pays de Sault in ELISA or in immunoblotting after SDS-PAGE (Table 1). IgE binding to the A/G fractions of the two genotypes, Récital and Engrain Pays de Sault, was quantified by ELISA for 18 sera (Table 3). For most sera, the binding of IgE was reduced with the Engrain Pays de Sault A/G fraction compared to Récital A/G. Five of them (no. 32, no. 35, no. 44, no. 269, and no. 450) exhibited almost the same level of IgE binding for both cultivars and only one (no. 18) was more reactive with A/G fraction of Engrain then Récital. The reduction in IgE binding varied according to sera, but the overwhelming majority of sera (10 from among a total of 18 sera) displayed concentrations in IgE specific for Engrain Pays de Sault A/G of less than 50% of those for Récital A/G (Table 3). Seven sera representative of the group were tested in immunoblotting for their IgE responses to A/G from the two genotypes (Fig. 1). The results obtained by immunoblotting are in agreement with those of ELISA, similar intensity to A/G from Engrain Pays de Sault and Récital was observed for serum no. 265, a small difference for serum no. 44, and larger differences in intensity or more components detected by sera no. 326, no. 479 and no. 451, notably at low molecular weights. Two sera (no. 47 and no. 55), not reactive in ELISA, clearly showed IgE reactivity to Récital A/G in immunoblotting, whereas lower or no reactivity was detected for Engrain Pays de Sault. Detected IgE-binding bands varied with the sera and ranged from 10 kDa to 81 kDa in both cultivars.

3.3. Allergen identification

The type and abundance of proteins in the salt soluble fraction of wheat vary greatly among genotypes and therefore led us to develop an 'allergenomic' approach to identify and compare allergens between Récital and Engrain Pays de Sault. Briefly, this allergenomic approach combines common proteomic approaches with immunodetection via patient sera. In a first step, two-dimensional electrophoresis was used to separate the salt soluble proteins. Figs. 2A and 3A show typical proteome maps obtained after CBB staining for Engrain Pays de Sault and Récital, respectively. The 2D maps obtained for both cultivars Engrain Pays de Sault reveal a similar number of spots, however their patterns were too different to permit any spot matching. It may be noted that many intense spots are visible in the lower part of Récital gels that don't appear in the gels obtained with Engrain Pays de Sault. The other three maps in Figs. 2 and 3 are immunoblots performed with the sera of three different patients. Numerous immunoreactive spots can be observed, ranging from 29 to 80 kDa,

Table 3 – Concentrations in IgE specific for A/G (ng/ml) from two wheat genotypes. Data represent mean $\pm$ SD of four replicates or mean of two replicates, <Q indicates that the mean value was below the limit of quantification calculated as 5 ± 2 .

No. serum	9	14	18	22	32	35	38	43	44	265	269	326	342	397	443	450	451	479
Récital	37	59 $\pm$ 6	28	34	8 $\pm$ 2	8	60	30 $\pm$ 1	12	98	16 $\pm$ 4	85	39 $\pm$ 5	62	105	11	120	56 $\pm$ 2
Engrain	21	11 $\pm$ 5	36	19	<Q	6	11	<Q	<Q	79 $\pm$ 2	15 $\pm$ 4	36	15 $\pm$ 3	31	30	8	66	9 $\pm$ 2

depending both on the serum and the genotype. Immunoblots performed with the two control sera didn't reveal any spots (not shown).

Among these numerous spots, we concentrated our efforts on the most reactive spots as revealed by their intensity in immunoblotting. By overlaying the membrane stained by Ponceau Red, its immunoblot and the corresponding CBB stained gel, we were able to accurately excise the spots revealed by IgE from the CBB stained gel in order to identify them by mass spectrometry. It should be noted that the smaller the number of spots detected was, the more difficult it was to match them and moreover, in some cases, no blue stained spot was visible at the calculated position. The MS identifications are listed in Table 4 and the best scoring proteins for each of these spots are reported. As a result of the analysis performed on both genotypes, we identified 39 proteins, most of them belonging to families (alpha amylase inhibitors, serpin, beta amylase, globulins etc.) in which allergens have been reported in various food or respiratory allergies. In the genotype Engrain Pays de Sault, 23 of the most reactive proteins detected with the three sera and identified with the best MASCOT scores were: serpin together with either enolase, aspartic proteinase or glucose 1 phosphate adenylyl-transferase (spots 17, 18, 19, 20, and 21), aspartate amino transferase and glyceraldehyde-3-phosphate dehydrogenase (spots 37, 38, 39, and 40), TC407122 homologous to a putative uncharacterized protein of *Oriza sativa* mixed with 14-3-3 protein (spots 3, 4, and 5), triosephosphate isomerase (spot 44) and globulin 3 (spots 14, 15, and 16). In spots presenting a

lower degree of reactivity, we identified ascorbate peroxidase together with dehydroascorbate reductase (spot 45), beta-glucosidase with globulins (spots 11, 12, and 24) and beta-D-glucan exohydrolase (spots 25, 26, and 27). The same sera also revealed many proteins in the genotype Récital within which 20 were identified, listed here by the order of intensity of reactivity: beta amylase mixed with enolase (spots R1 and R2), serpin mixed with beta amylase (R3, R4, and R5), alpha amylase inhibitors of five types (spots R39, R37, R43, R44, R45, R42, and R33), serpins (R25, R26, and R27) and globulins (spots R13, R14, R15, R16, R11, and R12) mixed with peroxidase and beta-glucosidase in spots R14 and R15.

Interestingly, the IgE of patient 44 detected only a few spots on Récital (Fig. 3C), mainly located at low molecular weights and belonging to the alpha amylase inhibitor family. IgE-binding spots of low intensity were detected in the same area in Engrain Pays de Sault however no clear correspondent blue stained spots were found and therefore they were not identified (Fig. 2C). In the case of serum 38 and serum 265, they revealed similar allergens for both genotypes although serpin and globulin 3 were found to be more highly expressed in Engrain Pays de Sault than in Récital (Figs. 2A and 3A).

In both genotypes, some of these more intense spots appeared as part of strings of spots that became visible when the revelation time was increased; they were identified and the results are reported in Table 4 (A for Engrain Pays de Sault and B for Récital). Most of the spots localized within such strings corresponded to isoforms belonging to the same family.

In most cases, one spot was found to be an envelope of two or more distinct proteins. This type of case leads to uncertainty as to the protein that is potentially allergenic. In fact, even if spectral counting [33–35] could also be used to assess the relative abundance of these proteins within a spot, we didn't use it since it is well known that minor proteins might be at the origin of the IgE binding. Therefore, except when one of these proteins has already been identified as an allergen in previous studies, we will only report them as putative allergens. The reactivity of these identified proteins has to be confirmed by further studies.

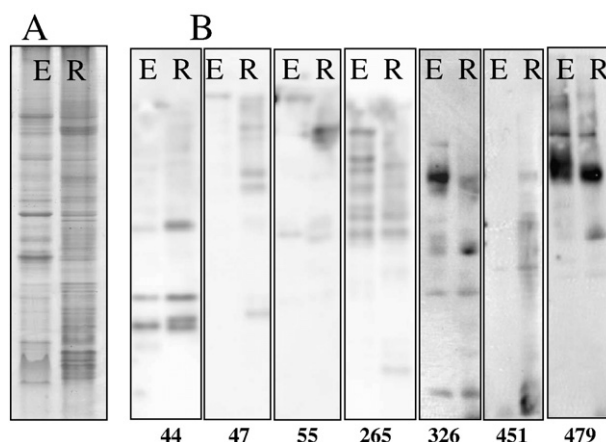


Fig. 1 – A/ SDS electrophoresis of A/G fractions extracted from Engrain (E) and Récital (R), 10 μ g of proteins loaded on each lane. B/ Reactivity in immunoblotting of IgE from patients suffering from wheat allergy with A/G fractions from Engrain (E) and Récital (R).

4. Discussion

In the present study, all 20 sera except one were less or equally reactive in ELISA to the A/G fraction of Engrain Pays de Sault than that of Récital. In order to explore differences in reactivity observed between these two genotypes, we developed an allergenomic analysis based on 1D, followed by 2D electrophoresis that has been proved to be a useful tool for the identification of allergens [20]. Among seven sera reactive in

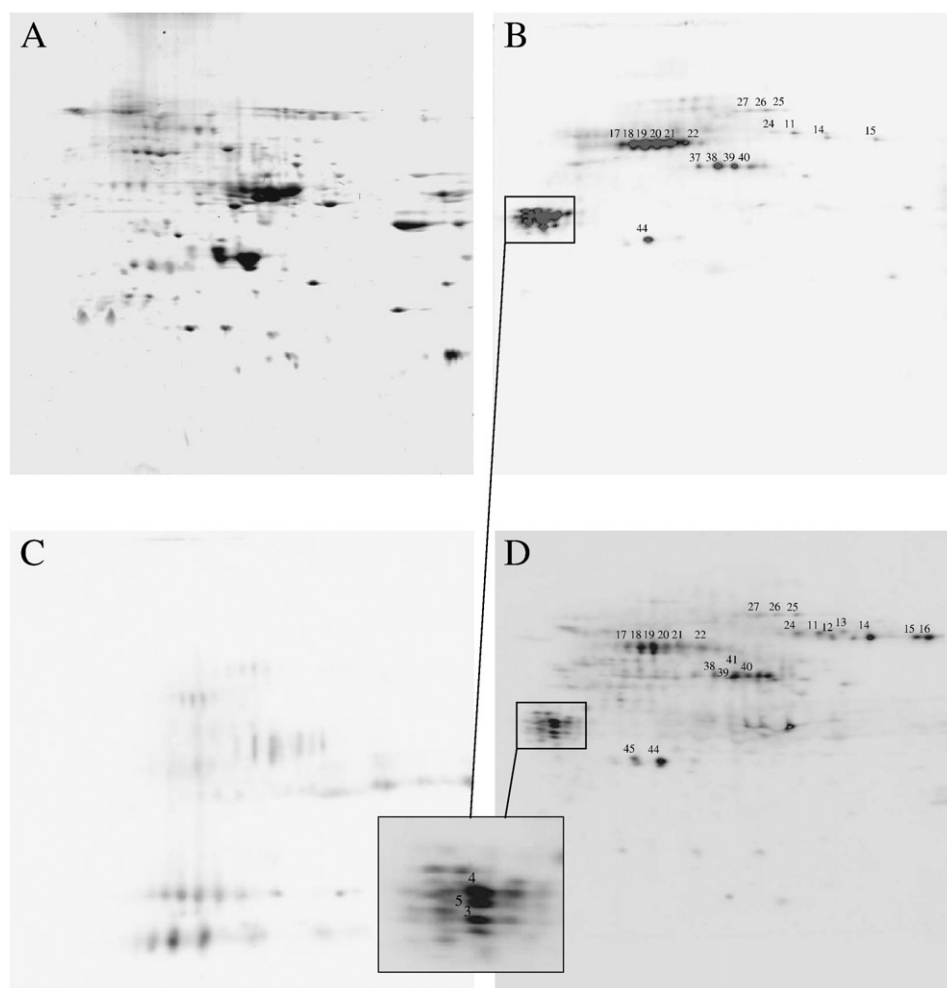


Fig. 2 – Two dimensional map of A/G extractable fraction of Engrain and immunoblotting with sera from wheat allergic patient. A/ Proteins spots were visualized by Coomassie staining. B/ Immunoblotting with serum 38. C/ Immunoblotting with serum 44. D/ Immunoblotting with serum 265. The identified and labeled spots are annotated according to the spots number used in Table 4.

1D immunoblotting, three were chosen because they recognized many different bands in both genotypes. The 2D immunoblotting patterns obtained for the three sera confirmed the high variability between individual patients with similar symptoms in the recognition of specific proteins by their IgE, regardless of the genotype concerned. It can be noted that serum 44 obtained from an adult patient (Table 1) give patterns very different from the two other sera. This result agrees with Sotkovsky's analysis performed within numerous hexaploid genotypes with many sera [11]. No clear correlation was found between reactivity in ELISA and the number of spots. However, serum 265, which is highly reactive in ELISA, exhibited the highest number of IgE-binding spots.

In agreement with previously published data on food or respiratory allergy on cereals, we identified alpha amylase inhibitors, beta amylase, serpin, globulin, beta-D-glucan hydrolase, etc. Some of them are clearly identified as allergens while others can only be considered to be potent allergens.

Proteins of the alpha amylase inhibitors were identified only on Récital with IgE-binding spots mainly located at low

molecular weights. It can be noted that the 2D blue stained gels of Engrain Pays de Sault present a few number of spots in the region of these inhibitors, some of them being slightly revealed by patients IgE thus allowing us to assume that these inhibitors are less expressed by the A genome. Indeed, the alpha amylase inhibitors reported earlier as allergens [18] were expression products of genes located on genomes B and D. Moreover, exploration of the Uniprot database (UniProt release 2010_11) revealed that only a few of these inhibitors were reported in *T. monococcum*, without evidence at protein and transcript levels. Among the tetrameric alpha amylase inhibitors found in Récital, CM16 has already been validated as a food allergen [12], while CM3, also previously reported [12,36] has not yet been validated. The 0.28 isoform has only been reported as potent allergen in baker's asthma [37]. Among the two dimeric alpha amylase inhibitor isoforms identified here, one is identical to a protein (AAV39519) found by Sotkovsky et al. [11] in *T. aestivum*. Our results on alpha amylase inhibitors point out two new putative allergens associated with wheat food allergy.

Beta amylase, beta-D-glucan hydrolase and beta-glucosidase belong to glycoside hydrolases and are present in seeds

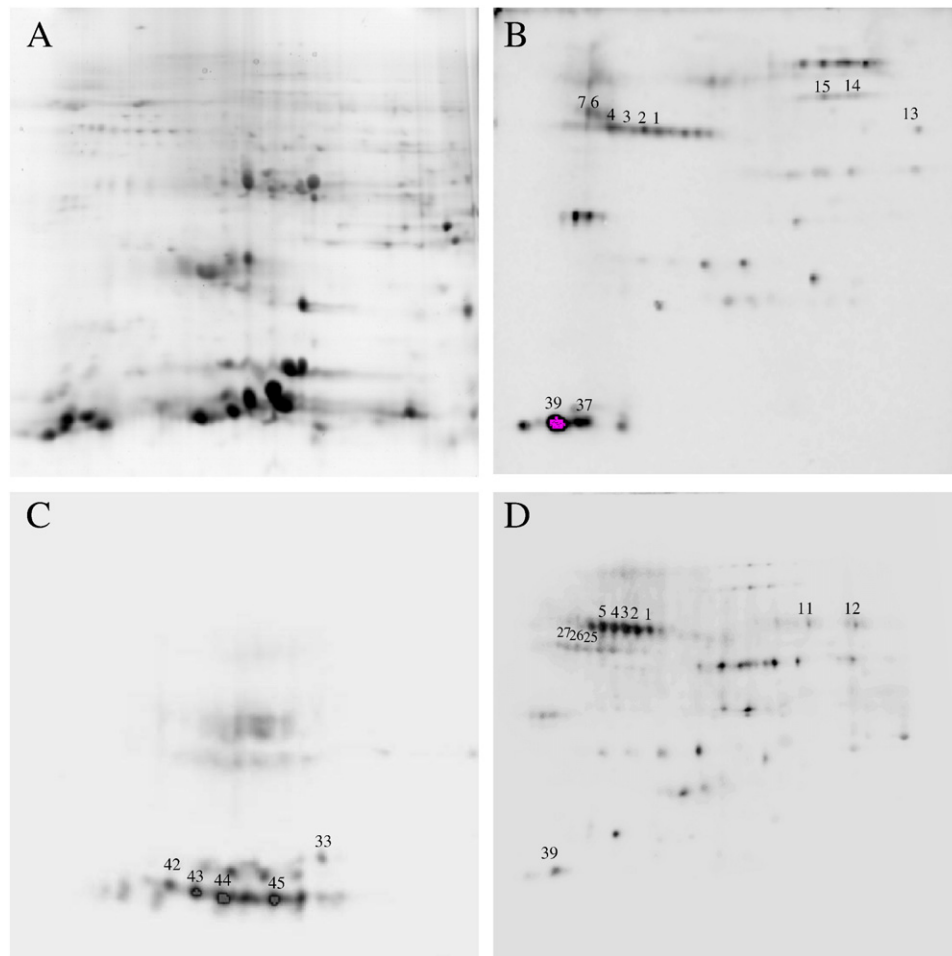


Fig. 3 – 2D gel of A/G fraction extracted from Récital and immunoblotting with sera from wheat allergic patient. A/ Proteins spots were visualized by Coomassie staining. B/ Immunoblotting with serum 38. C/ Immunoblotting with serum 44. D/Immunoblotting with serum 265. The identified and labeled spots are annotated according to the spots number used in Table 4.

containing starch as a food reserve. Despite the low content of beta amylase in barley flour, Sandiford [38] revealed its allergenicity in baker's asthma using RAST analysis and concluded as to their low cross-reactivity with fungal alpha amylase classically used in the baking industry [39]. Evidence for the allergenicity of beta amylase from other cereals such as wheat has not been proved despite the fact that they were identified twice in food allergenomic studies [11,12]. Beta-D-glucan hydrolase and beta-glucosidase can be considered as putative allergens, the second has never been yet identified in wheat. In our study this last protein has been found in IgE-binding spots for both genotypes.

We identified new IgE-binding proteins belonging to the cupin-2 superfamily. Globulin 3 was the most highly represented isoform in our identification and was found in many spots that were more abundant in Engrain Pays de Sault than in Récital. In both cases, their presence at different molecular weights and pI is not very surprising because previous studies on other seeds reported the presence of numerous isoforms of globulins, as well as the occurrence of proteolysis during seed maturation leading to several polypeptides [31,40]. This protein was found with two other globulins, globulin 3C and globulin 3B

with which it shares 88% and 72% identities respectively. All three can be categorized as belonging to the 7S globulin subfamily. This family is not very highly represented in cereals since they do not constitute the main storage proteins as they do in dicotyledonous plants. However, it appears that they are more highly represented in Engrain Pays de Sault than in Récital. A globulin 2 only identified in Engrain Pays de Sault is homologous to globulin 2 (S15675) found in maize A/G fraction that was shown to react with IgE patients [41]. Moreover, many proteins from the 7S globulin group are known allergens described in dicotyledonous plants. A major food allergen in peanuts, referred to as Ara h 1, on which 23 IgE-binding epitopes were mapped [42], was shown to share structurally-related IgE-binding epitopes with other 7S globulins of tree nuts [43]. Others have been described, including Ses i 3 from sesame and Gly m 5 from soybean [44]. Since the wheat globulins identified in this study belong to a family known to contain many allergens, they may be allergenic proteins. However, in some spots, beta amylase or peroxidase was also present and could therefore also be at the origin of the IgE reactivity.

Our 2D immunoblotting analysis also revealed numerous serpins. These proteins have already been reported as potent

Table 4 – List of identified proteins from 2D gels of Engrain (Fig. 2) and Récital (Fig. 3). The columns correspond to: Spot: assigned protein spot number corresponding to those indicated in Figs. 2 and 3, Prot Id: the protein identity as referred to in Uniprot or Wheat TIGR databank, Uniprot best homologue protein name: its corresponding protein or the Uniprot best homologue protein name, MW: its theoretical molecular weight, pI: its theoretical isoelectric point, %Cov: the per cent sequence coverage, Un pep: the number of unique peptides, Tot pep: the total number of peptides above the MASCOT individual ion score, and Score: the MASCOT total ion score.

Spot	Prot Id	Uniprot best homologue protein name	MW	pI	% Cov	Un pep	Tot pep	Score
<i>A. Engrain corresponding to Fig. 2</i>								
3	TC407122	OSJNBb0118P14.11 protein; <i>Oryza sativa</i> Japonica Group. 85% Id	19,139	5.21	58.9	7	39	499
	CD914053	Putative embryonic cell protein; <i>Oryza sativa</i> . Partial (48%). 88% Id	18,353	5.73	40	6	6	337
	Q08G36	14-3-3 protein — <i>Triticum aestivum</i>	29,388	4.83	43.3	10	11	328
4	TC407122	OSJNBb0118P14.11 protein; <i>Oryza sativa</i> Japonica Group. 85% Id	19,139	5.21	54.4	6	12	457
5	Q0JDG9	Os04g0404400 protein — <i>Oryza sativa</i>	31,329	4.9	5.8		12	713
	TC407122	OSJNBb0118P14.11 protein; <i>Oryza sativa</i> Japonica Group. 85% Id	19,139	5.21	56.1	7	38	521
	TC387595	14-3-3 protein — <i>Hordeum vulgare</i> . 99% Id	30,371	4.73	28.9	9	13	452
11	TC424895	Beta-glucosidase; <i>Hordeum vulgare</i> . Partial (95%). 97% Id	56,829	8.58	27.8	11	24	657
	TC425413	Globulin-2 precursor — <i>Zea mays</i> (Maize). Partial (47%). 72% Id	49,650	8.54	27.3	9	16	434
	TC404101	Endoglucanase 11 precursor <i>Oryza sativa</i> Japonica. Partial (91%). 80% Id	54,717	8.39	29	9	13	352
	B7U6L4	Globulin 3 — <i>Triticum aestivum</i>	66,652	7.78	8.2	4	5	119
12	TC424895	Beta-glucosidase; <i>Hordeum vulgare</i> . Partial (95%). 97% Id	56,829	8.58	20.5	10	33	522
	TC404101	Endoglucanase 11 precursor; <i>Oryza sativa</i> . Partial (91%). 80% Id	54,717	8.39	30.8	9	15	482
	TC425413	Globulin-2 precursor — <i>Zea mays</i> . Partial (47%). 72% Id	49,650	8.54	32.6	12	19	479
	B7U6L4	Globulin 3 — <i>Triticum aestivum</i>	66,652	7.78	18.2	9	12	253
13	TC425413	Globulin-2 precursor — <i>Zea mays</i> . Partial (47%). 72% Id	49,650	8.54	25.5	8	14	518
	B7U6L4	Globulin 3 — <i>Triticum aestivum</i>	66,652	7.78	24.1	12	17	329
14	B7U6L3	Globulin 3C (Fragment) — <i>Triticum aestivum</i>	38,634	9.15	16.6	4	8	254
	Q7DMU0	Storage protein — <i>Triticum aestivum</i>	72,551	6.8	11.5	8	10	223
	B7U6L5	Globulin 3B — <i>Triticum aestivum</i>	57,068	7.36	12.9	7	8	166
	B5A496	Beta-glucosidase — <i>Hordeum vulgare</i>	57,674	7.64	9.6	5	5	133
15	B7U6L4	Globulin 3 — <i>Triticum aestivum</i>	66,652	7.78	24.1	17	19	339
	B7U6L3	Globulin 3C (Fragment) — <i>Triticum aestivum</i>	38,634	9.15	16.6	8	8	201
	Q7DMU0	Storage protein — <i>Triticum aestivum</i>	72,551	6.8	11.5	8	9	170
16	B7U6L4	Globulin 3 — <i>Triticum aestivum</i>	66,652	7.78	23	11	16	335
	B7U6L3	Globulin 3C (Fragment) — <i>Triticum aestivum</i>	38,634	9.15	16.6	4	8	212
	Q7DMU0	Storage protein — <i>Triticum aestivum</i>	72,551	6.8	10.4	6	8	148
	B7U6L5	Globulin 3B — <i>Triticum aestivum</i>	57,068	7.36	11.5	6	7	146
	TC383884	Enolase — <i>Oryza sativa</i> . Complete	52,518	6.15	48.2	19	21	964
17	Q9ST58	Serpin-Z1C — <i>Triticum aestivum</i>	42,969	5.62	43.2	12	37	968
	TC383884	Enolase — <i>Oryza sativa</i> . Complete. 96% Id	52,518	6.15	48.2	14	21	964
	C0LF32	Serpin 3 — <i>Triticum aestivum</i>	43,227	5.56	41.6	12	25	679
18	Q0PG36	Glucose-1-phosphate adenylyltransferase — <i>Triticum aestivum</i>	58,399	6.12	44.1	18	19	611
	C0LF32	Serpin 3 — <i>Triticum aestivum</i>	43,227	5.56	38.3	11	20	523
	Q9ST58	Serpin-Z1C — <i>Triticum aestivum</i>	42,969	5.62	36.9	11	18	446
	TC383884	Enolase — <i>Oryza sativa</i> . Complete. 96% Id	52,518	6.15	33.3	14	15	485
	P08819	Serine carboxypeptidase 2 — <i>Triticum aestivum</i>	49,817	5.96	25.5	8	11	346
19	TC382510	Phytpsin precursor (Aspartic proteinase) — <i>Hordeum vulgare</i> . Partial (53%). 82% Id	29,405	5.48	41.3	10	26	853
	P93693	Serpin-Z1B — <i>Triticum aestivum</i>	43,120	5.44	43.6	14	20	560
	C0LF30	Serpin 1 — <i>Triticum aestivum</i>	43,261	5.44	41.4	13	18	536
20	P93692	Serpin-Z2B — <i>Triticum aestivum</i>	43,011	5.18	26.6	7	10	438
	C0LF30	Serpin 1 — <i>Triticum aestivum</i>	43,261	5.44	30.8	10	12	365
	TC382510	Phytpsin precursor (Aspartic proteinase) — <i>Hordeum vulgare</i> . Partial (53%). 82% Id	29,405	5.48	42.8	11	24	753
	TC387159	Aspartic proteinase — <i>Triticum aestivum</i> . Partial (58%). 79% Id	36,023	8.24	41.8	9	33	709
21	Q0PG36	Glucose-1-phosphate adenylyltransferase — <i>Triticum aestivum</i>	58,399	6.12	34.3	17	19	545
	Q9ST58	Serpin-Z1C — <i>Triticum aestivum</i>	42,969	5.62	29.6	13	9	264
	TC382510	Aspartic proteinase — <i>Triticum aestivum</i> . Partial (58%). 79% Id	29,405	5.48	49.1	12	42	596
24	TC424895	Beta-glucosidase — <i>Hordeum vulgare</i> . Partial (95%). 96% Id	56,829	8.58	29.8	14	35	1056
	TC425413	Globulin-2 precursor — <i>Zea mays</i> . Partial (47%). 72% Id	49,650	8.54	33.9	13	43	1020
	TC387479	Globulin-like protein — <i>Oryza sativa</i> . Partial (41%). 78% Id	45,770	7.26	23.8	8	18	409
25	Q8RWR5	Beta-D-glucan exohydrolase — <i>Triticum aestivum</i>	67,714	6.86	37.3	19	33	940
	B2CGM5	Triticin — <i>Triticum aestivum</i>	65,241	6.37	12.5	6	7	187
26	Q8RWR5	Beta-D-glucan exohydrolase — <i>Triticum aestivum</i>	67,714	6.86	38	21	27	834
27	Q8RWR5	Beta-D-glucan exohydrolase — <i>Triticum aestivum</i>	67,714	6.86	42.3	22	30	944
	B2CGM5	Triticin — <i>Triticum aestivum</i>	65,241	6.37	16.3	7	11	502
37	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase — <i>Triticum aestivum</i>	36,619	6.67	27	7	11	352
38	B0FRH4	Aspartate aminotransferase (Fragment) — <i>Triticum aestivum</i>	42,060	6.77	48.2	17	22	615
	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase — <i>Triticum aestivum</i>	36,619	6.67	40.1	10	13	401

Table 4 (continued)

Spot	Prot Id	Uniprot best homologue protein name	MW	pI	% Cov	Un. pep	Tot pep	Score
A. Engrain corresponding to Fig. 2								
39	B0FRH4	Aspartate aminotransferase (Fragment) — <i>Triticum aestivum</i>	42,060	6.77	55.8	23	33	859
	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase — <i>Triticum aestivum</i>	36,619	6.67	43	12	13	340
40	B0FRH4	Aspartate aminotransferase (Fragment) — <i>Triticum aestivum</i>	42,060	6.77	52.1	21	35	957
	P93692	Serpin-Z2B — <i>Triticum aestivum</i>	43,011	5.18	26.6	8	12	458
	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase — <i>Triticum aestivum</i>	36,619	6.67	32.6	9	10	304
41	B0FRH4	Aspartate aminotransferase (Fragment) — <i>Triticum aestivum</i>	42,060	6.77	23.4	11	13	330
	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase — <i>Triticum aestivum</i>	36,619	6.67	33.8	7	8	215
44	Q9FS79	Triosephosphate isomerase — <i>Triticum aestivum</i>	27,014	5.38	38.7	9	10	374
	C9EF64	Dehydroascorbate reductase — <i>Triticum aestivum</i>	23,457	5.88	50	8	13	309
45	O23983	Ascorbate peroxidase — <i>Hordeum vulgare</i>	27,532	5.85	38	8	11	330
	C9EF64	Dehydroascorbate reductase — <i>Triticum aestivum</i>	23,457	5.88	50	8	11	306
	Q9FS79	Triosephosphate isomerase — <i>Triticum aestivum</i>	27,014	5.38	32.4	7	8	250
B. Récital corresponding to Fig. 3								
R1	TC388221	Beta-amylase precursor; <i>Hordeum vulgare</i> 89% Id	38,254	5.86	41.2	13	36	571
	TC383884	Enolase; <i>Oryza sativa</i> 96% Id	52,518	6.15	20.7	7	9	425
R2	TC388221	Beta-amylase precursor; <i>Hordeum vulgare</i> 89% Id	38,254	5.86	35.9	11	22	589
	TC425761	Enolase; <i>Oryza sativa</i> 96% Id	52,758	5.8	10.2	4	4	227
R3	TC374294	Serpin; <i>Triticum aestivum</i>	45,523	5.81	26.6	9	9	533
	TC388221	Beta-amylase precursor; <i>Hordeum vulgare</i> 89% Id	38,254	5.86	33.8	10	34	476
	TC383884	Enolase; <i>Oryza sativa Japonica</i> 96% Id	52,518	6.15	13.9	5	6	297
R4	TC380640	Serpin; <i>Triticum aestivum</i>	44,790	5.37	26.8	9	13	605
	TC388221	Beta-amylase precursor; <i>Hordeum vulgare</i> 89% Id	38,254	5.86	39.1	12	41	556
	TC374294	Serpin; <i>Triticum aestivum</i>	45,523	5.81	26.6	9	10	540
R5	TC388221	Beta-amylase precursor; <i>Hordeum vulgare</i> 89% Id	38,254	5.86	35	10	37	528
	TC380640	Serpin; <i>Triticum aestivum</i>	44,790	5.37	24.4	7	12	478
R11	B7U6L4	Globulin 3 — <i>Triticum aestivum</i>	66,652	7.78	23.3	14	25	410
	B7U6L3	Globulin 3C (Fragment) — <i>Triticum aestivum</i>	38,634	9.15	20.1	7	13	248
	Q8LK23	Peroxidase — <i>Triticum aestivum</i>	39,255	8.14	11.7	4	4	109
R12	B7U6L4	Globulin 3 — <i>Triticum aestivum</i>	66,652	7.78	21.9	12	21	399
	B7U6L3	Globulin 3C (Fragment) — <i>Triticum aestivum</i>	38,634	9.15	16.9	6	9	213
R13	B7U6L4	Globulin 3 — <i>Triticum aestivum</i>	66,652	7.78	22.1	13	24	435
	B7U6L3	Globulin 3C (Fragment) — <i>Triticum aestivum</i>	38,634	9.15	14.6	6	10	271
	B7U6L5	Globulin 3B — <i>Triticum aestivum</i>	57,068	7.36	10.5	7	10	151
R14	B7U6L4	Globulin 3 — <i>Triticum aestivum</i>	66,652	7.78	17.5	12	12	415
	TC424895	Beta-glucosidase — <i>Hordeum vulgare</i>	56,829	8.58	15	9	9	266
	Q8LK23	Peroxidase — <i>Triticum aestivum</i>	39,255	8.14	6.1	2	2	84
R15	B7U6L4	Globulin 3 — <i>Triticum aestivum</i>	66,652	7.78	18.7	17	17	364
	B7U6L3	Globulin 3C (Fragment) — <i>Triticum aestivum</i>	38,634	9.15	14.6	6	6	181
	Q8LK23	Peroxidase — <i>Triticum aestivum</i>	39,255	8.14	9.5	4	4	108
R25	P93692	Serpin-Z2B — <i>Triticum aestivum</i>	43,011	5.18	30.7	10	32	1041
	Q9ST57	Serpin-Z2A — <i>Triticum aestivum</i>	43,341	5.46	33.2	11	30	749
	C0LF30	Serpin 1 — <i>Triticum aestivum</i>	43,261	5.44	22.3	9	12	382
R26	P93692	Serpin-Z2B — <i>Triticum aestivum</i>	43,011	5.18	28.4	9	42	1574
	Q9ST57	Serpin-Z2A — <i>Triticum aestivum</i>	43,341	5.46	30.9	9	25	552
	C0LF30	Serpin 1 — <i>Triticum aestivum</i>	43,261	5.44	20.1	8	10	316
R27	P93692	Serpin-Z2B — <i>Triticum aestivum</i>	43,011	5.18	39.4	11	48	2132
	Q9ST57	Serpin-Z2A — <i>Triticum aestivum</i>	43,341	5.46	33.2	10	21	489
	C0LF31	Serpin 2 — <i>Triticum aestivum</i>	43,518	5.11	24.2	11	14	346
	Q401N7	Aspartic proteinase — <i>Triticum aestivum</i>	54,965	5.14	4.9	2	3	130
R33	Q6S5B1	Alpha amylase inhibitor CM3 — <i>Triticum turgidum</i>	18,893	7.44	57.1	8	62	2401
R37	C3VWA4	Dimeric alpha amylase inhibitor — <i>Triticum turgidum</i>	15,605	5.01	75.2	11	18	651
R39	B5B0D5	Major allergen CM16 — <i>Triticum aestivum</i>	16,400	4.86	41.3	14	14	288
R42	C3VW80	Dimeric alpha amylase inhibitor — <i>Triticum turgidum</i>	13,743	6.49	89.5	10	19	793
	Q6S5B1	Alpha amylase inhibitor CM3 — <i>Triticum turgidum</i>	18,893	7.44	42.3	5	6	225
R43	C3VW80	Dimeric alpha amylase inhibitor — <i>Triticum turgidum</i>	15,588	5.28	69.5	10	11	442
	A4ZIU3	Monomeric alpha amylase inhibitor (Fragment) — <i>Triticum monococcum</i>	13,717	6.18	62	6	7	183
R44	A4ZIU3	Monomeric alpha amylase inhibitor (Fragment) — <i>Triticum monococcum</i>	13,717	6.18	92.6	13	98	2499
	TC435176	Alpha amylase inhibitor 0.28 precursor; <i>Triticum aestivum</i> 99% Id	17,530	6.73	63.6	12	84	608
R45	A4ZIU3	Monomeric alpha amylase inhibitor (Fragment) — <i>Triticum monococcum</i>	13,717	6.18	84.3	10	24	514
	C4P5B9	Monomeric alpha amylase inhibitor — <i>Triticum turgidum</i>	17,190	6.55	45	6	19	399
	TC431321	Dimeric alpha amylase inhibitor; <i>Triticum aestivum</i> 99% Id	18,467	8.03	52.1	6	7	498
	TC435176	Alpha amylase inhibitor 0.28 precursor; <i>Triticum aestivum</i> 99% Id	17,530	6.73	57.1	9	22	444

allergens in baker's asthma [45] and in food allergy [11,12] but the proof of their allergenicity must be shown. It is noteworthy that proteins belonging to the same family were shown to be involved in allergy, e.g., Gal d 2, the egg ovalbumin [46]. These serpins were found in IgE reactive spots of both genotypes, confirming the presence of their corresponding genes in genomes A, B and D, as reported earlier by Merlinot et al. [47]. At least three expressed isoforms were identified in each genotype. However, they were always escorted by either peroxidase or aspartic protease. Some enzymes belonging to these classes (EC 1.11.1 and EC 3.4.23) were reported as allergens, e.g., ascorbate peroxidase in food allergy to bell pepper [48,49] and aspartic proteases CPA63 in respiratory allergy to cedar pollen [50].

Triosephosphate isomerase was identified once and only in Engrain Pays de Sault. Until now, this protein has been reported once as a potent allergen associated to baker's asthma by Sander [16] who showed that it binds to the IgE of patients. In 2009, Pastor [51] purified a homologous protein from watermelon and reported it as one of the major allergens involved in allergic reactions to watermelon.

In conclusion, the allergenomic approach developed on the A/G fraction of two genotypes and our choice to use sera obtained from patients with similar symptoms but exhibiting a high heterogeneity succeed in revealing known allergens and many potent allergens. Only eight were found common to both genotypes probably due to their very different spot patterns, notably aspartic proteinase, beta-glucosidase, enolase, globulins 3, 3B, 3C and serpin 1, Z2B. The higher number of spots revealed in the upper part of the gels obtained with Engrain Pays de Sault has facilitated the matching and led to the identification of 17 additional potent allergens. Interestingly, different IgE responses between diploid and hexaploid genotypes were obtained for alpha amylase inhibitors, which are known allergens and were not identified in Engrain Pays de Sault, suggesting that these proteins are only expressed at very low levels in the diploid genotype. This may be one hypothesis to explain the global lower reactivity of sera towards Engrain Pays de Sault A/G in ELISA.

Concerning the potent allergens, some of them have already been described in wheat through the same experimental schemes that share the same analytical difficulties generally related to the spatial resolution of 2D electrophoresis and to the fact that these overviews are dominated by the most abundant proteins. All identified potent allergens belong to families in which some members were already reported as allergens in different species. These potent new allergens should be further studied as single proteins to identify their IgE-binding epitopes, T-cell epitopes involved in sensitisation and whether they may induce clinical allergic response.

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