Department of Agriculture, Forests, Nature and Energy

Tesi di Dottorato di ricerca in cotutela in
Biotecnologie Vegetali XIV Ciclo

Assessment of the allergenic potential of genetically modified wheats by comparison with their *wild type* genotypes and commonly grown cultivars through allergenomic and immunochemical approaches.

AGR07

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Résumé:

Le blé est l'une des céréales les plus cultivées dans le monde mais aussi la plus largement consommée. Les deux génotypes majoritairement cultivés sont le blé tendre (génome AABBDD) et le blé dur (génome AABB). Le blé tendre est couramment utilisé sous forme de farine en panification, en biscuiterie, tandis que le blé dur est surtout utilisé pour la préparation des pâtes. Cependant, le blé fait partie des six principaux aliments connus pour être allergène pour les sujets susceptibles. Selon les génotypes, les protéines du grain représentent entre 10 et 15 % de sa matière sèche. Elles sont classiquement séparées en fonction de leurs propriétés de solubilité en albumines (hydrosolubles), globulines (solubles en solution saline) et prolamines insolubles (glutenines et gliadines, constituant le gluten). Les fractions solubles dans l'eau et les sels (Albumines/Globulines, A/G) sont principalement constituées de protéines présentant des fonctions métaboliques ou structurales, alors que les protéines du gluten sont des protéines de réserve directement impliquées dans la qualité du blé. Ces deux fractions sont à l'origine d'allergies alimentaires et respiratoires alors que la fraction A/G est plutôt impliquée dans l'allergie respiratoire, l'asthme du boulanger, qui est l'une des allergies professionnelles les plus fréquentes en France. Ces allergies sont liées à une réponse immunitaire dirigée contre un allergène (une protéine) et impliquant les anticorps de type IgE. Les allergies sont en augmentation et sont désormais considérées comme un problème important de santé publique.

Les plantes génétiquement modifiées (GM) sont de plus en plus étudiées et certaines d'entre elles sont largement cultivées par exemple en Amérique du Nord et Sud mais pas en Europe. Elles entrent dès lors dans l'alimentation et peuvent représenter un risque pour la santé. En effet ces plantes génétiquement modifiées ne sont pas naturelles puisqu'on y a introduit un gène absent à l'état sauvage ou modifié l'expression d'un gène. Même si les cibles visées sont un gène unique, de nombreux travaux ont mis en évidence un impact plus ou moins important de ces transformations sur l'expression des autres gènes. Cet aspect retient beaucoup l'attention, aussi bien de la communauté scientifique que celle des consommateurs, et participe à leurs inquiétudes et leurs réticences vis à vis des plantes GM, essentiellement pour l'augmentation potentielle de la toxicité et/ou de l'allergénicité.

Pour les pays où la commercialisation de produits dérivés de plantes GM est acceptée, le principe qui s'applique est le principe d'équivalence substantielle.
Classiquement l'évaluation de la sécurité des plantes GM inclut une comparaison directe avec le génotype non-transformé correspondant la variabilité naturelle existante doit être également prise en compte.
Ce projet de doctorat s'insère dans ce contexte, avec comme 'objectif principal d'établir si la transformation du blé augmente le risque d'allergie, étant donné que le blé est déjà un aliment allergénique. L'approche utilisée dans cette étude permettra d'augmenter les connaissances sur les protéines impliquées dans les allergies au blé, particulièrement celles présentes dans la fraction soluble, qui ont été moins étudiées que les protéines insolubles du gluten.

Dans une première partie de la thèse, l'allergénicité des lignées de blé GM a été évaluée par une approche d'allergénomique et comparée à celle des génotypes parentaux. Dans une deuxième partie de la thèse, une étude plus large a été conduite pour comparer des lignées GM et leurs parents avec un groupe de variétés commerciales cultivées. Il s'agit d'observer si les variations d'allergénicité entre les blés GM et leurs parents sont du même ordre que celles existant entre des variétés obtenues par des méthodes de sélection conventionnelle.

Deux lignées de blé transgéniques (une lignée de blé dur cv Svevo, et une de blé tendre cv Bobwhite) ont été comparées aux génotypes correspondant non transformées, d'abord par ELISA (Enzyme Linked Immunoabsorbent Assay) avec vingt-un sérums de patients souffrant d'allergie alimentaire au blé ou d'asthme du boulanger, puis par une approche d'allergénomique.
Nous avons étudié une lignée de blé tendre sur-exprimant une sous-unité de gluténines de faible poids moléculaire (LMW-GS) et une lignée de blé dur sur-exprimant le gène Wx-B1 impliqué dans la synthèse de l'amylose. La comparaison de la teneur et de la composition en allergènes s'est focalisée sur les fractions albumines/globulines (A/G), car les transformations génétiques ont nettement modifié leurs quantités dans les blés GM : ont été changées suite aux transformations génétiques. En particulier, la fraction A/G de la lignée de blé tendre GM a diminué alors que celle de la lignée de blé dur transgénique montre une augmentation.

Les résultats obtenus par ELISA ne montrent que peu de différences significatives entre les lignées GM et leurs parents respectifs; en effet, nous avons mesuré des concentrations en IgE spécifiques des A/G qui diffèrent entre les blés GM et leurs parents seulement pour deux patients souffrant d'asthme du boulanger et pour six patients souffrant d'allergie alimentaire parmi les 21 sérums testés. Malgré ces différences, les concentrations d'IgE spécifiques des A/G mesurées pour la lignée de blé tendre GM et son génotype cultivé sont comparables pour 84% des sérums, et pour 70% des sérums en ce qui concerne la ligné de blé dur GM et la variété cultivée correspondante.

Des immunoblots bidimensionnels sur les fractions A/G des deux lignées GM et de leurs génotypes cultivés ont été réalisés avec trois sérums de patients allergiques alimentaires et un sérum d'un patient atteint d'asthme du boulanger. De nombreux polypeptides réagissant avec les IgE ont été détectés pour chaque génotype. Seulement quelques changements dans les profils de ces polypeptides réactifs ont été repérés entre les lignées transgéniques et leurs génotypes non transgéniques. Cent neuf spots ont été identifiés par spectrométrie de masse, la plupart d'entre eux ont été déjà décrits dans la littérature comme des allergènes ou allergènes potentiels, et peu d'entre eux sont spécifiques du génotype transgénique.

Pour la seconde partie de la thèse, nous avons réuni une collection de lignées de blé GM (2 génotypes de blé tendre et trois de blé dur) et les variétés commerciales et null-ségrégant correspondantes disponibles, ainsi que des blés tendres et des blés durs obtenus par des méthodes de sélection conventionnelle. Pour chaque blé, nous avons préparé différents extraits solubles : fractions A/G, Métaboliques et CM-like. Les concentrations en IgE spécifiques de chaque extrait ont été mesurées par ELISA dans les sérums de 24 patients allergiques alimentaires ou respiratoires au blé.

De plus, les fractions A/G, Métaboliques et CM-like ont été comparés par immunoblots monodimensionnels en utilisant des sérums de patients allergiques au blé et des anticorps spécifiques anti-LTP. Les polypeptides reconnus par les IgE ont été identifiés par spectrométrie de masse.

Les tests ELISA ont montré une large variation dans le groupe correspondant aux variétés commerciales cultivées, et les différences détectées entre les blés GM et leurs génotypes non-transformé sont inclues dans cette gamme de variation. De plus, les

valeurs les plus hautes de concentrations en IgE spécifiques ont été observées parmi les variétés commerciales cultivées.

Ces résultats ont montré que, au moins pour les génotypes transgéniques analysés ici, les concentrations en IgE spécifiques mesurées s'inscrivent dans la variation naturelle mesurée au sein des 20 variétés cultivées. Nous avons montré que l'allergénicité peut être soit augmentée soit diminuée selon la transformation, ce qui reflète un effet aléatoire de la transformation sur l'expression de gènes non ciblés.

Même si l'étude est concentrée quelques lignées de blé transgéniques et ne permet donc pas de tirer une conclusion générale sur l'équivalence substantielle de blés transgéniques, elle montre clairement la nécessité d'effectuer des évaluations de risque au cas par cas.

Il est important de réaliser toutes les évaluations nécessaires avant la commercialisation de lignées GM, pour contribuer à l'acceptabilité publique. Bien sûr ceci devrait être fait par des institutions de recherche publiques, indépendantes et non pas par des sociétés de biotechnologie, afin d'éviter les conflits d'intérêts.

Riassunto

Il problema delle allergie ha suscitato una crescente attenzione soprattutto riguardo al tema della Sostanziale Equivalenza delle piante GM. È in questo contesto che si è inserito il presente lavoro di dottorato, il quale si è prefissato due obiettivi:verificare eventuali cambiamenti nella risposta allergica di linee di frumento GM rispetto ai corrispondenti genotipi *wt* e verificare come tali cambiamenti rientrano nelle differenze di reattività osservate tra differenti cv di frumenti. Per questa prima parte del lavoro è stato condotto uno screening tramite ELISA utilizzando dei sieri di pazienti allergici. Parallelamente a questo si è voluto verificare se la trasformazione genetica possa aver avuto delle influenze sul potenziale allergico e/o la comparsa di nuovi polipeptidi riconosciuti come allergeni. Per questa parte del progetto si è scelto di utilizzare un approccio proteomico su 2 linee di frumento trasgeniche, e i corrispondenti genotipi *wt*. I risultati relativi alla prima parte del lavoro hanno dimostrato che le differenze osservate in termini di concentrazioni di IgE specifiche tra le linee di frumento trasgeniche e i corrispondenti genotipi *wt* sono inseribili nella variabilità di risposte osservate all'interno di una collezzione di frumenti coltivati. Anche per la seconda parte del lavoro non sono state osservate differenze significative tra le linee di frumento GM e le corrispondenti linee *wt*.

ABSTRACT

Wheat is one of the world's most popular and cultivated crops and is also one of the six majour foods allergens. The kernel proteins are typically classified according to their solubility properties into albumins (water soluble), globulins (salt soluble) and insoluble prolamins (gluten proteins). The salt-soluble fraction includes proteins with metabolic activity or structural functions, while gluten proteins are directly involved in wheat quality, and both fractions cause food allergy and Baker's asthma. These proteins accounting for about 10-15% of the grain dry weight. Since allergies are a major health concern and seem increasing, much attention is now being focused on foods from genetically modified (GM) plants because of the postulated health risk. This concern includes the perception that the insertion of transgenes into host plant genomes may result in unpredicted effects on the expression of other genes and effects on plant phenotype (e.g. increases in toxicity and allergy). If this is the case, transgenic crops could not be considered "substantially equivalent" to non-GM crops.

The classical safety assessment of GM plants includes a direct comparison with the corresponding untrasformed genotype. Thus, the first approach carried out in this work was a comparison by pairs of GM wheat lines and their untransformed (wild type, *wt*) genotypes, followed by a with a larger group of cultivars.

Two GM wheat lines (a durum and bread wheat) were compared to untransformed counterparts, first by Enzyme-Linked Immunoabsorbent Assay (ELISA) with twenty-one sera from patients suffering from food allergy to wheat and Baker's asthma, and second by allergenomic approach. In particular we investigated a bread wheat line overexpressing a LMW-GS and a durum wheat line overexpressing the *Wx-B1* gene. The comparison was focused on the albumins/globulins (A/G) fractions, because their amounts were changed as a result of the two transformation events. The results obtained by ELISA revealed significant differences for only two patients suffering from Baker's asthma and for six patients affected from food allergy among the 21 tested. Thus, the concentrations of A/G specific IgE measured for GM bread wheat and its *wt* genotype and GM durum line and its wt genotype were comparable for 84% and 70% of sera. Two dimensional immunoblots were performed by using three sera from patients affected from food allergy and one serum from a patient with Baker's asthma on A/G fractions of the two GM lines and their *wt* genotypes. Numerous IgE-binding

polypeptides were detected for each genotype and the profiles of these reactive polypeptides between GM wheat and its untransformed genotypes were similar. Only few changes in allergenic profiles between the transgenic lines and their wt genotype were detected. Hundred nine spots were identified by mass spectrometry as IgE binding proteins; most of them have been already described as allergens or potential allergens, and few of them are peculiar either of the GM or the wt genotype.

A comparison based on ELISA between the different water-salt soluble extracts obtained from a collection of GM wheat lines and commercial varieties of wheat obtained by conventional breeding methods was performed. Moreover, the different water-salt soluble extracts were compared by one dimensional immunoblots performed by using sera from patients allergic to wheat and specific anti-LTP antibodies. The IgE-binding polypeptides from different extracts were compared by mass spectrometry.

The ELISA test showed a wide variation in the group corresponding to the commercial cultivars, and the differences detected between GM wheats and their *wt* genotypes were included in this range of variation. Moreover, the highest values were observed in commercial cultivars compared to the GM lines.

These results showed that, at least for the GM genotypes here analysed, the differences in specific IgE concentrations can be considered within natural variation.

Context of the work

Wheat is indisputably an important part of the daily diet of millions of people, and also one of the major crops cultivated in the world, with a total production of about 600 million tons each year globally, 75% of which is destined to food use. Moreover, it has always been a target of choice for classical breeding and biotechnological programs. Unfortunately, this popular and cultivated crop is also one of the six major foods involved in food allergy. Allergic reactions to wheat are of two types; they may arise after ingestion of food containing flour, but also from flour and dust inhalation during grain processing, as in baker's asthma. This latter is one of the most common occupational disease in Europe.

The kernel proteins are typically classified according to their solubility properties into albumins (water soluble), globulins (salt soluble), gliadins (soluble in concentrate alcohol), glutenins (soluble in diluted acid or alkaline solutions). Gliadins and glutenins together make up the gluten.

The water/salt-soluble fraction includes proteins with metabolic activity or structural functions, while gluten proteins are directly involved in wheat quality, and both fractions cause food allergy and Baker's asthma. These proteins account for about 10-15% of the grain dry weight.

Because food allergies seem increasing this aspect is considered now as a significant public health concern, in particular in regard to GM foods. In fact, one of the major issues about plant genetic modifications is the possibility of unpredicted effects caused by the insertion of transgenes into the host plant genome that might cause changes of gene expression, included those coding for allergenic or toxic compounds. At this regard, a comparative assessment between GM plants and their traditional parental controls is requested to evaluate applicability ofthe "substantial equivalence" principle.

Aim of the work

The main aim of this work was to establish if genetic transformation of wheat increases the allergy risk, since wheat is *per se* one of the most allergenic foods. Moreover, the approaches used allowed to increase the knowledge on proteins involved in wheat allergies, especially those present in the water/salt-soluble fraction, that have been less intensively studied with respect to the insoluble gluten proteins. This because these latter are directly involved in quality of wheat end-use products, and thus have been characterized in more detail.

For assessment of allergenicity of GM wheat lines compared to their parental genotypes, an allergenomic approach was first used. In a second step, a wider study was undertaken in order to compare the GM lines and their parents with a large of group of commercial cultivars, in order to establish if variations in allergenicity could be of the same order as that present in varieties obtained by conventional breeding methods.

Articles and Poster published during this PhD

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Lupi R., Denery-Papini S.,Rogniaux H., Lafiandra D., Rizzi C., M. De Carli M., Moneret-Vautrin D. A., Masci S.[*], Larré C.[*] "An allergenomic approach to evaluate the impact of genetic transformation in bread and durum wheat". Submitted

Lupi R., Masci S., Lafiandra D., Rogniaux H., Tranquet O., Moneret-Vautrin, Rizzi C., De Carli M., Denery S., Larré C. "Immunochemical analyses for the assessment of the variation in the amount of allergenic polypeptides of GM genotypes in comparison to commercial durum and bread wheat". Will be submitted.

Short Paper:

Lupi R., Pineau F., Deshayes G., Moneret-Vautrin D.A., Denery S., Popineau Y., D'Ovidio R., Lafiandra D., Anderson O.D., Masci S., Larré C. **(2009)** "Characterization of allergens present in the seed soluble protein fraction of transgenic and untransformed wheat lines", Proceedings of **Xth International Gluten Workshop**, Clermont-Ferrand.

Poster and oral communication

Lupi R., Denery S., Lafiandra D., Larrè C., Masci S."How much does the transgenesis affect the allergenicity?" Joint Meeting AGI-SIBV-SIGA, Assisi, September 2011 ISBN 978-88-904570-2-9 **(oral presentation).**

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Lupi R., Denery S., Masci S., Larré C. "Allergens of the salt-soluble fraction of the grain are not modified in a GM line over-expressing a glutenin low molecular weight subunit". *SIGA, 54°Annual Congress*, Matera, September **2010 (poster).**

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Abbreviations

1D electrophoresis: One dimensional electrophoresis

2D electrophoresis: Two dimensional electrophoresis

A/G: Albumins/Globulins

AD: Atopic Dermatitis

ADP: Adenosine Di-Phosphate

AEDS: Atopic eczema dermatitis syndrome

AM: AMylose

ANOVA: ANalyis Of VAriance

AP: AmyloPectin

A-PAGE: Acid-PolyAcrylamide Gel Eletrophoresis

APC: Antigen Presenting Cell

APT: Atopy Patch Test

AS: Anaphylactic shock

AT: Asthma

ATP: Adenosine Tri-Phosphate

BA: Baker's Asthma

CD: Coeliac Disease

CD4: Cluster of Differentiation 4

CHU: Centre Hospitalier Universitaire

CM-like proteins: Chloroform-Methanol like proteins

CV: cultivated variety

DBPCFC: Double-Blind Placebo-Controlled Food Challenge

DNA: DeoxyriboNucleic Acid

Dpa: Days post anthesis

DTT: DiThioThreitol

EDTA: EthyleneDiamineTetraacetic Acid

EFSA: European Food Safety Authority

ELISA: Enzyme Linked ImmunoSorbent Assay

EU: European Union

FA: Food Allergy

FAO: Food and Agriculture Organization

FmPG: *Fusarium moniliforme* endopolygalacturonase

GBSS: Granule-Bound Starch Synthase

GI: Gastro-intestinal symptoms

GM: Genetically Modified

HLA: human histocompatibility leucocyte antigen

HMW-GS: High Molecular Weight Glutenin Subunit

HWP: Hydrolyzed Wheat Proteins

IEF: IsoElectric Focusing

Ig : Immunoglobuline

IgE: Immunoglobulin E

IgG: Immunoglobulin G

ISAC: Immuno-Sorbent Allergen Chip

ISAC: International Society for Advancement of Cytometry

IUIS: International Union of Immunological Societies

KDa: Kilo Dalton

LC-MS/MS: Liquid Chromatography Mass Spectrometry/Mass Spectrometry

LMW-GS: Low Molecular Weight Glutenin Subunit

M: Molar

MHC: Major histocompatibility complex

MS: Mass Spectrometry

MW: Molecular weight

NIAID: National Institute of Allergy and Infectious Diseases

NMR: Nuclear Magnetic Resonance

nsLTP : non-specific Lipid Transfer Protein

OAS: Oral allergy syndrome

OECD: Organisation for Economic Co-operation and Development

PAGE : Poly Acrylamide Gel Eletrophoresis

PBS: Phosphate Buffer Salin

PBST: Phosphate Buffered Saline Tween

PGIP: PolyGalacturonase Inhibitor Protein

pI: Isoelectric point

PVDF: PolyVinylidene DiFluoride

PVP: PolyVinylPyrrolidone

R: Rhinitis

RAST: Radio Allergo Sorbent Test

Rev.: Reviewed

RNAi: RiboNucleic Acid interference

RP-HPLC: Reversed Phase High Performance Liquid Chromatography

SBE: Starch Branching Enzymes

SDS-PAGE: Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

Serpin: serine protease inhibitor

SIC: Specific Inhalation Challenge

SIT: Allergen-Specific ImmunoTherapy

SPT: Skin Prick Test

SS: Starch Synthases

T lymphocytes: Thymus Lymphocytes

T-DNA: Transferred DNA

TG2: enzyme tissue transglutaminase

Th: T helper

Ti: Tumor inducing plasmid

TLP: Thaumatin-Like Protein

UK: United Kingdom

Urt: Urticaria

WDEIA: wheat-dependent exercise-induced anaphylaxis

WHO: World Health Organization

wt: Wild-type

Amino Acids List

Glycine: Gli, G

Alanine: Ala, A

Serine: Ser, S

Threonine: Thr, T

Cysteine: Cys, C

Valine: Val, V

Leucine: Leu, L

Isoleucine: Ile, I

Methionine: Met, M

Proline: Pro, P

Phenylalanine: Phe, F

Tyrosine: Tyr, Y

Tryptophan: Trp, W

Aspartic Acid: Asp, D

Glutamic Acid: Glu, E

Asparagine: Asn, N

Glutamine: Gln, Q

Histidine: His, H

Lysine: Lys, K

Arginine: Arg, R

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Chapter I:

Table 7: List of identified specific proteins from 2D gel of Svevo *wt* (Fig. 5). Spot Number: assigned protein spot number corresponding to those indicated in Fig. 5, Sub-group: Sub-group to which the protein belongs. All the proteins in a sub-group are identi ed with the same valid peptides, 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, 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, Serum: serum for which the IgE-binding spot was detected .

Table 8: List of identified common proteins from 2D gels between Bobwhite *wt* (Fig. 8) and Bobwhite-GM line (Fig. 9). Spot Number: assigned protein spot number corresponding to those indicated in Figs. 5 and 6, Sub-group: Sub-group to which the protein belongs. All the proteins in a sub-group are identi ed with the same valid peptides, 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, 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, Serum: serum for which the IgE-binding spot was detected .

Table 9: List of identified specific proteins from 2D gel of Bobwhite-GM line (Fig. 9). Spot Number: assigned protein spot number corresponding to those indicated in Figs. 5 and 6, Sub-group: Sub-group to which the protein belongs. All the proteins in a sub-group are identi ed with the same valid peptides, 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, 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, Serum: serum for which the IgE-binding spot was detected .

Table 10: List of identified specific proteins from 2D gel of Bobwhite *wt* (Fig. 8). Spot Number: assigned protein spot number corresponding to those indicated in Figs. 5 and 6, Sub-group: Sub-group to which the protein belongs. All the proteins in a sub-group are identi ed with the same valid peptides, 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, 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, Serum: serum for which the IgE-binding spot was detected .

Chapter II:

Table 1: Wheat genotypes included in this study

Table 2: Clinical characteristics of patients and their IgE reactivity against A/G in ELISA or immunoblotting. AEDS: atopic eczema dermatitis syndrome, GI=Gastro-intestinal symptoms AS: anaphylactic shock, R=Rhinitis, AT= Asthma and nd: not done, +: = response at the lower limit of quantification, neg: not reactivity.

Annex-Chapter II

Table 3: List of identified proteins from 1D gel of Bobwhite *wt* (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 Wheat TIGR databank, Uniprot 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.

Introduction

1 Wheat

The cereals are a major source of dietary calories, among which wheat provides about one-fifth of the calories consumed by humans. The world cereal production was 2432 million tons in 2010, 650 million tons of which corresponded to wheat. FAO's data for Italian and French wheat production in 2010 was 6.9 and 38.207 million tonnes respectively with an area harvested corresponding to 1,865,000 hectares in Italy and 5,426,000 hectares in France (http://faostat.fao.org/site/567/default.aspx#ancor).

Tab. 1: Wheat production and area harvest in the world and in different continents (FAOSTAT data, 2010).

	2010 Wheat production (tonnes)	**2010 Wheat area harvest (hectares)**
World	2432236739	216974683
Africa	22016718	9501565
North America	83269400	27546900
South America	25731739	8150346
Asia	292441446	101657580
Europe	201149388	55870270
Oceania	22582901	13561772

1.1 Origin of the wheat genomes

Cultivated wheats and their close wild relatives belong to the genus *Triticum* L. of the family *Gramineae* and are a member of the tribe Triticeae, which contains ~300 species, forming a polyploid series, with a basic chromosome number (x) equal to 7, comprising diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42) wheats. The wheat genus *Triticum* has a relatively small number of species (six species) with wild taxa occurring in the Middle East and Transcaucasus region. The genus *Triticum* consists of six species: *Triticum monococcum* L. (AA genome); *Triticum urartu* Tumanian ex Gandilyan (AA genome); *Triticum turgidum* L. (AABB genome); *Triticum timopheevii* (Zhuk.) Zhuk (AAGG genome); *Triticum aestivum* L. (AABBDD genome); and *Triticum zhukovskyi* Menabde & Ericz. (AAAAGG genome) (Matsuoka, 2011).

Nowadays, the commercial wheat, tetraploid and hexaploid wheats, contain two and three homeologous genomes, respectively. These genomes are named A, B, D and G, according to the donor species, each of which consists of seven pairs of chromosomes numbered 1 to 7. In the hybridisation process, spontaneous crosses between wild species with different chromosomes have been followed by spontaneous doubling of chromosomes to originate a fertile allopolyploid.

Over the last 10,000 years, crop domestication has been the single most important human cultural development. Despite the independent domestication of the four major cereal complexes (maize in America; wheat, barley, oats and rye in the Near East; rice in Asia; sorghum and millet in Africa), the same sets of traits were searched. In the case of wheat domestication, the desired traits were non-brittle rachis, naked grain often associated to shorter dormancy, flowering time and grain size (Buckler et al., 2001; Charmet, 2011).

The first cultivation of wheat occurred about 10,000 years ago, as part of the ‘Neolithic Revolution’, which saw a transition from hunting and gathering of food to settled agriculture (Shewry, 2009). Tetraploid forms of current domesticated wheats are derived from a wild tetraploid progenitor, identified as the wild emmer *Triticum turgidum* ssp. *dicoccoides*. This species has an allotetraploid genome (AABB) resulting from spontaneous amphiploidization between the diploid wild wheat *Triticum urartu* (AA genome) and an unidentified diploid *Aegilops* species (BB genome) (Haudry et al., 2007) (Fig. 1). Today, the cultivated form of the durum wheat (*T. durum*) is widely

grown in regions with relatively dry conditions as Mediterranean area, and consumed as pasta and semolina products (Matsuoka, 2011).

The hexaploid wheats originated some 6,000-7,000 years ago by natural hybridisation of tetraploid wheat, (AABB genome) with the diploid wild grass *Aegilops squarrosa* (DD) (*T. tauschii*) (Miller, 1987) (Fig. 2). The resulting hexaploid species *T. aestivum* (AABBDD), the common "bread wheat", is the dominant species in world agriculture. The hexaploid wheat species *T. zhukovskyi*, whose genomic composition is AAAAGG, could have been originated recently by interspecific hybridization of cultivated tetraploid *T. timopheevvii* (AAGG) with the cultivated diploid *T. monococcum* (AA).

Today, about 95% of the wheat crop is hexaploid (*T. aestivum*), whereas the remaining 5% is durum wheat. Compared with tetraploid wheat, *T. aestivum* has broader adaptability to different photoperiod and vernalization requirements; improved tolerance to salt, low pH, aluminum, and frost; better resistance to several pests and diseases; and extended potential to make different food products, as bread, cookies and pastries (Dubcovsky and Dvorak 2007).

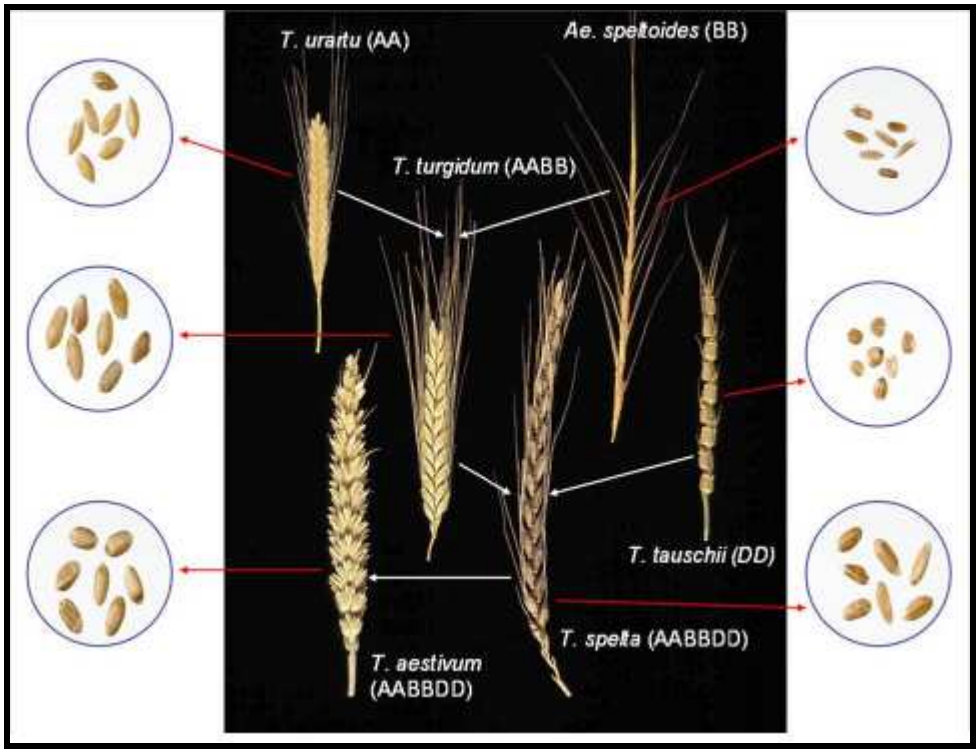

Fig. 1: The evolutionary and genome relationships between cultivated bread and durum wheats and related wild diploid grasses, showing examples of spikes and grain (Taken from Shewry, 2009).

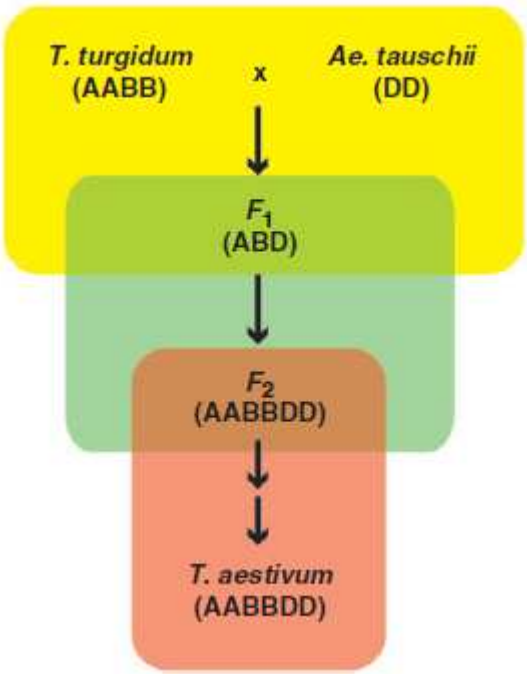

Fig. 2: Critical reproductive and genetic events in the allopolyploid speciation of *T. aestivum*: *T. turgidum–Ae. tauschii* natural hybridization (yellow); normal growth of fertile triploid F1 hybrids (green); and genetic and epigenetic changes in the allohexaploid genomes of F2 and later generations (red).Taken from Matsuoka, 2011 .

1.2 Wheat caryopsis: anatomy and composition

The wheat grain is botanically a single-seeded fruit, called “caryopsis” or “kernel”. It is a composite of different tissues, each with a unique temporal pattern of gene expression during grain fill. Technically, the mature grain is a caryopsis, with an outer testa closely appended to the seed. It develops within floral envelopes (the “lemma” and “palea”), which are actually modified leaves. At maturity, the wheat kernel averages ~2.5-3.0 mm thick (or higher as it stands on its base), ~3.0-3.5 mm wide, ~6.0-7.0 mm in length, with an average weight of ~30-40 mg. The seed is constituted by three distinct parts: the bran, the starchy endosperm and the embryo or germ (Fig 3). They account for 13-17%, 80-85% and 2-3% of the dry weight of the seed respectively. The embryo, aleurone, and pericarp plus testa are removed during milling, leaving the starchy endosperm as the principal contributor to white flour (Dupont and Altenbach 2003; Belderok, 2000). This mealy endosperm is composed of 82% carbohydrates (mainly starch), 13% proteins and 1.5% fats but a low content in minerals and dietary fibers.

The contents of minerals (ash) and of dietary fibres are low, 0.5% and 1.5%, respectively. More than half of the bran consists of fiber components (53%) and interestingly its mineral content is rather high (7.2%). The rather high level of proteins and carbohydrates in the bran is coming from the aleurone layer, the cells of which are filled with living protoplasts. Finally, the germ exhibits the more equilibrated composition with proteins, fats, carbohydrates and dietary fibres (Belderok, 2000).

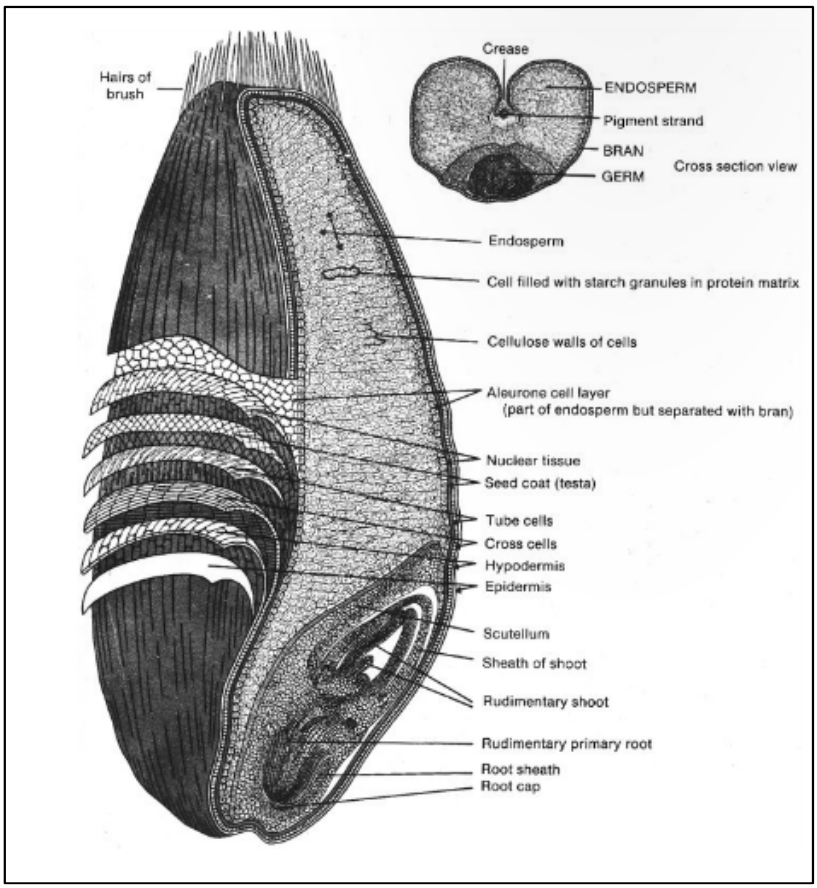

Fig. 3: The wheat kernel

Tab. 3: Chemical composition of wheat grain and its different parts (converted to percentage on a dry matter basis). Taken from Belderok, 2000.

	Whole grain	Mealy endosperm	Bran	Germ
Proteins	16	13	16	22
Fats	2	1.5	5	7
Carbohydrates	68	82	16	40
Dietary fibers	11	1.5	53	25
Minerals (ash)	1.8	0.5	7.2	4.5
Other components	1.2	1.5	2.8	1.5
Total	100	100	100	100

1.3 Wheat bran

The bran of the wheat grain is composed by a series of different cell layers. The "pericarp" (fruit coat) surrounds the entire seed and consists of two portions, the outer pericarp and the inner pericarp. The outer pericarp is composed by the epidermis (epicarp), the hypodermis, and by the innermost layer, called the remnants of thin-walled cells. The inner pericarp, adjacent to the remnants, is composed of intermediate cells (cross cells and tube cells). A further inner layer of cells is the seedcoat (also called "testa") where the pigments confer the grain colors. Bran is particularly rich in dietary fiber and contains significant quantities of starch, protein, vitamins, and dietary minerals.

1.4 The embryo

The wheat germ makes up 2-3% of the kernel. It consists of two major parts, the embryonic axis and the scutellum, which functions as a storage organ. The germ is relativity high in protein (25%) carbohydrates (40%), oil and ash. It contains a rather high amount in B and E vitamins and contains many enzymes. No starch was found in this organ, the sugars are mainly sucrose and raffinose.

1.5 Endosperm development

The development of cereal endosperms has been largely investigated. The best known and most widely used scale for the recording of growth stages of cereals is probably the scale designed by Feekes (Large, 1954). The Feekes' scale is well suited to the small grain cereals in North West Europe, especially wheat, but it can also be applied to wheat, barley, rye, oats, and to in other parts of the world. But also the decimal code developed by Zadoks et al. (1974), Chang and Konzak has been widely recognized as the best scale available (Chang et al., 1974).

This subjective scale divide grain development into early, medium and late milk stages, and early, soft and hard dough stages (Fig. 4). The temporal pattern of grain development also can be described in terms of transition points in the accumulation of total dry matter, starch, protein, and water, in order to pinpoint times in grain development when changes in gene expression and protein accumulation are likely to occur (Fig. 5) (Dupont and Altenbach 2003).

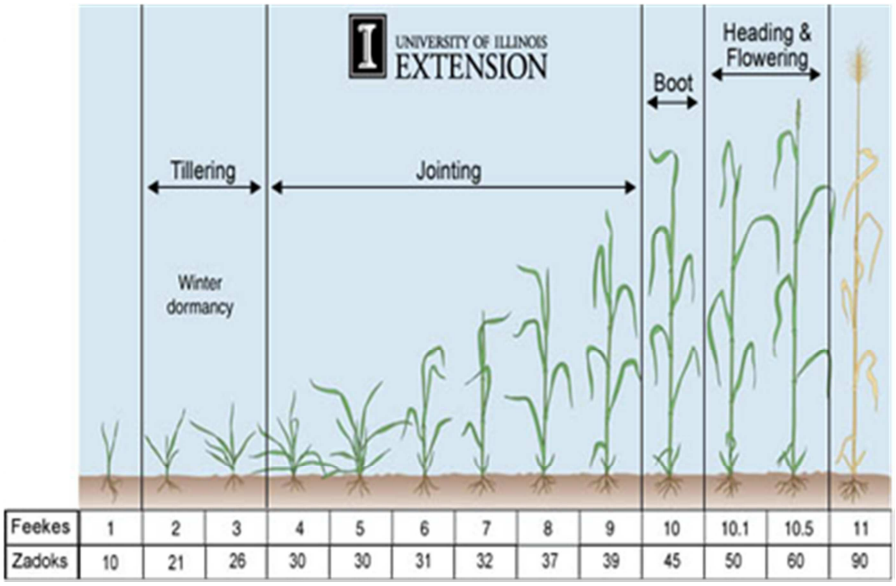

Fig. 4: Schematic representation of Feekes' and Zadoks scales.

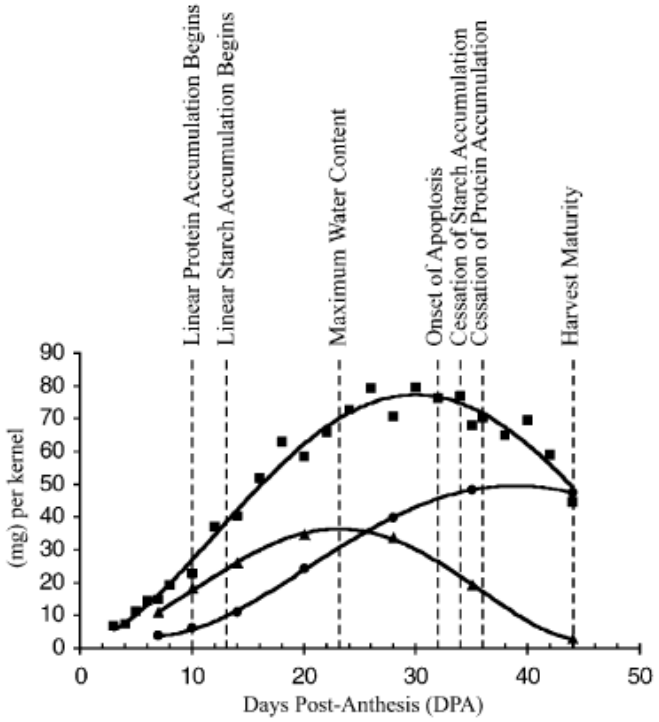

Fig. 5: Temporal patterns in the accumulation of starch and protein on the basis of water content (►), fresh weight (■) and dry weight (●) during the grain development. Proteins were measured by a nitrogen combustion analysis, starch was measured by a specific assay kit, and the onset of apoptosis was estimated by the analysis of fragmented genomic DNA. Plants grown under a 24°C/17°C day/night regimen. Taken from Dupont and Altenbach (2003).

The endosperm is the result of the fertilization of two polar nuclei in the central cell of the embryo sac by one sperm cell nucleus, which generates a triploid (3n, 3C) nucleus, whereas the diploid (2n, 2C) embryo originates from fertilization of the egg cell by the second sperm cell nucleus (Sabelli and Larkins 2009).

In the caseof endosperm, cell wall formation starts from the periphery on day 4 after pollination, via the formation of open-ended alveoli that grow towards the centre of the endosperm, and is completed by day 7. Cell divisions continue until 12–14 d post-anthesis (dpa). This so-called nuclear type of endosperm development, which is characterized by a limited or permanent phase of free-nuclear division, is found in cereals, as well in other plants (Wegel et al., 2005).

Following is a period of cell expansion in which water content increases and starch and protein reserves accumulate. The maximum amounts of starch and protein that accumulate in each grain depend on the number of endosperm cells, determined early in grain fill, and the final size of the cells, which is influenced by water uptake, cell-wall extensibility and rate and duration of grain fill (Dupont and Altenbach 2003).

The inner daughter cells of the formative division represent starchy endosperm cell initials. In wheat, the outermost endosperm cell layer differentiates as aleurone and the cells on the inside develop as starch- and protein-storing cells. The endosperm cell layers located adjacent to the nucellar projection are morphologically distinct, while the adjacent nucellar cells form a symplastic transport system and facilitate the transport of sucrose from the vascular system to the endosperm, where it is converted into starch. The starchy endosperm cells of maturing grains die, as a result of programmed cell death (Fábián et al., 2011). Finally, kernels desiccate rapidly, losing all but 10–15% of their water content, at which time they are ready for harvest.

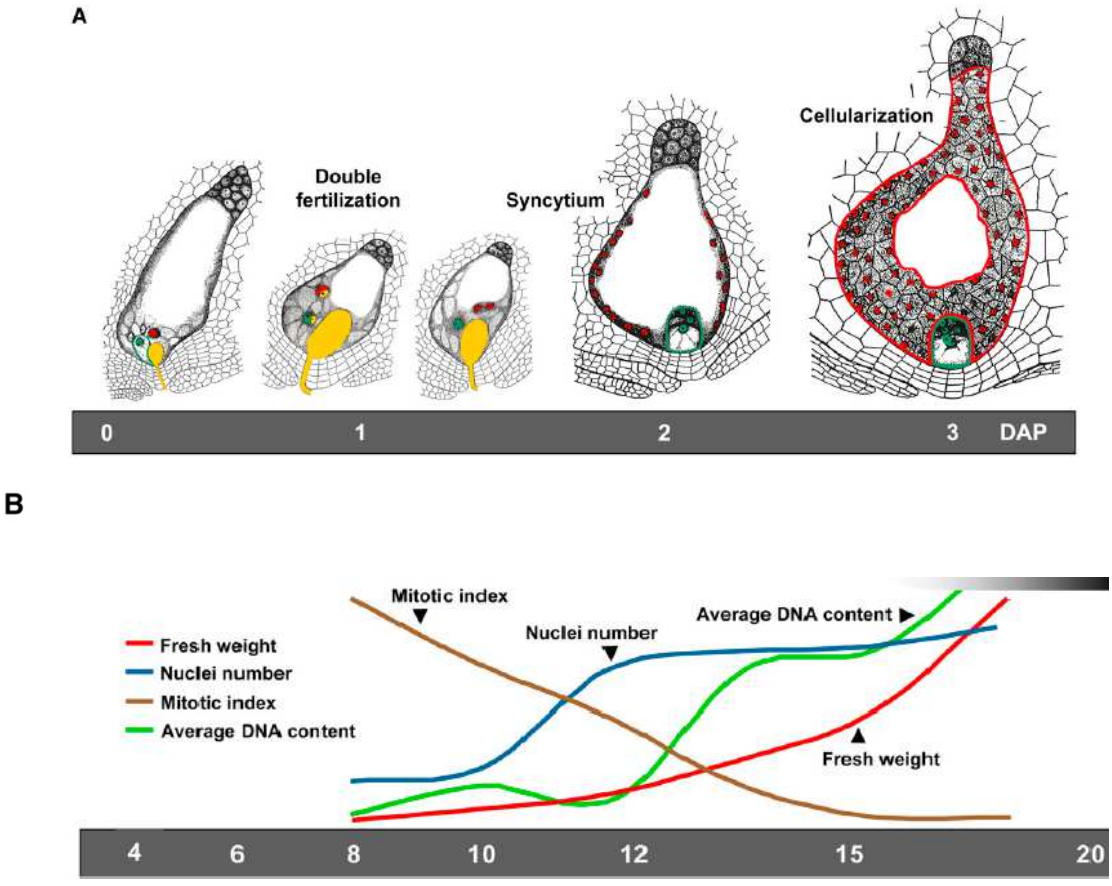

Fig. 6: Phases in endosperm development. Although this figure refers to maize, it is a good example of endosperm development in other grasses as well. **A**, Double fertilization, syncytium formation, and cellularization of the endosperm occur within 3 to 4 DAP. The pollen tube and sperm nuclei are shown in yellow, polar nuclei in the central cell of the female gametophyte and endosperm nuclei are shown in red, and the egg cell nucleus and embryo nuclei are shown in green. Outlines of the multicellular endosperm and embryo are drawn in red and green, respectively. **B**, The dynamics of key parameters during mid endosperm development, such as fresh weight (red line), nuclei number (blue line), mitotic index (brown line), and average DNA content (C value; green line), are shown at bottom. Taken from Sabelli and Larkins 2009.

1.6 Main components of wheat endosperm

1.6.1 Starch

Starch is a major storage product (Denyer et al., 2001). In addition to its use in a non-processed form, starch is processed in many different ways. Processed starch is subsequently used in multiple forms, for example in high-fructose syrup, as stabilizers and fat-replacements in the food industry It is also used for various technical processes based on the fact that, as a soluble macromolecule, it exhibits high viscosity and adhesive properties for example in glues, paper, textiles, pharmaceutical and biodegradable plastics.

1.6.1.1 Starch structure

Wheat starch is composed only of glucose units; the glucose units are linked α-1,4 to form linear chains and branches are formed through the connection of α-1,4 linked chains via α-1,6 linkages. Starch is generally described as containing two broad classes of molecules, amylose (AM) and amylopectin (AP), that differ in degree of polymerisation and branch frequency (Rahman et al., 2000).
In most common types of cereal endosperm starches, the relative weight percentages of amylose and amylopectin range between 72 and 82% amylopectin, and 18 and 33% amylose (Buléon et al., 1998).

1.6.1.2 Amylose and Amylopectin

Amylose is essentially a linear molecule, in which glucosyl monomers are joined via α-1,4 linkages and contributes about 30% of storage starches. Amylopectin, the more abundant polymer in starch (70% of storage starches), contains linear chains of various lengths (Smith et al., 1997). Approximately 5% of the glucosyl units in amylopectin are joined via α-1,6 linkages, which introduce chain branches. Amylopectin has a high degree of structural organization, as exemplified by the non-random distribution of linear chains and the clustered positioning of branch linkages. Regions of high-branch frequency alternate with regions that are devoid of branches, enabling intervening linear

chains to align in parallel arrays of double helices. This conserved architecture is responsible for the semi-crystalline nature of starch granules, which allows the dense packaging of glucose units. A higher-order organization in amylopectin gives rise to two types of crystalline structure, A-type and B-type, which differ with respect to the symmetry and packing of short amylopectin chains (James et al., 2003). Large type A granule, of 10-30 pm in diameter which are lenticular in shape, are initiated about 4-7 DPA, and smaller type B granules generally spherical (10 pm) appear around 10-12 DPA. Many of the physical and chemical properties of starch are determined by the relative amounts, size, chain length and branch frequency of these two types of polymer (Burton et al., 1995; Morell et al., 1995).

1.6.1.3 Starch biosynthesis

In plants, four enzymes control starch biosynthesis. Within the amyloplast, ADP-glucose pyrophosphorylase converts glucose-phosphate to ADP-glucose in the presence of ATP. ADP-glucose then is polymerized into α-1,4 linked chains by multiple isoforms of starch synthase. The first group of starch synthase contains granule-bound starch synthase (GBSS), and includes GBSSI and GBSSII. GBSSI is encoded by the *Waxy* locus in cereals, located on each of the wheat genomes, and functioning specifically to elongate amylose. It is found essentially within the granule matrix (one of the so-called granule-associated proteins). In *Triticum aestivum* these isoforms are encoded on chromosome 7 of genome A *(Wx-Al),* chromosome 4 of genome A (*Wx-Bl*), and chromosome 7 of genome D *(Wx*-Dl) (Graybosch, 1998). In durum wheat only *Wx-Al* and *Wx-Bl* are present. By cross-breeding of mutant lines that show one or more functional *Wx* genes, wheat genotypes with varying numbers of functional GBSSI isoforms can be obtained (in Lafiandra et al., 2010). Wheat genotypes can be classified as: wild-type (all three isoforms are present), one gene null (any one isoform lacking), two gene null (any two isoforms lacking), or three gene null (all three isoforms lacking). A drastic decrease of amylose content is observed in the complete three waxy-null line (Lafiandra et al., 2010).

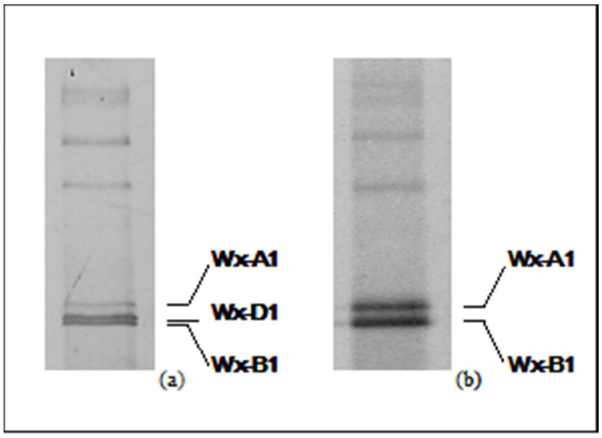

Fig.7: 1D SDS-PAGE of *waxy* proteins in bread wheat (a) and durum wheat (b).

Expression of GBSSI appears to be mostly confined to storage tissues, and a second form of GBSS (GBSSII), which is encoded by a separate gene, is thought to be responsible for amylose synthesis in leaves and other non-storage tissues which accumulate transient starch (Tetlow et al., 2004).

Soluble starch synthase produces linear polymers that are the substrate for addition of α-1,6 branches to form amylopectin. Branching of amylopectin is the result of the balanced activities of starch synthases (SSI, SSII, SSIII), starch branching enzymes (SBEI, SBEIIa and SBEIIb) and starch debranching enzymes (McCUE et al., 2002). In monocot there are three types of branching enzymes: SBE I, SBE-IIa and SBE-IIb. At the nucleic acid level there is about 65% sequence identity between types I and II in the central portion of the molecules. Although SBE-I and SBE-II catalyse identical reactions, evidence from mutational and gene suppression experiments demonstrate that the enzymes differ in their roles, and biochemical evidence suggests that they also differ in their patterns of action (Rahman et al., 1999).

In maize, rice and pea, suppression of SBEIIb leads to amylose-extender (ae) phenotype, with a very high amylose content (>50%), in contrast suppression of SBEI or SBEIIa has no effect on the amount of amylose. In wheat, SBEIIa is the predominant isoform present in the soluble phase of the endosperm, whereas in maize and rice endosperm SBEIIb is the predominant isoform involved in amylopectin biosynthesis. In contrast to other cereals, the silencing of *SBEIIb* genes has no effect on amylose content and starch granule shape; whereas silencing of *SBEIIa* genes results in a strong increase in amylose content (>70%) and granule deformation (Sestili et al., 2010).

Particular interest is associated at the production of low and high amylose starches, because they are associated with industrial and processing properties as well as with human health and nutrition. The manipulation of starch composition has mostly interested bread wheat, whereas durum wheat has been investigated less (Lafiandra et al., 2010). For example, Regina et al (2006) showed a strong increase of amylose content in bread wheat through the silencing of *SBEIIa* gene by RNAi approach. The same technique has been used in durum wheat cv Svevo (Sestili et al., 2010) with an increase of amylose content of 70%.

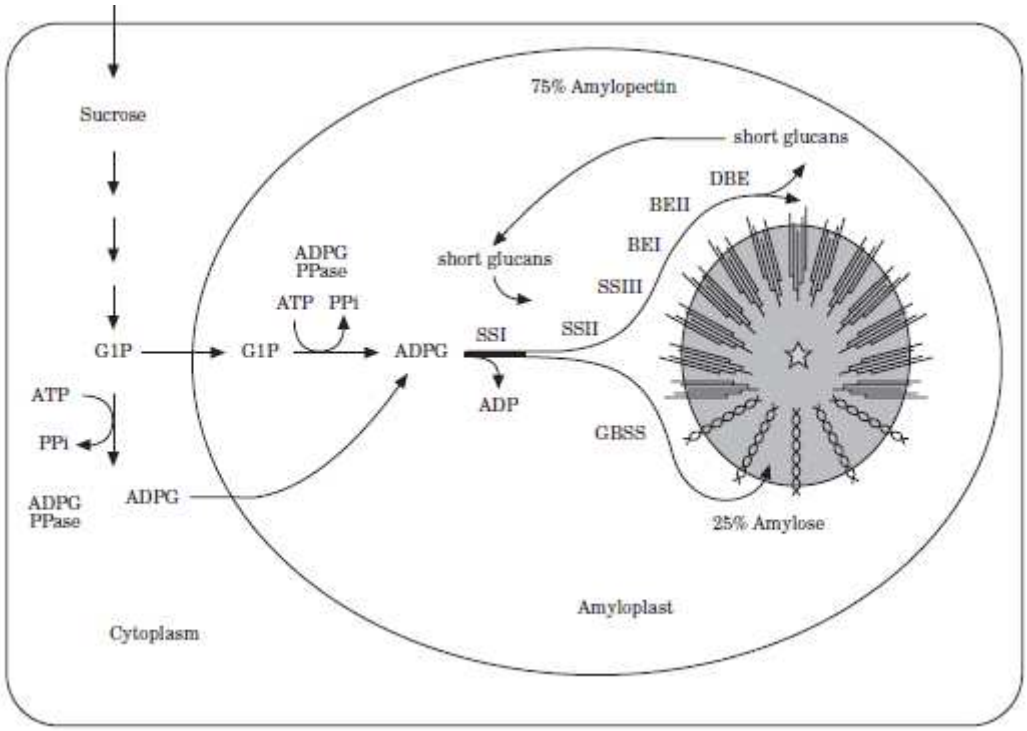

Fig. 8: General scheme for starch biosynthesis in cereals. Sucrose is transformed to glucose-1-phosphate through the action of invertase, sucrose synthase, UDP-glucose pyrophosphorylase, hexokinases, and phosphoglucomutase (not shown). Taken from Rahman et al., 2000.

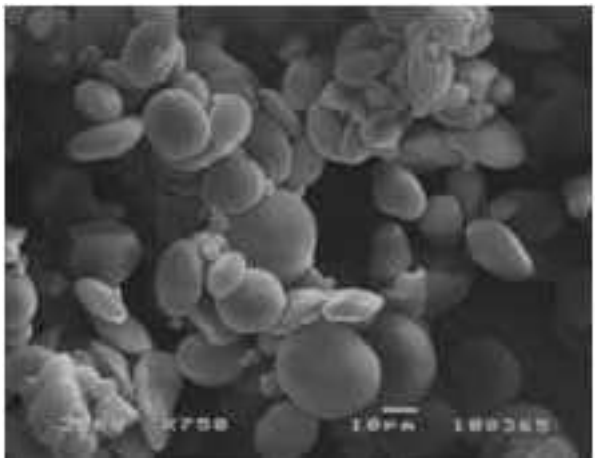

Fig. 9: Scanning electron micrographs of isolated starch granules from durum wheat cv Svevo. Taken from Sestili et al., 2010.

1.6.2 Proteins

1.6.2.1 Seed storage proteins

The study of cereal grain proteins extends back to 1745, with the pioneering work of Professor Giacomo Beccari. He was the first one to describe and call “gluten” that cohesive protein mass obtained after washing wheat flour with water (Beccari, 1745). However, the detailed study of seed storage proteins dates from the turn of the century, when Osborne (1924) classified them into groups on the basis of their extraction and solubility in water (albumins), dilute saline (globulins), alcohol hater mixtures (gliadins), and dilute acid or alkali (glutenins). The major seed storage proteins include albumins, globulins, and prolamins (Shewry et al., 1995).

1.6.2.1.1 Prolamin storage proteins

The prolamins constitute up to 80% of total flour protein, and confer properties of elasticity and extensibility that are essential for functionality of wheat flours (Dupont, and Altenbach, 2003). They are unique in terms of their amino acid compositions, which are characterized by high contents of glutamine and proline and by low contents of amino acids with charged side groups. They are constituted from two fractions, gliadins and glutenins, both are important contributors to the rheological properties of dough, but their functions are divergent. The gliadins have little elasticity and are less

cohesive than glutenins; they contribute mainly to the viscosity and extensibility of the dough system. In contrast, the glutenins are both cohesive and elastic and are responsible for dough strength and elasticity (Wieser, 2007). The gliadins and glutenins form the gluten. The gluten is traditionally prepared by gently washing wheat dough in water or dilute salt solution, leaving a cohesive mass, the remainder being mainly starch granules which are trapped in the protein matrix (Shewry, 2009).

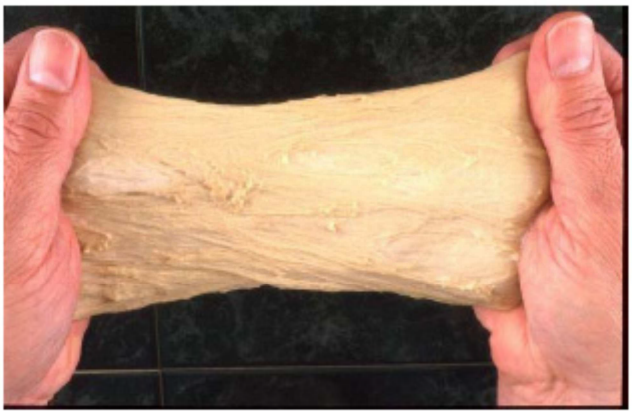

Fig. 10: After kneading, dough can be washed to recover the gluten network as a cohesive mass which is stretched in the photograph to demonstrate its viscoelastic properties. Taken from Shewry, 2009.

Gluten contains hundreds of proteins which are present either as monomers (gliadins) or, linked by interchain disulphide bonds, as oligo- and polymers (glutenins). These proteins were very early characterized according to their electrophoretic mobility (Kasarda et al., 1983; Lafiandra and Kasarda 1985; Tatham and Shewry 1985).

The gliadins are separated into α, β, γ, and ω-type by electrophoresis at low pH (Woychik et al., 1961). Later studies on amino acid sequences, however, have shown that the electrophoretic mobility does not always reflect the protein relationships and that α- and β-gliadins fall into one group (α/β-type) (Altenbach et al., 2002; Wieser, 2007). Also the glutenins can be separated into two groups after reduction, high molecular weight (HMW) and low molecular weight (LMW) subunits (Rev. in D'Ovidio and Masci 2004).

A more recent classification based on amino acids composition defines three groups of prolamins: 1) the high molecular weight prolamins, which comprise only the HMW subunits of glutenin polymers; 2) the sulfur-poor (S-poor) prolamins, which comprise

ω-gliadins and LMW subunits of ω-type (D-type of glutenin polymers), and 3) the S-rich prolamins, which comprise α/β-type gliadins, γ-type gliadins, and the LMW subunits of glutenin polymers (Shewry et al., 1986).

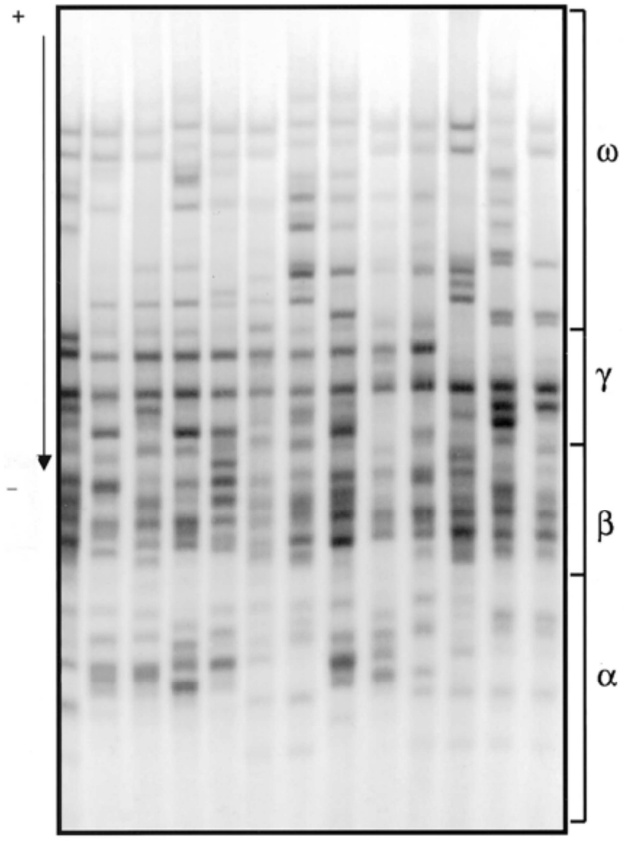

Fig. 11: Typical acid-PAGE separation of gliadins of hexaploid wheats. Arrow indicates direction of acid-PAGE run. Gliadins are divided on basis of electrophoretic mobilities into ω-,γ-, β- and α-gliadins. Taken from Gianibelli et al., 2001.

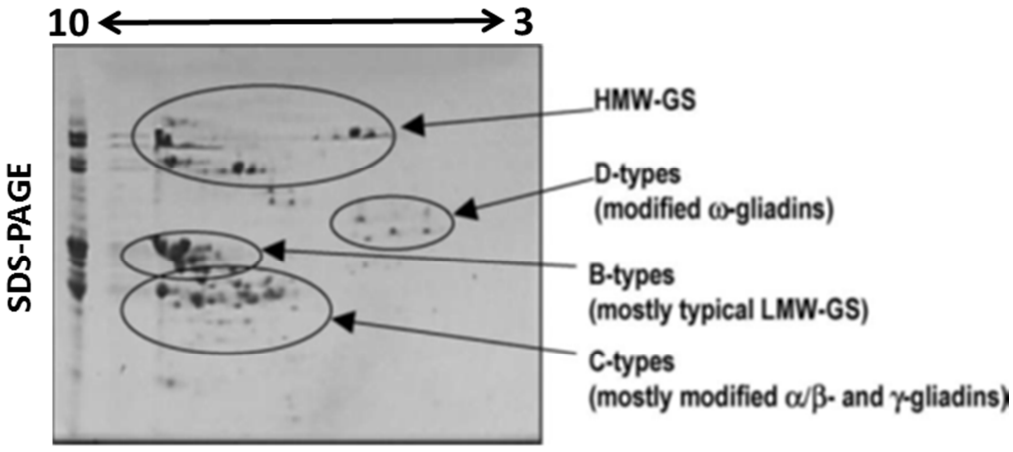

Fig. 12: Two-dimensional electrophoresis of glutenin subunits of the bread wheat (cv Chinese Spring). The HMW-GS and the B-, C-, and D-type groups of LMW-GS are indicated. Taken from D'Ovidio and Masci 2004.

1.6.2.1.1.1 The gliadins

The gliadins, accounting for ~40–50% of the total endosperm proteins, are soluble in 70% alcohol, range in size from ~30 to 70 kDa and are encoded by large complex gene families (Altenbach et al., 2002). Genes coding the gliadins are located on the short arms of group 1 and 6 chromosomes, and they are controlled by gene cluster at the Gli-1, Gli-2 loci, and some other single genes separated from them. *Gli-1* genes code for all the ω- and most of the γ-gliadins. In particular ω-1 and ω-2 are encoded on the 1A and 1D chromosome (DuPont et al., 2004) respectively, while ω-5 is encoded on the chromosome 1B (DuPont et al., 2000). *Gli-2* genes code for all the α-, most of the β-, and some of the γ-gliadins (Rev. in Gianibelli et al., 2001).

Several studies performed with 1D electrophoresis on a single wheat grain, showed that gliadins can be separated into 20–25 components (Bushuk and Zillman 1978; Autran et al., 1979; Wrigley et al., 1982; Metakovsky et al., 1984), whereas by 2D electrophoresis they can be separated separated into up to 50 components (Wrigley, 1970; Payne et al., 1982; Pogna et al., 1990). Due to their extensive polymorphism, these proteins have been widely used for cultivar identification in hexaploid and tetraploid wheats (Rev. in Gianibelli et al., 2001).

Based on A-PAGE gel, Sapirstein and Bushuk (1985) showed that the gliadins electrophoretic mobility correspond to: ω <40.4KDa, γ- between 40.4 and 53.2 KDa, β- between 53.2 and 68.6 KDa, and α >68.6 KDa. Later studies on amino acid sequences, however, have shown that the electrophoretic mobility does not always reflect the protein relationships and that α- and β-gliadins fall into one group (α/β-type) (Altenbach et al., 2002; Wieser, 2007).

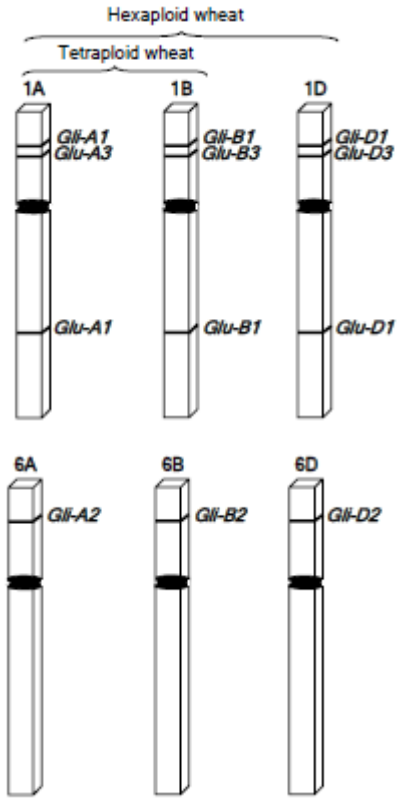

Fig. 13: Schematic representation of the chromosomal locations for the genes encoding the gluten proteins of tetraploid and hexaploid wheats.

ω-gliandins show a high content of glutamine (40-50 mol %), proline (20-30 mol %) and phenylalanine (7-9 mol %), and they contain no cysteine residues (S-poor prolamins), and have low contents of charged amino acid residues (Rev. in Tatham and Shewry 2012).

The absence of cysteine residues also means that the ω-gliadins are not participants in intra- and inter-molecular disulfide bond formation as are the other wheat prolamins (Hsia and Anderson 2001). ω-gliadins account only for 6 to 20% of total gliadins (Wieser et al., 1994).

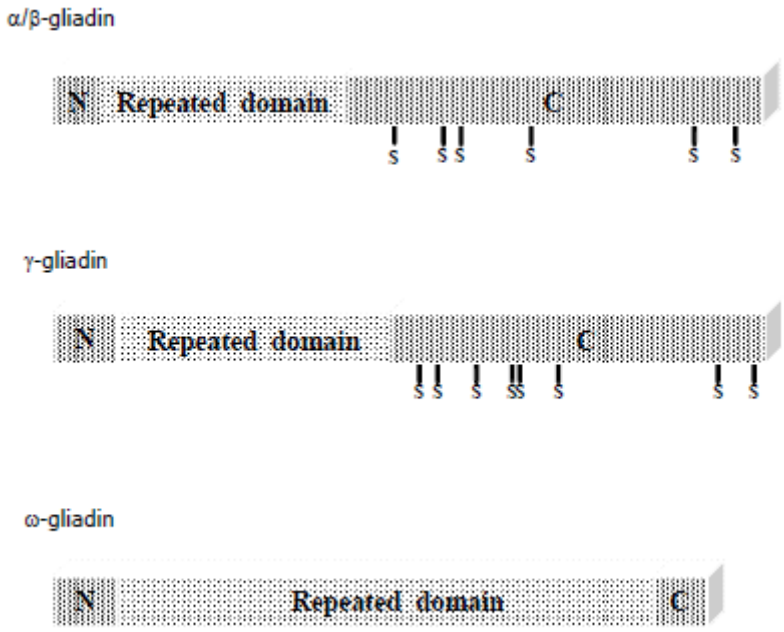

Fig. 14: Schematic illustration of α/β-, γ-, and ω-gliadins. Black bars with letter s indicate the approximate position of cysteine residues that are all involved in intramolecular disulphide bonds.

On the basis of the first three amino acids of N-terminal sequences, Kasarda et al., (1983) and Tatham and Shewry (1995) reclassified ω-gliadins into four types named: AREL, ARQ-, KEL-, and SRL-types. The ARQ type is thought to be the ancestral S-poor sequence type. The KEL-type differs from the ARQ-type in its lack of the first eight residues and DuPont et al., (2004) suggested that the KELQ-type ω-gliadins result from post-translational proteolysis of the ARQ type. The SRL type of ω- gliadin N-terminal sequence is characteristic of ω-gliadins encoded by chromosome 1B, the first eight residues showing a number of substitution when compared to the ARQ and KEL type (Tatham and Shewry 1995). The AREL and ARQL-type ω-gliadins encoded by chromosomes 1A and 1D are referred to as ω-1and ω-2 types, respectively; while the SRLL-type ω-gliadins encoded by chromosome 1B are referred to as ω-5 types (DuPont et al., 2004).

The S-poor prolamins include C hordeins of barley, the ω-secalins of rye, and the ω-gliadins of wheat. In all cases, the encoded proteins consist almost entirely of repeats of the octapeptide motif Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln that are flanked at the N-terminal side by short unique sequences of 12 residues and at the C-terminal side by short unique sequences of either six residues (Shewry et al., 1995). However, ω-gliadins

encoded at chromosome 1B are characterized by different internal peptides rich in glutamine such as QQXP, QQQXP, and QQQQXP, where X is F, I, or L in order of predominance (DuPont et al., 2000).

The ω-gliadins do not have a compact structure, and circular dichroism spectra indicate β-turns and only low levels of α-helices and β- sheets (Tatham and Shewry 1985).

Unlike other wheat gliadins, α-gliadins are present only in wheat and its closely related species, not in rye and barley. The α-gliadin genes on chromosome 6 probably originated from the gliadin genes on chromosome 1 through a duplication and/or translocation event. The γ-gliadins are considered to be the most ancient of the wheat prolamin family (Qi et al., 2006).

The α/β and γ-gliadins are characterized by high levels of glutamine and proline, (with 90% of the glutamic and aspartic acid residues amidated) (Bietz et al., 1977; Ewart 1983; Kasarda et al., 1983). These proteins are also relatively high in leucine and low in basic amino acid. The α/β- and γ-gliadins are rich in sulfur with six and eight cysteine residues (Kasarda et al., 1984; Köhler et al., 1993; Müller and Wieser 1995, 1997). As a result three and four intramolecular disulfide bonds are formed (Rev in Gianibelli et al 2001). N-terminal sequences of the α/β-gliadins are represented by small sequence of five amino acid residues (VRVPV) (Bietz et al., 1977). Peptide motifs based on the pentapeptides PQQQP and PQQPY are always present in a repetitive region that follows the N-terminal region of the α/β-gliadin proteins (Shewry et al., 1986). While the N-terminal region of the γ-gliadins is formed by 12 amino acid residues (NMQVDPSGQVQW) that precedes a series of repeats based on the consensus motif PQQPFPQ (Autran et al., 1979; Kasarda et al., 1983; Shewry and Tatham 1990).

1.6.2.1.1.2 The glutenins

The glutenins are the largest natural polymers in nature (Wrigley, 1996). They are insoluble polymers that consist of high molecular weight glutenin subunits (HMW-GS) with a range between 70,000 and 90,000 Da and low molecular weight glutenin subunits (LMW-GS) with a range between 20,000 and 45,000 Da linked by interchain disulfide bonds.

1.6.2.1.1.2.1 **High Molecular Weight Glutenin Subunits (HMW-GS***)*

Even if the HMW-GS are minor components in terms of quantity, they are key factors in the process of breadmaking because they are major determinants of gluten elasticity (Tatham et al., 1985a).

The molecular weights of HMW-GS estimated by SDS-PAGE are 80,000-130,000 Da, but showed lower molecular weights (60,000-90,000 Da) when calculated from derived amino acid sequences (Anderson et al., 1988, 1989; Anderson and Green 1989).
The HMW-GS are encoded at the loci *Glu-A1*, *Glu-B1,* and *Glu-D1*on the long arms of group 1 chromosomes (1A, 1B, and 1D) (Bietz et al., 1975; Payne et al., 1980). Each locus includes two genes linked together encoding two types of HMW-GS, x- and y-type subunits. The x-type subunits generally have a slower electrophoretic mobility in SDS-PAGE and higher molecular weight than the y-type subunits (Payne et al., 1981; Shewry et al., 1992).

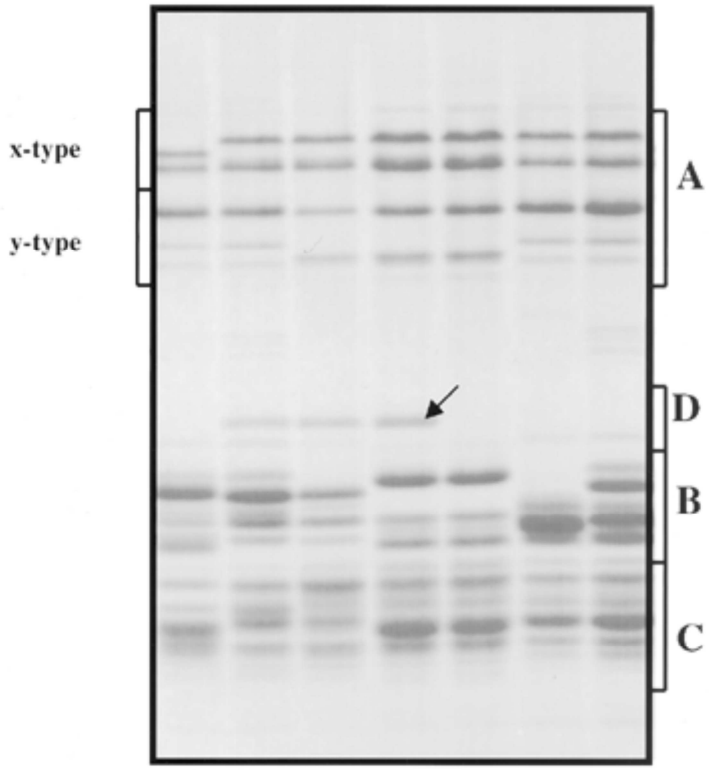

Fig. 15: SDS-PAGE of polymeric protein (after reduction to subunits), Group A: HMW glutenin subunits showing x- and y-type glutenin subunits. Groups B-, C-, D-: LMW glutenin subunits. Arrow indicates subunit D. Taken from Gianibelli 2001.

Electrophoretic studies have revealed there is allelic variation in the subunits encoded by each Glu-1 locus in bread wheat cultivars (Lawrence and Shepherd 1980) and in durum wheats (Branlard et al., 1989; Waines and Payne 1987). In particular there are three allelic forms at the *Glu-1A*, 11 alleles at the *Glu-1B*, and six alleles at the *Glu-1D* (Payne and Lawrence 1983).

Hexaploid wheat could contain six different HMW-GS but gene silence observed in bread and durum wheat resulted in a variable expression, from three to five subunit in bread wheat and one to three subunits in durum wheats. More recently, some bread wheats with six HMW-GS have been reported (Johansson et al., 1993).

Lawrence et al. (1988) before, and Lafiandra et al. (2000) after, have developed a set of bread wheat lines in which the number of subunits increased progressively from zero to five, and wheat lines with single x- or y-type subunits respectively. This material has proved useful in determining the relative effects of individual HMW subunits on flour breadmaking properties and provided the possibility of developing wheats suitable for different end uses.

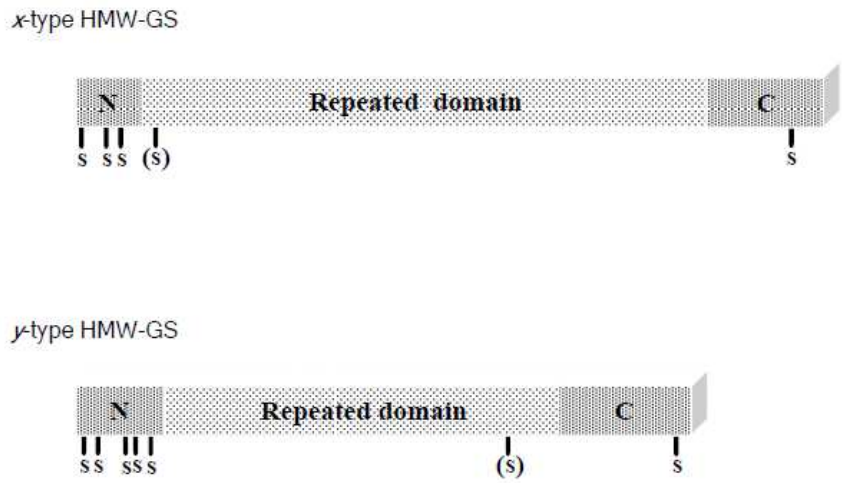

Fig. 16: Structure of the *x*- and *y*-type HMW-GS. Black bars with letters represent cysteine residues which may be involved in either an inter- or intramolecular disulphide bond. (s) indicates the cysteine residues not found in some *x* and *y*-type HMW-GS.

Shewry et al. (1989) has investigated the amino acid composition of HMW-GS and has shown the hydrophilic nature of the central repetitive domain and the hydrophobic characteristics of the N- and C-terminal domains. The central repetitive domain is composed of short amino acid motifs (PGQGQQ, GYYPTSPQQ) that composed up to 85 % of the protein sequence, and non-repetitive terminal domains that contain the majority of the cysteine residues (Blechl and Anderson 1996). Both x- and y-type are predicted to adopt a β-turn conformation (Tatham et al., 1990) whereas Miles et al. (1991) and Shewry et al. (1992) also proposed a α-helical arrangement of the amino acids for both N- and C-terminal regions. The high level of glutamine residues in the central repetitive domain has a very high capacity to form both intra- and intermolecular hydrogen bonds and this feature could therefore be involved in elasticity through formation of intermolecular hydrogen bonds. In the dough, some of these bonds break on stretching, giving rise to unbonded mobile regions (loops) and bonded regions (trains). Thus, the loops can be stretched and then reform when the stress is removed, which accounts for the elastic restoring force of the dough, as in rubber elasticity (Belton et al., 1994).

1.6.2.1.1.2.2 Low Molecular Weight – Glutenin Subunits (LMW-GS)

The LMW-GS represent about ≈60% of total glutenins and about 20-30% of the total seed proteins (Bietz and Wall 1973). LMW-GS contributed greatly to dough resistence and extensibility (Metakovskii et al., 1990; Andrews et al., 1994; Cornish et al., 2001), and also to pasta-making quality of tetraploid durum wheat (Masci et al., 2000a). Whereas HMW-GS comprise only a few components and have been widely studied, LMW-GS include a large number of polypeptides and their structure; organization and relationship to grain processing quality have not yet been investigated to the same degree as for the HMW-GS (Rev. in D'Ovidio and Masci 2004). This has been mainly due to the difficulty in identifying them in one dimensional SDS-PAGE gels, since LMW-GS largely overlap with gliadins. Jackson et al. (1983), has classified LMW-GS in B-, C-, and D-subunits on the basis of electrophoretic mobility in SDS-PAGE. Their molecular weight range is between 20,000 and 45,000 Da (Rev. in D'Ovidio and Masci 2004). While most of the LMW-GS belong to the B group ("typical" LMW-GS) subsequent analyses showed that the D group is composed of modified ω-gliadin components that have acquired a cysteine residue (ω-gliadins lack this amino acid residue). This finding was the first evidence that gliadin-like subunits were present and incorporated in the glutenin polymers (Masci et al., 1993, 1999). LMW-GS with α- and γ-type gliadin-like N-terminal sequences are the most abundant proteins in the so-called C group, with at least thirty components being detected by two-dimensional analyses. As for the D subunits, it is probable that they form part of the glutenin fraction because the numbers of cysteine residues is different from that in typical α- and γ- gliadins (Masci et al., 2002).

On the basis of the N-terminal sequences, the typical LMW-GS have been divided into two groups: LMW-m and LMW-s type. The –m and -s refers to the first amino acid in the sequence, methionine and serine, respectively. The N-terminal amino acid sequence of LMW-s type subunit (more common than LMW-m) is SHIPGL, whereas the N-terminal sequences of LMW-m type subunits are more variable and include METSHIGPL-, METSRIPGL-, and METSCIPGL- (Kasarda et al., 1988; Lew et al., 1992; Masci et al., 1995). Both LMW-s and LMW-m type subunits contain eight cysteine residues, two of which are involved in intermolecular disulphide bonds. Almost all B-type subunits have LMW-m or LMW-s N-terminal sequences (Rev. in Gianibelli et al., 2001).

Payne et al. (1984, 1985) has established that LMW-GS (B, C, and D group) are controlled by genes at the complex *Gli-1* loci, in particular Glu-A3, Glu-B3, and Glu-D3 (that also encoded γ- and ω-gliadins) on the short arms of chromosome 1, and some C-type LMW-GS are encoded on the group 6 chromosomes (that correspond to α-type LMW-GS) (Masci et al., 2002).

The general structure of a typical LMW-GS shows four main structural regions including a signal peptide of 20 amino acids, a short N-terminal region (13 amino acids) that usually contains the first cysteine residue, a repetitive domain rich in glutamine codons and a C-terminal region. Cassidy et al. (1998) suggested that the C-terminal region can be subdivided into three distinctive regions: a cysteine-rich region containing five cysteine residues, a glutamine-rich region containing a cysteine residue and stretches of glutamine residues, and a C-terminal conserved sequence containing the last cysteine residue. Most of the full-length genes vary from 909 bp to 1167 bp, in size with the molecular masses of the encoded mature proteins ranging from about 32,000 to 42,800 Da. The number of repeats present in the repetitive domain is mainly responsible for this length variation, ranging between about 12 and 25. This variation can result from deletion and/or insertion of repeat units (D'Ovidio et al., 1999), or be caused by unequal crossing-over and/or slippage during replication as suggested for the evolution of other prolamins (Shewry et al., 1989).

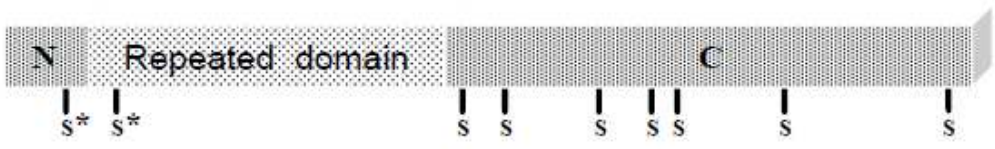

Fig. 17: Schematic illustration of a B-type LMW-GS. Black bars with letter s represent cysteine residues available for intermolecular or intramolecular disulphide bonds. s* indicates the first cysteine residue alternatively located in the N-terminal region or at the start of repetitive domain.

The general hydrophilic character of LMW-GS is mainly influenced by the repetitive domain. The secondary structures of LMW-GS, except for the D-subunits, show a similarity with the structure of the α and γ-gliadins (Tatham et al., 1987; Thompson et al., 1993, 1994). The N-terminal domains are rich in β-turns, while the short non-repetitive C-terminal domains are rich in α-helices and appear to be more compact (Thomson et al., 1992; Masci et al., 1998).

1.6.2.2 Sequences and structures homologies of wheat proteins

There is 30 % of homology between the sequences of gliadins and glutenins, 50 % between α/β- and γ-gliadins and until 95 % between that of the different HMW-GS.
All prolamins of wheat show the same organization: a short N-terminal domain specific of each family, followed by a repetitive domain. The repetitive domain of ω-gliadins and HMW-GS, represents almost totality of the sequence whereas αβ-, γ-gliadins and the LMW-GS show an important C-terminal not repetitive domain.
Important homologies were found in the repetitive domains (Tab. 4). The repetitive sequences of α/β-, γ- and ω-gliadins as well as those of LMW-GS is richer in proline. This suggested a common origin of these sequences. The repetitive sequences in HMW-GS are different and are richer in glycine.

Tab. 4: Comparison of prolamins repetitive sequences

Prolamins family	**Repetitive sequences**
S-Poor : ω-gliadines	PQQPFPQQ
S-Rich: α/β-gliadines	PQQPY, QPQPFP
γ-gliadines	PQQPFPFQ
LMW-GS	PQQQPPFS, QQQQPVL
HMW-GS : x-type	GYYPTSPQQ, PGQGQQ, GQQ
y-type	GYYPTSLQQ, PGQGQQ

In the primary structure of α/β-, γ-gliadins and LMW-GS the position of some cysteine residues is conserved (Shewry et al., 1994). These conserved cysteine residues are involved in intra-molecular disulphide bounds; moreover in LMW-GS there are two other cysteine residues available for inter-molecular bond (Shewry et al., 1994).
The conserved cysteine residues were also found in some albumins, such as α-amylase inhibitors and trypsic inhibitors (Egorov et al., 1996).
The C-terminus non-repetitive domains of α/β, γ-gliadins and LWM-GS show three regions of 20-35 residues. These regions known as A, B, C contains cysteine residues separated from variable regions. The A, B, C domains was found also in LMW-GS; the A and B regions were found on non-repetitive N-terminal domain of HMW-GS, whereas the C region on their C-terminal.

Similar A, B, C sequences were also found in storage proteins from barley, rye, maize and some other albumins and globulins, for instance, inhibitor enzymes.

1.6.2.3 Albumins and Globulins

Fewer studies have been done on albumins and globulins even if they accounted for 20% of the total proteins and despite they include most of metabolic and structural proteins, involved in different pathways and functions. This is probably because the role of albumins and globulins in flour quality is not as well defined as that of the gliadins and glutenins.

According to the classifications of storage proteins based on solubility, the albumins and globulins are soluble in water and in salt solutions respectively, and usually albumins are more abundant than globulins. Some of these proteins are located in the embryo and aleurone layers, and others are distributed throughout the endosperm. The albumins contain enzyme inhibitors, metabolic enzymes that survived dehydration, and 2S type albumins which act as storage proteins in dicots. The globulins contain some hydrolytic enzymes necessary for germination (Payne and Rhodes 1982). The globulins have sedimentation coefficients of about 7 and they have similar structures and properties to the 7S vicilins of legumes and other dicotyledonous plant (Kriz, 1999). Furthermore, although the aleurone and embryo are rich in proteins, the globulins in these tissues have limited impact on the end use properties of the grain. Albumins and globulins have a lower amount of glutamic acid and more lysine than prolamins, and due to this lysine content, these proteins have a good amino acid composition for the dietary requirements of humans and monogastric animals. Unfortunately, because they are present in the wheat endosperm in minor proportions, their presence it is not enough to overcome the lack of lysine in wheat flour. Singh and Shepherd (1985) showed that the soluble fraction also contains other proteins related to legumins (the seed storage proteins of legumes), called “triticins”, which represent 5% of the total seed proteins and are located in the protein bodies of the starchy endosperm. Their solubility properties are comparable to those of globulins; no important link with pasta or breadmaking quality was found. The major albumins and globulins are controlled by genes at chromosome groups 3, 4, 5, 6, and 7 (Garcìa-Olmedo et al., 1982).

Vensel et al. (2005) by using a combined 2D electrophoresis-mass spectrometry approach on soluble fraction of wheat (*Triticum aestivum* L., cv. Butte 86) has identified over 250 proteins grouped in 13 different biochemical processes of the endosperm: ATP interconversion reactions, carbohydrate metabolism, cell division, cytoskeleton, lipid metabolism, nitrogen metabolism, protein synthesis/ assembly, protein turnover, signal transduction, protein storage, stress/defense, transcription/ translation, and transport (Amiour et al., 2002; Vensel et al., 2005). A comparative 2DE-MS approach of the salt soluble proteins from imbred lines enables Merlino et al. (2009) to identify 54 proteins and to map 120 spots on 21 chromosomes (Merlino et al., 2009; Debiton et al., 2011).

Vensel et al. 2005 showed that carbohydrate metabolism, transcription/translation, and protein synthesis/assembly were the principal endosperm functions in the early stages of grain development (10 dpa) followed by nitrogen metabolism, protein turnover, cytoskeleton, cell division, signal transduction, and lipid metabolism. Moreover, Carbohydrate metabolism and protein synthesis/assembly were also major functions in the later stages of grain development (36 dpa), but stress/defense and storage were predominant (Vensel et al., 2005).

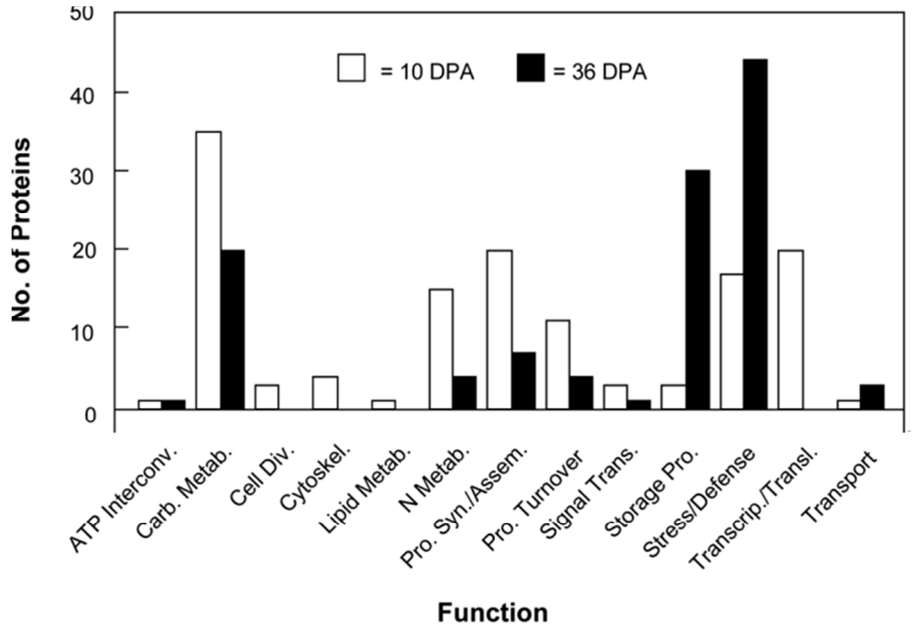

Fig. 18: Timing of biochemical processes of wheat endosperm during grain development. Profiles based on protein number. Taken from Vensel et al., 2005.

2 Bread and pasta making

Most of the wheat consumed by humans is processed from white flour, which is produced by milling to remove the germ (embryo) and bran (pericarp, testa, nucellar layer, and aleurone layer) (Shewry and Halford 2002). Wheat is the basic ingredient of many types of food, such as bread, pasta, biscuits and cakes. They all require flour with different characteristics. The general terms “baking quality” and “pasta quality” usually refer to the specific properties required for the production of leavened bread or pasta. Pasta with high quality must have a yellow color and maintain its shape when cooked in boiling water; moreover it should give a firm bite (known as ‘al dente’) and its surface should not be sticky after it is cooked. Furthermore water should be free of starch and the pasta should be resistant to over-cooking. Qualitative characteristics of wheat dough such as strength, elasticity and extensibility are very important to have a good end product, and they are largely determined by protein content and composition, in particular the gluten proteins. These macromolecules (gliadins and glutenins) are capable of imbibing water even up to 3 times their weight, and under mechanical stress, rendering a viscoelastic network that is responsible for gas retention during leavening and the further expansion of loaves during baking (Marchetti et al., 2012). Very important are the glutenin polymers, especially HMW-GS to obtain strong (highly viscoelastic) doughs (Field et al., 1983). Also Payne et al. (1987) showed that allelic variation in the composition of the HMW-GS was strongly correlated with differences in the breadmaking quality of European bread wheats (Payne et al., 1987).

The right combination of the dough properties is critical to produce food products with the optimum quality. For instance, bread and pasta making require gluten with a good balance of elasticity and extensibility, while pasta making also requires strong gluten to retain starch during cooking.

3 Genetic engineering compared to classical breeding

Cultivated plants have been selected over thousands of years, to improve disease resistance, growth and other useful characteristics. Wheat has been a target of plant breeding, traditionally using extensive crossing programs with the objective of increase grain yield, improve the quality of wheat end-use products, minimize crop loss due to unfavorable environmental conditions and with the aim of introducing resistances against various pests and pathogens (Rakszegi et al., 2001, Sahrawat et al., 2003; Jones, 2005, Shewry and Jones 2005).

The rapidly growing of world population causes an increase of food demands. Especially for wheat, it is estimated that demand will be 40% higher in the year 2020. This increase is not feasible with conventional plant breeding approach, whereas the recombinant DNA technology can allow the increase ofwheat production (Rev. in Razzaq et al., 2010).

The two mostly used techniques for introduction of genes into plants are the biolistic method, which permits direct insertion of DNA segment via particle bombardment, and direct insertion of genes via *Agrobacterium tumefasciens* by T-DNA (Rev. in Shewry and Jones 2005).

Agrobacterium tumefaciens is a soil bacterium that naturally infects dicots and causes tumorous growth resulting in crown gall disease. The tumor is caused by incorporation of T-DNA (small DNA molecule outside the bacterial genome called tumor inducing plasmid, Ti). The *Vir* genes are located on Ti plasmid and their expression isstimulated by phenolic compounds exuded from infected plants. The *Vir* genes are responsible of Ti excision, transfer and integration into plant genome. In plant transformation the natural capability of *Agrobacterium* is manipulated by replacing the genes causing tumorous growth by genes of interest (Rev. in Shewry and Jones 2005).

For the first time, tungsten particle bombardments were used by Sanford et al. (1987) for transformation of rice and wheat, monocots that are not susceptible to *Agrobacterium* infection. This physical method of transformation uses high-pressure gas to drive metal particles coated with DNA intoplant tissues. In this method there is no biological vector for the insertion of the transgene construct, because is directly transferred into plant material. Several studies have analyzed transgenic cereal lines

obtained by bombardments (biolistic method), which have shown that several copies of transgene are typically transferred (usually 5-15) and also that insertion rearrangements are possible (Makarevitch et al., 2003; Rooke et al., 2003). Moreover an unpredictable gene silencing can beobserved caused by high copies integration (Anand et al., 2003; Howarth et al., 2005; Danilova, 2007). Cheng et al (1997) used for the first time the transformation mediated by *Agrobacterium* on the bread wheat model variety, Bobwhite. Several studies have been performed to compare transgene copy number between wheat lines obtained by biolistic and *Agrobacterium* methods. Cheng et al (1997) showed that only 17% of the wheat lines obtained by biolistic technique contained a single transgene copy compared with 35% of those transformed with *Agrobacterium*. Wu et al. (2003) obtained the same results; in particular, 60% of lines produced via *Agrobacterium* showed a single copy of transgene compared with 10 % of lines produced by bombardment.

These analyses revealed that gene transfer by *Agrobacterium* has remarkable advantage respect to biolistic method, due to the low copy number of transgene, and also fewer problems of co-suppression and instability. Moreover *Agrobacterium* approach is reproducible, and also, as shown by Hu et al. (2003) this method has higher transformation efficiency respect to particle bombardment for wheat (Hu et al., 2003).

However, the use of *Agrobacterium* for monocots is very recent, and still needs to be completely set up, thus the biolistic one is still the method of choice in most laboratories.

3.1.1 Wheat transformation

Although, at present, no GM wheat is grown anywhere in the world, there is an increasing interest in this procedure, either as an alternative to classical wheat breeding or as a powerful tool for functional genomics. In wheat, targets of transgenesis include the increased resistance to biotic and abiotic stresses, the increase of grain yield, to minimize crop loss due to unfavourable environmental conditions, the improvement of dough quality properties and the modulation of starch composition (Rakszegi et al., 2001; Sahrawat et al., 2003; Jones, 2005; Shewry and Jones 2005).

3.1.2 Some examples of wheat transformation

In order to investigate the possibility to manipulate the gluten strength and elasticity by changing the ratio between HMW-GS and LMW-GS, Masci et al. (2003) have transformed the bread wheat cv Bobwhite with LMW-GS gene under control of its own promoter . The transformation of Bobwhite was performed on immature embryos by biolistic method. Two plasmids were used for co-transformation: UBI:BAR (Cornejo et al., 1993) and pLMWF23A, which contains a LMW-GS gene coded at the *Glu-D3* locus isolated from the bread wheat cultivar Cheyenne (Cassidy et al., 1998). The transgenic LMW-GS was estimated to be twelve or sixteen times overexpressed with respect to endogenous LMW-GS, depending on the method used (densitometric analysis of SDS-PAGE gel or RP-HPLC). This strong over-expression is probably due to insertion of multiple transgenes (Carrozza et al., 2005). Masci et al. (2003) showed that gluten polymer composition was altered by overexpression of the transgenic LMW-GS and that such changes affect wheat end-use properties.

Sestili et al. (submitted) have produced a durum wheat (cv Svevo) overexpressing the *Wx-B1* gene, involved in amylose synthesis. The transformation was performed by biolistic method. The *Wx-B1* gene was inserted in the vector pRDPT under the promoter of Dx5 high molecular weight glutenin subunit (Tosi et al., 2004). The plasmid pAHC20 (Christensen and Quail 1996), carrying the *bar* gene, was co-bombarded with pRDPT+Wx-B1 for the transformation. The authors showed that the transformation event did not result in an increase of the amylose content, although the amount of the Wx-B1 protein increased up to 4-fold, as compared to the amount present in the control. Moreover, no differences in the viscosity properties of starch were found between transgenic plants and control plants

Sestili et al. (2010) have used another transgenic strategy in order to increase the amylose content in durum wheat seeds. In particular, the effects of *SBEIIa* gene silencing were investigated in terms of amylose content, transcript accumulation and protein profile of the enzymes involved in starch biosynthesis (Sestili et al., 2010). Biolistic method was used for transformation of immature embryos of the durum wheat *cv* Svevo. Co-transformation was performed by using pRDPT+ *SBEIIa*(RNAi) and *bar* selectable marker plasmids. In addition, *Triticum durum* cv Ofanto was transformed

with pGUB-G + *SBEIIa* (RNAi) by *Agrobacterium* method. Sestili et al. (2010) showed that the silencing of *SBEIIa* genes in durum wheat causes alterations in granule morphology and starch composition, leading to high amylose wheat. The authors also showed that the two different methods of silencing (transformation either with *Agrobacterium* or with the biolistic method) in the two durum wheat cultivars gave the same results.

Tab. 2: Other examples of genes transferred into wheat by biolistic method. Taken from Sahrawat et al., 2003, modified.

Target tissue	Source of the gene	Gene	Marker	Phenotype	Reference
Immature embryo	*T. aestivum*	High molecular weight glutenin subunit 1Ax1	*bar*	Accumulation of glutenin subunit 1Ax1	Altpeter et al. 1996b
Immature embryo	*T. aestivum*	High molecular weight glutenin chimaeric subunit	*bar*	Accumulation of chimaeric glutenin subunit Dy10:Dx5	Blechl & Anderson, 1996
Immature embryo	*T. aestivum*	High molecular weight glutenin subunits 1Dx5 and 1Ax1	*bar*	Increased dough elasticity	Barro et al. 1997
Immature embryo	*T. aestivum*	High molecular weight glutenin subunit 1Dx5	*bar*	Increased dough strength	Rooke et al. 1999
Scutellum derived calli	*T. turgidum*	Epitope -tagged Low molecular weight glutenin subunit	*bar, uid*	Accumulation of epitope-tagged low molecular weight glutenin subunit	Tosi et al. 2004
Immature embryo	*Oryza sativa*	Thaumatin-like protein (TLP), chitinase (chiII)	*bar, hpt*	Resistance to fungus *F. graminearum*	Chen et al. 1999
Immature embryo	*Hordeum vulgare*	Trypsin inhibitor (CMe)	*bar*	Increased insect resistance	Altpeter et al. 1999
Immature embryo	*T. aestivum*	Protein puroindoline (PinB)	*bar*	Increased friabilin levels and decreased kernel hardness	Beecher et al. 2002

4 Allergy: symptoms and diagnostics

In developed countries, more than 40% of the population may be affected by allergies. While the "first wave" of allergic disease (asthma and allergic rhinitis) appeared about 50 years ago, a "second wave" of food allergy has emerged in the last 10-15 years (Prescott and Allen 2011).

Food allergy affects more than 1-2% but less than 10% of the population. Even if hundreds of epidemiologic studies have been published, it is still challenging to determine food allergy with certainty because of multiple variations in methodologies and also a variety of methodologic concerns. Despite these difficulties, all these reports clearly show that food allergy is a significant public health concern (Sicherer, 2011).

The European Academy of Allergy and Clinical Immunology (EAACI) proposed to classify adverse reactions to food as reported in Figure 23. Food allergy is one of these aberrant reactions; it is defined as a hypersensitive response most often mediated by an Immunoglobulin E mechanism:

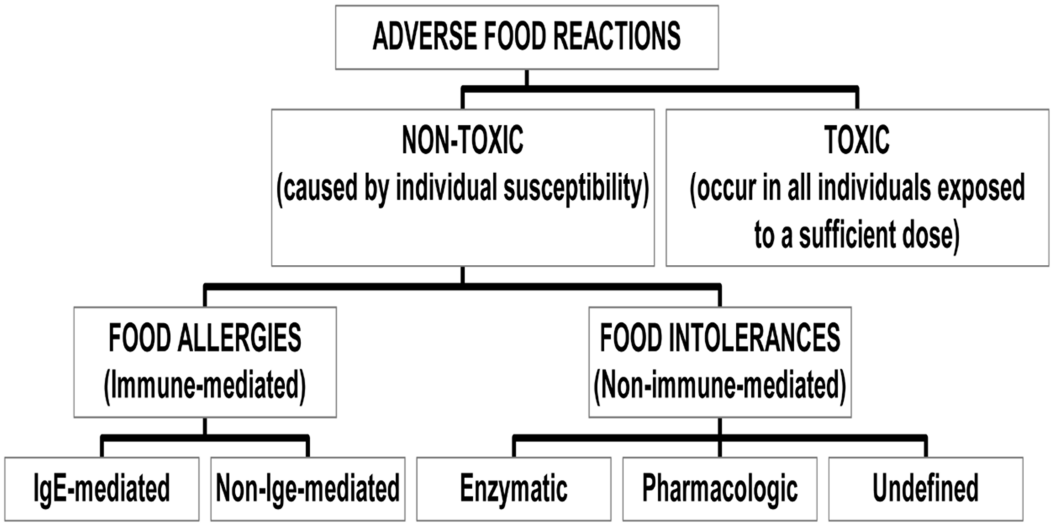

Fig. 19: Classification and terminology for food allergy most frequently used in Europe. Taken from Asero et al., 2007.

The general prevalence of food allergies is approximately 6–8% in children during the first 3 years of life (Woods at al., 2001). In most cases, food allergy in infants is caused by cows' milk and hen's egg. Fortunately, up to 85% of them outgrow their allergy in the first 5–10 years of life; however the naturel recovery may take several years. In adults, food allergies are less prevalent than in young children; in Europe about about 2–4% of adults have food allergy (Kanny et al., 2001). Only a limited number of foods are responsible for the majority of food allergies and within these foods only few molecules (allergens) are capable of eliciting an allergic response in in predisposed individuals.

While adults tend to be allergic to fish, crustaceans, peanuts and tree nuts, children tend to be allergic to cow's milk, egg white, wheat and soy more frequently (Ebo and Stevens 2001). Moreover, plant allergens are more frequently involved in adult allergies because of cross-reactions with some pollen allergens. However, variations occur according to countries. In particular in the United States, there is a high prevalence of peanut allergy, whereas soy, cow's milk and hen's egg are the most frequent food allergens in Japan, and in France, there are more allergies to mustard than in other countries. In Portugal and Spain frequent food allergens are fish, in Italy seafood and cow's milk, and in Scandinavia tree nuts (Ferrari and Eng 2011).

A few studies on the prevalence of plant food allergies included food challenge tests: the gold standard for diagnostic. They indicated prevalences ranging from 0.1% to 4.3% of the general population for allergies to fruits or tree nuts, 0.1% to 1.4% for vegetables and < 1% for wheat and soy (Zuidmeer et al., 2008).

Food allergies cause a number of clinical conditions involving the gastrointestinal tract, the skin, the airways or the most dangerous of all allergic reactions, anaphylaxis. These reactions are dependent on the physical/chemical characteristics of the allergen responsible for sensitization, dose (amount ingested), whether the food was ingested alone or in combination with other foods that may delay its absorption, the association with alcoholic beverages, aspirin or exercise and importantly, ill-defined, host' factors (Asero et al., 2007). The food allergy sensitization can occur through the intestinal tract and is often caused by stable proteins, but it may also be a consequence of sensitization to airborne allergens (Breiteneder and Ebner 2000). The diagnosis starts with a medical and dietary history and physical examination, supported by a series of both in vivo and in vitro tests. Detecting the offending food is certainly essential, but in some instances

the identification of the responsible protein(s) is even more important as distinct foods containing homologous, crossreacting allergens might pose a risk of further allergic reactions (Asero et al., 2007).

4.1 Pathophysiology

Exposure to food allergen generally occurs via the gut where non-immunologic and immunologic mechanisms prevent intact food antigens to enter the body. However, some food antigens remain immunologically active, pass through the epithelium and enter the circulation. Generally, these antigens do not elicit reactions because highly efficient mechanisms exist for suppression of immune responses to food allergens, i.e. mechanisms of oral tolerance (Ebo and Stevens 2001).

Typically, the immunological mechanism in food allergies is an immunoglobulin IgE-mediated Type-I response. Other mechanisms of Type-II (IgG mediated) and Type-IV (T cell-mediated) may also be involved. The development of an IgE-mediated response to an allergen is the result of a series of molecular and cellular interactions involving antigen presenting cells (APCs), T cells, and B cells.

Cytokines produced by the CD4 subgroup of T lymphocytes (helper T cells) mediate a wide range of pro-inflammatory and anti-inflammatory responses. Most CD4 T cells belong to Th1 and Th2 subgroups producing Th1 and Th2 cytokines respectively. Interleukins 4, 5, 10, and 13 are the principal Th2 cytokines and mediate IgE and eosinophilic responses in atopic diseases. In the first step, APCs present small peptide fragments (T-cell epitopes) from the allergen, in conjunction with MHC class II molecules to T cells. T cells bearing the appropriate complementary T-cell receptor will bind to the peptide-MHC complex. This interactive “first signal” leads to T-cell proliferation and cytokine generation (IL12) and the generation of a “second” signal, which promotes then TH2-like cell activation for an IgE response. These cells and their products, in turn, interact with B cells bearing appropriate antigen-specific receptors, leading to isotype switching and the generation of antigen- specific IgE. At all stages, a number of specific cytokines are secreted, which modulate the cell interactions. The antigen-specific IgE then binds to surface receptors of mast cells, basophils, macrophages, arming the immune system for an allergic reaction with the next encounter of the specific antigen (Sampson, 1999). When IgE molecules interact with

specific antigens on mast cells in the gastrointestinal tract or other tissues, there is degranulation of these cells. This leads to the release of vasoactive amines and cytokines and synthesis of a variety of arachidonic acid derived inflammatory mediators (Baral and Hourihane 2005). IgE mediated reactions typically have a rapid onset within minutes to two hours from the time of ingestion of the offending agent (Guandalini and Newland 2011).

The IgE are normally found in very low concentrations (ng/mL) in the serum and only a small proportion of the plasma cells in the body synthesis this immunoglobulin. The expression of atopy (i.e. the atopic phenotype) is characterized by high concentrations of total IgE in serum and by the presence of IgE antibodies specific to ordinarily harmless environmental antigens. These occur when IgE antibodies are produced in response to allergen exposure.

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In summary, the IgE-mediated responses are characteristically divided in two phases:

- The induction phase during which the sensitization to an allergen occurs
- The effector phase during which the clinical manifestations of allergy are triggered and expressed (Fig. 24).

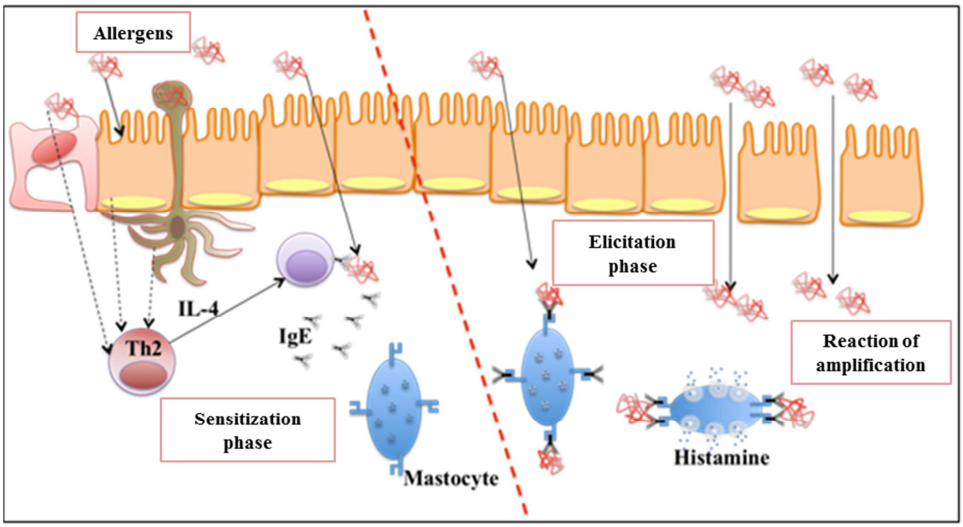

Fig. 20: **General mechanism of allergic reaction:** During the sensitization phase, an allergen molecule crosses the epithelial barrier and is captured by an antigen presenting cell (APC). The allergen is presented to a naive T cell that differentiates into Th2 lymphocytes and secretes IL-4. The production of allergen-specific IgE by B cells is activated. These IgE antibodies then bind to mast cells. During the effector phase of allergy, a new contact with the allergen binding to IgE on the surface of mast cells induces their degranulation, releasing many inflammatory mediators such as histamine.

4.2 Clinical manifestations

Food allergies cause different clinical conditions, can involve many organ systems, as gastrointestinal tract, the skin, the airways or anaphylaxis (the most dangerous of all allergic reaction). In severe cases, patients may present cardiovascular symptoms including hypotension, shock, cardiac dysrhythmias, and patients often report a "sense of doom" (Ebo and Stevens 2001).

4.2.1 Gastrointestinal symptoms

Gastrointestinal disorder may present different symptoms such as, nausea, abdominal pain due to colonic spasms, vomiting, gastric retention, intestinal hyper-motility, and diarrhea (Sampson, 1999). Symptoms usually develop within minutes to 2 h of the ingestion of the offending food. Food allergens causing gastrointestinal symptoms are generally pepsin-stable, and hence able to reach the gastrointestinal tract in an almost unmodified form or as (assembled) fragments with sufficient residual allergenicity. Immediate gastrointestinal hypersensitivity reactions are rarely isolated, and most often accompanied by allergic symptoms in other target organs (skin, nose, lungs and eyes) (Asero et al., 2007).

4.2.2 Skin disorders

Frequently, IgE-mediated food allergy involves the skin with immediate cutaneous symptoms as pruritus, urticaria, and angioedema or morbilliform rashes. Especially in adult patients, the most common skin disorder is acute urticaria, with or without angioedema (Sampson, 2003a). As IgE-reactive components from foods are rapidly absorbed, urticaria may appear within minutes of ingesting the offending food and may last for some hours. Contact urticaria is a rather common disorder associated with the handling of foods; in some instances, patients with contact urticaria have food allergy as well. The most common food involved in contact urticaria are fruits, fish, vegetable and also raw meats.

Food allergy can also appear as a chronic inflammatory skin disorder, known as atopic dermatitis (AD) which is common in children but rare in adults (Asero et al., 2007).

4.2.3 Oral allergy syndrome (OAS)

The OAS is a particular type of IgE mediated reaction, particularly frequent in adult with food allergy. The organs involved are lips, oral mucosa, and pharynx (Mari et al., 2005). Typically, the symptoms appeared after few minutes with local itching of lips, tongue, palate, throat, and/or ears and nose and/or swelling (angioedema) of the same areas. Only in some cases the clinical course is dramatic with a lethal swelling of pharynx or with anaphylactic reaction; in fact in the most cases the clinical course is mild and resolving within 1h (Sampson, 2005).Generally OAS is associated with sensitization to heat-labile/pepsin-labile plant-derived proteins in patients with pollen-related food allergy, due to the cross-reactivity between homologous plant-derived proteins in pollen and vegetable food. A wide majority of birch pollen-allergic patients after ingestion of kiwi, apple, nuts, celery and carrot may experience oral allergy, due to the homologous proteins in these food with Bet v 1 which is the major birch pollen allergen (Rev. in Asero, 2005). Moreover, the majority of patients with OAS can ingest offending foods after cooking or thermic process because most allergens involved in cross-reactivity reaction are easily destroyed by heat or after pepsin digestion (Asero et al., 2007).

4.2.4 Respiratory disorders

Patients with food allergy can show respiratory symptoms, after ingestion of the offending foods, as rhinoconjunctivitis and bronchospasm. These disorders are often associated with gastrointestinal and skin disorders but are rarely present as the only symptom (Sampson, 2003a). Bronchospasm or rhinoconjunctivitis following inhalation of food dusts or vapours are common in patients with food allergy and especially have been associated fish, crustaceans and legumes legumes and can be observed also with wheat flour or pasta cooking.

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4.2.5 Anaphylaxis

Anaphylaxis is the most severe allergic reaction, and occurs in 17% to 37% of allergic patients admitted to hospital emergency units (Moneret-Vautrin et al., 2004, Brown et al., 2001). Food anaphylaxis affects both sexes equally (Bock et al., 2001) and can cause the death (due to serious acute asthma) usually within 30 minutes in young adults or adolescents, as opposed to drug or venom anaphylaxis, which usually manifests as a cardiovascular collapse within a shorter period. The most implicated foods in anaphylaxis disease are peanuts, tree nuts, seafood and hard-shelled fruit (Sampson, 2003b). Other factors can influence anaphylaxis. Food associated, exercise-induced anaphylaxis occurs in susceptible individuals when they ingest the triggering food within 2–4 h of exercise (Wang and Sampson 2007). This is a particular case of anaphylaxis because symptoms do not occur in the absence of exercise or if the food is not ingested before exercise. Males are less affected that females, and the majority of cases occur in individual <30 years of age (Sampson, 2003b). The period between food intake and the clinical reaction, like the effort period, varies from 30 minutes to 3 hours. All foods can be involved, with a marked predominance of wheat flour (Kano et al., 2000). Some substances as alcohol, aspirin, β-blockers, angiotensin converting enzyme inhibitors, monoamine oxidase inhibitors, tricyclic antidepressants and concurrent infection are additional factors that may increase the severity of anaphylactic reactions or diminish the efficacy of epinephrine (Pumphrey, 2004).

4.3 In vivo tests in the diagnosis of food allergy

4.3.1 Skin prick test (SPT)

SPT is an easy, safe, cheap and rapid (15 min) test, frequently used to screen food specific IgE (Beyer and Teuber, 2005). The accuracy of this test depends on the quality of the food allergen extracts, in fact many commercially food extracts are not standardized nowadays, but the legislation evolves towards an accurate characterization of these extracts. Sampson and Ho (1997) showed that in children with AD and food allergy to egg, milk, peanut and fish, SPT with these foods have an excellent sensitivity and negative predictive accuracy (generally >90%), but poor specificity and positive predictive accuracy (50–85%). For many plant allergen commercial extracts, SPT test show a low sensitivity resulting in a high rate of false-negative results. This phenomenon is related to the low abundance or the lack of stability of several allergens to endogenous enzymatic processes taking place in plant food extracts. It's for this reason that *in vivo* tests with fruits and vegetables were performed with native foods by the prick-prick technique, which shows a superior performance (Rancé et al., 1997). In this test, the lancet is plunged several times into the food immediately before pricking patient's skin (Fig). This test shows some drawbacks as, the impossibility to standardize the allergen source, and its dependence on the availability of the fresh food in question.

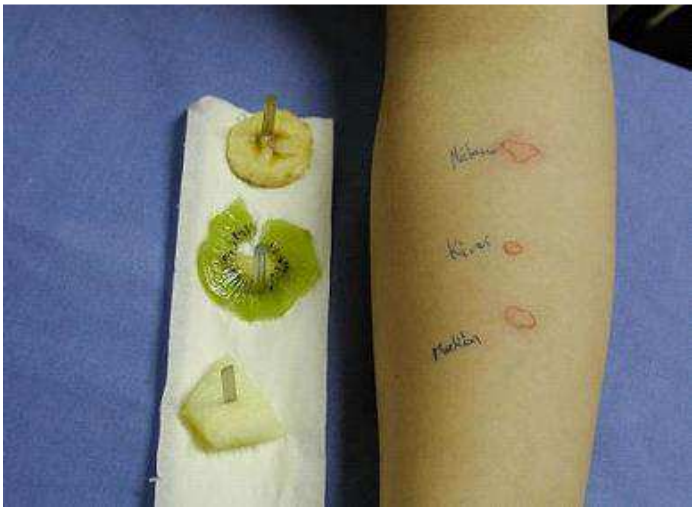

Fig. 21: SPT test with fresh food carried out by the prick–prick technique.

4.3.2 Atopy patch test (APT)

In the APT test, allergens extracts are applied epicutaneously and the induced eczematous skin lesions are evaluated 48 hours later. It is used to observe delayed reactions. While some people consider the APT a high specificity test, in particularly in combination with the measurement of specific serum IgE, and a useful tool in the diagnostic work-up of food allergy in infants and children with AD (Niggemann, 2002), in contrast, other people reported that the APT has a poor reliability and does not increase the diagnostic accuracy in food allergy (Osterballe et al., 2004). This different point of view might be explained by the fact that the APT is difficult to interpret, particularly by non-dermatologists, as non-specific reactions frequently occur (Asero et al., 2007).

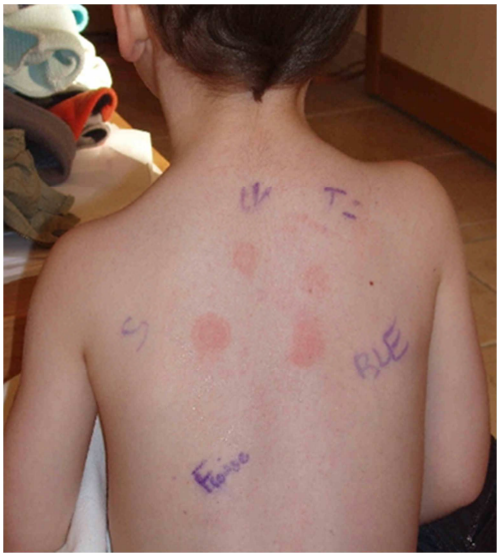

Fig. 22: Atopy patch test with allergens extracts applied on skin and reaction observed 48 h later.

4.3.3 Food challenges

The oral challenge is a diagnostic test which provides strong evidence of a food allergy, and allows the clinician to recommend a correct elimination diet. It is not necessary in patients with a history of severe immediate systemic reaction (anaphylaxis) after the ingestion of an isolated food to which specific IgE is demonstrated (Asero et al., 2007).

Oral food challenges can be performed with single-blind or double-blind (the gold standard), and blinded challenges are controlled by placebo. Nevertheless, DBPCFC (double-blind placebo-controlled food challenge) is expensive; time consuming (Asero et al., 2007; Guandalini and Newland 2011). This test is applied only in patients with permanents avoidance of foods essential to diet such as milk, eggs, wheat etc. The food to be tested is administered dried or lyophilized in opaque capsules, or alternatively in its natural form masked by an inert base. The placebo consists of a capsule of the same appearance containing dextrose or an inert base consisting of foods, which are tolerated by the patient and allow an adequate masking of the food to be administered (Ortolani and Pastorello 2006).

4.3.4 In vitro quantification of specific IgE

Radioallergosorbent Test (RAST) and Enzyme Linked Immunosorbent Assay (ELISA) were two in *vitro* test used to evaluate suspected IgE-mediated food allergy. At present, test as CAP system fluorescent enzyme immunoassay instead of the RAST radioallergosorbent test, are used because more sensitive in detecting low levels of allergen-specific IgE. In the CAP system the allergen is fixed on a solid support and incubated with the patient's serum and the binding between specific IgE and the allergen is revealed by anti-IgE fluorescent or enzymatic marker.

SPTs are generally favoured because they are highly reproducible and less costly in comparison to *in-vitro* tests but these latter may provide better quantitative results (i.e. exact values of specific IgE), and may therefore be more useful for monitoring specific IgE levels over time (Rev. in Gerez et al., 2010).

The micro array technology (ISAC) is a component-resolved diagnosis allowing the analysis of about 100 allergens. It has still some limitations at the moment, such as the poor performance of some allergens (wheat) or absence of some allergen sources; however, in a near future, it could be applied to epidemiologic studies and be helpful in the search of cross-reactions (Rev. in. Moneret-Vautrin et al., 2011).

4.4 Cross-reactivity: Cross-allergy

Allergen cross-reactivity is the phenomenon that occurs when IgE antibodies originally directed at the epitopes of one allergenic source recognize similar structures in another allergenic source (Ortolani and Pastorello 2006); this phenomenon may be *in vitro* or *in vivo* caused by chemical or functional homology. To date, 160 allergens originating from 56 plant foods are listed by the IUIS (International Union of Immunological Societies) in an allergen database (http://www.allergen.org). Jenkins et al. (2005) showed that 65% of plant food allergens belong to only four structural families, the cereal prolamin superfamily, the cupins, the Bet-v 1 homologues and the profilins. This could lead to extensive IgE cross-reactivity even among allergens belonging to taxonomically unrelated plants. Additionally, cross-reactive IgE binding can be retained by up to 35–40% of the amino acid sequence if the conformational structure is preserved. However, sometimes, IgE cross-reactivity can give rise to many false positive results in diagnostic tests. Therefore it is important to have lists of foods which are cross-reactive with a given pollen or, even worse, foods containing one of the so-called ‘panallergens’(Ortolani and Pastorello 2006).

Tab. 5: Clusters of cross-immunity/cross-allergy (Taken from Ebo and Stevens 2001)

ALLERGEN	***SOURCE***
Defense proteins	
Bet v 1 homologues (protease / PRP class 10)	Pollen : alder, birch, hazelnut Food : apple, almond, carrot, cherry, celery, hazelnut, kiwi, peach, pear, plum, potato
Art v 1 homologues (Pectate lyase)	Pollen : birch, Japanese cedar, mugwort, ragweed Food : anise, apple, carrot, celery, dill, fennel, parsley, tomato
Thaumatin – like proteins (Permeatins, osmotins / PRP 5)	Apple, bell peppers, cherry, wheat, oat, tomato
Hevein – like domain (class I chitinases / PRP 3)	Natural Rubber Latex (Hevea brasiliensis) Food : avocado, banana, chestnut
Lipid transfer proteins (PRP 14)	Apple, apricot, broccoli, carrot, celery, cereals, kiwi, melon, peach, peanut, pistachio, tomato, walnut
α-amylase inhibitors	Barley, Indian millet, maize, rice, wheat
Lysozymes (α-lactalbumine)	Cow's milk, egg white, meat (beef)
Seed storage proteins	
2S albumin	Mustard, nuts, peanuts
Vicilins	Cacao, cotton seed, nuts, peanuts, soybean
Muscle proteins	
Parvalbumin	Amphibians, carp, codfish, eel, perch, salmon, tuna
Tropomyosin	crab, crayfish, insects, lobster, mites, molluscs, shrimps, snails, cockroach
Miscellaneous	
Albumin (Serum protein)	Egg yolk, sera, meat, feathers, epithelia (cat)
Profilins (Actin binding cytoskeleton protein)	Pollen : birch, mugwort, ragweed Food : apple, bell peppers (paprika), carrot, celery, coriander, fennel, peanut, potato, tomato

5 Wheat allergy

Wheat is an important protein source for the human diet, but unfortunately belongs to the six major food allergens (together with milk, egg, peanut, soybean, and fish). Wheat proteins, ingested or inhalated in the form of raw flour or cooked products can be responsible of adverse reactions. These adverse reactions to wheat can have a broad spectrum of clinical manifestations due to the different affected pathways as: wheat gluten enteropathy (coeliac disease), respiratory allergy to wheat flour, and wheat food allergy (Battais et al., 2008). Hydrolyzed wheat proteins (HWP) can also be involved in sensitization by contactand. This is rare, but can induce severe allergic reaction (Laurière et al., 2006).

5.1 Hypersensitive reactions to wheat

5.1.1 Baker's asthma

Baker's asthma is one of the most common forms of occupational asthma and its affects between 4 and 10% of bakery workers in Europe. In particular in France is the first type of occupational asthma (Ameille et al., 2003) and the second in UK (McDonald et al., 2000). Several studies showed a strong association between flour dust exposure and sensitization to wheat allergens, but no evidence was found for the existence of an exposure threshold for wheat sensitization of work-related symptoms (Cullinan et al., 2001; Peretz et al., 2005). In a recent study, Jacobs et al. (2008) showed that the prevalence of wheat sensitization, work-related respiratory symptoms and asthma increased till average wheat exposure levels of approximately 25–30 µg/m3, leveled off and decreased at higher exposure concentrations. Moreover the authors confirmed the exposure–response relationship especially for cumulative wheat allergens exposure with sensitization, allergic respiratory symptoms and asthma.

The baker's asthma diagnosis can be performed with SPT, and skin reactivity is related to the quality, potency, and standardization of allergen extracts, which are often poorly defined for cereal and other occupational allergens (Rev. in Salcedo et al., 2011).

Wheat and rye extracts for SPT from 3 companies were compared by Sander et al. (2004), which showed that different protein concentration caused different SPT results.
The authors compared also the sensitivity of specific IgE measurements (by either IgE-Enzyme Allergosorbent test or Phadia CAP-system) respect to SPT, and showed that Phadia CAP-system sensibility was higher than SPT with commercial cereal (wheat and rye) extracts (Sander et al., 2004). Anhoter test used for diagnosis of baker's asthma is SIC (Specific Inhalation Challenge), which is considered the gold standard (Rev in Salcedo et al., 2011). Allergen-specific immunotherapy (SIT) and other immunomodulatory treatments, such as anti-IgE monoclonal antibodies play an important role in treatment of baker's asthma. The efficacy of wheat flour SIT in baker's asthma was show by Swaminatha and Heddle (2007). Cirla et al. (2007) performed a study on 41 sensitized bakers with underwent subcutaneous SIT with wheat flour extract for 4 or more years, without having to stop work. Thirtyfour subjects out of 41 had an acceptable quality of life and were able to work normally (Cirla et al., 2007).

5.1.2 Food allergy to wheat

Food allergy affects about 6% of young children and 3-4% of adults suffering from food allergy (Sicherer and Sampson 2006). The prevalence of food allergy to wheat in the general population has been estimated for children by oral challenge tests in the range of 0.2-0.5% of children (Zuidmeer et al., 2008). In adults, the sensitization to wheat (presence of wheat specific IgE in the serum) was estimated between 0.4 and 3.6 % of the general population (Zuidmeer et al., 2008)
The diseases caused by wheat allergy are the same as those of other food allergy. Children and adults show different symptoms, in particular children show mainly atopic dermatitis (AD), sometimes associated with respiratory symptoms and digestive problems, whereas adults show anaphylactic shock, angioedema, irritable bowel syndrome (Rasanen et al., 1994; Sicherer et al., 2000).

5.1.3 Coeliac disease

The gluten fraction of wheat, along with barley and rye are responsible for coeliac disease (Sollid, 2002; Mowat, 2003). Coeliac disease affects ~1% of the population in Europe, North and South America, North Africa and the Indian subcontinent (Hischenhuber et al., 2006). While some studies suggested that patients suffering from coeliac disease tolerate oat (Janatuinen et al., 1995, 2002; Srinivasan et al., 1996; Lundin et al., 2003) this cereal remains in the list gluten-containing cereals.

Coeliac disease (CD) is a genetically chronic inflammatory intestinal disorder (non IgE mediated – T-cell mediated), which is more frequent in women than man. Generally, CD appears with diarrhea, loose stools, vomiting, general weakness, a distended abdomen.

In coeliac patients, the CD4+T l lymphocytes react to gluten peptides presented by antigen-presenting cells in the context of class 2 histocompatibility molecules. The recognition of these peptides allows the activation and release of inflammatory cytokines characterized by a Th1 pattern (Nilsen et al., 1998; Salvati et al., 2002). Celiac disease is also considered an auto-immune disease, because auto-antibodies directed against the enzyme tissue transglutaminase (TG2) are found in patients. Seemingly, TG2 increases the affinity between the hydrolysed gliadin peptides and human histocompatibility leucocyte antigen (HLA) class 2 DQ2 or DQ8 molecules on antigen-presenting cells, by a process of selective deamidation of these peptides (Hischenhuber et al., 2006).

The gliadins fraction, in particular α-gliadins are the most important polypeptides involved in CD, but several T cell stimulatory peptides from γ-gliadin, and glutenins were identified using mass spectrometry analysis or by screening large peptide libraries (Tollefsen et al., 2006). Camarca et al. (2009) found that intestinal T cell lines were frequently and strongly stimulated by the ω-gliadin-derived peptide, DQ2- ω-1 (QPQQPFPQPQQPFPWQP). The results obtained by Camarca et al. (2009) showed that there is a substantial heterogeneity in intestinal T cell responses to gluten and highlighted the relevance of γ- and ω-gliadin peptides for CD pathogenesis. Moreover the authors showed α-gliadin, γ-gliadin and ω-gliadin were the most active gluten peptides in $DQ2^{+}$ celiac patients (Camarca et al., 2009).

5.2 Wheat allergens

A“food allergen”is a protein or chemical hapten that is recognized by allergen-specific immune cells and elicits specific immunologic reactions (mediated by IgE) resulting in characteristic symptoms (Boyce et al., 2010).

Due to the diversity and variability of the human IgE response, all of the allergenic proteins are not always recognised by all individuals allergic to this food. Those allergens that are recognised by more than 50% of a population of individuals allergic to the food are called major allergens. This concept relates only to the frequency of recognition by IgE antibodies, and it is not related to the severity of the clinical manifestations of an allergic reaction. Major allergens may constitute a small proportion of the total protein content of the food concerned (EFSA, 2010).

A systematic nomenclature for allergenic proteins has been developped by The WHO/IUIS Allergen Nomenclature Sub-committee. This allergen database (http://www.allergen.org) contains approved and officially recognized allergens Table 6 is taken from this website and includes all recognized wheat allergens.

Table 6: List of recognized wheat allergens by the IUIS Allergen Nomenclature Sub-Committee

Allergen	Biochemical name	MW(SDS-PAGE)	Food Allergen	Entry Date	Modified Date
Tri a 12	Profilin	14	Yes	2010-04-29 16:57:55	0000-00-00 00:00:00
Tri a 14	Non-specific lipid transfer protein 1	9	Yes	2010-04-29 16:57:55	0000-00-00 00:00:00
Tri a 15	Monomeric alpha-amylase inhibitor 0.28		No	2011-04-07 07:45:43	2011-04-07 07:45:43
Tri a 18	Agglutinin isolectin 1		Yes	2010-04-29 16:57:55	0000-00-00 00:00:00
Tri a 19	Omega-5 gliadin, seed storage protein	65	Yes	2010-04-29 16:57:55	0000-00-00 00:00:00
Tri a 21	Alpha-beta-gliadin		No	2011-04-07 07:52:47	2011-04-07 07:52:47
Tri a 25	Thioredoxin		Yes	2010-04-29 16:57:55	2011-04-07 08:58:48
Tri a 26	High molecular weight glutenin	88	Yes	2010-04-29 16:57:55	2011-05-25 09:19:54
Tri a 27	Thiol reductase homologue	27	No	2010-04-29	0000-00-00

				16:57:55	00:00:00
Tri a 28	Dimeric alpha-amylase inhibitor 0.19	13	No	2010-04-29 16:57:55	0000-00-00 00:00:00
Tri a 29	Tetrameric alpha-amylase inhibitor CM1/CM2	13	No	2010-04-29 16:57:55	0000-00-00 00:00:00
Tri a 30	Tetrameric alpha-amylase inhibitor CM3	16	No	2010-04-29 16:57:55	0000-00-00 00:00:00
Tri a 31	Triosephosphate-isomerase		No	2011-04-07 08:02:08	2011-04-07 08:02:08
Tri a 32	1-cys-peroxiredoxin		No	2011-04-07 08:08:50	2011-04-07 08:08:50
Tri a 33	Serpin		No	2011-04-07 08:12:13	2011-04-07 08:12:13
Tri a 34	Glyceraldehyde-3-phosphate-dehydrogenase		No	2011-04-07 08:48:03	2011-04-07 08:48:03
Tri a 35	Dehydrin		No	2011-04-07 08:54:18	2011-04-07 08:54:18
Tri a 36	Low molecular weight glutenin GluB3-23	40 kDa	Yes	2011-05-23 08:56:36	2011-05-23 08:56:36
Tri a 37	Alpha purothionin	12 kDa	Yes	2011-11-08 03:46:02	2011-11-08 03:46:02

5.2.1 Storage proteins

Several studies showed that the gluten fraction was involved in adverse reactions to wheat (Battais et al., 2006; Palosuo, 2003; Pastorello et al., 2007, Mittag et al., 2004), in particular IgE-binding against gliadins and LMW-GS were found in adults and children with food allergy to wheat (Battais et al 2003). α- and ω-5 gliadins were also found as minor allergens in Baker's asthma (Sandiford et al., 1997). Battais et al. (2005) carried out a detailed study with 60 patients, which showed that different allergenic profiles could be detected in wheat food allergy. In fact, for the children with AD the major allergens were some water-salt soluble proteins and α/β- and γ-gliadins were also involved. ω-5 gliadins were the major allergens for the 100% of adults with wheat-dependent exercise-induced anaphylaxis (WDEIA), and/or with anaphylaxis, and for the 55% for those with urticaria. A precedent study of Morita et al. (2003) showed that ω-5 gliadins (Tri a 19) was the major allergen in WDEIA. While B-type LMW-GS was considered important allergens in adults (Pastorello et al., 2007; Battais et al., 2005) and children (Akagawa et al., 2007) with anaphylaxis, the HMW-GS were considered minor allergens.

5.2.1.1 Salt Soluble proteins

In addition to gluten proteins, several salt-soluble wheat proteins were also identified as allergenic proteins, among these: fructose-biphosphate aldolase, serine protease inhibitor (serpin), α-amylase inhibitor, LTP, acyl-coenzyme A oxidase, and wheat flour peroxidase (Sánchez-Monge et al., 1997; Weiss et al., 1997; Sander et al., 2011).

The most important of these salt-soluble allergens involved in Baker's asthma was α-amylase inhibitors. Letho et al. (2010) also showed that Thaumatin-like protein was allergen in Baker's asthma. Another important wheat allergen is nsLTP, which was involved in food allergy (Pastorello et al., 2007; Battais et al., 2005) but also in Baker's asthma (Palacin et al., 2009).

Pastorello et al. (2007) also identified other IgE-binding proteins from patients with food allergy to wheat. Several of these (wheat germ agglutinin, peroxidase, serpin, β-amylase, thioredoxin *h* B) were reported as allergens in Bakers' asthma while others (globulin, β-purothionin, puroindolines a and b, tritin, granule-bound starch synthase) have not so far been identified in other studies; Akagawa et al. (2007) found also serpins, whereas β-D-glucan exohydrolase was found by Šotkovský et al. (2008) in a proteomic analysis with sera from patients with food allergy.

This diversity could be explained by differences in populations or in the different approaches used to identify the IgE-binding proteins (Tatham and Shewry 2008).

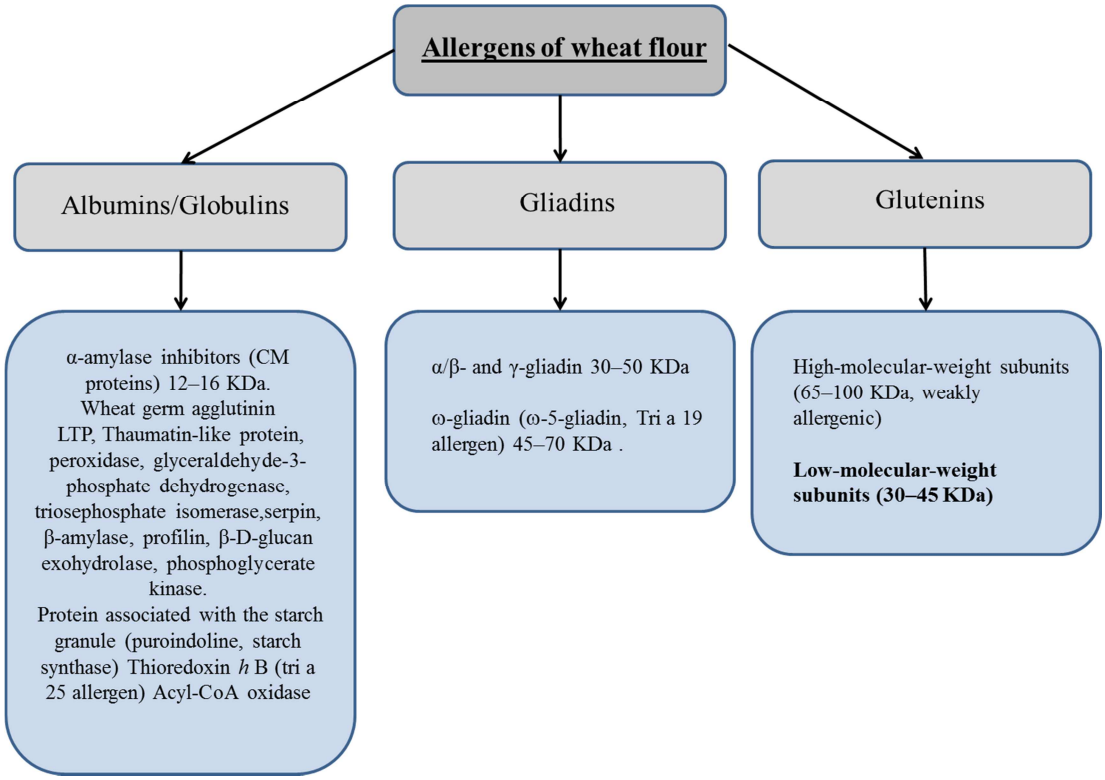

Fig. 23: Classification of the different groups of allergenic proteins identified in wheat flour. Taken from Mamone et al., 2011.

5.3 Examples of wheat allergens of the salt soluble proteins

5.3.1 LTP- lipid transfer protein

Plant non specific LTPs have anti-microbial properties and in the absence of immune system, play a role in the defence mechanism of plant against their pathogens. These proteins were found initially in wheat seed and lateralso in barley, rye and oats (Douliez et al., 2000), and were isolated from soluble fraction where they represent approximately 5-10% of the total soluble proteins (Kader, 1996). The family of nsLTPs is ubiquitous in plant and includes monomeric proteins that are stabilized together by four disulfide bonds to form a hydrophobic tunnel (Breiteneder and Radauer 2004). Two main families have been isolated: nsLTP1 and nsLTP2 with a molecular mass of 9 and 7 KDa respectively.

Even if, both families are characterized by pI around 9 and a conserved pattern of 8 cysteine residues involved in disulphide bonds, they exhibit low overall amino acid

sequence similarity (30%). The two families also share a common structural architecture of a hydrophobic cavity enclosed by four α-helices, held in a compact fold by four disulfide bonds (Yeats and Jocelyn 2008). No Tryptophan residues are found in the sequences of LTP and phenylalanine is rare (Douliez et al., 2000).

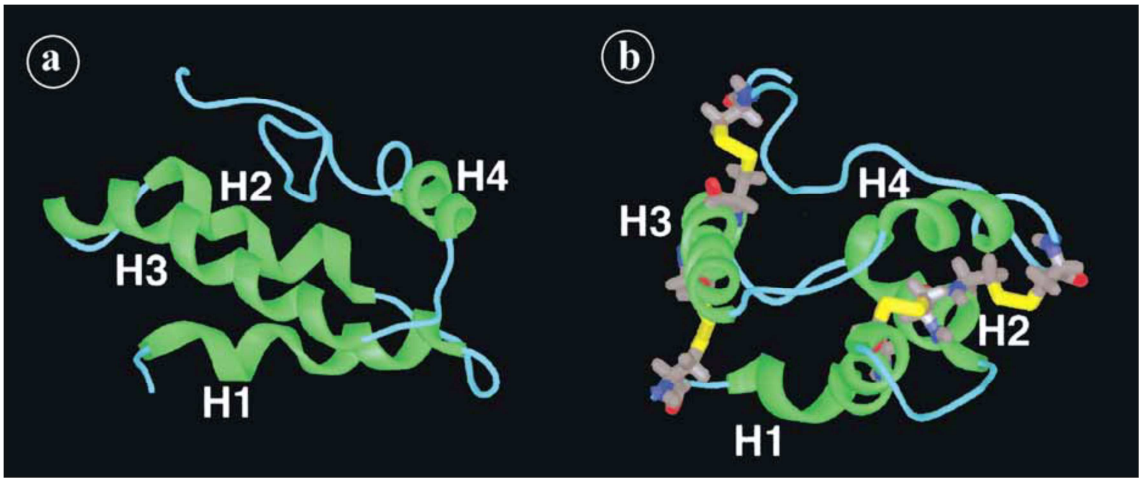

Fig 24: Structure of the wheat nsLTP1 showing the helix bundle (a) and disulphide bonds (b). Taken from Douliez et al., 2000.

Data obtained by NMR (Simorre et al., 1991; Gincel et al., 1994; Poznanski et al., 1999) and X-ray crystallography (Lee et al., 1998; Charvolin et al., 1999) from wheat and maize showed that LTP1 is characterized by a 4 α-helix bundle surrounded in part by a C-terminus formed by turns that confer a saxophone shape. This fold forms a large internal cavity which is partially covered by the C-terminal region (Lee et al., 1998).

By comparing structure of the various LTP1, the size of this cavity can vary between different isoform of LTP1; this confers also different ability to lipid binding and transfer.

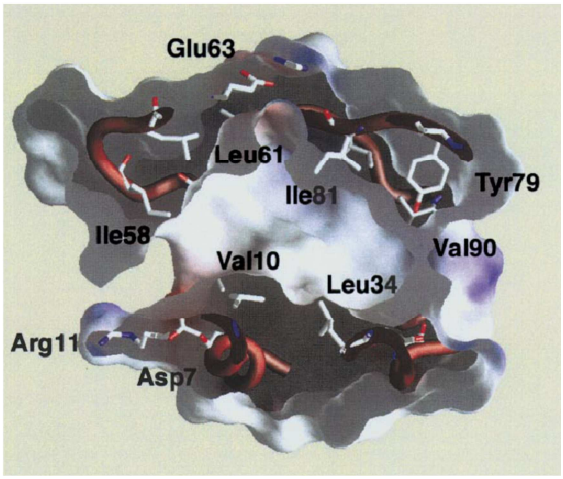

Fig. 25: Molecular model of LTP1 internal cavity

The plant LTPs also show large sequences homology (Fig. 21), whereas less homology was found between plant LTPs and mammalian LTPs. Other phylogenetic and homology studies revealed that these LTPs belong to the plant prolamins superfamily (Kreis et al., 1985; Shewry et al., 2002). The superfamily existence was proposed by Kreis et al (1985) by comparison of amino acids sequences which show a preserved cysteine skeleton with eight cysteine residues spaced out in the following way:

$$- C\text{-}X_n\text{-}C\text{-}X_n\text{-}CC\text{-}X_n\text{-}CXC\text{-}X_n\text{-}C\text{-}X_n\text{-}C$$

Non-specific LTPs have been identified as allergens in a range of fruits, principally those of the Rosaceae family, vegetables and even nuts. In wheat, nsLTP (Tri a 14) was described as an important food allergen (Battais et al., 2005; Pastorello et al., 2007) and as a allergen associated with baker's asthma in Spain (Palacin et al., 2009). A limited cross-reactivity of LTP Tri a 14 with the peach LTP Pru p 3 was found (Tordesillas et al., 2009, Palacin et al., 2007) however, the 3-dimensional modeling of both LTP proteins reveals differences in the regions mapped as IgE-binding epitopes of Pru p 3 and Tri a 14 .

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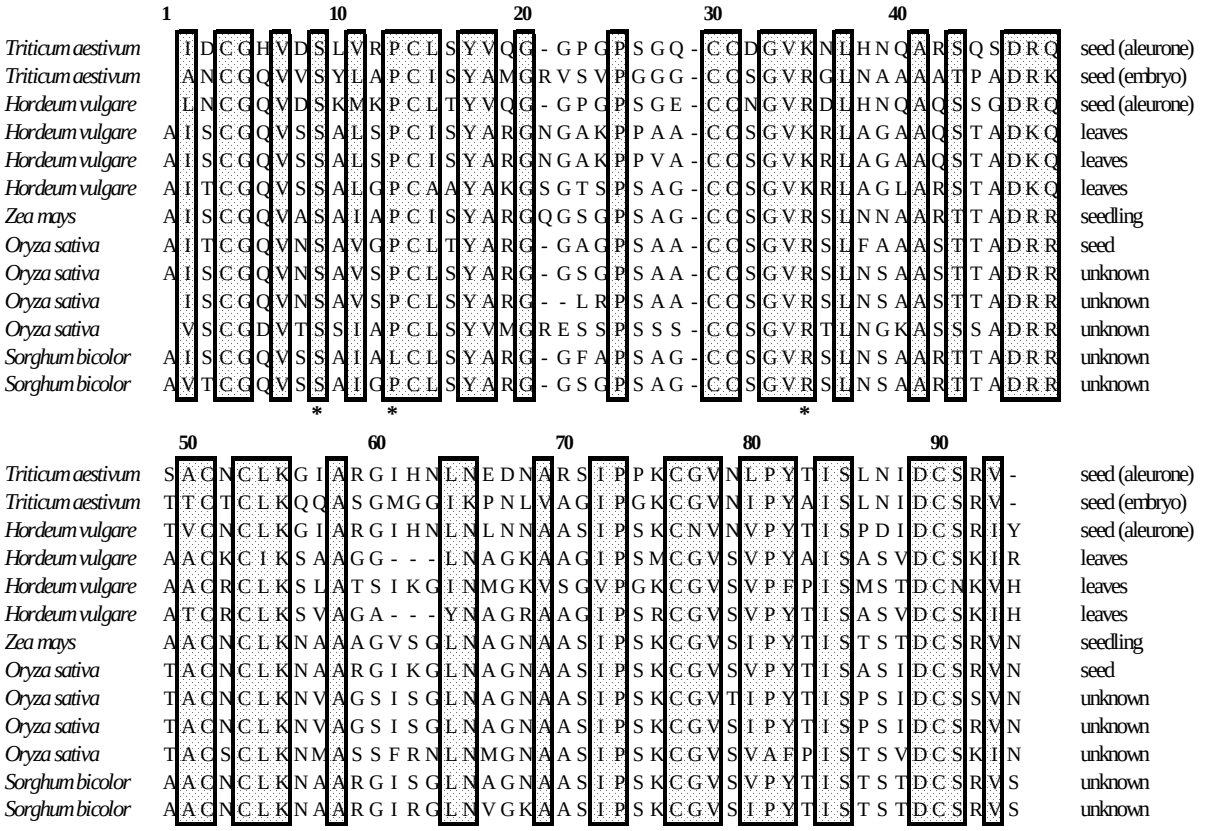

Fig. 26: Sequences alignment of cereal LTP1 proteins. Taken from Douliez et al., 2000

5.4 α-amylase /trypsin inhibitors

Severe crop losses worldwide are caused by insect pests that feed on plant tissues. For the defense, the plants have evolved different mechanisms of protection, through the production of defense compounds and proteins, including α-amylase inhibitors (Franco et al., 2002). The family of α-amylase includes enzymes that hydrolyze α-D-(1,4)-glucan linkages and play an important role in the carbohydrate metabolism of many organisms which use α-amylase primarily to digest starch in their food sources (MacGregor et al., 2001). Several kinds of α-amylase and proteinase inhibitors in seeds and vegetative organs act to regulate the numbers of phytophagous insects (Wang et al., 2008). These α-amylase inhibitors that can also possess proteinase inhibitory activities are attractive candidates for the control of seed weevils because of interferes with the digestion of plant starches and proteins by impeding insect gut enzymes (Breiteneder and Radauer 2004). This family of enzymes is found in wheat, barley, rice and corn (Garcìa-Olmedo et al., 1992). In cereal seeds, trypsin inhibitor, as well α-amylase

inhibitor, can be grouped into one large family on the basis of the homology between their amino acid sequences.

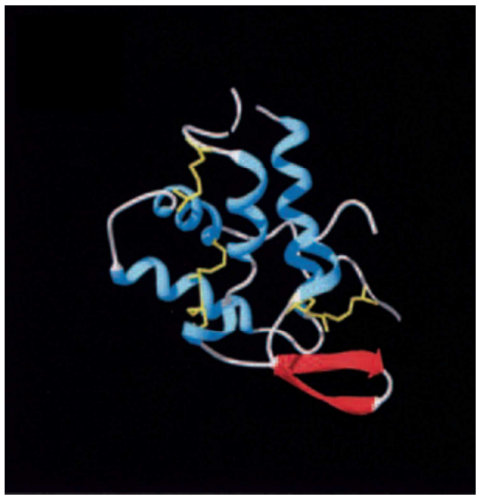

Fig. 27: Wheat α-amylase inhibitor (1HSS). Blue, a-helices; red, b-strands; yellow, disulfide bonds. Taken from Breiteneder and Radauer (2004).

They have subunits of approximately 120 to 160 amino acid residues, contain 4 disulfide bonds (Fig) and exist as monomers, dimers, or tetramers (James et al., 1997).
In wheat, a few α-amylase-inhibitor are found in the albumins and globulins fraction. These proteins are most likely phylogenetically related and are encoded by a multigene family which is dispersed over several chromosomes; in particular they have been assigned to the short and long arms of the chromosomes 3, 4, 6 and 7 of B and D genome (Carbonero and Garcìa-Olmedo 1999; Islam et al., 2003).
The α-amylase/trypsin inhibitor family has been shown to be the main culprit of baker's asthma. Allergenic members of this family are capable of sensitizing susceptible atopic patients through inhalation but also ingestion, they are therefore also involved in food allergy. Allergens within the cereal superfamily of inhibitors include many inhibitor subunits (WDAI-2, WTAI-CM1, WTAI-CM2, WTAI-CM3, WTAI-CM16), the homologous barley allergens CMb*, Hor v 15 (Hor v 1/BMAI-1), and barley dimeric protein; Sec c 1 from rye flour; and the rice dimeric a-amylase inhibitors RDAI-1 and RDAI-3 (Breiteneder and Radauer 2004). The subunits of the tetrameric CM16* inhibitor from wheat were described to exhibit glycosylated forms. Despite some authors (Garcìa-Casado et al., 1996) reported a prominent role of the attached N-

complex glycans in the allergenic potency of CM16, their capacity to potentiate the allergenicity of the inhibitor subunits is still uncertain.

5.5 β-amylase

β-amylases are found in the major cereal crops and exist in two distinct forms which differ in their expression patterns: one form is specific to the endosperm, while the other has a tissue-ubiquitous pattern of expression (Ziegler, 1999).

The β-amylases are water soluble enzymes (classified as albumins) with high molecular weight (~ 60KDa) in reduction condition, which are encoded at the β-*Amy-1* loci on the chromosome arms 4DL, 4AL, and 5AL. These proteins were rapidly degraded during seed germination like the storage proteins of wheat. They were found not to be present in the protein bodies from developing wheat endosperm, however (Gupta et al., 1991).

An insoluble complex between β-amylases and glutenins was observed (Rothfus et al., 1970) in fact disulfide bonds were found between β-amylases and LMW-GS (Peruffo et al., 1996). Moreover, Curioni et al. (1996) have shown a correlation between the size of glutenin macropolymers and the β-amylases quantity.

Using RAST analysis, Sandiford et al. (1994) revealed the allergenicity of barley beta-amylase in baker's asthma and also concluded as to their low cross-reactivity with fungal alpha amylase classically used in the baking industry. Despite beta amylases from wheat have been identified as potential allergens using food allergenomic approaches (Pastorello et al 2007, Šotkovský et al 2008, Larré et al 2011) and despites their homology with other cereal beta-amylases, no direct evidence of their capacity in eliciciting allergies was given.

5.6 *Ser*ine *p*rotease *in*hibitors

*Ser*ine *p*rotease *in*hibitors, also known as serpins, are member of superfamily most of which have protolitic activity. Generally, they show a molecular weight range between 40 and 50 KDa. Serpins are ubiquitous in the plant kindom and have members in the animal kindom; over 2000 serpins were found in various species (Rawlings et al., 2010). More particularly; Hejgaard and Roberts (2007) have identified these proteins in *Hordeum vulgare* L., *Secale cereale* L., *Avena sativa* L. and *Triticum aestivum* L. They are abundant salt-soluble proteins and represent up to 4% of the total protein in the mature endosperme of cereal grain (Østergaard et al., 2000). Seven serpin isoforms were recently identified by Wu et al. (2012) their study reveals five unique expression patterns of these serpin among 196 varieties. Even if their physiological role remains unclear, their activity suggests their role in the inhibition of endogenous proteins (Rev. in Roberts and Hejgaard 2008). Moreover, they showed an amino acid sequence motifs in their structure similar to the prolamins Gln-rich repeat sequences and also they form complexes by intermolecular disulfide bridges between serpins and between serpins and β-amylase protein (Rev. in Roberts and Hejgaard 2008). Several studies have shown their involvement as minor allergens in baker's asthma (Sander et al., 2011) and food allergy (Maméri et al., personal communication), mainly by proteomics (Sander, 2001; Pastorello et al 2007; Akagawa et al 2007; Šotkovský, 2008, 2011; Larré et al., 2011) and recently with ImmunoCAPs (Sander et al 2011).

5.7 Effects of processing on allergenicity

Wheat and other cereals are processed (for example through cooking) before human consumption. These processes impact the food components by inducing chemical and physical modifications. These modifications occur at a molecular level and can have an effect on allergenicity of the proteins by inducing modifications on the epitopes (the portion continuous or discontinuous of an antigen capable of eliciting an immune response) which are generally composed of 8 to 12 aminoacids. Thermal treatments may result in a number of modifications including denaturation, hydrolysis of peptide bonds, aggregation by non-covalent and disulphide bonding; reaction with other food molecules, such as sugars lipids and carbohydrates can also occur. Biochemical processes, including a range of enzyme-mediated reactions including proteolysis, oxidation or reactions with transglutaminases (Tatham and Shewry 2008) can also be part of the process, these one are generally deliberate

Sutton et al. (1982) showed that heating decreased the allergenicity of gluten extract; Varjonen et al. (1996) also showed that albumins/globulins, gliadins, hordeins and secalins of wheat, barley and rye flours, had a reduced IgE-binding capacity after heating. In opposition Simonato et al. (2001) showed that the allergenicity of wheat prolamins was increased by cooking. In particular the authors showed a reduced ability of enzymes to digest allergenic epitopes in vitro in bread, in comparison with dough and suggested that baking increases the resistance of allergens to digestion in vivo, allowing them to reach the small intestine and elicit allergenic responses (Simonato et al., 2001).

A reduced digestibility of pasta cooked at very high temperatures was also observed by Petitot et al., (2009) probably due to the formation of highly-aggregated proteins linked by very strong covalent bonds.

6 "Allergenomics"

The term "allergenomics" was proposed as particular application of proteomics technique to identify food allergens (Yagami et al., 2004, González-Buitrago et al., 2007). In allergenomics the first step isextraction and solubilization of proteins from allergenic source followed from separation by two dimensional electrophoresis (2-DE), which was the critical point. Hundreds of proteins are separated according to charge (first dimension) by IEF and size, typically by second dimension SDS-PAGE gel. After 2DE the proteins are transferred to a membrane but, due to the heterogeneous nature of the proteins the complete transfer is not possible.

The second step is the detection of IgE-binding antigens by an immunodetection involving incubation in presence of allergy serum followed by a labelled anti-IgE antibody. In the last step, the IgE-binding polypeptides is cut out from the gels, manually or by robotic spot-picker and hydrolyzed by enzymatic digestion. Finally the proteins are identified by MS analysis of the fragmented peptides and database research, even if only ideally the identification for one spot will correspond to an individual protein. After identification and isolation, the allergen may be used for immunological studies to establish its physiological effects, and physicochemical approaches as spectroscopic technique and NMR, may be used to define the structure and conformation of the allergen (Rev. in De Angelis et al., 2010). The food allergens characterization is a first step that can help to identified epitopes involved in sensitization and to explain the cross reactions (Sanchez-Monge et al., 2005). This approach can be used to identify allergens in traditional crops, in genetically modified plants or also in novel foods; moreover can also be useful for breeding or biotechnology based approaches aimed at silencing allergen expression.

Numerous constituents of the A/G fraction have been reported as IgE-binding proteins following allergonomics approaches in both baker's asthma and food allergy to wheat (Akagawa et al., 2007; Larré et al 2011; Sander et al., 2001; Šotkovský et al., 2008; Pastorello et al., 2007)

Larré et al. (2011) used allergonomics approach in order to compare the allergenicity of an ancient diploid wheat cv Engrain Pays de Sault (genome AA) with a hexaploid wheat cv Récital (genome AABBDD). Some allegens of A/G fraction were identified in both genotypes and interestingly, different IgE responses between diploid and hexaploid genotypes were obtained for α-amylase inhibitors, which were not identified in Engrain

Pays de Sault. This result suggested that these proteins were expressed at very low levels in the diploid genotype. This could be a hypothesis to explain the global lower reactivity of sera towards Engrain Pays de Sault against A/G in ELISA (Larré et al., 2011).

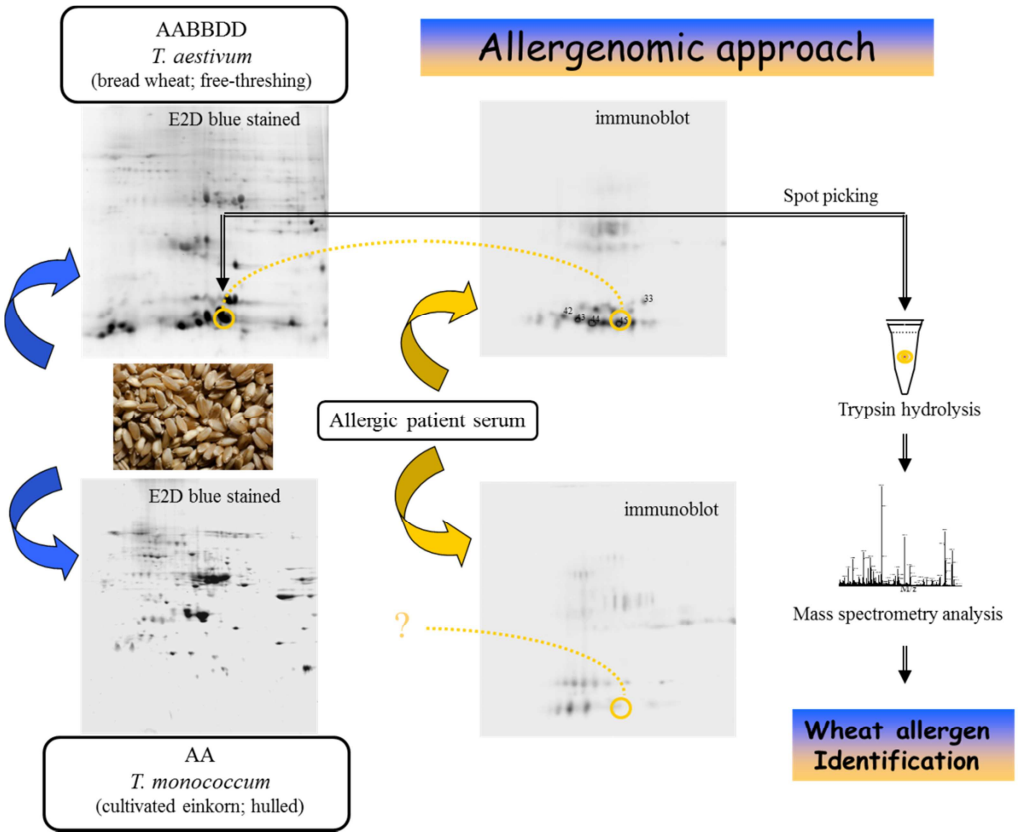

Fig 28: Schematic representation of "allergenomics" approach, which combines the protein separation by 2D electrophoresis with IgE immunorecognition by immunoblotting. Allergens are immunolabelled with serum of allergic patients. In this strategy, MS, supported by bioinformatic and database analyses, has the pivotal task for protein identification. Taken from Larré et al., 2011.

7 Substantial equivalence of Genetically Modified Plants and Natural Variation

A considerable debate derived from the GM plants safety assessment, started for the first time at the Asilomar Conferencein 1975 (in Vennaria et al., 2008) and continued up to present (Goldman, 2000; Hodgson, 2001) with particular attention for allergenicity of these (Kimber and Dearman, 2002, Batista et al., 2007). However, it is important to consider that humans were exposed to many thousands of proteins of which only a small proportion of these were allergens (Kimber and Dearman, 2002).

Also FAO and WHO recommends assessment of foods derived from GM plants by *in vivo* and *in vitro* methods before products commercializing (Poulsen et al., 2004). The GM plants allergenicity can be caused because a protein known as allergenic was introduced, because there was a change in the level of intrinsic allergens or because the product of a gene introduced may have the capacity to induce de novo sensitization in susceptible subjects. The principle applied in the case of the GM plants and their derived foods was that of "substantial equivalence" according to OECD (2006), and WHO (2005) guidelines which the GM plant food or feed was compared with parental or isogenic line (within naturally occurring variability) in order to establish possible difference and to evaluate the safety for humans and animals. The question was born from the possibility that the acquisition of transgene lead to unintended effects as change in metabolic pathways, alteration in nutritional value, potential toxicity, antibiotic resistance, potential allergenicity and carcinogenicity (Ricroch et al., 2011).

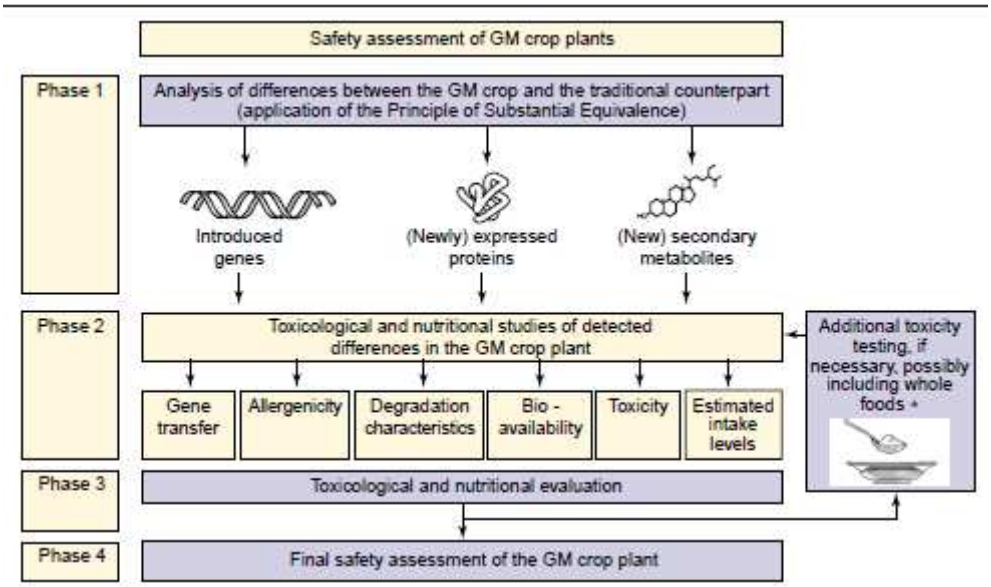

Fig. 29: Safety assessment strategies for genetically modified (GM)-crop-derived foods. Taken from Kok and Kuiper 2003.

In order to investigate safety assessment of genetic tranformant wheats, Shewry et al. (2007) compared GM wheats with conventional counterparts under both field and glasshouse conditions, over four years, and on two sites with contrasting climates. Genomic, trascriptomic, proteomic, metabolomic, and also functional properties were considered in the study (Fig. 29). The authors performed several measurements on the grain as dry weight, nitrogen content, and proteic composition by SDS-PAGE, dough mixing properties by Mixograph and metabolite profiles. The results obtained by Shewry et al. (2007) showed that the GM line and control line have the agronomic performance and grain functional properties similar. Moreover, gene expression profiles between transgenic lines and parental genotype are more similar to those of the parental lines than are the profiles of lines produced by conventional plant breeding. The variation observed on metabolite profiles between GM wheats and parental lines are within the range of variation which is observed between genotypes grown under different environmental conditions (Shewry et al., 2007).

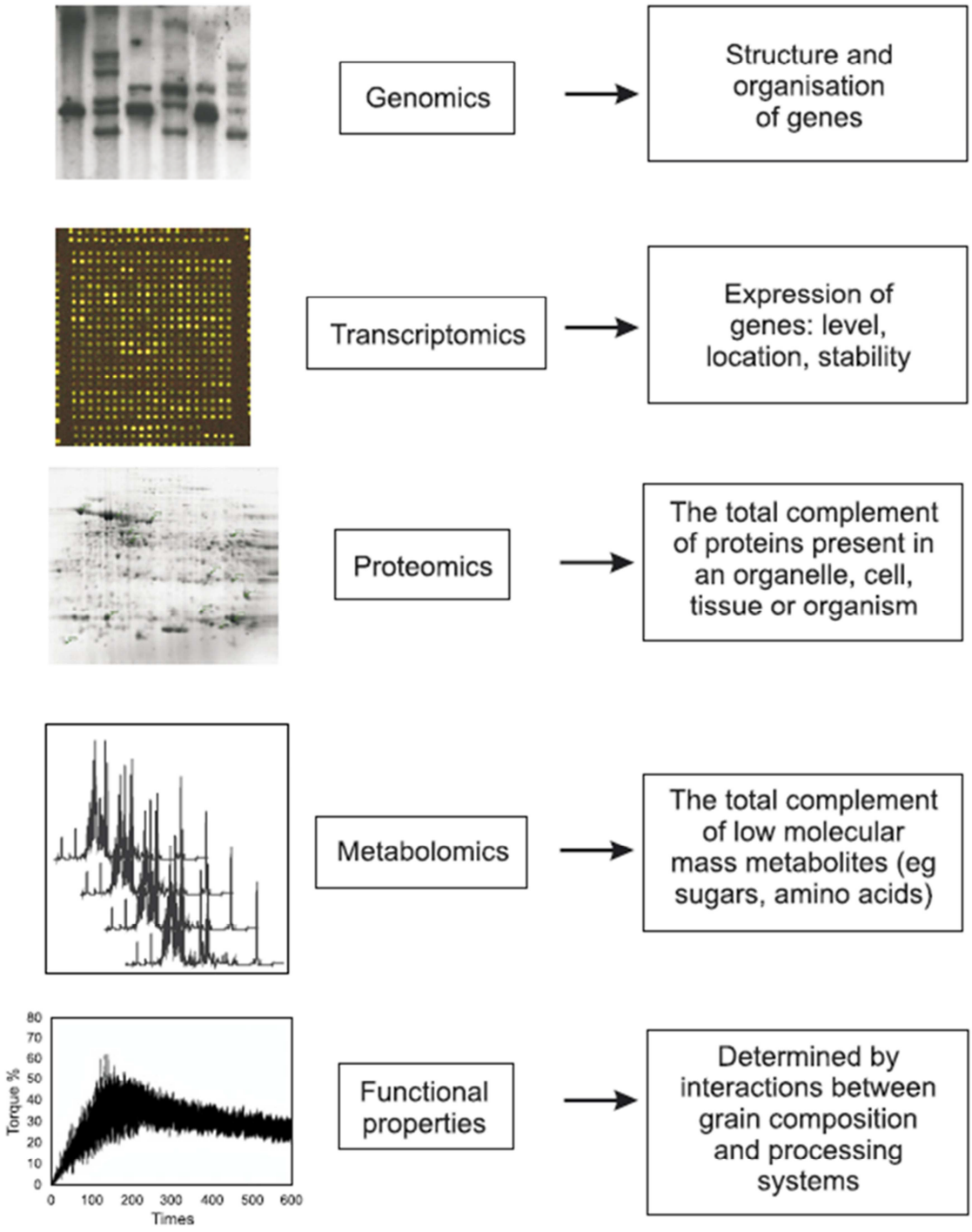

Fig. 30: Analysis performed in Shewry's study in order to define substantial equivalence of GM wheats. Taken from Shewry et al., 2007.

Harrison et al. (1996) has study the effects of CP4 EPSPS protein introduced in soybean with the objective of to produce an herbicide tolerant plant. The author showed that the transgenic proteins was readily degraded in simulated digestive fluids, moreover the protein was non-toxic when administered to mice with a dose 1000s of times higher than potential human exposure to CP4 EPSPS in foods, also investigation of amino acid sequence homology with known allergens was found.

Three transgenic wheats line over-expressing HMW-GS and corresponding parental and null segregant line were compared with metabolomic approach in order to establish their substantial equivalence (Baker et al., 2006). The genotypes were grown in two UK site and an increase of the maltose and/or sucrose levels for one transgenic line was observed. Nevertheless, the differences between the control and GM lines were within the same range as the differences observed between the control lines grown on different sites (Baker et al., 2006).

Batista et al. (2005) performed a study in order to evaluate the potential allergenicity of proteins introduced in GM plant. In this article 4 transgenic maize (MON810, Bt11, T25, Bt176) and soya (Roundup Ready) were investigated. Skin prick test on subjects with food and respiratory allergy showed that none of the individuals reacted differentially to the transgenic and control samples. Also immunoblotting with allergic patient's sera tested for 5 GM plants and also for pure transgenic proteins (CryIA[b] and CP4 5-enolpyruvylshikimate- 3-phosphate synthase) showed that the patients did not react differentially to the transgenic and non-transgenic samples, and then the transgenic products showed the same allergenic potential in comparison to control samples (Batista et al., 2005).

Transcriptomic approach and microarray analysis were performed in order to establish the substantial equivalence at the level of RNA expression between several transgenic and conventional bread wheat lines, expressing additional genes encoding HMW-GS (Baudo et al., 2006). The authors showed that difference in transcriptome profile between GM line and its untransformed genotype was very small, while much larger difference was observed in gene expression between conventionally bread lines then between transgenic and untransformed lines exhibiting the same complements of gluten subunits (Baudo et al., 2006).

Brandão et al. (2010) used a proteomic approach to evaluated protein expression between transgenic and non-transgenic soybean seed. Ten proteins showed significant

difference which were analyzed by mass spectrometry. A systematic increase on volume and/or intensity was observed for those proteins from transgenic soybean.

Another recent study of Coll et al. (2010) has evaluated transcriptional differences between commercial MON810 GM maize and non-transgenic crops in field conditions by microarray technique. These authors showed that MON810 and comparable non-GM varieties grown in the field had very few differences in terms of expression, being comparable to that present in different varieties). In general, even if some difference between GM crop lines and conventional lines are present, the variation is wider between varieties rather than between GM and untransformed lines, and environmental factors have greater influence than genetic transformation (Ricroch et al., 2011).

These are some of the examples concerning assessment of GM plants. EFSA proposes guidelines for safety assessment of GM plants (EFSA, 2010), but no validated approach for the routine transgenic plants is available yet).

8 Hypoallergenic crops obteined by genetic transformation

In the last years transgenensis was used for to obtain hypoallergenic crops, even if at present no hypoallergenic crops are commercially available (Rev. in Riascos et al., 2010). Several studies was performed on allegens target in rice, tomato and in order to reduce allergenic content such as R-amylase inhibitor (Tada et al., 2003), profilin, nsLTP (Le et al., 2006), and Mal d 1 (Gilissen et al., 2005). In legume crops, varieties were obtained with reduced content of P34/Glym Bd 30K (a cysteine protease) in soybean and Ara h 2 (a 2S albumin) in peanut (Rev. in Riascos et al., 2010).

Herman et al. (2003) have transformed soybean using a cosuppression approach in order to reduce the expression of P34 (major soybean allergen) in cotyledons. This protein was completely eliminated in transgenic plant. By comparison between GM lines and its corresponding genotype no differences in growth, development, reproduction, seed set, and seed maturation were observed. No compensatory effects due to the transgenesis were observed in GM lines's proteomes. Immunoblots performed with pool sera from allergic patients to soy revealed that the proteins from GM line and wt genotype were recognized by IgE and no differences in binding were observed, apart from P34's absence in the transgenic line (Herman et al., 2003).

Peanut was transformed by RNAi technology with the objective to generate plants with reduced levels of Ara h 2 (one of the major allergen togheter Ara h1 and Ara h 3) Dodo et al. (2008). The authors sowed that the accumulation of this protein was reduced a maximum of 25% from its original levels. The overall IgE-binding capacity of crude peanut extracts in the transgenic lines also showed a significant decrease when compared to the control nontransformed counterpart (Dodo et al., 2008).

The genes Ara h 2 and Ara h 6 in penut were silencing by biolistic RNAi technique (Chu et al., 2008). Sera from allergic patients to peanut exhibited decrease recognition against these proteins. Moreover, no change in the protein composition and phenotypic difference was observed (Chu et al., 2008).

References

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Chapter I

An allergenomic approach to evaluate the impact of genetic transformation in bread and durum wheat

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Wheat kernel proteins, albumins/globulins (A/G) and gluten proteins are responsible of food allergy and Baker's asthma for atopic subjects. Even if no genetically modified wheat are grown today for food uses, they are under study and their allergenicity needs to be tested.

In order to establish the safety assessment of genetic transformation, two GM wheat lines are compared to untransformed counterparts (*wt*), first by ELISA with sera from patients suffering from food allergy to wheat and Baker's asthma and second by allergenomic approach. The ELISA analysis revealed that the amount of specific IgE against the A/G fraction between GM wheat lines and its *wt* genotypes was quite comparable. 2D immunoblots performed by using sera from patients affected from food allergy and Baker's asthma on (A/G) fraction of the four lines (GM and *wt*) revealed very comparable IgE-binding profiles. Hundred nine IgE-binding spots were analysed by mass spectrometry; most of the identified proteins were already described as allergens or potential allergens. Only few IgE binding proteins were identified specifically in one or another line.

Introduction

Wheat is an important part of the daily diet of millions of people, with a total production of about 600 million tons each year globally, 75% of which is destined to food use (Rev. in Shewry, 2009). However, this staple food is capable of eliciting IgE-mediated allergic responses in atopic individuals. Allergic reactions to wheat may arise after ingestion of food containing flour, but also from flour and dust inhalation during grain processing, as in baker's asthma. Wheat is considered a major source of food allergies (Herman and Burks 2011, Zuidmeer et al., 2008), and it accounts for approximately 6% of food allergies in children and adults, and baker's asthma is one of the most common types of occupational allergies (Jacobs et al., 2008). The allergy mechanism occurs through polypeptides able to induce specific IgE antibodies production, and causes the release of inflammatory mediators by bridging surface-bound IgE on mast cells or basophils (Mari et al. 2009). This mechanism is distinct from other adverse responses to food, such as celiac disease or food intolerances (Guandalini and Newland 2011).

The symptoms of allergic responses to the ingestion of wheat include urticaria, atopic eczema/dermatitis, but also more severe reactions such as wheat-dependent exercise-induced anaphylaxis (WDEIA) (Hischenhuber et al., 2005). Allergic rhinitis and asthma are the main symptoms of baker's asthma.

Currently about 95% of the wheat cultivated in the world is hexaploid bread wheat, *Triticum aestivum* (2n = 6x = 42, AABBDD), whereas 5% is tetraploid durum wheat *T. durum* (2n = 4x = 28, AABB) (Peng et al. 2011). This latter is more adapted to the dry Mediterranean climate than bread wheat and is often called pasta wheat to reflect its major end-use. The distinctive feature that makes wheat unique is the visco-elastic property of its storage proteins (Rev. in Shewry 2009).

Wheat grain proteins are classically subdivided into the water/salt-soluble fraction (including albumins and globulins) which represents about 20% of the total amount of proteins and the water/salt-insoluble gluten, containing gliadins and glutenins (Shewry and Thatam 1990). The gliadins are monomeric proteins classified into three groups on the basis of their electrophoretic mobility at low pH: these are α/βgliadins (fast), γ-gliadins (intermediate) and ω-gliadins (slow). The glutenins are polymers of individual proteins linked by interchain disulphide bonds. After reduction of disulphide bonds, the

component subunits that are released are classified into high (HMW) and low molecular weight (LMW) glutenin subunits, typically separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS– PAGE) (Tatham and Shewry 2008).

Numerous IgE-binding polypeptides have been identified in wheat. Eighty-two wheat allergens are reported in the Allergome Database (http://www.allergome.org/ as updated at December 2011) which contains IUIS allergens, as well as putative allergens annotated using sequence similarity. Most of gluten proteins, along with some of the albumin/globulin fraction are responsible for triggering food allergic reactions (Tatham and Shewry, 2008; Battais et al. 2005, De Gregorio et al 2009, Šotkovský et al 2011, Mamone et al 2001, Simonato et al 2004, Akagawa et al 2007). Baker's asthma is mostly triggered by proteins belonging to the albumin/globulin fraction, such as the α-amylase/trypsin inhibitor family, lipid transfer proteins (LTP), peroxidase, thioredoxin, serine proteinase inhibitor, thaumatin-like protein (Palacin et al. 2007, Salcedo et al. 2011). Even if some prolamins were also identified as respiratory allergens (Bittner et al, 2008) they are less relevant than those found in the A/G fraction.

The past decades were marked by a notable interest in food allergies considered now as a significant public health concern as reported in the NIAID report (Boyce et al. 2010) and also by the widespread of genetically modified organisms. The safety assessment of genetic modified food plants is regulated by The Codex Alimentarius (ftp://ftp.fao.org/es/esn/food/guide_plants_en.pdf) it includes a specific paragraph addressing the question of allergenicity, modified content of known allergens or appearance of new and unknown allergens. The general approach is to verify if the GM food can be considered as safe, namely "substantially equivalent", as conventional food (Vennaria et al. 2008). Particularly, the EFSA recommends comparing the GM food with its appropriate comparator, when the recipient of the introduced gene is known to be allergenic. This recommendation is based on the possibility that the genetic modification might have induced an unintended effect, e.g. resulting in an over-expression of natural endogenous allergen (EFSA Journal 2010).

Although at present, no GM wheat is grown anywhere in the world, there is an increasing interest in this procedure, either as an alternative to classical wheat breeding or as a powerful tool for functional genomics. In wheat, targets of transgenesis include the increased resistance to biotic and abiotic stresses, the improvement of dough quality properties and the modulation of starch composition.

Wheat represents a staple food for a large majority of humans and thus has a huge emotional impact on public opinion, the assessment of the safety of new GM genotypes is therefore essential. The objective of the present work was to evaluate the effect the over-expression of two endogenous wheat genes coding for a LMW-GS protein of the storage fraction and a granule bound starch synthase (the Wx-B1 polypeptide) on the allergenicity of the transformants. Because numerous constituents of the albumin/globulin fraction were reported as IgE-binding proteins both in baker's asthma and wheat food allergy, this study focused particularly on these proteins. The protein content of each fraction was characterized and the Ig-E binding potential of their salt-soluble fractions was measured with sera obtained from patients suffering from food and respiratory wheat allergies. In a second step, we used an allergenomic approach to explore the potential allergens in the salt-soluble fraction of transgenic lines in comparison with their wild type (*wt*) parents.

Materials and Methods

Patients' sera

Sera were obtained from 21 adults and children with clinically-documented allergy to wheat, 9 adults with Baker's asthma, and 12 (10 children and 2 adults) with food allergy. Clinical data of the patients (symptoms, age, wheat-specific IgE) are summarized in Table 1. Sera were obtained from the Service of Clinical Immunology and Allergology at the Hospital of Epinal, France, and the University Hospital of Udine, Italy with the informed consent of the patient. Control sera were obtained from healthy volunteers.

Wheat samples

Wheat flours were prepared from mature kernels of two transformed lines and their wild type parents as controls: the first line was *Triticum aestivum* L. (bread wheat, AABBDD genome) cultivar Bobwhite (carrying the 1B-1R translocation) strongly over-expressing a transgenic low molecular weight glutenin subunit gene (LMW-GS), as described by Masci et al. (2003), along with its corresponding control genotype. The transgenic genotype used corresponds to T_5 generation. The second line was *Triticum durum* (durum wheat, AABB genome) cultivar Svevo over-expressing the *Wx-B1* gene

involved in amylose synthesis, as described by Sestili et al. (submitted), with its corresponding control genotype. The transgenic genotype used corresponds to T_4 generation.

Both the two transgenic lines were obtained by biolistic method as described in Masci et al (2003) and Sestili et al (submitted).

Wheat protein extraction

For each genotype, salt-soluble protein fraction were extracted from 3 gr of wheat flour that were mixed with 80 mL of extraction buffer containing 0.05M phosphate buffer/0.1M NaCl, pH 7.8 for 2h at 4°C. After centrifugation at 8,000 *g* for 15 min at 4°C, the supernatant corresponding to the Albumins/Globulins (A/G) was collected, dialyzed at 4°C against water for 3 days.

The remaining pellet corresponding to the salt-insoluble fraction was washed three times with 0.05M phosphate buffer/0.1M NaCl, pH 7.8. After centrifugation at 8,000 *g* for 15 min at room temperature, the gliadin fraction was extracted from the pellet with 80 mL of ethanol 50% for 1h and the supernatant was collected.

In order to extract glutenin subunits, the residue pellet obtained after A/G and gliadin extraction, was washed three times with ethanol 50% and, after centrifugation at 12,000 *g* for 10 minutes, the glutenin subunits were extracted with 30 mL of extraction buffer containing propanol 50%/1M Tris-HCl (pH 8.5)/1%DTT/1.4% (v/v) 4-Vinylpyridine for 1h at 60°C. After centrifugation at 12,000 *g* for 20 minutes the supernatant containing the glutenin fraction was collected. Gliadins and glutenin subunits thus obtained were dialyzed at 4°C against acetic acid for 3 days (Nicolas et al 1998).

The protein content was measured after freeze-dry according to Kejdahl method.

Enzyme-linked immunosorbent assay (ELISA)

ELISA tests were performed both for determining the concentrations of specific IgE against the four A/G fractions, and for detecting possible differences in the presence of α/β–, γ–, ω2–, ω5-gliadins, HMW-GS and LMW-GS in the four wheat genotypes here considered. Moreover, ELISA was used to characterize patients' sera by using A/G, gliadins, and LMW-GS extracted from the bread wheat cultivar Récital, with the same extraction procedure reported above.

As regards the ELISA with patients' sera, the wells on microtiter plates (Immobilizer Amino, Nunc) were coated with 5µg/mL in 100mM carbonate buffer (pH 9.6) of antigen (A/G) for 2h at room temperature. A standard curve was made with a serial dilution from 160 ng/mL to 0.08 ng/mL of IgE standard. The plates were blocked with PBS-0.1%Tween 20 and 0.5% porcine gelatin (G2500 SIGMA) for 1 hour at 37°C. After washes with PBS-0.1%Tween 20, patients' sera were diluted 1:10 with PBS-0.5% gelatin-0.1 % Tween 20 and incubated for 15h at 37°C. Goat anti-human IgE antibodies (ε-chain specific-Alkaline phosphatase developed in goat - Affinity isolated antigen Specific antibody - A3525-SIGMA) diluted 1:500 in 0.5% G–PBST was incubated for 2h at 37°C. The fluorescent substrate (4-Methylumbelliferyl phosphate M3168-SIGMA) diluted 1:5 in 1M Tris/HCl pH 9.8 was added for 90 min at room temperature and in the dark as described by Bodinier et al. (2008). The fluorescence was measured at 440 nm (excitation 360 nm) and the concentration of specific IgE binding to the antigen (A/G) was calculated using a standard curve. Four replicates and four controls wells with 10mM 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 parental genotypes. Differences were considered significant when *p*-value<0.01.

As regards the gluten protein fractions, indirect ELISA was performed. The wells on microtiter plates (MaxiSorp, Nunc) were coated with 5µg/mL in 100mM carbonate buffer (pH 9.6) of antigen for 1h at room temperature. After three washes with PBS-0.5% Tween 20, the plates were blocked with PBS-milk 2% for 1h at 37 °C. The plates were washed three times with PBS-0.5% Tween 20, and incubated for 1h at 37 °C with anti-peptide antibodies (specific for α/β-, γ-, ω2-, ω5-, LMW-GS or HMW-GS) (Denery-Papini et al, 1994, 1995). The curves were obtained with a serial dilution of antibodies from 1:500 to 1:8000 in PBS. Goat anti-rabbit IgG antibody (H+L) Horseradish peroxidase conjugate Human IgG adsorbed (BioRad) diluted 1:3000 in PBS was incubated for 1h at 37°C after three washing with PBS-0.5% Tween 20. The colorimetric substrate (OPD, *o*-Phenylenediamine/ H_2O_2) in 0.05 M citrate buffer, pH 5.5 was added for 30 minutes at room temperature. The OPD reaction was stopped with H_2SO_4 4N and read at 490-630 nm. For each antibody dilutions two replicates were performed and the average calculated.

Gliadins and glutenin subunits separation by SDS-PAGE and Immunoblotting

This procedure was performed on the two bread wheat genotypes, Bobwhite *wt* and Bobwhite GM-line. The gliadin and glutenin subunits extracts obtained as described above, were solubilised in Tris–HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, bromophenol blue, (and β-mercaptoethanol 4% for the glutenin subunits), adjusted at a concentration of 1mg/ml and 10 µl were loaded on the 1D SDS-PAGE, 15% mini-gels (BioRad). Polypeptides were electroblotted to nitrocellulose membranes (0.2 µm, Sartorius, Germany), in 25mM Tris, 192mM glycine, 0.1%SDS with a semidry transfer for 45min at 300mA. The membranes were blocked with PBS containing 0.1% Tween 20 (PBST) and 4%PVP (-SIGMA) for 4h at room temperature. After washing with PBST/2%PVP three times, the membranes were incubated overnight with serum of patient. Sera were diluted from 1:20 to 1:50, depending on the level of wheat-specific IgE, in PBST and 2%PVP. The membranes were washed three times with PBST- 2%PVP and incubated for 1h with peroxidase-labelled rabbit anti-human IgE (P0295, Dako, Denmark) diluted 1:100000. After three washing with PBST-2%PVP 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 a camera (Luminescent Image Analyser LAS 3000; Fujifilm) (Larré et al. 2011).

Protein separation by two-dimensional gel Electrophoresis (IEF vs SDS-PAGE)

Before solubilisation of proteins, the freeze-dried A/G extracts were desalted by precipitation with 2mL of acetone at -20°C. The A/G pellet was then solubilised in 8M urea, 2M Thiourea 2% CHAPS 2%Triton X and 1% IPG-Buffer pH 3-10 (GE Healthcare). For 2D gel electrophoresis 600µg of proteins were loaded on immobilized pH gradient gel strips (13 cm Immobiline Dry Strip pH 3-10, GE Healthcare); they were rehydrated overnight at 20 °C and isoelectrofocalization (IEF) was performed with Ettan IPGphor 3 (GE Healthcare) for 19.5h with linear voltage at 30V for 12.5h, 300V for 1h, 500V for 1h, 1000V for 1h, 5000V for 1h, 8000V for 2h. After IEF, the strips were equilibrated two times for 15 min with 50mM Tris-HCl buffer pH 8.8, 6M Urea, 30% v/v Glycerol, 2% SDS. The strips were placed on top of 15% polyacrylamide midi-

gels (SE600-Hoeffer, 13 cm) and the second dimension was run. After 2D-E the gels were stained with Coomassie Brilliant Blue G-250 (Devouge et al., 2007) or transferred for immunoblotting, as reported in the previous paragraph. The stained gels were scanned with an Imaging Densitometer (GE Healthcare) at 300 dpi. The scanned images were stored in TIFF image file.

Two-dimensional Immunoblotting with anti-peptide antibodies specific for α/β- and γ-gliadins, and LMW-GS

This analysis was performed in order to detect the possible contamination of the A/G fraction with some of the gluten proteins (α/β- and γ-gliadins, and LMW-GS).
The non-stained gels with A/G fraction of Bobwhite *wt*, Bobwhite GM-line, Svevo *wt*, and Svevo GM-line were transferred to nitrocellulose membrane with semidry system at 300mA for 1 h. The membranes were blocked with PBS-milk 5% for 1h at room temperature. After three washing with PBS-Tween 20 0.05%, the membranes were incubated for 1h with the antibody. The polyclonal antibodies (anti α/β- and γ-gliadins) were diluted 1:20000 and monoclonal antibodies (anti LMW-GS) 1:8000, in PBS-milk 2%. The membranes were washed 3 times with PBS-Tween 20 0.05%, and incubated for 1h with anti-Rabbit IgG (H+L) Horseradish Peroxidase Conjugate Human IgG Adsorbed (Bio-Rad), for polyclonal antibodies, and anti-Mouse IgG (H+L) Horseradish Peroxidase Conjugate Human IgG Adsorbed (Bio-Rad), for monoclonal antibody. Both antibodies were diluted 1:100000 in PBS-milk 2%. After three washing with PBS-Tween 20 0.05% and two with PBS, the membranes were incubated with 4mL of chemiluminescent substrate (Super Signal West Dura Extended Duration Substrate, 34076, Pierce, USA) according to the manufacturer's protocol. The bound IgG-antibodies were detected with a camera (Luminescent Image Analyser LAS 3000; Fujifilm).

Detection of IgE binding polypeptides by two-dimensional Immunoblotting

The non-stained gels with A/G fraction of Bobwhite *wt*, Bobwhite GM-line, Svevo *wt*, and Svevo-GM-line were transferred to nitrocellulose membrane for 1h. Because of the scarce availability of sera, only the portion of the gel between pI 4 and 9 (10 cm x10 cm) in which the major amount of polypeptides are present, was electroblotted and immunoblotting with patient's sera was performed as described in the previous

paragraph. It was not possible to perform replicas, because the availability of patients' sera is strongly limiting. Sera were diluted from 1:20 to 1:80, depending on the level of wheat-specific IgE, in PBST and 2%PVP. The serum from a non-allergic subject was used as a negative control (Ahn et al, 2009).

Protein identification by mass spectrometry

Protein spots were picked up manually and prepared for mass spectrometry. In-gel digestion was performed using trypsin hydrolysis according to Larré (2010).

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 2µm 100A, 75-µm i.d. x 15-cm length, Dionex) at a flow rate of 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. 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 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.

Databank searches and interpretation

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://compbio.dfci.harvard.edu/tgi/, release 12 from April 2010). A database search was performed with XTandem 2008.02.01 (http://www.thegpm.org/TANDEM/) via a pipeline developed by B. Valot (B. Valot. 2001) and available at http://pappso.inra.fr/bioinfo/xtandempipeline/.Fixed modification of cysteine residues by iodoacetamide was considered, as well as oxidized methionins. Precursor mass and fragment mass tolerance were 2.0 and 0.8 D, respectively. One missed trypsin cleavage was set for databank searches with a mass tolerance of 0.0005. Proteins were considered when a minimum of three unique peptides with an E value below 0.001were matched in their sequence and when their E value was below 10^{-4}. Protein identifications were compared in the two databanks, best matches were validated and when the results were identical in the two banks, the identification of UniProt was chosen.

Results

Characteristics of patients

Table 1A: Clinical profiles of the patients with food allergy to wheat and their concentrations (ng/ml) in IgE specific for A/G, gliadins, and LMW-GS of the bread wheat cv Récital as tested by ELISA. AEDS=Atopic eczema dermatitis syndrome, Urt=Urticaria, GI=Gastro-intestinal symptoms, AS=Anaphylactic shock, nd: not done, +: = response at the lower limit of quantification, neg=negative. **= sera used in 2D immunoblotting.

Patient serum n°	Age	Symptoms	IgE specific for A/G	IgE specific for gliadins	IgE specific for LMW-GS
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

Table 1B: Clinical profiles of the patients with Baker's asthma and their concentrations (ng/ml) in IgE specific for A/G, gliadins and LMW-GS of the bread wheat cv Récital as tested by ELISA. R=Rhinitis, AT= Asthma, +: = response at the lower limit of quantification, neg: negative. **= sera used in 2D immunoblotting.

Patient serum n°	Age	Symptoms	IgE specific for A/G	IgE specific for gliadins	IgE specific for LMW-GS
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

Sera were obtained from 12 patients with food allergy (10 of which were children 1.5-14 years old) and 9 adult patients with baker's asthma. In a first step, the amount of specific IgE was determined by ELISA for the three major classes of wheat allergens: A/G, gliadins and LMW-GS (bread wheat variety Récital). In the group of patients suffering from Baker's asthma (Table 1A), all the sera contained IgE specific for the A/G fraction, with a range of variability among patients, from 2 to 75ng/mL. Fewer responses were observed towards gliadins and LMW-GS, with respectively four and one serums reacting with these proteins. In the group, of patients suffering food allergies (Table 1B), 11 sera were reactive with the A/G fraction, with a range of variability from 10 to 127ng/mL. Responses to gluten proteins were higher for these patients with eleven sera that possessed IgE specific for gliadins, and eight sera that reacted against LMW-GS.

After sequential extraction, the amounts of the major classes of seed proteins, A/G, gliadins and glutenins were compared between the two GM lines and their corresponding *wt* genotypes to investigate a possible change of protein accumulation caused by the genetic transformation. The percentages referred to the three proteins fraction for the bread wheat and for the durum wheat genotypes are reported in Fig. 1. As expected, the Bobwhite GM line shows a strong increase of the glutenin fraction (45%), accompanied by a decrease of 49% and 30% for the gliadin and A/G fractions, respectively. This behavior was also reported by Scossa et al (2008). The comparison of the three protein fractions between this specific GM durum wheat line and its parental genotype showed a decrease of gliadin and glutenin fractions (10 % and 27% respectively), whereas an increase (37%) of the soluble fraction in comparison to Svevo *wt* was observed (Fig. 1).

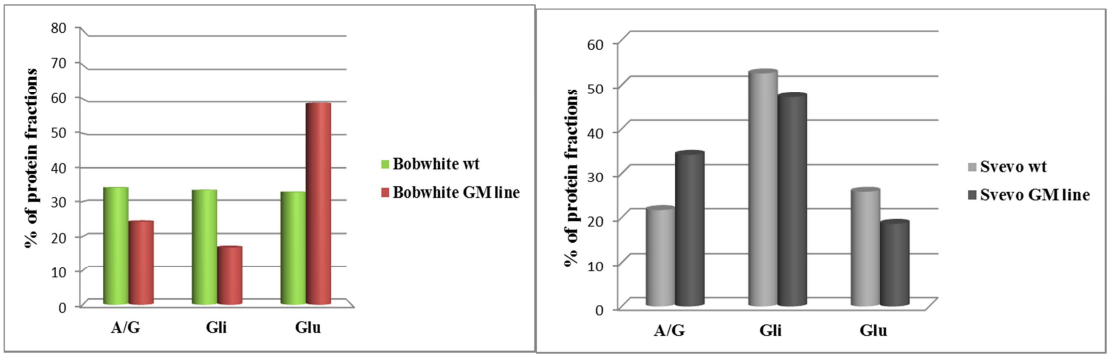

Fig.1: Percentage of major protein fractions after sequential extraction for Bobwhite-GM line and the wild type genotype (left), and Svevo-GM line and the wild type genotype (right).

As we used a standard protocol for the sequential extraction of the three fractions, the possible contamination with gliadins and glutenins of the A/G fraction was checked in 2D Western blot using anti-peptide antibodies specific for α/β- and γ-gliadins, and LMW-GS. No contamination with LMW-GS was observed, nevertheless very low amounts of α/β andγ-gliadin contaminants were detected (one example of results was show in Supplementary Data, Fig. S1), confirming the effectiveness of the separation procedure.

Comparison of the composition of gliadin and glutenin fractions between the GM lines and their wt *genotypes*

Numerous food allergens are present among the gliadins and glutenins. The two GM lines and their parents varied in the proportion of these fractions; therefore a brief characterization of their composition was undertaken to estimate the impact of the transgenesis on these storage proteins. An antigen-coated-plate ELISA was performed with anti-peptide antibodies specific for the different gliadin and glutenin classes: α/β-, γ-, ω-2-, ω-5-gliadins, LMW-GS and HMW-GS (Results shown in supplementary data, Figs S2a and S2b). Concerning gliadins, no difference in the detection of γ-gliadins between the two GM wheat lines and their untransformed genotypes was observed, and slightly lower responses were seen for α-gliadins in the two GM lines than in their parents. As the Bobwhite cultivars carry the 1B-1R translocation, nearly no ω5-gliadins could be revealed in their gliadin extracts; a lower response of the anti-ω5-gliadin antibody was measured for Svevo GM line compared to Svevo *wt*. Very few ω2-

gliadins were detected in the two Svevo genotypes (no genome D) and equivalent signals were obtained in the two Bobwhite genotypes. Equal detection of HMW-GS was obtained in the four wheat genotypes, and equal detection of LMW-GS in the two Svevo genotypes. Detection of LMW-GS with an antibody of broad reactivity to LMW-GS indicated a global increase of these GS in the GM bread wheat line compared to its parent cultivar. Although this is a semi-quantitative test, this latter difference is very likely authentic, since it was expected because of the huge over-expression of the transgene (Masci et al, 2003; Scossa et al, 2008). Nonetheless, another antibody with a reactivity restricted to some LMW-GS (Denery-Papini et al., 1996) gave a decreased intensity in the GM line.

Comparison of IgE-binding to gliadin and glutenin fractions between Bobwhite GM line and the wild type *genotype*

Four sera from patients with clinically-documented food allergy to wheat were tested in one-dimensional immunoblotting after SDS-PAGE of gliadins and glutenins of Bobwhite GM-line and its *wt* line. Because sera availability was scarce, we decided to perform this analysis only on the bread wheat lines, since the amount of their respective Gli and Glu fractions were greatly affected and, moreover, the transgene encodes a polypeptide known to be involved in wheat allergies (the LMW-GS).

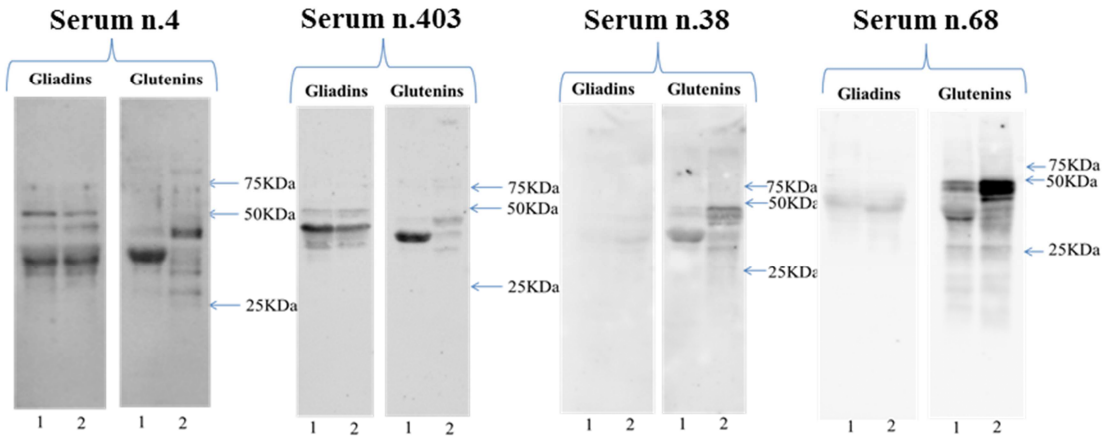

Fig. 2: 1D Immunoblotting. Reactivity of IgE towards gliadin and glutenin fractions of Bobwhite-GM line (1) and Bobwhite *wt* (2). Sera from patients affected from food allergy to wheat.

IgE reactivity against the gliadin fractions was the same for both GM and *wt* bread wheat lines in food allergy (Fig. 2) with detection of the same IgE-binding proteins between 30 and 75 KDa, with quite similar intensity. No difference was observed among gliadin patterns between the GM-line and its *wt* cultivar.

Differently from gliadins, IgE binding to the glutenin fractions by all four sera tested showed different profiles between the GM line and the parental (*wt*) genotype. Among the glutenin subunits of the *wt* genotype, IgE reacted with different polypeptides in the molecular weight range 20-80 KDa, with various intensities according to sera (Fig. 4). On the contrary, all sera reacted strongly with a 40 KDa band of the LMW-GS present in the GM line. Additional bands corresponding to components already detected in the parent line were weakly detected by some sera. Such different behavior between GM and *wt* genotypes is due to the strong over-expression of the transgenic LMW-GS that displays a high IgE-binding potential and causes a decrease of expression of some other glutenin polypeptides (Scossa et al, 2008).

Comparison by ELISA of IgE reactivity towards A/G fractions between the GM and the wt *durum and bread wheat genotypes*

Eighteen out of the 21 sera that were tested by ELISA against the A/G fractions of the two GM wheat genotypes and their two untransformed counterparts; in particular, 8 sera of patients suffering from Baker's asthma (Fig. 3) and 10 from food allergy (Fig. 4).

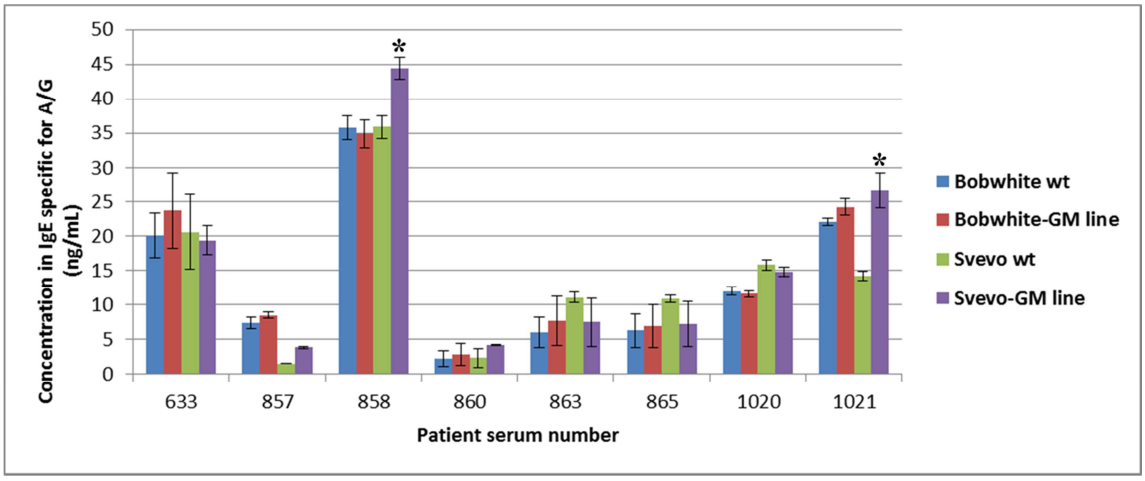

***Fig. 3*:** Concentration in IgE specific for A/G fraction (ng/mL) extracted from four wheat genotypes. Sera of patients suffering from Baker's asthma. Asterisks indicate patients'sera showing significative differences between the GM line and its untransformed counterpart.

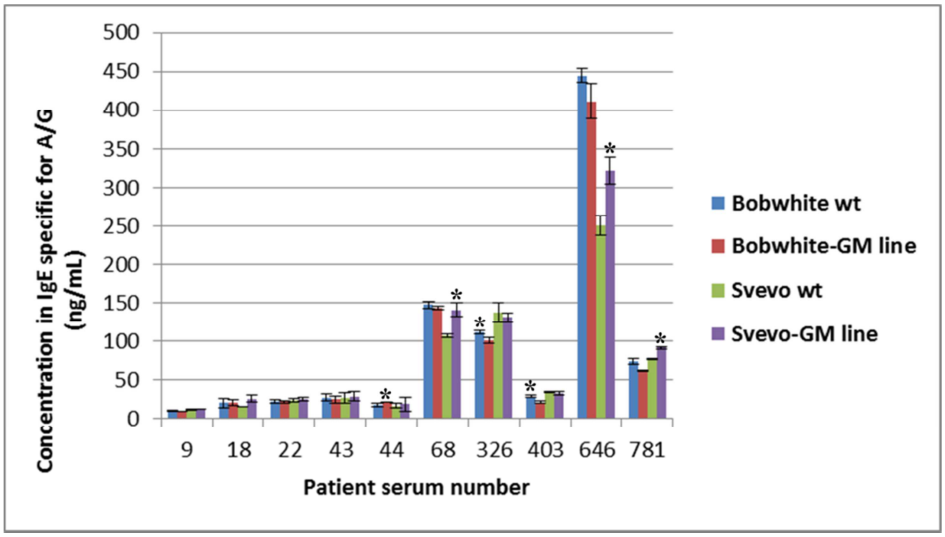

***Fig. 4*:** Concentration of IgE specific for A/G fraction (ng/mL) extracted from four wheat genotypes. Sera of patients suffering from food allergy. Asterisks indicate patients'sera showing significative differences between the GM line and its untransformed counterpart.

The comparison of IgE binding levels between GM genotypes and their untransformed counterparts revealed significant differences for only two patients affected from Baker's asthma and for six patients suffering from food allergy. Among them, five sera (68, 646, 781, 858, 1021) displayed an increased reactivity (increase between 14% and 45%) towards Svevo-GM line A/G in comparison with Svevo *wt* A/G. Only three sera of patients with food allergy (numbers 44, 326 and 403) displayed small differences in specific IgE concentrations for A/G fraction between Bobwhite-GM line and its *wt* genotype: decrease of 5 or 9% or increase of 19%. Nevertheless, the IgE binding capacities to A/G fractions relative to the sera tested were mostly comparable between Svevo *wt* and the GM line and between Bobwhite *wt* and Bobwhite-GM line, showing 70% and 84% of common IgE binding, respectively.

Comparison of IgE-binding to A/G fractions by 2D immunoblotting and allergen identification in transgenic wheat lines and their untransformed genotypes

In order to identify the wheat flour salt-soluble allergens, 2D immunoblots were performed by using three sera from patients affected from food allergy and one serum patients with Baker's asthma (summarized in Tab. 2), on A/G fraction of Svevo *wt*, Svevo-GM line, Bobwhite *wt* and Bobwhite-GM line.

Tab. 2: Sera from patients suffering from food allergy (FA) and Baker's asthma (BA) used for 2D immunoblotting on A/G fraction of Svevo *wt*, Svevo-GM line, Bobwhite *wt*, and Bobwhite-GM line.

Immunoblotting on Svevo *wt*	Immunoblotting on Svevo-GM line	Immunoblotting on Bobwhite *wt*	Immunoblotting on Bobwhite-GM line
a: serum **68**(FA)	**d**: serum **68**(FA)	**g**: serum **68**(FA)	**l**: serum **68**(FA)
b: serum**781**(FA)	**e**: serum **781**(FA)	**h**: serum **38**(FA)	**m**: serum **38** (FA)
c: serum **458**(BA)	**f**: serum **458**(BA)	**i**: serum **458**(BA)	**n**: serum **458**(BA)

These sera were chosen on the basis of their reactivity and the amount available. Given the difficulties in obtaining sufficient volumes of serum, we chose to use them for a cross-comparison between the two GM lines and their untransformed genotypes, but not for replicates. For each line, an immunoblot control was performed with the sera of a non-atopic subject, and in all cases no spots were detected (figures not shown).
The well-known variability of the expressed proteins between genotypes leads us to perform the analysis by genotypes.

Comparison between Svevo wt *and Svevo-GM line*

As shown in Figs. 5 and 6, many IgE binding polypeptides were revealed on A/G fraction of Svevo *wt* and Svevo-GM line using the three sera 68, 781, and 458. As shown in table 3, more than 82% of the IgE binding polypeptides were common between the GM and its parent line. With reference to the same serum, a few numbers of different spots were detected between the two lines, that can be specific either to the *wt* genotype or the GM line.
In the case of serum 68, seven polypeptides were specifically detected in Svevo *wt*, but absent from the equivalent blot on its transgenic line (an example is shown in Fig. 7). As for serum 781 and 458, three and two specific polypeptides, respectively, were detected in Svevo *wt*, whereas six and three specific polypeptides were found in Svevo-GM line. Twenty-seven spots in Svevo-GM line and 31 in Svevo *wt* were identified by mass spectrometry, 17 of which have shown a common identification between the two lines (Tab. 3).

Tab. 3:Numbers of IgE-binding polypeptides, S: serum.

	# of IgE-binding polypeptides for Svevo *wt*	# of IgE-binding polypeptides for Svevo-GM line	# of common polypeptides between Svevo *wt* and Svevo-GM line	# of common proteins identified
S. **68**	47	40	40	17
S. **781**	17	20	14	
S. **458**	29	30	27	

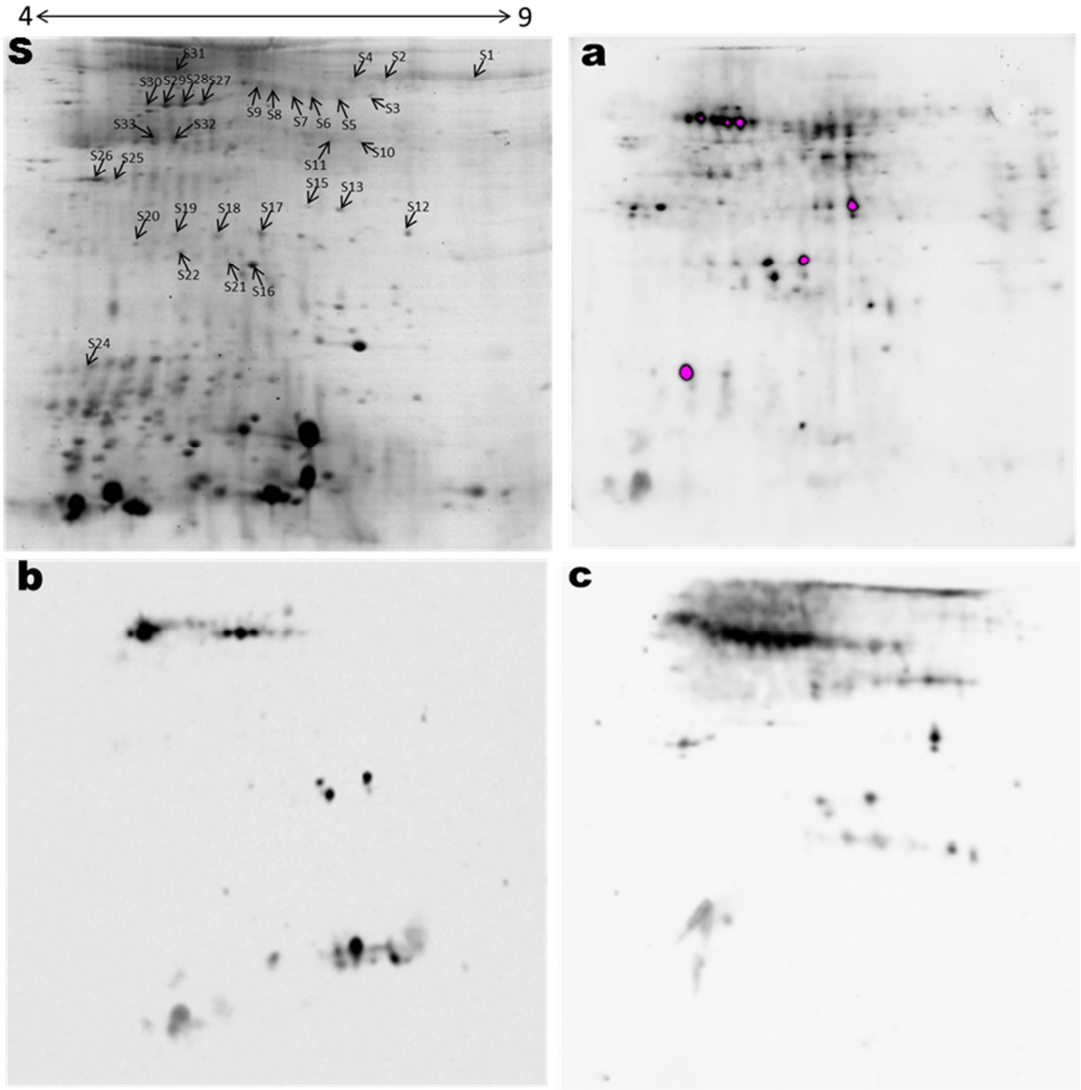

Fig. 5: 2D-Immunoblotting of Svevo *wt* with sera from allergic patient to wheat. **S**: 2D-SDS-PAGE of Svevo*wt*'s A/G fraction.Immunoblotting with serum **68 (a)**; serum **781(b)**; serum **458 (c)**.

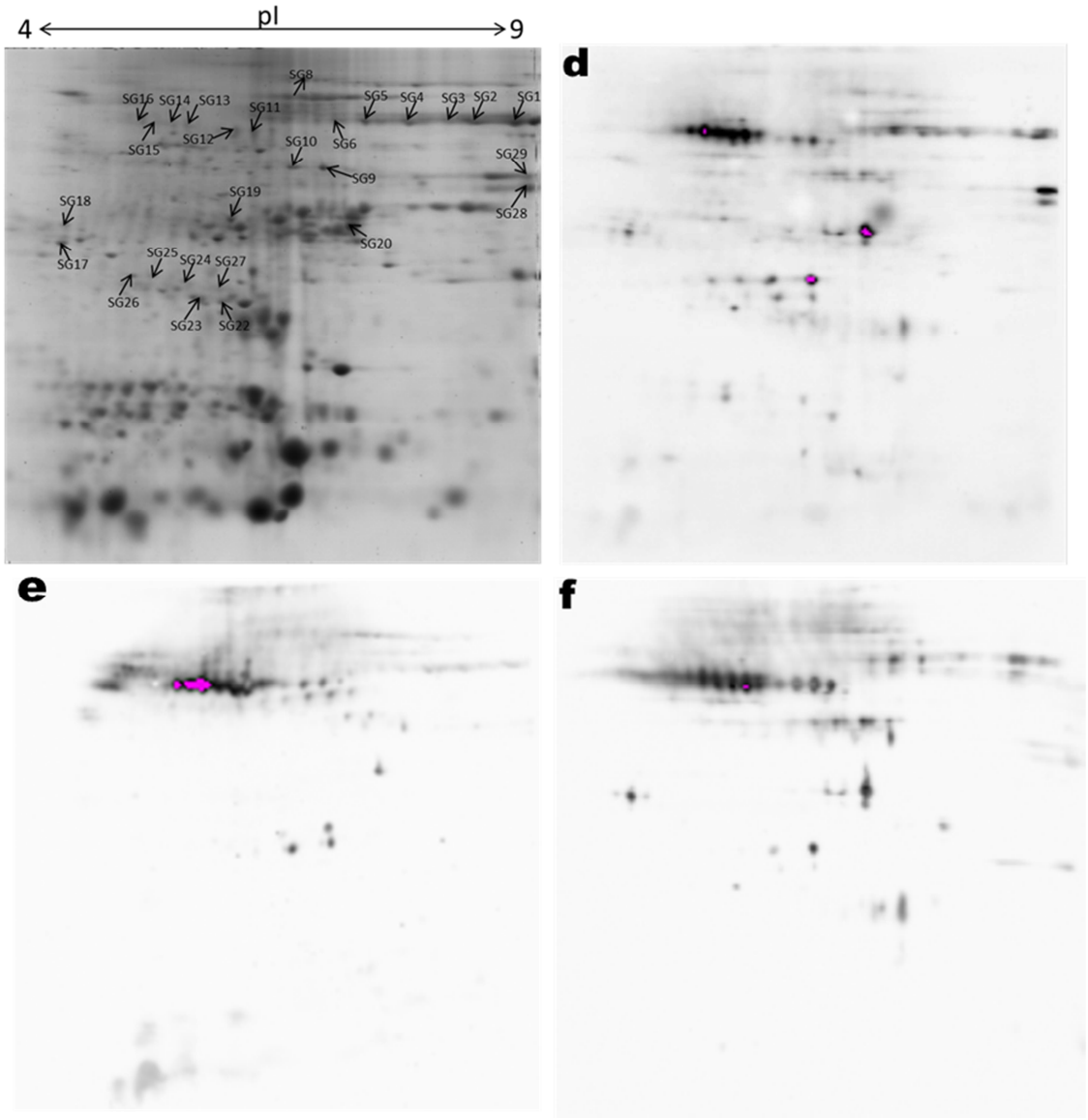

Fig. 6: 2D-Immunoblotting of Svevo-GM line with sera from allergic patient to wheat. **SG**: 2D-SDS-PAGE of Svevo-GM line's A/G fraction. Immunoblotting with serum **68 (d)**; serum **781(e)**; serum **458 (f)**.

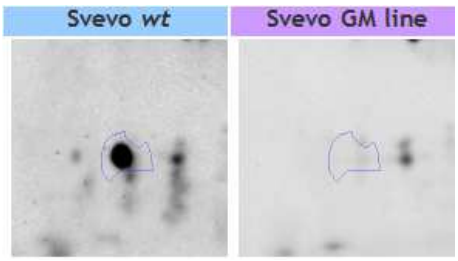

Fig. 7: Specific IgE-binding polypeptide (S24) for the serum s68 detected in Svevo *wt* and absent from the equivalent blot of Svevo-GM line

Comparison between Bobwhite wt *and Bobwhite-GM line*

As shown in Figs. 8 and 9, a high number of IgE-binding polypeptides were revealed on A/G fraction of Bobwhite *wt* and Bobwhite-GM line using the three sera 68, 38, and 458. More than 90% of the IgE-binding polypeptides were common between Bobwhite *wt* and Bobwhite GM-line (Tab. 4). Some polypeptides were specific of one genotype. In the case of the sera 68 and 38, six and seven specific polypeptides were found in Bobwhite-GM line and two and five in the *wt* genotype, respectively. Whereas four specific polypeptides were detected in Bobwhite *wt* and one in Bobwhite-GM line by immunoblotting with serum 458. Twenty-seven IgE-binding polypeptides were identified for Bobwhite *wt* and twenty-four for Bobwhite-GM line by mass spectrometry, of which 17 have shown a common identification between the two lines (Tab. 4).

Tab. 4: Numbers of IgE-binding polypeptides, S: serum.

	# of IgE-binding polypeptides for Bobwhite *wt*	# of IgE-binding polypeptides for Bobwhite-GM line	# of common polypeptides between Bobwhite *wt* and Bobwhite-GM line	# of common proteins identified
S. **68**	62	66	60	17
S. **38**	95	97	90	
S. **458**	53	50	49	

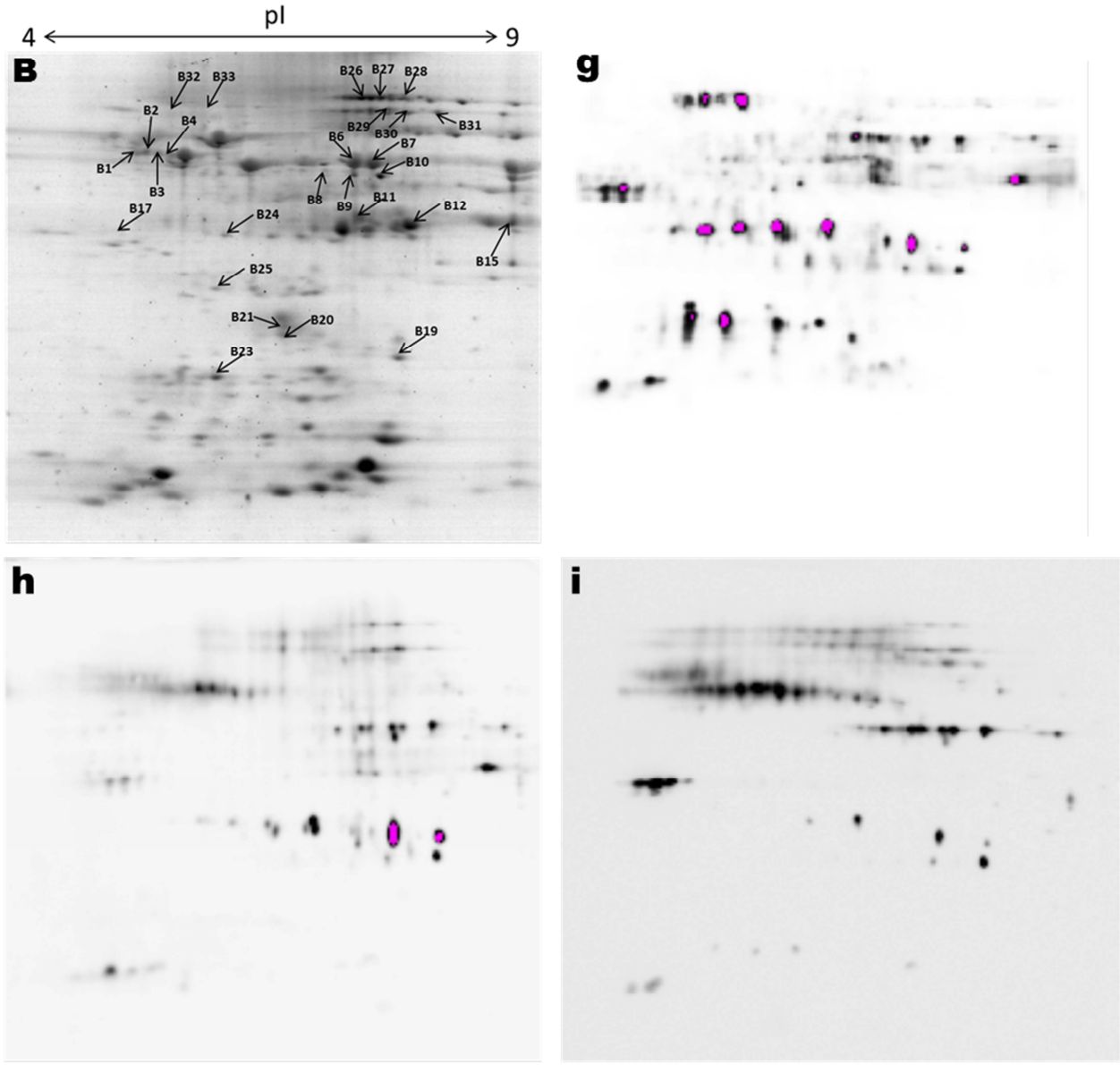

Fig. 8: 2D-Immunoblotting of A/G from Bobwhite *wt* with sera from patient allergic to wheat. **B**:2D-SDS-PAGE of Bobwhite *wt*'s A/G fraction. Immunoblotting with serum **68 (g)**; serum **38(h)**; serum **458 (i)**.

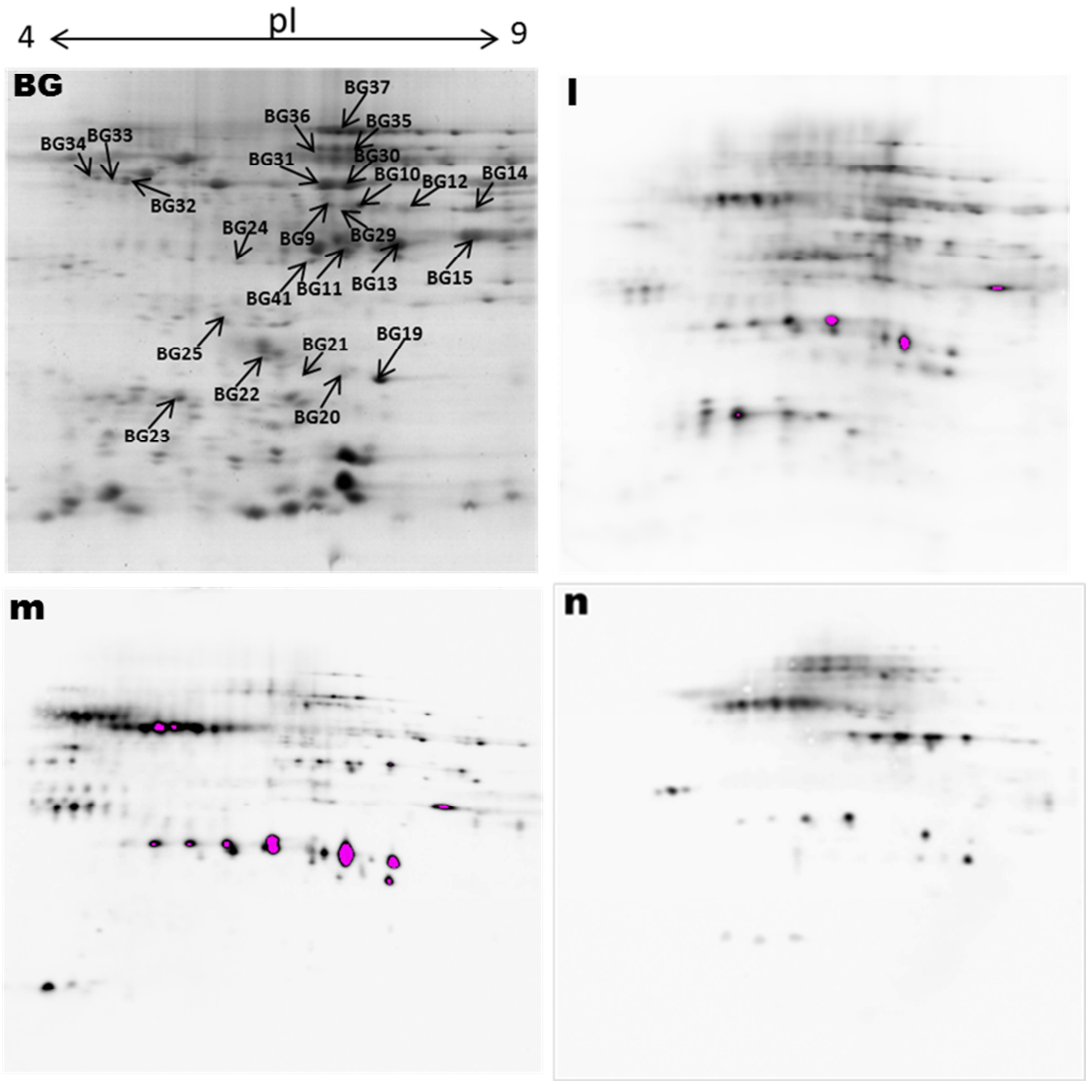

Fig. 9: 2D-Immunoblotting of A/G from Bobwhite-GM line with sera from patient allergic to wheat.BG:2D-SDS-PAGE of Bobwhite-GM line's A/G fraction. Immunoblotting with serum **68 (l)**; serum **38(m)**; serum **458 (n)**.

Discussion

Allergens are present in all wheat kernel protein fractions. These are typically represented by soluble proteins (albumins and globulins, A/G) and insoluble gluten proteins (gliadins and glutenins). Gluten proteins are mostly involved in food allergy, whereas A/G are involved both in food and respiratory allergies.

In the present study, we have performed a comparative study of two cultivars along with two of their GM lines: the durum wheat cultivar Svevo and the bread wheat cultivar Bobwhite, (Masci et al, 2003; Sestili et al, submitted). A comparative allergenomic analysis was focused on the A/G fraction involved in either type of allergy, while only a brief comparison was made for gluten proteins.

The durum GM wheat line, Svevo (MJ 11-12) was obtained by Sestili et al (submitted) with the objective to produce starches with novel properties and new industrial applications. In fact the granule bound starch synthase (GBSSI also known as waxy protein or Wx), catalyzes the amylose synthesis. In durum wheat there are two waxy genes, Wx-A1 and Wx-B1, with this latter having a strong effect on amylose content and starch properties. In order to increase amylose content and to study the effect on starch properties of the *Wx-B1* gene, Sestili et al (submitted) produced a transgenic wheat line overexpressing this gene, although even if the level of *Wx-B1* transcript was 10-fold higher in the transgenic line than in the parental genotype, the amylose content of the GM line did not result increased (Sestili et al, submitted).

The bread wheat cultivar Bobwhite was transformed with a LMW-GS transgene in order to increase the amount of these polypeptides (Masci et al, 2003). In fact, LMW-GS represent about 30-40% of the gluten proteins and their amount, along with their structure, influence technological properties of wheat dough. Because a strong overexpression of the transgenic polypeptide was obtained, likely because of a high number of transformation events (Scossa et al., 2008), a drastic decrease in the amount of all endogenous proteins, including CM-like proteins, with respect to the *wt* genotype, was observed as an unintended effect of genetic transformation. This was confirmed in our study: we observed a huge increase of the glutenin fraction characterized by a large amount of LMW-GS and a simultaneous decrease of the gliadins and the A/G fraction. The composition and IgE-binding potential of LMW-GS was also affected by the transformation, while such effects were not observed for gliadins.

In the case of the GM durum wheat genotype produced by Sestili et al (submitted), we observed a decrease of gliadin and glutenin fractions balanced by an increase of the A/G fraction.

A set of 21 sera from patients with food or respiratory allergy to wheat was chosen mainly on the basis of their reactivity against A/G. Patients with Baker's asthma were all adults, while patients with food allergy were mostly children and showed also the highest levels of specific IgE. This result is consistent with Battais et al. (2005) that also highlighted the frequent IgE-binding to A/G for children. These sera were used to investigate the impact of wheat genetic transformation on the allergens present in their A/G fractions (after controlling the absence of contamination of this fraction with gliadins). In a first step a quantitative ELISA allowed to compare levels of IgE-binding to A/G. All four A/G fractions obtained by extraction from the GM lines and wt

cultivars were recognized by serum IgE from patients suffering from Baker's asthma and from food allergy. The wide variation in response intensities reflects the well-known inter individual variations in patients with the same pathology. Focusing on the variations observed between the GM and their *wild type* counterparts, significant increases of the serum reactivity to Svevo-GM A/G were measured for only five sera out of the 18 tested and varied between 14 and 45% of the values measured for Svevo *wt*. A different situation was observed in the bread wheat lines: significative differences were found only in three sera (all from food allergic patients) out of the 18 sera, with higher or lower reactivity against Bobwhite GM-line on the serum used; however, such differences, although significative, are small (maximum 10 ng/ml).

In order to better describe at the molecular level the allergens and compare them between GM lines and their *wt* counterparts, we carried out a comparative allergenomics approach in which polypeptides detected in immunoblots using patients' sera were identified by mass spectrometry. This approach has been recently proposed in EFSA guidelines for the assessment of allergenicity of GM organisms (EFSA 2010).

Two-dimensional immunoblottings were performed on each of the GM or *wt* lines of durum and bread wheat, using sera from patients affected with food allergy (two sera) or Baker's asthma (one serum). It has to be stressed that large volumes of sera are required in such analyses, which led us to select only two sera to compare the reactivity of the four lines. The total number of IgE-binding spots revealed a higher reactivity towards Bobwhite genotypes with respect to Svevo genotypes for serum 68 (patient with food allergy) and serum 458 (patient with Baker's Asthma). The effect of the wheat genotype on the number of IgE-binding spots was already reported (Larré et al, 2010) and might be related to the different levels of ploidy, which induces large variations in the expressed proteins and, among them, of salt-soluble A/G proteins. Most of the proteins identified in this study have been previously reported as allergens or putative allergens in wheat or other species (Salcedo et al 2011, Breitener et al, 2004; Tatham and Shewry, 2008). Whether we consider the genotypes Bobwhite or Svevo, the allergens have been identified either in the GM lines or in their *wt* counterparts.

Whatever the allergy considered, numerous spots were identified as globulin3 or globulin2 which belong to the cupin-2 superfamily and are related to the predominant seed storage proteins of 7S globulin class from dicotyleneous plants (Breiteneder and Radauer 2004). Globulins were identified in many spots, most of them common between the *wt* genotypes and their respective GM lines (11 out of 16 spots for Svevo,

and 9 out of 17 spots for Bobwhite). On another hand, globulins were also predominant amongst the spots specifically detected for a given line: in the case of Svevo GM line, they represented 5 out of 10 specific spots induced by the transformation; similar results were obtained for Bobwhite GM line, with 6 out of 10 specific spots identified as globulins. Globulin 2 proteins are encoded by a gene family consisting of three members and globulins 3 by three genes present as multiple copies. Consistently, their expressed polypeptides are detected as numerous spots in 2D electrophoresis, also reflecting the occurrence of post-translational modifications as described elsewhere (Altenbach et al, 2009; Loit et al., 2009, Larré et al 2011). This confirms the finding of Larré et al (2011) which identified many globulins among the reactive polypeptides present in*T. monococcum,* a diploid wheat, and in the bread wheat cv. Récital.

Many serine protease inhibitors (serpins) were detected as IgE-binding proteins in the four lines studied. This is in agreement with previous findings, in which serpins were already identified using patient's sera suffering from Baker's asthma (Amano et al., 1998, Sander et al., 2001, Akagawa et al., 2007, Constantin et al 2008) and from food allergy (Šotkovský et al., 2008, Pastorello et al., 2007; Larré et al., 2011). Recently, the serpin WSZ2a was expressed as a fusion protein containing MBP in *E. coli* and was recognized by 2 out of 40 sera of patients suffering from Bakers' asthma (Sander et al., 2011), confirming its role in respiratory allergy. The involvement of serpins in food allergy still needs to be established.

As expected, when using the serum obtained from the patient affected from Baker's asthma, we predominantly identified polypeptides corresponding to α-amylase inhibitors, since this class of proteins is known to be amongst the major allergens in this pathology (Pastorello et al., 2007). Alpha-amylase inhibitors (WTAI) are represented by heterogeneous and numerous polypeptides, showing different pI and molecular weights (Altenbach et al., 2011). Only three forms of α-amylase inhibitors were identified in this study: endogenous α-amylase/subtilisin inhibitor and WTAI-0.19 were found in Bobwhite genotypes, whereas WTAI-CM3 was identified in Svevo genotypes. Although these polypeptides are mostly described for respiratory allergies, we also found them with sera from patients suffering of food allergies. This was also reported previously by Šotkovský et al. (2008) and Larré et al. (2011). The serum which was chosen in our study as representative of patients suffering of Baker'asthma seems particular, in the sense that it responds poorly to proteins of low molecular weight, among which the WTAI are found. This can be related to the large well-known

variability in the IgE response of patients, as also reported by Sander et al (2008) in a group of fifty patients suffering from Bakers'asthma.

Wheat contains three classes of xylanases inhibitors (XIs): *T. aestivum* xylanase inhibitor (TAXI), xylanase-inhibiting protein (XIP) and thaumatin-like xylanase inhibitor (TLXI). They are believed to act as a defensive barrier against pathogens. Two XIPs (XIP1 and XIP-III) were identified in Bobwhite GM and Svevo respectively. XIP1 was already identified as a potential allergen in Bakers'asthma by Lehto et al (2010) and, recently, in wheat food allergy by Šotkovský et al. (2011). The XIP-III we identified in Svevo has not been previously identified as an allergen; however, it shares 86% identity with XIP1 let us hypothesize that also this protein can have a role in triggering allergies. Thaumatin-like protein (TLP) was only found in the Bobwhite genotypes. This protein belongs to the glycosyl hydrolase family, in which another member (TLP PWIR2) was identified as one of the allergens responsible for Baker's asthma using IgE binding and skin print test performed with purified extracts (Lehto et al 2010). Yet, it is not currently described in the recognized IUIS allergens. Although the protein identified in this study shares only 40% similarity with TLP PWIR2, it may be a potential allergen as it has been detected in sera from patient suffering from Baker's asthma.

Enolase was identified as many spots common to Svevo *wt* and its GM line, but also in specific spots of Svevo *wt*, recognized from all three sera. This protein was also found in Bobwhite *wt*, but in this case it was recognized only from the patient affected from Baker's asthma. In all cases, this protein was identified together with another allergen in the same spot. However, enolases constitute a highly conserved family in which many members have been described as fungal and latex allergens (Postigo et al., 2011). Nothing is known in the case of wheat enolase, but it shares 88% identity with latex enolase.

Barley β-amylase was described as an important allergen in occupational allergies, among which Baker's asthma (Sandiford et al, 1994). Wheat β-amylase which shares 89% identity with its barley homologue was identified in all four genotypes. Its detection with all sera types, confirms its involvement in both food and respiratory allergies, as already reports by Šotkovský et al (2008) and Larré et al (2011). .

The proteins described above as allergens or potential allergens are present in the majority of the spots of Svevo and Bobwhite genotypes but never as a single

identification. It is obvious that the separation of spots by 2D electrophoresis is far from complete, which leads to the identification of more than one protein per spot. Other proteins were identified in these spots and it is clear that the simultaneous presence of an allergen cannot completely exclude a potential allergenicity of these proteins.

Only three spots specific of the Svevo GM genotype were identified: seed maturation protein, 1-Cys peroxiredoxin, and guanine nucleotide binding protein (subunit beta like).Only 1-Cys peroxiredoxin was described as potential allergen by Phar et al (2012).

Table 5: List of identified common proteins from 2D gels between Svevo wt (Fig. 5) and Svevo-GM line (Fig. 6). Spot Number: assigned protein spot number corresponding to those indicated in Figs. 5 and 6, Sub-group: Sub-group to which the protein belongs. All the proteins in a sub-group are identi ed with the same valid peptides, 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, 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, Serum: serum for which the IgE-binding spot was detected .

Spot Number	Sub-group	Prot Id TC or Uniprot	Best homologue protein name (Common Protein)	log (E-value)	Coverage	MW	Total Unique Peptides	Serum
S3=SG3	1.01	B7U6L4	Globulin 3 OS=Triticum aestivum GN=glo-3A PE=4 SV=1	-26,763792	17	66,2	6	68
	2.01	TC425413	Globulin-2 precursor; n=1; Zea mays	-16,727694	12	49,3	3	
S5=SG4	1.01	B7U6L4	Globulin 3 OS=Triticum aestivum GN=glo-3A PE=4 SV=1	-43,39569	19	66,2	8	68
	2.01	TC425413	Globulin-2 precursor; n=1; Zea mays|	-36,012054	22	49,3	7	
S6=SG5	1.01	B7U6L4	Globulin 3 OS=Triticum aestivum GN=glo-3A PE=4 SV=1	-45,197323	16	66,2	8	68
	2.01	TC425413	Globulin-2 precursor; n=1; Zea mays|	-34,735435	24	49,3	5	
S7=SG6	1.01	B7U6L4	Globulin 3 OS=Triticum aestivum GN=glo-3A PE=4 SV=1	-50,35024	21	66,2	8	68
	2.01	TC425413	Globulin-2 precursor; n=1; Zea mays	-21,588572	15	49,3	5	
	3.01	TC373663	Aspartate aminotransferase (Fragment) OS=Triticum aestivum PE=2 SV=1	-24,51145	17	41,8	4	
S9=SG11	1.01	B7U6L4	Globulin 3 OS=Triticum aestivum GN=glo-3A PE=4 SV=1	-47,53899	26	66,2	9	68
	2.01	TC382510	Phytepsin precursor (Aspartic proteinase) [Contains: Phytepsin 32 kDa subunit; Phytepsin 29 kDa subunit; Phytepsin 16 kDa subunit; Phytepsin 11 kDa subunit]; n=1; Hordeum vulgare	-18,882378	24	28,8	4	

S10=SG9	2.01	TC425413	Globulin-2 precursor; n=1; Zea mays	-44,65452	30	49,3	10	68
	3.01	TC373663	Aspartate aminotransferase (Fragment) OS=Triticum aestivum PE=2 SV=1	-30,263525	22	41,8	4	
S11=SG10	2.01	TC425413	Globulin-2 precursor; n=1; *Zea mays*	-43,054245	27	49,3	8	68
	3.01	TC373663	Aspartate aminotransferase, cytoplasmic; n=5 *Oryza sativa* subsp. japonica (Rice)	-20,458721	14	42,4	4	
	4.01	TC402211	Alpha-amylase/trypsin inhibitor CM3 precursor; n=3; Triticum	-21,47958	38	18,29	4	
S13=SG20	2.01	TC425413	Globulin-2 precursor; n=1; *Zea mays*	-13,796424	7	49,3	3	68
	4.01	TC377861	Beta-amylase; n=3; *Hordeum vulgare*	-18,281	21	30,2	4	
S16=SG22	1.01	P93606	Superoxide dismutase OS=*Triticum aestivum* GN=SOD3.2 PE=2 SV=1	-50,354847	51	25,2	10	68
	3.01	TC402211	Alpha-amylase/trypsin inhibitor CM3 precursor; n=3; *Triticum*	-30,129051	47	18,1	5	
S17=SG27	1.01	TC403071	Cupin family protein, expressed; n=2*; Oryza sativa* Japonica Group (Rice)	-51,99327	35	27,7	7	68, 781, 458
	2.01	TC428590	Triosephosphate isomerase; n=1; *Triticum aestivum* (Wheat)	-24,44944	26	32,3	6	
	7.01	TC402211	Alpha-amylase/trypsin inhibitor CM3 precursor; n=3; *Triticum*	-31,500242	46	18,29	5	
S18=SG24	1.01	TC403071	Cupin family protein, expressed; n=2; *Oryza sativa* Japonica Group (Rice)	-39,18621	42	27,7	8	68, 781, 458
	2.02	TC428590	Triosephosphate isomerase; n=1; *Triticum aestivum* (Wheat)	-27,222885	33	32,3	7	
			Dehydroascorbate reductase OS=*Triticum aestivum* GN=DHAR PE=2 SV=1					

	3.01	TC369624		-14,411169	23	23,3	3	
S19=SG25	2.02	TC428590	Triosephosphate isomerase; n=1; *Triticum aestivum* (Wheat)	-22,305655	28	32,3	7	68
	3.01	TC392505	Cupin family protein, expressed; n=2; *Oryza sativa Japonica* Group (Rice)	-17,475735	24	27,7	3	
S20=SG26	3.01	T389920	Triosephosphate isomerase; n=1; *Triticum aestivum* (Wheat)	-19,99038	22	32,3	4	68
	4.02	TC369624	Dehydroascorbate reductase OS=*Triticum aestivum* GN=DHAR PE=2 SV=1	-24,331146	36	23,3	4	
S27=SG13	1.01	Q9ST57	Serpin-Z2A OS=*Triticum aestivum* PE=1 SV=1	-38,037704	28	43,2	6	68, 781, 458
	4.01	TC383884	Enolase; n=2; Oryza sativa Japonica Group (Rice)	-36,734863	21	52	6	
S28=SG14	1.01	TC374294	Serpin-Z2A OS=*Triticum aestivum* PE=1 SV=1	-46,66467	33	43,2	7	68, 781, 458
	2.01	TC383884	Enolase; n=2; *Oryza sativa* Japonica Group (Rice)	-45,53541	26	52	8	
S29=SG15	1.01	TC374294	Serpin-Z2A OS=*Triticum aestivum* PE=1 SV=1	-45,04376	29	43,2	8	68, 781, 458
	2.01	TC383884	Enolase; n=2; *Oryza sativa* Japonica Group	-50,33027	26	52	9	
S30=SG16	1.01	TC374294	Serpin-Z2A OS=*Triticum aestivum* PE=1 SV=1	-34,948277	28	43,2	7	68, 458
	2.01	TC383884	Enolase; n=2; *Oryza sativa* Japonica Group (Rice)	-56,550793	29	52	9	

Table 6: List of identified specific proteins from 2D gel of Svevo-GM line (Fig. 6). Spot Number: assigned protein spot number corresponding to those indicated in Fig. 6, Sub-group: Sub-group to which the protein belongs. All the proteins in a sub-group are identi ed with the same valid peptides, 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, 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, Serum: serum for which the IgE-binding spot was detected .

Spot Number	Sub-group	Prot Id TC or Uniprot	Best homologue protein name (Specific for Svevo-GM line)	log (E-value)	Coverage	MW	Total Unique Peptides	Serum
SG1	1.01	TC389327	Embryo globulin; n=2; Triticeae	-55,515205	31	45,09	9	68, 781, 458
SG2	1.01	TC389327	Embryo globulin; n=2; Triticeae	-34,790607	16	45,09	6	68, 781, 458
SG8	1.01	TC399106	Embryo globulin; n=2; *Triticeae* (Barley)	-27,99566	21	40,1	6	781
	2.01	TC425413	Globulin-2 precursor; n=1; *Zea mays* (Maize)	-28,908264	22	49,3	6	
SG12	1.01	TC382510	Phytepsin precursor (Aspartic proteinase) [Contains: Phytepsin 32 kDa subunit; Phytepsin 29 kDa subunit; Phytepsin 16 kDa subunit; Phytepsin 11 kDa subunit]; n=1; *Hordeum vulgare* (Barley)	-25,939905	32	28,8	5	68, 781, 458
	2.02	TC421662	Hydroxyproline-rich glycoprotein DZ-HRGP precursor; n=1; Volvox carteri f. nagariensis	-28,08037	16	38,6	6	
	5.01	P93693	Serpin-Z1B OS=*Triticum aestivum* PE=1 SV=1	-13,838033	6	42,9	3	
SG17	1.01	CD914053	Seed maturation protein [*Oryza sativa* Japonica Group]	-30,760145	49	18,3	5	68, 458
SG18	1.01	CD914053	Seed maturation protein [*Oryza sativa* Japonica Group]	-30,760145	49	18,3	5	68
	3.01	TC368606	Protein disulfide isomerase 2 precursor; n=3; Triticum	-15,335358	10	58,09	4	

SG19	1.01	B7U6L4	Globulin 3 OS=*Triticum aestivum* GN=glo-3A PE=4 SV=1	-28,378788	10	66,2	5	68
	3.01	TC373687	Guanine nucleotide-binding protein subunit beta-like protein; n=4; *Oryza sativa* (Rice),	-21,451077	31	32,2	3	
SG23	1.01	Q6W8Q2	1-Cys peroxiredoxin PER1 OS=*Triticum aestivum* GN=PER1 PE=2 SV=1	-32,82117	40	23,9	8	68, 781
	2.01	TC394284	Dehydroascorbate reductase OS=*Triticum aestivum* GN=DHAR PE=2 SV=1	-24,212635	36	23,3	6	
	3.01	TC417836	Superoxide dismutase OS=*Triticum aestivum* GN=SOD3.2 PE=2 SV=1	-30,172518	35	25,2	7	
SG28	1.01		Embryo globulin; n=2; Triticeae	-30,550413	20	45,09	6	68
	2.01	Q8H0K8	Xylanase inhibitor OS=*Triticum aestivum* GN=xiI PE=1 SV=1	-15,543633	12	40,8	3	
SG29	1.01	TC383936	Embryo globulin; n=2; *Triticeae Hordeum vulgare* (Barley)	-31,811724	13	45,09	6	68
	2.01	Q8H0K8	Xylanase inhibitor OS=*Triticum aestivum* GN=xiI PE=1 SV=1	-15,023329	12	40,79	3	

Table 7: List of identified specific proteins from 2D gel of Svevo *wt* (Fig. 5). Spot Number: assigned protein spot number corresponding to those indicated in Fig. 5, Sub-group: Sub-group to which the protein belongs. All the proteins in a sub-group are identi ed with the same valid peptides, 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, 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, Serum: serum for which the IgE-binding spot was detected .

Spot Number	Sub-group	Prot Id TC or Uniprot	Best homologue protein name (Specific for Svevo wt)	log (E-value)	Coverage	MW	Total Unique Peptides	Serum
S1	1.01	B7U6L4	Globulin 3 OS=*Triticum aestivum* GN=glo-3A PE=4 SV=1	-24,010012	14	66,2	5	68
	3.01	TC404101	Endo-1,4-beta-glucanase; n=1; *Malus x domestica*	-16,315155	15	54,3	4	
S2	1.01	B7U6L4	Globulin 3 OS=*Triticum aestivum* GN=glo-3A PE=4 SV=1	-26,382036	16	66,2	6	68
	3.01	TC404101	Endo-1,4-beta-glucanase; n=1; *Malus x domestica*	-16,568462	14	54,3	3	
S4	1.01	B7U6L4	Globulin 3 OS=*Triticum aestivum* GN=glo-3A PE=4 SV=1	-38,01104	16	66,2	7	68
	2.01	TC425413	Globulin-2 precursor; n=1; *Zea mays* (Maize)	-27,167355	19	49,3	6	
	5.01	TC373663	Aspartate aminotransferase (Fragment) OS=*Triticum aestivum* PE=2 SV=1	-17,369188	14	41,8	4	
S8	1.01	B7U6L4	Globulin 3 OS=*Triticum aestivum* GN=glo-3A PE=4 SV=1	-55,864117	29	66,2	13	68
	2.01	TC425413	Globulin-2 precursor; n=1*; Zea mays* (Maize)	-35,652462	30	49,3	7	
S12	1.01	TC369421	Xylanase inhibitor XIP-III; n=1; *Triticum aestivum* (Wheat)	-45,262867	40	33,7	8	68
			Embryo globulin; n=2; Triticeae					

	2.01	TC389327		-20,565174	17	45,09	4	
	3.01	TC425413	Globulin-2 precursor; n=1; *Zea mays* (Maize)	-22,926228	15	49,3	5	
S15	1.01	TC408407	Glucose and ribitol dehydrogenase homolog Barley	-33,897038	27	36,29	6	68
	2.01	TC425413	Globulin-2 precursor; n=1; *Zea mays* (Maize)	-33,449093	22	49,3	6	
	4.01	TC408407	Alpha-amylase/trypsin inhibitor CM3 precursor; n=3; Triticum	-28,436291	46	18,29	5	
S21	1.01	TC428590	Triosephosphate isomerase; n=1; *Triticum aestivum* (Wheat)	-15,235525	17	32,3	4	68, 781, 458
	2.01	TC403071	Cupin family protein, expressed; n=2; *Oryza sativa* Japonica Group (Rice)	-18,966576	24	27,7	3	
S22	1.01	TC428590	Triosephosphate isomerase; n=1; *Triticum aestivum* (Wheat)	-46,468967	47	32,3	10	68
	2.01	TC374136	Glutathione transferase OS=*Triticum aestivum* GN=gstu1B PE=2 SV=1	-16,240936	16	24,9	3	
	7.01	TC369624	Dehydroascorbate reductase OS=Triticum aestivum GN=DHAR PE=2 SV=1	-22,285723	36	23,3	5	
S24	1.01	B7U6L4	Globulin 3 OS=*Triticum aestivum* GN=glo-3A PE=4 SV=1	-19,055517	5	66,2	3	68
	2.01	TC428590	Triosephosphate isomerase; n=1; *Triticum*	-9,3902187	13	32,29	3	
S25	1.01	CK215488	Lactoylglutathione lyase; n=4; Oryza	-29,020706	44	19,5	6	68, 458
	2.01	CJ541649	Embryo globulin; n=2; Triticeae	-14,209434	35	10	3	
	4.01	Q41593	Serpin-Z1A OS=*Triticum aestivum* GN=WZCI PE=1 SV=1	-16,455736	13	43	4	

S26	1.01	TC374296	Lactoylglutathione lyase; n=4; *Oryza sativa* (Rice)	-17,51442	17	32,9	4	68, 458
	2.01	CJ541649	Embryo globulin; n=2; Triticeae	-12,871924	35	10	3	
	3.01	Q9ST58	Serpin-Z1C OS=*Triticum aestivum* PE=1 SV=1	-12,374276	10	42,8	3	
S31	1.01	TC374294	Serpin; n=1; *Triticum aestivum* (Wheat)	-16,712109	11	45,40	4	458
	3.01	TC369723	Enolase; n=2; *Oryza sativa Japonica* Group (Rice)	-18,658802	13	30	4	
S32	1.01	TC380640	Serpin-Z2B OS=*Triticum aestivum* PE=1 SV=1	-24,102434	15	42,9	5	68
	2.02	TC380730	Beta-amylase precursor; n=1; *Hordeum vulgare* subsp. spontaneum (Barley)	-33,465237	30	37	6	
	5.01	TC383884	Enolase; n=2; *Oryza sativa* Japonica Group (Rice)	-34,937714	22	52	7	
	6.01	Q9ZR33	Glycosyltransferase 75 OS=*Triticum aestivum* GN=rgp PE=2 SV=1	-18,573215	15	41,4	3	
S33	1.01	P93692	Serpin-Z2B OS=*Triticum aestivum* PE=1 SV=1	-24,885109	15	42,9	6	68
	2.03	TC380730	Beta-amylase precursor; n=1; *Hordeum vulgare* subsp. spontaneum (Barley)	-22,879698	19	36,8	5	
	4.01	TC383884	Enolase; n=2; *Oryza sativa* Japonica Group (Rice)	-44,08806	26	52	8	

Table 8: List of identified common proteins from 2D gels between Bobwhite *wt* (Fig. 8) and Bobwhite-GM line (Fig. 9). Spot Number: assigned protein spot number corresponding to those indicated in Figs. 5 and 6, Sub-group: Sub-group to which the protein belongs. All the proteins in a sub-group are identi ed with the same valid peptides, 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, 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, Serum: serum for which the IgE-binding spot was detected .

Spot Number	Sub-group	Prot Id TC or Uniprot	Best homologue protein name (Common proteins)	log (E-value)	Coverage	MW	Total Unique Peptides	Serum
B1=BG34	1.01	TC380640	Serpin; n=2; *Triticum aestivum* (Wheat)	-29,709045	20	44,59	7	68
	3.03	TC386671	Beta-amylase precursor; n=1; *Hordeum vulgare* subsp. Spontaneum (Barley)	-16,513548	17	32,90	3	
	5.01	TC390079	Aspartic proteinase; n=1; *Triticum aestivum* (Wheat)	-17,190969	16	46,09	4	
B2=BG33	1.01	TC380640	Serpin; n=2; *Triticum aestivum* (Wheat)	-21,472857	14	44,59	5	68
B3=BG32	1.01	TC380640	Serpin; n=2; *Triticum aestivum* (Wheat)	-18,069317	14	44,59	4	68
	2.01	P12783	Phosphoglycerate kinase, cytosolic OS=*Triticum aestivum* PE=2 SV=1	-34,307091	51	42	7	
B6=BG31	1.01	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase OS=*Triticum aestivum* GN=ga3pd PE=2 SV=1	-20,316355	24	36,40	4	68
	2.02	TC372049	Fructose-bisphosphate aldolase; n=1; *Triticum aestivum* (Wheat)	-20,59034	41	23,60	5	
B7=BG30	1.01	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase OS=*Triticum aestivum* GN=ga3pd PE=2 SV=1	-36,989994	52	36,40	4	68
	2.01	TC425413	Globulin-2 precursor; n=1; *Zea mays* (Maize)	-39,999851	28	49,29	7	
B8=BG9	1.01	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase OS=*Triticum aestivum* GN=ga3pd PE=2 SV=1	-32,133533	43	36,40	6	68, 458

	1.02	TC432185	Fructose-bisphosphate aldolase cytoplasmic isozyme; n=2; Oryza sativa Japonica Group (Rice)	-14,387746	12	46,70	3	
B9=BG29	1.01	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase OS=*Triticum aestivum* GN=ga3pd PE=2 SV=1	-25,684849	36	36,40	5	68, 458
	2.01	TC425413	Globulin-2 precursor; n=1; *Zea mays* (Maize)	-33,795158	25	49,29	6	
	4.01	TC373663	Aspartate aminotransferase, cytoplasmic; n=5; *Magnoliophyta* (Rice)	-22,06262	30	42,40	4	
B10=BG10	2.01	TC414845	Globulin-2 precursor; n=1; *Zea mays* (Maize)	-12,345824	19	27,89	3	68
	3.01	TC382425	Aldose reductase; n=1; Hordeum vulgare|Rep: Aldose reductase - Hordeum vulgare (Barley), partial (80%)	-14,266401	17	28,60	3	
B15=BG15	1.02	TC422366	26 kDa endochitinase 1 precursor; n=1; *Hordeum vulgare* (Barley)	-16,547998	22	30,79	4	68
	2.01	Q8LK23	Peroxidase OS=*Triticum aestivum* GN=WSP1 PE=2 SV=1	-13,640544	11	38,70	3	
	3.01	TC387479	Globulin-like protein; n=1; *Oryza sativa* Japonica Group (Rice)	-11,064286	12	45,29	3	
B17=BG17	1.01	TC408407	Glucose and ribitol dehydrogenase homolog Barley	-14,644011	41	13,89	3	38, 68, 458
B19=BG19	1.01	P16347	Endogenous alpha-amylase/subtilisin inhibitor OS=*Triticum aestivum* PE=1 SV=1	-60,213375	76	19,5	13	68, 458
	2.01	Q8S4P7	Thaumatin-like protein OS=*Triticum aestivum* PE=2 SV=1	-11,87844	16	23,5	3	
B21=BG22	1.01	A4GFQ9	Dimeric alpha-amylase inhibitor OS=*Triticum turgidum* subsp. dicoccoides PE=4 SV=1	-15,351416	36	13,19	3	68, 458
B24=BG24	2.01	TC385921	Embryo globulin; n=2; *Triticeae* (Barley)	-21,063662	18	29,89	5	68
			22.0 kDa class IV heat shock protein precursor; n=1; *Glycine max* (Soybean)					

B25=BG25	1.01	TC387936		-11,598599	11	28,20	3	38, 68
	1.02	B7U6L4	Globulin 3 OS=*Triticum aestivum* GN=glo-3A PE=4 SV=1	-12,615199	7	66,19	3	
B28=BG37	1.02	B7U6L4	Globulin 3 OS=*Triticum aestivum* GN=glo-3A PE=4 SV=1	-23,394577	14	66,19	6	38, 458
B29=BG36	1.01	TC425413	Globulin-2 precursor; n=1; *Zea mays* (Maize)	-41,618752	24	49,29	8	38, 458
	2.04	TC397230	Embryo globulin; n=2; *Triticeae*	-14,819015	11	59	3	
	3.02	TC421662	Hydroxyproline-rich glycoprotein DZ-HRGP precursor; n=1; Volvox carteri f. nagariensis	-31,44507	16	38,59	6	
B30=BG35	1.01	TC425413	Globulin-2 precursor; n=1; *Zea mays* (Maize)	-27,56671	19	49,29	6	38, 458
	2.02	TC419611	Embryo globulin; n=2; *Triticeae* (Barley)	-17,105364	12	55,59	4	

Table 9: List of identified specific proteins from 2D gel of Bobwhite-GM line (Fig. 9). Spot Number: assigned protein spot number corresponding to those indicated in Figs. 5 and 6, Sub-group: Sub-group to which the protein belongs. All the proteins in a sub-group are identi ed with the same valid peptides, 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, 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, Serum: serum for which the IgE-binding spot was detected .

Spot Number	Sub-group	Prot Id TC or Uniprot	Best homologue protein name (Specific for Bobwhite-GM line)	log (E-value)	Coverage	MW	Total Unique Peptides	Serum
BG12	1.01	TC408407	Glucose and robitol dehydrogenase homolog Barley	-17,499018	15	36,29	4	38, 68, 458
	2.02	TC382451	Lipoprotein-like; n=2; *Oryza sativa* (Rice)	-15,071277	27	26,29	3	
BG41	1.02	TC408407	Glucose and robitol dehydrogenase homolog Barley	-36,71529	44	36,29	8	38, 68, 458
	2.02	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase OS=*Triticum aestivum* GN=GAPC PE=2 SV=1	-16,853962	29	36,40	4	
	3.02	TC382451	Lipoprotein-like; n=2; *Oryza sativa* (Rice)	-20,18667	35	26,29	4	
	7.01	Q8L5C6	Xylanase inhibitor protein 1 OS=*Triticum aestivum* GN=XIPI PE=1 SV=2	-10,553384	18	33,20	3	
BG13	2.01	Q8LK23	Peroxidase OS=*Triticum aestivum* GN=WSP1 PE=2 SV=1	-19,061914	15	38,70	4	68
BG20	1.01	P16347	Endogenous alpha-amylase/subtilisin inhibitor OS=*Triticum aestivum* PE=1 SV=1	-24,214748	30	19,50	5	38, 68, 458
BG23	1.01	TC371775	16.9 kDa class I heat shock protein; n=1; *Triticum aestivum* (Wheat)	-27,38588	40	19,60	5	38, 68, 458
BG25	1.01	TC400242	Embryo globulin; n=2; *Triticeae* (Barley)	-22,995527	13	36,20	5	68

	2.01	P12810	16.9 kDa class I heat shock protein 1 OS=*Triticum aestivum* GN=hsp16.9A PE=2 SV=1	-15,08445	39	16,79	4	

Table 10: List of identified specific proteins from 2D gel of Bobwhite *wt* (Fig. 8). Spot Number: assigned protein spot number corresponding to those indicated in Figs. 5 and 6, Sub-group: Sub-group to which the protein belongs. All the proteins in a sub-group are identi ed with the same valid peptides, 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, 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, Serum: serum for which the IgE-binding spot was detected .

Spot Number	**Sub-group**	**Prot Id TC or Uniprot**	**Best homologue protein name (Specific for Bobwhite *wt*)**	**log (E-value)**	**Coverage**	**MW**	**Total Unique Peptides**	**Serum**
B4	1.01	TC380640	Serpin; n=2; *Triticum aestivum* (Wheat)	-15,275397	10	44,59	3	68
B11	1.01	A5YVV3	Glyceraldehyde-3-phosphate dehydrogenase OS=*Triticum aestivum* GN=GAPC PE=2 SV=1	-22,186485	28	36,5	5	68, 458
	2.01	TC382425	Aldose reductase; n=1; Hordeum vulgare (Barley)	-18,119759	26	28,60	4	
B12	1.01	TC386688	Globulin-like protein; n=1; *Oryza sativa* Japonica Group (Rice)	-9,9739161	15	32,79	3	68
B20	2.01	A4GFQ9	Dimeric alpha-amylase inhibitor (Fragment) OS=*Triticum turgidum* subsp. *dicoccoides* PE=4 SV=1	-16,549534	36	13,19	3	68, 458
B23	1.02	TC374459	16.9 kDa class I heat shock protein; n=1; *Triticum aestivum* (Wheat)	-15,724823	29	19,60	4	68
	2.01	TC385921	Embryo globulin; n=2; *Triticeae* (Barley)	-13,710233	14	29,89	3	
B26	1.01	TC385921	Embryo globulin; n=2; *Triticeae* (Barley)	-14,930702	14	29,89	3	38, 458

B27	1.01	TC397230	Embryo globulin; n=2; *Triticeae* (Barley)	-27,714376	11	59	6	38, 458
	1.02	B7U6L4	Globulin 3 OS=*Triticum aestivum* GN=glo-3A PE=4 SV=1	-12,615199	7	66,19	3	
B31	1.01	TC425413	Globulin-2 precursor; n=1; *Zea mays* (Maize)	-34,863571	21	49,29	7	38, 458

Conclusion

Our propose an allergenomic approach in order to assessment the safety of two GM wheat line in comparison to its untransformed genotypes.

Few differences were observed in the amount of specific IgE-binding polypeptides between *wt* and GM lines of Svevo and Bobwhite. This shows that the two transformation events here considered slightly influence the global allergenic potential. As already reported in transcriptomic or proteomic studies, in which a comparison was performed between GM lines of different plant species and their controls, only minor effects were observed in our study, and this despites the sensitivity of IgE-binding method. Using a comparative allergenomic approach, the great majority of the polypeptides identified corresponded to already known potential allergens, although minor polypeptides were not known.

In conclusion, at least for the two specific GM lines here considered, we did not find any significant changes in the allergenic profiles relative to the A/G fractions between the transgenic lines and their cultivated counterpart genotypes, and the allergenomic approach here used allows an extensive characterization of the allergens present.

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Chapter I: Supplementary data

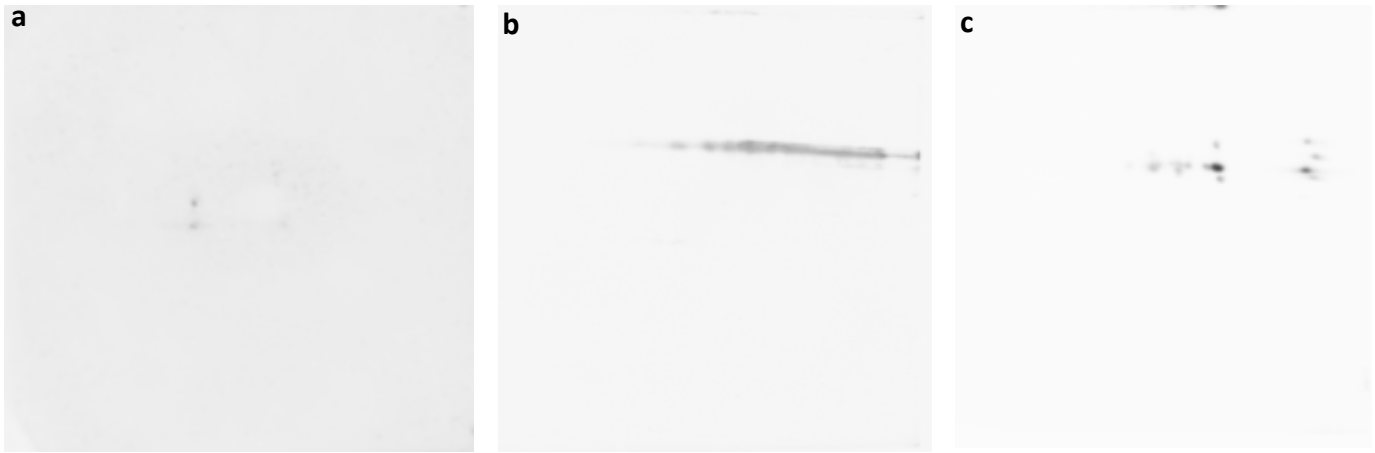

Fig S1: 2D-Immunoblotting performed on A/G fraction of Bobwhite-GM line with anti-peptide antibodies specific for N-terminal sequences of LMW-GS (**a**), γ-gliadins (**b**) and α/β-gliadins (**c**).

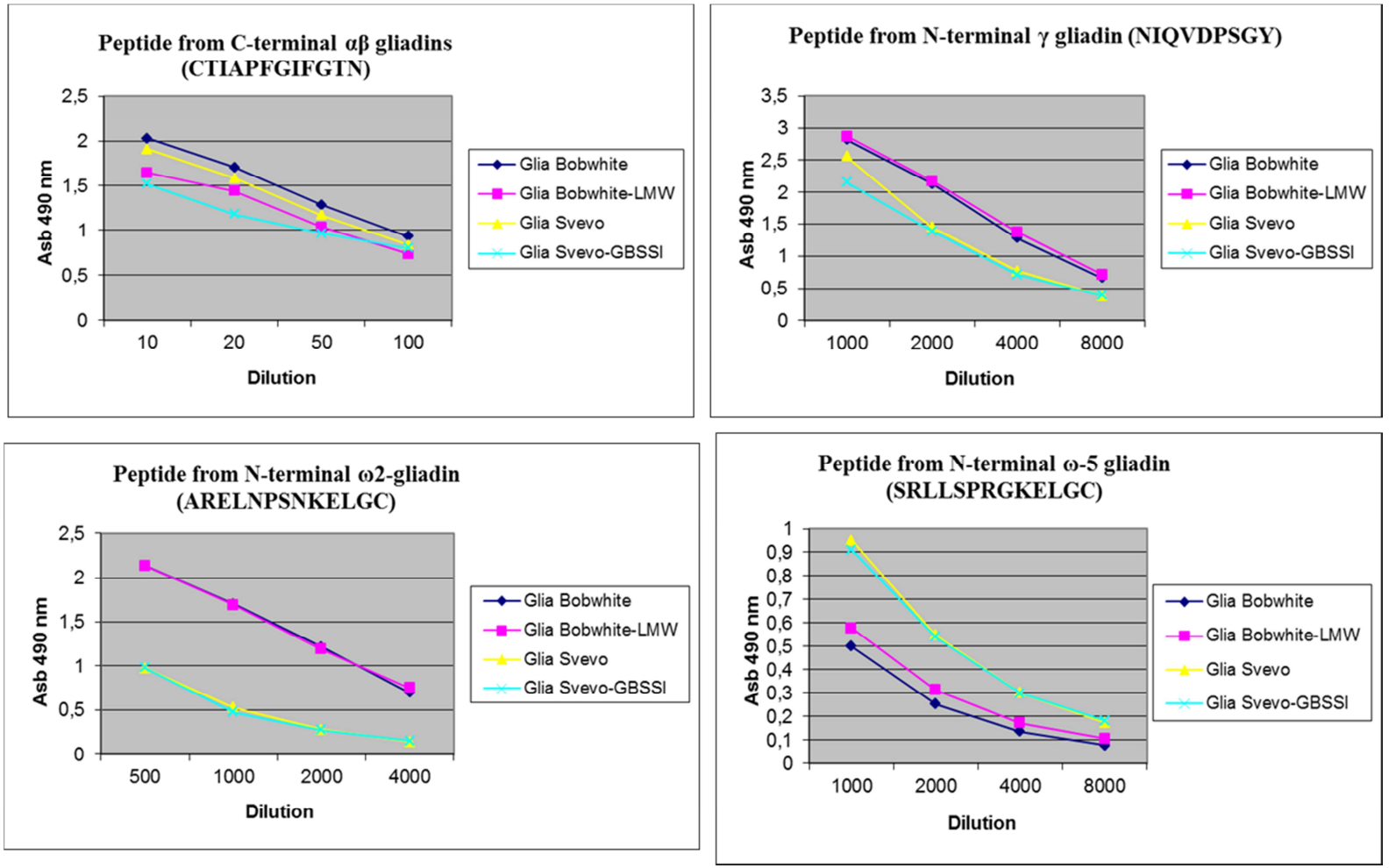

Fig. S2a: ELISA performed with anti-peptide antibodies specific for the different gliadin classes: α/β-, γ-, ω-2-, ω-5-gliadins.

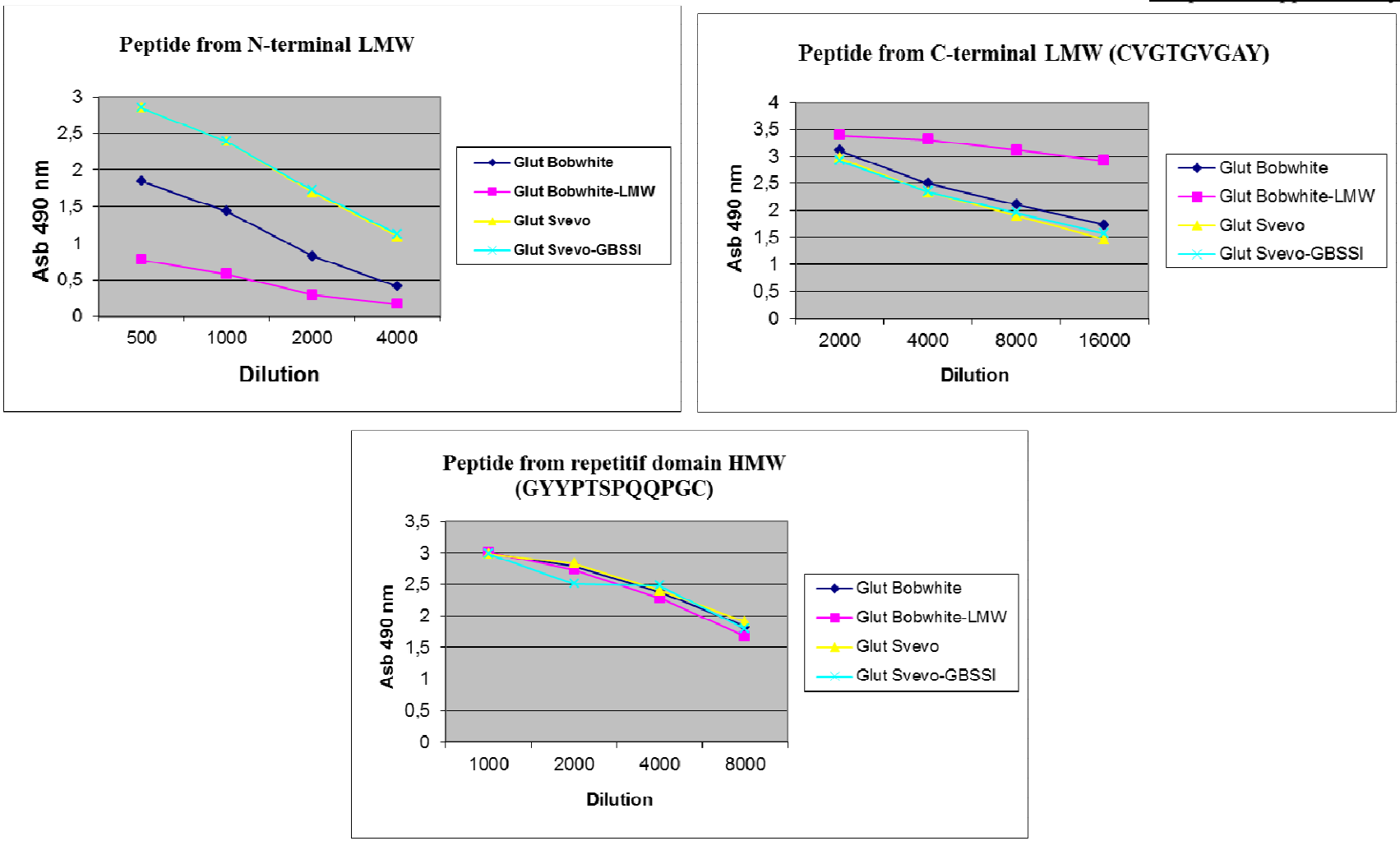

Fig. S2b: ELISA performed with anti-peptide antibodies specific for the different glutenin classes: LMW-GS and HMW-GS.

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**B7U6L4**	**S3=SG3**	AKDQQDEGFVAGPEQQQEHER		-	-	1.01	9,4E-07	2	2426,095947	2426,097168	-0,0011	-0,453403115
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	-	-	1.01	0,00065	2	2138,0625	2138,066406	-0,0038	-1,777306795
1			QASEGDQGHHWPLPPFR	Q1:-17.0265	-	-	1.01	0,00049	3	1941,894775	1941,899536	-0,0047	-2,420310497
1			GSSNLQVVCFEINAER	C9:+57.0215	3	4	1.01 1.02 1.03	6,1E-06	2	1822,87439	1822,87561	-0,0012	-0,658300579
1			VAVANITPGSMTAPYLNTQSFK		3	4	1.01 1.02 1.03	8,2E-07	2	2310,175781	2310,18042	-0,0044	-1,904613137
1			AFVVPGLTDADGVGYVAQGEGVLTVIENGEKR		-	-	1.01	6,8E-05	3	3261,67041	3260,679932	0,99000001	303,6176453
2	**TC425413**		SKGEGEIYEASEEQIR		2	2	2.01 2.02	3,1E-06	2	1824,859863	1824,86145	-0,0015	-0,821980238
2			AFLQPSHHDADEIAFVR		-	-	2.01	9,3E-06	2	1952,959595	1952,961792	-0,0021	-1,075289845
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	2,7E-05	2	2098,093506	2098,093262	0,0004	0,190649286
3	**TC404101**		LYVGVGDADADHR		2	2	3.01 3.02	0,00059	2	1387,662842	1387,660522	0,0024	1,729529738
3			SNSAMSDGSAANVDLTGGYYDGGNNVK	M5:+15.9949	2	2	3.01 3.02	4,4E-07	3	2680,141602	2680,143311	-0,0015	-0,559671521
3			SYLNAPGPNPNVHTGAVVGGPDENDAFPDDR		-	-	3.01	2,2E-06	3	3192,45874	3192,462158	-0,0033	-1,033684969
3			QVDYVLGDNPLGMSYMVGYGAR	M13:+15.9949 - M16:+15.9949	-	-	3.01	6,2E-05	3	2437,10791	2437,116699	-0,0087	-3,569792271
1	**B7U6L4**	**S5=SG4**	AKDQQDEGFVAGPEQQQEHERGDR		-	-	1.01	0,00042	3	2754,244873	2754,246582	-0,0017	-0,617228687
1			SFHALAQHDVR		4	5	1.01 1.03 1.04 1.05	0,00027	2	1280,650391	1280,649902	0,0006	0,468512148
1			VLTAALKTSDER		-	-	1.01	6E-05	2	1303,720825	1303,722046	-0,0012	-0,920441687
1			QGKEEEEKSISIVR	Q1:-17.0265	-	-	1.01	0,00012	2	1614,833862	1614,83374	1E-04	0,061925881
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	2	5	1.01 1.02	1,3E-06	2	2138,066162	2138,066406	-1E-04	-0,046771232
1			QASEGGQGHHWPLPPFR		2	5	1.02 1.05	1,1E-05	2	1900,918457	1900,920654	-0,0021	-1,104727864
1			SYTVRQGDVIVAPAGSIMHLANTDGR	M18:+15.9949	2	5	1.01 1.02	0,00019	3	2744,378418	2744,378906	-0,0003	-0,10931436
1			GSAFVVPPGHPVVEIASSR		3	5	1.01 1.04 1.05	0,00022	2	1906,015625	1906,018555	-0,0029	-1,521496177
2	**TC425413**		GGGGSGSEKEDIQPR		-	-	2.01	0,00012	2	1473,692871	1473,693359	-0,0003	-0,203570187
2			FHQITGDQCHHLR	C9:+57.0215	2	2	2.01 2.02	1,9E-05	2	1648,772339	1648,776489	-0,0042	-2,547343493
2			AFLQPSHHDADEIAFVR		-	-	2.01	2,2E-06	2	1952,958618	1952,961792	-0,0031	-1,587332726
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	6,5E-05	2	2098,090088	2098,093262	-0,003	-1,429869771
2			IYVVVEGRDGYFEMACPHISSSGR	C16:+57.0215	-	-	2.01	3,3E-06	3	2729,28125	2729,281494	-0,0002	-0,073279358
2			VVMFINPVSTPGR		-	-	2.01	0,00061	2	1416,76709	1416,767212	-1E-04	-0,070583224
2			FQEFFLIGSGDERPQSFLSVFSDEVIQAALNTR		-	-	2.01	1,4E-08	3	3747,868652	3747,865723	0,0029	0,77377373
1	**B7U6L4**	**S6=SG4**	AKDQQDEGFVAGPEQQQEHER		-	-	1.01	2,1E-05	3	2426,098389	2426,097168	0,0012	0,494621575
1			DQQDEGFVAGPEQQQEHER		-	-	1.01	2,5E-09	2	2226,963379	2226,965088	-0,0016	-0,71846652
1			QASEGDQGHHWPLPPFR	Q1:-17.0265	-	-	1.01	0,00028	3	1941,894043	1941,899536	-0,0054	-2,780782461
1			GSAFVVPPGHPVVEIASSR		3	5	1.01 1.02 1.04	9,2E-05	2	1906,015381	1906,018555	-0,0031	-1,626426935
1			QGDVIVAPAGSIMHLANTDGR		2	5	1.01 1.03	0,00024	3	2122,073242	2122,071289	0,0018	0,848227859
1			GSSNLQVVCFEINAER	C9:+57.0215	3	5	1.01 1.02 1.04	0,00018	2	1822,876221	1822,87561	0,0006	0,329150289
1			AFVVPGLTDADGVGYVAQGEGVLTVIENGEKR		-	-	1.01	2,8E-05	3	3260,684326	3260,680176	0,0041	1,257406354
1			LAVVLEGEGEVEIVCPHLGR	C15:+57.0215	-	-	1.01	0,00027	3	2176,144775	2176,143555	0,0013	0,597387016
2	**TC425413**		GGGGSGSEKEDIQPR		2	2	2.01 2.02	0,00066	2	1473,692627	1473,693359	-0,0006	-0,407140374
2			SKGEGEIYEASEEQIR		2	2	2.01 2.02	3,1E-06	2	1824,859863	1824,86145	-0,0015	-0,821980238
2			GEGEIYEASEEQIR		2	2	2.01 2.02	0,00075	2	1609,734741	1609,734375	0,0003	0,186366171
2			AFLQPSHHDADEIAFVR		-	-	2.01	9,3E-06	2	1952,959595	1952,961792	-0,0021	-1,075289845
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	2,7E-05	2	2098,093506	2098,093262	0,0004	0,190649286

Group	Description	Sample	Sequence	Modifs	Used	on a total of	Sub-groups	E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**B7U6L4**	**S7=SG6**	AKDQQDEGFVAGPEQQQEHER		-	-	1.01	2,1E-05	3	2426,098389	2426,097168	0,0012	0,494621575
1			ILHTISVPGK		2	5	1.01 1.03	0,00051	2	1064,645996	1064,646729	-0,0006	-0,563567281
1			DQQDEGFVAGPEQQQEHER		-	-	1.01	2,5E-09	2	2226,963379	2226,965088	-0,0016	-0,71846652
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949 - Q1:-17.0265	2	5	1.01 1.03	0,00088	3	2122,036377	2121,040039	0,99699998	470,0524292
1			QASEGDQGHHWPLPPFR	Q1:-17.0265	-	-	1.01	0,00028	3	1941,894043	1941,899536	-0,0054	-2,780782461
1			GSAFVVPPGHPVVEIASSR		3	5	1.01 1.02 1.04	9,2E-05	2	1906,015381	1906,018555	-0,0031	-1,626426935
1			AFVVPGLTDADGVGYVAQGEGVLTVIENGEKR		-	-	1.01	2,8E-05	3	3260,684326	3260,680176	0,0041	1,257406354
1			LAVVLEGEGEVEIVCPHLGR	C15:+57.0215	-	-	1.01	0,00027	3	2176,144775	2176,143555	0,0013	0,597387016
2	**TC425413**		WQEGGDEGR		-	-	2.01	0,00028	2	1033,434326	1033,433838	0,0006	0,580588758
2			SKGEGEIYEASEEQIR		2	3	2.01 2.02	2,8E-06	2	1824,86145	1824,86145	1E-04	0,054798678
2			DGYFEMACPHISSSGR	C8:+57.0215 - M6:+15.9949	2	3	2.01 2.02	2,6E-09	2	1829,753662	1829,758545	-0,0049	-2,677948952
2			AFLQPSHHDADEIAFVR		-	-	2.01	0,0002	2	1952,95874	1952,961792	-0,003	-1,536128402
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	0,00012	2	2098,09668	2098,093262	0,0036	1,715843558
3	**TC373663**		VATVQCLSGTGSLR	C6:+57.0215	-	-	3.01	4,7E-06	2	1448,752808	1448,753052	-1E-04	-0,069024868
3			LIFGADSPAIQENR		-	-	3.01	0,00012	2	1530,788574	1530,791504	-0,0029	-1,894444704
3			MFVADGGELLMAQSYAK	M1:+15.9949	-	-	3.01	6,1E-06	2	1846,875488	1846,871826	0,0037	2,00338769
3			EYLPITGLADFNK		-	-	3.01	0,00055	2	1480,769287	1480,768555	0,0007	0,472727478
1	**B7U6L4**	**S9=SG11**	AKDQQDEGFVAGPEQQQEHERGDR		-	-	1.01	0,00042	3	2754,244873	2754,246582	-0,0017	-0,617228687
1			VLTAALKTSDER		-	-	1.01	6E-05	2	1303,720825	1303,722046	-0,0012	-0,920441687
1			QGKEEEEKSISIVR	Q1:-17.0265	-	-	1.01	0,00012	2	1614,833862	1614,83374	1E-04	0,061925881
1			SYTVRQGDVIVAPAGSIMHLANTDGR	M18:+15.9949	2	5	1.01 1.02	0,00019	3	2744,378418	2744,378906	-0,0003	-0,10931436
1			GSAFVVPPGHPVVEIASSR		3	5	1.01 1.04 1.05	0,00022	2	1906,015625	1906,018555	-0,0029	-1,521496177
1			GSSNLQVVCFEINAER	C9:+57.0215	3	5	1.01 1.04 1.05	5,3E-05	2	1822,873779	1822,87561	-0,0018	-0,987450778
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949 - Q1:-17.0265	3	5	1.01 1.02 1.03	4,7E-06	2	2121,039551	2121,039795	-1E-04	-0,047146685
1			LAVVLEGEGEVEIVCPHLGR	C15:+57.0215	-	-	1.01	0,00014	3	2176,145996	2176,143555	0,0026	1,194774032
1			AFVVPGLTDADGVGYVAQGEGVLTVIENGEK		2	5	1.01 1.03	1,6E-07	3	3104,578857	3104,579102	-0,0002	-0,064420968
2	**TC382510**		IGAPGVVSQECK	C11:+57.0215	-	-	2.01	0,00071	2	1244,62793	1244,630859	-0,0028	-2,249662876
2			IPSPMGESSVDCGR	C12:+57.0215	-	-	2.01	6,5E-06	2	1491,658569	1491,657104	0,0016	1,072632551
2			LASMPDIAFSIGGK	M4:+15.9949	-	-	2.01	9,7E-05	2	1422,728149	1422,730103	-0,0019	-1,335460663
2			CAAIADSGTSLLSGPTAIITQINEK	C1:+57.0215	-	-	2.01	4,9E-07	2	2531,301758	2531,30249	-0,0008	-0,316042811
2	**TC425413**	**S10=SG9**	GGGGSGSEKEDIQPR		2	3	2.01 2.02	0,00033	2	1473,692749	1473,693359	-0,0004	-0,271426857
2			WQEGGDEGR		-	-	2.01	0,00028	2	1033,434326	1033,433838	0,0006	0,580588758
2			FHQITGDQCHHLR	C9:+57.0215	3	3	2.01 2.02 2.03	1,9E-06	2	1648,775024	1648,776489	-0,0015	-0,909765482
2			SKGEGEIYEASEEQIR		2	3	2.01 2.02	2,3E-05	2	1824,85791	1824,86145	-0,0035	-1,917953849
2			GEGEIYEASEEQIR		2	3	2.01 2.02	0,00011	2	1609,734253	1609,734375	-1E-04	-0,062122047
2			DGYFEMACPHISSSGR	C8:+57.0215 - M6:+15.9949	2	3	2.01 2.02	2,6E-09	2	1829,753662	1829,758545	-0,0049	-2,677948952
2			AFLQPSHHDADEIAFVR		-	-	2.01	0,0002	2	1952,95874	1952,961792	-0,003	-1,536128402
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	0,00012	2	2098,09668	2098,093262	0,0036	1,715843558
2			EGDVIVIPAGSIVYSANTHR	E1:-18.0106	-	-	2.01	7,3E-05	2	2080,080078	2080,08252	-0,0025	-1,201875448
2			VVMFINPVSTPGR		2	3	2.01 2.02	0,00062	2	1416,769287	1416,767212	0,0021	1,48224771
3	**TC373663**		VATVQCLSGTGSLR	C6:+57.0215	-	-	3.01	4,7E-06	2	1448,752808	1448,753052	-1E-04	-0,069024868
3			LIFGADSPAIQENR		-	-	3.01	0,00012	2	1530,788574	1530,791504	-0,0029	-1,894444704
3			MFVADGGELLMAQSYAK	M1:+15.9949	-	-	3.01	6,1E-06	2	1846,875488	1846,871826	0,0037	2,00338769
3			EYLPITGLADFNK		-	-	3.01	0,00055	2	1480,769287	1480,768555	0,0007	0,472727478

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
2	**TC425413**	**S11=SG10**	GGGGSGSEKEDIQPR		2	2	2.01 2.02	0,00026	2	1473,692505	1473,693359	-0,0007	-0,474997014
2			SKGEGEIYEASEEQIR		2	2	2.01 2.02	2,5E-06	2	1824,8573	1824,86145	-0,0041	-2,246745825
2			DGYFEMACPHISSSGR	C8:+57.0215 - M6:+15.9949	2	2	2.01 2.02	6,9E-13	2	1829,753662	1829,758545	-0,0049	-2,677948952
2			GEGEIYEASEEQIR		2	2	2.01 2.02	0,00057	2	1609,733032	1609,734375	-0,0014	-0,869708657
2			AFLQPSHHDADEIAFVR		-	-	2.01	4,2E-07	2	1952,959351	1952,961792	-0,0024	-1,228902817
2			DGYFEMACPHISSSGR	C8:+57.0215	2	2	2.01 2.02	1,2E-05	2	1813,757324	1813,763672	-0,0063	-3,473440409
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	0,00027	2	2098,086914	2098,093262	-0,0062	-2,955064297
			VVMFINPVSTPGR		2	2	2.01 2.02	0,00087	2	1416,766602	1416,767212	-0,0006	-0,423499376
3	**TC373663**		VATVQCLSGTGSLR	C6:+57.0215	-	-	3.01	2E-06	2	1448,752563	1448,753052	-0,0004	-0,276099473
3			LIFGADSPAIQENR		-	-	3.01	2,6E-05	2	1530,789429	1530,791504	-0,002	-1,306513667
3			MFVADGGELLMAQSYAK	M1:+15.9949	-	-	3.01	4,7E-06	2	1846,866821	1846,871826	-0,0049	-2,653135061
3			ALLPFFDSAYQGFASGSLDK		-	-	3.01	5,6E-05	3	2134,04834	2134,049561	-0,0011	-0,515451908
4	**TC402211**		DYVLQQTCGTFTPGSK	C8:+57.0215	-	-	4.01	5,2E-09	2	1801,840942	1801,842896	-0,002	-1,109974742
4			LYCCQELAEISQQCR	C3:+57.0215 - C4:+57.0215 - C14:+57.0215	-	-	4.01	6,9E-05	2	1957,854248	1957,856934	-0,0026	-1,327982664
4			SGNVGESGLIDLPGCPR	C15:+57.0215	-	-	4.01	1,4E-05	2	1727,837524	1727,838501	-0,001	-0,578757823
4			YFIALPVPSQPVDPR		-	-	4.01	2,7E-06	2	1698,919556	1698,921753	-0,0022	-1,294939041
2	**TC425413**	**S13=SG20**	SKGEGEIYEASEEQIR		-	-	2.01	1,8E-06	2	1824,865356	1824,86145	0,004	2,191947222
2			AFLQPSHHDADEIAFVR		-	-	2.01	2,9E-05	2	1952,958008	1952,961792	-0,0037	-1,894558311
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	5,6E-07	2	2098,090088	2098,093262	-0,003	-1,429869771
4	**TC377861**		YDPTAYNTILR		2	2	4.01 4.02	1E-04	2	1326,670044	1326,669312	0,0008	0,603013873
4			LSNQLVEGQNYVNFK		-	-	4.01	2,8E-05	2	1752,888306	1752,891968	-0,0036	-2,053748846
4			SGPELTIEMILQAAQPK	M9:+15.9949	2	2	4.01 4.02	1,3E-05	2	1841,967529	1841,96814	-0,0006	-0,325738549
4			SAPEELVQQVLSAGWR		-	-	4.01	1,8E-05	2	1769,917603	1769,918457	-0,0009	-0,508497953
1	**P93606**	**S16=SG22**	NLKPISEGGGEPPHGK		-	-	1.01	3,3E-06	3	1616,835938	1616,839478	-0,0036	-2,226566076
1			HHATYVANYNK		3	4	1.01 1.02 1.03	0,00056	3	1317,633423	1317,633911	-0,0005	-0,379468083
1			GDASAVVHLQSAIK		3	4	1.01 1.02 1.03	1,7E-06	2	1395,758911	1395,759521	-0,0005	-0,358227909
1			KLSVETTPNQDPLVTK		-	-	1.01	2,4E-06	2	1769,963135	1769,964844	-0,0016	-0,903972745
1			ALEQLDAAVSK		2	4	1.01 1.02	0,00031	2	1144,620117	1144,621338	-0,0011	-0,961016536
1			LSVETTPNQDPLVTK		4	4	1.01 1.02 1.03 1.04	1,1E-05	2	1641,870117	1641,869751	0,0003	0,18271853
1			LGWAIDEDFGSIEK		3	4	1.01 1.02 1.03	5,3E-05	2	1579,763916	1579,764282	-0,0003	-0,189901754
1			KMNAEGAALQGSGWVWLALDK	M2:+15.9949	3	4	1.01 1.02 1.03	7,8E-06	3	2261,139648	2261,138672	0,001	0,44225508
1			LPYDFGALEPAVSGEIMR		-	-	1.01	7,3E-05	2	1964,981445	1964,979126	0,0024	1,221387029
1			TLPDLPYDFGALEPAVSGEIMR		-	-	1.01	7,7E-06	2	2391,1875	2391,19043	-0,003	-1,254605174
3	**TC402211**		LLVAPGQCNLATIHNVR	C8:+57.0215	-	-	3.01	5,1E-07	2	1876,023315	1876,022583	0,0008	0,4264341
3			DYVLQQTCGTFTPGSK	C8:+57.0215	-	-	3.01	2,4E-08	2	1801,840698	1801,842896	-0,0022	-1,22097218
3			LYCCQELAEISQQCR	C3:+57.0215 - C4:+57.0215 - C14:+57.0215	-	-	3.01	1E-06	2	1957,850342	1957,856934	-0,0065	-3,319956779
3			SGNVGESGLIDLPGCPR	C15:+57.0215	-	-	3.01	3,4E-06	2	1727,837524	1727,838501	-0,001	-0,578757823
3			YFIALPVPSQPVDPR		-	-	3.01	5,7E-05	2	1698,9198	1698,921753	-0,0019	-1,118356466

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**TC403071**	**S17=SG27**	AGAAVGGQVVEKER		-	-	1.01	0,00029	2	1370,738037	1370,739136	-0,001	-0,729533434
1			VADAAGTVYR		-	-	1.01	0,00081	2	1022,526978	1022,526978	0	0
1			GKVTYIQEGGSETSSLEVQR		2	2	1.01 1.02	0,00013	3	2168,08667	2168,083496	0,0033	1,522081614
1			VTYIQEGGSETSSLEVQR		2	2	1.01 1.02	2,5E-07	2	1982,965576	1982,967041	-0,0013	-0,655583262
1			VVAEGEAGTVTATDVADAAGTVYR		-	-	1.01	5,8E-05	3	2323,139893	2323,141602	-0,0017	-0,731767714
1			RVVAEGEAGTVTATDVADAAGTVYR		-	-	1.01	1,2E-05	3	2479,242432	2479,242676	-0,0004	-0,161339581
			IYAIFTSNAINSDDPSHPTSEAYSSVSNLLR		-	-	1.01	4,8E-07	3	3369,619873	3369,623779	-0,0038	-1,127722383
2	**TC428590**		EAGSTMAVVAEQTK	M6:+15.9949	-	-	2.01	7,7E-05	2	1437,688477	1437,689453	-0,0009	-0,626004457
2			VATPAQAQEVHANLR		3	3	2.01 2.02 2.03	0,0003	2	1604,847778	1604,85083	-0,0029	-1,807021499
2			SLMGESSEFVGEK	M3:+15.9949	2	3	2.01 2.02	0,00019	2	1415,636719	1415,636353	0,0005	0,353198051
2			IIYGGSVTGASCK	C12:+57.0215	3	3	2.01 2.02 2.03	1,8E-05	2	1312,657593	1312,656982	0,0007	0,533269525
2			VIACVGETLEQR	C4:+57.0215	2	3	2.01 2.02	0,00077	2	1374,706177	1374,704956	0,0012	0,872914612
2			LRPEIQVAAQNCWVK	C12:+57.0215	2	3	2.01 2.02	0,00048	2	1811,959106	1811,958862	0,0002	0,110377774
7	**TC402211**		LLVAPGQCNLATIHNVR	C8:+57.0215	-	-	7.01	3,6E-07	2	1876,022217	1876,022583	-0,0003	-0,159912795
7			LYCCQELAEISQQCR	C3:+57.0215 - C4:+57.0215 - C14:+57.0215	-	-	7.01	1,5E-06	2	1957,855713	1957,856934	-0,0011	-0,561838806
7			DYVLQQTCGTFTPGSK	C8:+57.0215	-	-	7.01	1,3E-06	2	1801,844482	1801,842896	0,0016	0,887979746
7			SGNVGESGLIDLPGCPR	C15:+57.0215	-	-	7.01	5,9E-06	2	1727,837158	1727,838501	-0,0013	-0,752385139
7			YFIALPVPSQPVDPR		-	-	7.01	6,2E-05	2	1698,923706	1698,921753	0,002	1,177217364
1	**TC403071**	**S18=SG24**	AGAAVGGQVVEKER		-	-	1.01	0,00029	2	1370,738037	1370,739136	-0,001	-0,729533434
1			VADAAGTVYR		-	-	1.01	0,00081	2	1022,526978	1022,526978	0	0
1			GKVTYIQEGGSETSSLEVQR		2	2	1.01 1.02	0,00013	3	2168,08667	2168,083496	0,0033	1,522081614
1			VTYIQEGGSETSSLEVQR		2	2	1.01 1.02	2,5E-07	2	1982,965576	1982,967041	-0,0013	-0,655583262
1			VVAEGEAGTVTATDVADAAGTVYR		-	-	1.01	5,8E-05	3	2323,139893	2323,141602	-0,0017	-0,731767714
1			GTVTATDVADAAGTVYR		-	-	1.01	2,6E-07	2	1667,82605	1667,823853	0,0022	1,319084167
1			RVVAEGEAGTVTATDVADAAGTVYR		-	-	1.01	1,2E-05	3	2479,242432	2479,242676	-0,0004	-0,161339581
1			IYAIFTSNAINSDDPSHPTSEAYSSVSNLLR		-	-	1.01	4,8E-07	3	3369,619873	3369,623779	-0,0038	-1,127722383
2			VATPAQAQEVHANLR		3	3	2.01 2.02 2.03	1,3E-05	2	1604,849609	1604,85083	-0,0011	-0,685422003
2	**TC428590**		EAGSTMAVVAEQTK		-	-	2.01	0,00011	2	1421,695801	1421,694458	0,0013	0,914401829
2			SLMGESSEFVGEK	M3:+15.9949	2	3	2.01 2.02	0,00019	2	1415,636719	1415,636353	0,0005	0,353198051
2			IIYGGSVTGASCK	C12:+57.0215	3	3	2.01 2.02 2.03	1,8E-05	2	1312,657593	1312,656982	0,0007	0,533269525
2			VIACVGETLEQR	C4:+57.0215	2	3	2.01 2.02	0,00077	2	1374,706177	1374,704956	0,0012	0,872914612
2			LRPEIQVAAQNCWVK	C12:+57.0215	2	3	2.01 2.02	0,00048	2	1811,959106	1811,958862	0,0002	0,110377774
2			VIACVGETLEQR	C4:+57.0215	2	3	2.01 2.02	0,00045	2	1374,706421	1374,704956	0,0015	1,091143131
3	**TC369624**		AAVGHPDTLGDCPFSQR	C12:+57.0215	-	-	3.01	0,00014	2	1827,844849	1827,844727	0,0002	0,109418482
3			YPTPSLVTPAEYASVGSK		-	-	3.01	7,1E-07	2	1866,948364	1866,948853	-0,0004	-0,214253321
3			WIADSDVITQVIEEK		-	-	3.01	2,6E-05	2	1745,895874	1745,895996	-1E-04	-0,05727718

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
2	**TC428590**	**S19=SG25**	EAGSTMEVVAEQTK	M6:+15.9949	-	-	2.02	0,00048	2	1495,693115	1495,694946	-0,0017	-1,136595368
2			VATPAQAQEVHANLR		3	3	2.01 2.02 2.03	1,3E-05	2	1604,849609	1604,85083	-0,0011	-0,685422003
2			SLMGESSEFVGEK	M3:+15.9949	2	3	2.01 2.02	0,00019	2	1415,636719	1415,636353	0,0005	0,353198051
2			IIYGGSVTGASCK	C12:+57.0215	3	3	2.01 2.02 2.03	1,8E-05	2	1312,657593	1312,656982	0,0007	0,533269525
2			VIACVGETLEQR	C4:+57.0215	2	3	2.01 2.02	0,00077	2	1374,706177	1374,704956	0,0012	0,872914612
2			LRPEIQVAAQNCWVK	C12:+57.0215	2	3	2.01 2.02	0,00048	2	1811,959106	1811,958862	0,0002	0,110377774
2			VIACVGETLEQR	C4:+57.0215	2	3	2.01 2.02	0,00045	2	1374,706421	1374,704956	0,0015	1,091143131
3	**TC392505**		VASPEQAQEVHAAVR		2	2	3.01 3.02	2,6E-06	2	1591,819092	1591,819092	0	0
3			HVIGEDDQFIGK		-	-	3.01	0,00067	2	1357,677002	1357,675049	0,002	1,473106623
3			IIYGGSVNAANCAELAK	C12:+57.0215	2	2	3.01 3.02	6,1E-05	2	1750,87793	1750,879761	-0,0017	-0,97094053
3	**TC389920**	**S20=SG26**	VASPEQAQEVHAAVR		2	2	3.01 3.02	2,6E-06	2	1591,819092	1591,819092	0	0
3			HVIGEDDQFIGK		-	-	3.01	0,00067	2	1357,677002	1357,675049	0,002	1,473106623
3			IIYGGSVNAANCAELAK	C12:+57.0215	2	2	3.01 3.02	6,1E-05	2	1750,87793	1750,879761	-0,0017	-0,97094053
3			GPDFATICNSVTSK	C8:+57.0215	2	2	3.01 3.02	5E-05	2	1496,701294	1496,705444	-0,004	-2,67253685
4	**TC369624**		YPTPSLVTPPEYASVGSK		-	-	4.01	0,00061	2	1892,962402	1892,964355	-0,002	-1,056543946
4			YPTPSLVTPAEYASVGSK		-	-	4.02	1,4E-06	2	1866,945557	1866,948853	-0,0032	-1,71402657
4			WIADSDVITQVIEEK		2	2	4.01 4.02	2,3E-05	2	1745,888672	1745,895996	-0,0073	-4,181233883
4			ALVDELQALEEHLK		2	2	4.01 4.02	3,6E-05	2	1607,862427	1607,864258	-0,0019	-1,181691766
1	**Q9ST57**	**S27=SG13**	DQLVATLGEGEAER		-	-	1.01	0,00049	2	1487,733887	1487,734131	-1E-04	-0,067216314
1			TFVEVNETGTEAAAATIAK		-	-	1.01	8,1E-08	2	1922,969238	1922,970947	-0,0017	-0,88404876
1			VALSLITAGAGGATR		2	2	1.01 1.02	6,5E-05	2	1357,778076	1357,780151	-0,0021	-1,546642065
1			HLGLQLPFSDEADLSEMVDSPMPQGLR	M17:+15.9949 - M22:+15.9949	-	-	1.01	7,7E-05	3	3014,423828	3014,423828	1E-04	0,033173833
1			EDTSGVVLFIGHVVNPLR		-	-	1.01	5E-07	2	1952,056641	1952,060425	-0,0037	-1,895433068
1			LHALAEQVVQFVLADASYADSPR		-	-	1.01	0,00053	3	2500,285645	2500,283447	0,0023	0,919895768
4	**TC383884**		TYDLNFKEENNDGSQK		-	-	4.01	7,9E-06	2	1901,853027	1901,851563	0,0015	0,788705111
4			KYGQDATNVGDEGGFAPNIQENK		2	2	4.01 4.02	7,6E-05	3	2452,13916	2452,137939	0,0013	0,530149639
4			YGQDATNVGDEGGFAPNIQENK		2	2	4.01 4.02	2,8E-07	2	2324,041016	2324,042969	-0,0019	-0,817540824
4			LAMQEFMILPTGAASFK	M3:+15.9949 - M7:+15.9949	-	-	4.01	4,2E-06	2	1886,937256	1886,939453	-0,0022	-1,165909052
4			DPTAQTELDNFMVQQLDGTK		-	-	4.01	4,9E-09	2	2251,054688	2251,055176	-0,0004	-0,17769444
4			QLVLPVPAFNVINGGSHAGNK	Q1:-17.0265	-	-	4.01	8,5E-06	2	2115,128906	2115,13501	-0,006	-2,836698294

Group	Description	Sample	Sequence	Modifs	Used	on a total of	Sub-groups	E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**TC374294**	**S28=SG14**	YKAEAQSVDFQTK		2	2	1.01 1.02	2,1E-06	2	1514,747192	1514,749023	-0,0017	-1,122298121
1			AEAQSVDFQTK		2	2	1.01 1.02	0,00079	2	1223,590698	1223,590698	1E-04	0,081726678
1			DQLVATLGEGEAER		-	-	1.01	0,00018	2	1487,738525	1487,734131	0,0045	3,024733782
1			TFVEVNETGTEAAAATIAK		-	-	1.01	4,6E-07	2	1922,969727	1922,970947	-0,0012	-0,624034464
1			HLGLQLPFSDEADLSEMVDSPMPQGLR	M17:+15.9949 - M22:+15.9949	-	-	1.01	5,8E-05	3	3014,421631	3014,423828	-0,0023	-0,762998223
1			EDTSGVVLFIGHVVNPLR		-	-	1.01	2,7E-06	2	1952,063599	1952,060425	0,0032	1,639293432
1			LHALAEQVVQFVLADASYADSPR		-	-	1.01	0,00019	3	2500,281982	2500,283447	-0,0014	-0,559936523
2	**TC383884**		TYDLNFKEENNDGSQK		-	-	2.01	1,5E-05	2	1901,852783	1901,851563	0,0012	0,6309641
2			YGQDATNVGDEGGFAPNIQENK		-	-	2.01	5E-05	3	2324,040039	2324,042969	-0,0029	-1,247825503
2			KIPLYQHIANLAGNK		-	-	2.01	0,00051	2	1679,957642	1679,959595	-0,0019	-1,130979657
2			IPLYQHIANLAGNK		-	-	2.01	0,00081	2	1551,860352	1551,864624	-0,0042	-2,706421614
2			VNQIGSVTESIEAVK		-	-	2.01	8E-06	2	1573,842651	1573,843628	-0,0009	-0,571848452
2			LAMQEFMILPTGAASFK	M3:+15.9949 - M7:+15.9949	-	-	2.01	2,7E-07	2	1886,938843	1886,939453	-0,0006	-0,317975223
2			DPTAQTELDNFMVQQLDGTK		-	-	2.01	4,9E-09	2	2251,053955	2251,055176	-0,0011	-0,488659739
2			QLVLPVPAFNVINGGSHAGNK	Q1:-17.0265	-	-	2.01	3,9E-05	2	2115,125488	2115,13501	-0,0094	-4,444160938
1	**TC374294**	**S29=SG15**	YKAEAQSVDFQTK		2	2	1.01 1.02	4,6E-05	2	1514,74707	1514,749023	-0,0019	-1,254333258
1			AEAQSVDFQTK		2	2	1.01 1.02	0,00031	2	1223,59021	1223,590698	-0,0004	-0,326906711
1			DQLVATLGEGEAER		-	-	1.01	6,6E-05	2	1487,735352	1487,734131	0,0014	0,941028297
1			GAWTDQFDSR		-	-	1.01	0,00022	2	1182,517944	1182,517944	1E-04	0,084565304
1			DQLVATLGEGEAER		-	-	1.01	8,1E-05	2	1487,733765	1487,734131	-0,0002	-0,134432629
1			TFVEVNETGTEAAAATIAK		-	-	1.01	6,3E-08	2	1922,969482	1922,970947	-0,0015	-0,780042946
1			EDTSGVVLFIGHVVNPLR		-	-	1.01	8,8E-07	2	1952,05896	1952,060425	-0,0014	-0,717190921
1			LHALAEQVVQFVLADASYADSPR		-	-	1.01	3,4E-11	2	2500,289063	2500,283447	0,0056	2,239746094
2	**TC383884**		DGGSDYLGK		2	2	2.01 2.02	0,00027	2	911,4105225	911,4108887	-0,0004	-0,438879967
2			TYDLNFKEENNDGSQK		-	-	2.01	4,4E-07	2	1901,848877	1901,851563	-0,0027	-1,419669151
2			KYGQDATNVGDEGGFAPNIQENK		2	2	2.01 2.02	1,7E-06	3	2452,137451	2452,137939	-0,0005	-0,203903705
2			YGQDATNVGDEGGFAPNIQENK		2	2	2.01 2.02	0,00023	3	2324,039307	2324,042969	-0,0038	-1,635081649
2			VVIGMDVAASEFYNDKDK	M5:+15.9949	-	-	2.01	3,4E-06	2	2016,955688	2016,95874	-0,003	-1,487387896
2			VNQIGSVTESIEAVK		-	-	2.01	1,3E-05	2	1573,842163	1573,843628	-0,0014	-0,889541984
2			LAMQEFMILPTGAASFK	M3:+15.9949 - M7:+15.9949	2	2	2.01 2.02	1,7E-06	2	1886,937256	1886,939453	-0,0022	-1,165909052
2			DPTAQTELDNFMVQQLDGTK		-	-	2.01	5,4E-10	2	2251,050537	2251,055176	-0,0045	-1,9990623
2			QLVLPVPAFNVINGGSHAGNK	Q1:-17.0265	2	2	2.01 2.02	6,7E-07	2	2115,132568	2115,13501	-0,0024	-1,134679437
1	**TC374294**	**S30=SG16**	YKAEAQSVDFQTK		2	2	1.01 1.02	2,1E-06	2	1514,747192	1514,749023	-0,0017	-1,122298121
1			AEAQSVDFQTK		2	2	1.01 1.02	0,00079	2	1223,590698	1223,590698	1E-04	0,081726678
1			DQLVATLGEGEAER		-	-	1.01	0,00018	2	1487,738525	1487,734131	0,0045	3,024733782
1			TFVEVNETGTEAAAATIAK		-	-	1.01	4,6E-07	2	1922,969727	1922,970947	-0,0012	-0,624034464
1			HLGLQLPFSDEADLSEMVDSPMPQGLR	M17:+15.9949 - M22:+15.9949	-	-	1.01	5,8E-05	3	3014,421631	3014,423828	-0,0023	-0,762998223
1			EDTSGVVLFIGHVVNPLR		-	-	1.01	2,7E-06	2	1952,063599	1952,060425	0,0032	1,639293432
1			LHALAEQVVQFVLADASYADSPR		-	-	1.01	0,00019	3	2500,281982	2500,283447	-0,0014	-0,559936523
2	**TC383884**		DGGSDYLGK		2	2	2.01 2.02	0,00027	2	911,4105225	911,4108887	-0,0004	-0,438879967
2			TYDLNFKEENNDGSQK		-	-	2.01	4,4E-07	2	1901,848877	1901,851563	-0,0027	-1,419669151
2			KYGQDATNVGDEGGFAPNIQENK		2	2	2.01 2.02	1,7E-06	3	2452,137451	2452,137939	-0,0005	-0,203903705
2			YGQDATNVGDEGGFAPNIQENK		2	2	2.01 2.02	0,00023	3	2324,039307	2324,042969	-0,0038	-1,635081649
2			VVIGMDVAASEFYNDKDK	M5:+15.9949	-	-	2.01	3,4E-06	2	2016,955688	2016,95874	-0,003	-1,487387896
2			VNQIGSVTESIEAVK		-	-	2.01	1,3E-05	2	1573,842163	1573,843628	-0,0014	-0,889541984
2			LAMQEFMILPTGAASFK	M7:+15.9949	2	2	2.01 2.02	5,3E-07	2	1870,94397	1870,94458	-0,0006	-0,320693642
2			DPTAQTELDNFMVQQLDGTK		-	-	2.01	5,4E-10	2	2251,050537	2251,055176	-0,0045	-1,9990623
2			QLVLPVPAFNVINGGSHAGNK	Q1:-17.0265	2	2	2.01 2.02	6,7E-07	2	2115,132568	2115,13501	-0,0024	-1,134679437

Group	Description	Sample	Sequence	Modifs	Used	on a total of	Sub-groups	E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**B7U6L4**	**S1**	AKDQQDEGFVAGPEQQQEHER		-	-	1.01	9,4E-07	2	2426,095947	2426,097168	-0,0011	-0,453403115
1			DQQDEGFVAGPEQQQEHER		-	-	1.01	1,8E-08	2	2226,965088	2226,965088	1E-04	0,044904158
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949 - Q1:-17.0265	-	-	1.01	6,5E-08	2	2121,037354	2121,039795	-0,0023	-1,084373832
1			VAVANITPGSMTAPYLNTQSFK		3	4	1.01 1.02 1.03	8,2E-07	2	2310,175781	2310,18042	-0,0044	-1,904613137
1			AFVVPGLTDADGVGYVAQGEGVLTVIENGEKR		-	-	1.01	6,8E-05	3	3261,67041	3260,679932	0,99000001	303,6176453
3	**TC404101**		LYVGVGDADADHR		2	2	3.01 3.02	0,00059	2	1387,662842	1387,660522	0,0024	1,729529738
3			SNSAMSDGSAANVDLTGGYYDGGNNVK	M5:+15.9949	2	2	3.01 3.02	4,4E-07	3	2680,141602	2680,143311	-0,0015	-0,559671521
3			DVMAFAWQHQGK		-	-	3.02	0,00011	2	1417,668335	1417,668579	-0,0002	-0,141076699
3			QVDYVLGDNPLGMSYMVGYGAR	M13:+15.9949 - M16:+15.9949	-	-	3.01	6,2E-05	3	2437,10791	2437,116699	-0,0087	-3,569792271
1	**B7U6L4**	**S2**	AKDQQDEGFVAGPEQQQEHER		-	-	1.01	9,4E-07	2	2426,095947	2426,097168	-0,0011	-0,453403115
1			DQQDEGFVAGPEQQQEHER		-	-	1.01	1,8E-08	2	2226,965088	2226,965088	1E-04	0,044904158
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	-	-	1.01	1,5E-06	2	2138,063232	2138,066406	-0,003	-1,403136969
1			QASEGDQGHHWPLPPFR	Q1:-17.0265	-	-	1.01	5,2E-06	2	1941,901245	1941,899536	0,0018	0,926927447
1			GSSNLQVVCFEINAER	C9:+57.0215	3	4	1.01 1.02 1.03	6,1E-06	2	1822,87439	1822,87561	-0,0012	-0,658300579
1			VAVANITPGSMTAPYLNTQSFK		3	4	1.01 1.02 1.03	8,2E-07	2	2310,175781	2310,18042	-0,0044	-1,904613137
3	**TC404101**		LYVGVGDADADHR		2	2	3.01 3.02	7,9E-05	2	1387,658691	1387,660522	-0,0017	-1,22508359
3			SNSAMSDGSAANVDLTGGYYDGGNNVK	M5:+15.9949	2	2	3.01 3.02	4,8E-07	3	2680,138428	2680,143311	-0,0048	-1,790949106
3			SYLNAPGPNPNVHTGAVVGGPDENDAFPDDR		-	-	3.01	5,2E-07	3	3192,454102	3192,462158	-0,008	-2,505903006
1	**B7U6L4**	**S4**	AKDQQDEGFVAGPEQQQEHER		-	-	1.01	9,4E-07	2	2426,095947	2426,097168	-0,0011	-0,453403115
1			DQQDEGFVAGPEQQQEHER		-	-	1.01	1,8E-08	2	2226,965088	2226,965088	1E-04	0,044904158
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	-	-	1.01	1,5E-06	2	2138,063232	2138,066406	-0,003	-1,403136969
1			QASEGDQGHHWPLPPFR	Q1:-17.0265	-	-	1.01	5,2E-06	2	1941,901245	1941,899536	0,0018	0,926927447
1			GSSNLQVVCFEINAER	C9:+57.0215	3	4	1.01 1.02 1.03	6,1E-06	2	1822,87439	1822,87561	-0,0012	-0,658300579
1			VAVANITPGSMTAPYLNTQSFK		3	4	1.01 1.02 1.03	8,2E-07	2	2310,175781	2310,18042	-0,0044	-1,904613137
1			AFVVPGLTDADGVGYVAQGEGVLTVIENGEKR		-	-	1.01	6,8E-05	3	3261,67041	3260,679932	0,99000001	303,6176453
2	**TC425413**		GGGGSGSEKEDIQPR		-	-	2.01	0,00012	2	1473,692871	1473,693359	-0,0003	-0,203570187
2			FHQITGDQCHHLR	C9:+57.0215	2	2	2.01 2.02	1,9E-05	2	1648,772339	1648,776489	-0,0042	-2,547343493
2			AFLQPSHHDADEIAFVR		-	-	2.01	2,2E-06	2	1952,958618	1952,961792	-0,0031	-1,587332726
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	6,5E-05	2	2098,090088	2098,093262	-0,003	-1,429869771
2			VVMFINPVSTPGR		-	-	2.01	0,00061	2	1416,76709	1416,767212	-1E-04	-0,070583224
2			FQEFFLIGSGDERPQSFLSVFSDEVIQAALNTR		-	-	2.01	1,4E-08	3	3747,868652	3747,865723	0,0029	0,77377373
5	**TC373663**		VATVQCLSGTGSLR	C6:+57.0215	-	-	5.01	2,8E-06	2	1448,751709	1448,753052	-0,0012	-0,828298569
5			LIFGADSPAIQENR		-	-	5.01	8,5E-05	2	1530,789673	1530,791504	-0,0018	-1,175862312
5			MFVADGGELLMAQSYAK	M1:+15.9949	-	-	5.01	3,3E-07	2	1846,871704	1846,871826	-1E-04	-0,054145608
5			ALLPFFDSAYQGFASGSLDKDAQSVR		-	-	5.01	0,00088	3	2790,367676	2790,373779	-0,0061	-2,186086893

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**B7U6L4**	**S8**	DQQDEGFVAGPEQQQEHER		-	-	1.01	1,8E-08	2	2226,965088	2226,965088	1E-04	0,044904158
1			QASEGDQGHHWPLPPFR	Q1:-17.0265	-	-	1.01	5,2E-06	2	1941,901245	1941,899536	0,0018	0,926927447
1			GSSNLQVVCFEINAER	C9:+57.0215	3	4	1.01 1.02 1.03	6,1E-06	2	1822,87439	1822,87561	-0,0012	-0,658300579
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949 - Q1:-17.0265	-	-	1.01	6,5E-08	2	2121,039063	2121,039795	-0,0006	-0,282880127
1			VAVANITPGSMTAPYLNTQSFK		3	4	1.01 1.02 1.03	8,2E-07	2	2310,175781	2310,18042	-0,0044	-1,904613137
1			AFVVPGLTDADGVGYVAQGEGVLTVIENGEKR		-	-	1.01	6,8E-05	3	3261,67041	3260,679932	0,99000001	303,6176453
1			AKDQQDEGFVAGPEQQQEHERGDR		-	-	1.01	0,00042	3	2754,244873	2754,246582	-0,0017	-0,617228687
1			SFHALAQHDVR		4	5	1.01 1.03 1.04 1.05	0,00027	2	1280,650391	1280,649902	0,0006	0,468512148
1			VLTAALKTSDER		-	-	1.01	6E-05	2	1303,720825	1303,722046	-0,0012	-0,920441687
1			AKDQQDEGFVAGPEQQSR		-	-	1.05	3,8E-06	2	1989,923096	1989,926514	-0,0034	-1,708605886
1			AKDQQDEGFVAGPEQQQEHER		-	-	1.01	9,6E-05	3	2426,092529	2426,097168	-0,0046	-1,89604938
1			QGKEEEEKSISIVR	Q1:-17.0265	-	-	1.01	0,00012	2	1614,833862	1614,83374	1E-04	0,061925881
			LAVVLEGEGEVEIVCPHLGR	C15:+57.0215	-	-	1.01	0,00014	3	2176,145996	2176,143555	0,0026	1,194774032
2	**TC425413**		GGGGSGSEKEDIQPR		-	-	2.01	0,00012	2	1473,692871	1473,693359	-0,0003	-0,203570187
2			FHQITGDQCHHLR	C9:+57.0215	2	2	2.01 2.02	1,9E-05	2	1648,772339	1648,776489	-0,0042	-2,547343493
2			AFLQPSHHDADEIAFVR		-	-	2.01	2,2E-06	2	1952,958618	1952,961792	-0,0031	-1,587332726
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	6,5E-05	2	2098,090088	2098,093262	-0,003	-1,429869771
2			IYVVVEGRDGYFEMACPHISSSGR	C16:+57.0215	-	-	2.01	3,3E-06	3	2729,28125	2729,281494	-0,0002	-0,073279358
2			VVMFINPVSTPGR		-	-	2.01	0,00061	2	1416,76709	1416,767212	-1E-04	-0,070583224
2			FQEFFLIGSGDERPQSFLSVFSDEVIQAALNTR		-	-	2.01	1,4E-08	3	3747,868652	3747,865723	0,0029	0,77377373
1	**TC369421**	**S12**	GGPGKPLHLTATVR		2	2	1.01 1.02	0,00085	2	1403,813843	1403,812256	0,0017	1,210988164
1			CGYPPAAHVGR	C1:+57.0215 - C1:-17.0265	2	2	1.01 1.02	0,00012	2	1167,536133	1167,536743	-0,0006	-0,513902485
1			YFDKQSDYSSYIK		-	-	1.01	7,3E-06	2	1643,755493	1643,759155	-0,0037	-2,250937939
1			YHLDLSGHDLSAVGTDIK		-	-	1.01	1,3E-08	2	1940,971802	1940,97168	0,0002	0,103041172
1			GVPVSLSVGGYGTGYSLPSNR		-	-	1.01	1,4E-07	2	2067,04834	2067,051025	-0,0026	-1,25783062
1			ACNQYGAWEEAWDR	C2:+57.0215	-	-	1.01	0,00012	2	1755,717285	1755,718384	-0,0011	-0,62652421
1			DNYGGVMLWDR	M7:+15.9949	-	-	1.01	0,00042	2	1342,59082	1341,589966	1,00100005	746,1296387
1			EACDSGMYTMVTMSFLDVFGAK	- M7:+15.9949 - M10:+15.9949 - M	-	-	1.01	8,2E-05	2	2508,041992	2508,044189	-0,0021	-0,837305784
2	**TC389327**		AKDQQDEGFVAGPEQQQEHER		-	-	2.01	0,00015	3	2426,096924	2426,097168	-0,0003	-0,123655394
2			DQQDEGFVAGPEQQEQER		-	-	2.02	0,00082	2	2089,913086	2089,90625	0,007	3,349432707
2			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	-	-	2.01	7,6E-07	2	2138,064453	2138,066406	-0,0018	-0,841882169
2			LAVVLEGEGEVEIVCPHLGR	C15:+57.0215	-	-	2.01	0,00021	3	2176,143555	2176,143555	0,0002	0,091905698
3	**TC425413**		SKGEGEIYEASEEQIR		-	-	3.01	8,5E-06	2	1824,853638	1824,86145	-0,0078	-4,274296761
3			GEGEIYEASEEQIR		-	-	3.01	0,00022	2	1609,732544	1609,734375	-0,0019	-1,180319071
3			DGYFEMACPHISSSGR	C8:+57.0215 - M6:+15.9949	-	-	3.01	6,5E-08	2	1829,751953	1829,758545	-0,0066	-3,607033253
3			VVMFINPVSTPGR	M3:+15.9949	-	-	3.01	0,00023	2	1432,759766	1432,762085	-0,0023	-1,605291009
3			AFLQPSHHDADEIAFVR		-	-	3.01	0,00015	2	1952,962036	1952,961792	0,0003	0,153612852

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**TC408407**	**S15**	VVEEVANAHGGR		2	2	1.01 1.02	0,00037	2	1237,627686	1237,628784	-0,0011	-0,888796389
1			GHEDKDAEETLQALR		-	-	1.01	1,5E-06	2	1711,833252	1711,824951	0,0083	4,848626137
1			VALVTGGDSGIGR		-	-	1.01	0,00089	2	1201,654175	1201,654053	0,0003	0,249655902
1			ALAGDLGYEENCR	C12:+57.0215	-	-	1.01	6E-07	2	1467,653076	1467,653687	-0,0006	-0,408815831
1			HMGPGSSIINTTSVNAYK		-	-	1.01	2,4E-06	2	1876,923706	1876,922607	0,0012	0,639344454
1			VNGVAPGPIWTPLIPASFPEEK		2	2	1.01 1.02	2,6E-05	2	2319,237061	2319,23877	-0,0017	-0,732999146
2	**TC425413**		GGGGSGSEKEDIQPR		2	2	2.01 2.02	0,00062	2	1473,692749	1473,693359	-0,0004	-0,271426857
2			SKGEGEIYEASEEQIR		2	2	2.01 2.02	4,6E-05	2	1824,860596	1824,86145	-0,0008	-0,438389421
2			GEGEIYEASEEQIR		2	2	2.01 2.02	0,00013	2	1609,733887	1609,734375	-0,0005	-0,310610265
2			AFLQPSHHDADEIAFVR		-	-	2.01	2,3E-06	2	1952,960083	1952,961792	-0,0016	-0,819268465
2			DGYFEMACPHISSSGR	C8:+57.0215	2	2	2.01 2.02	4E-06	2	1813,760132	1813,763672	-0,0035	-1,929689288
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	6,8E-08	2	2098,091797	2098,093262	-0,0013	-0,61961019
4	**TC402211**		LLVAPGQCNLATIHNVR	C8:+57.0215	-	-	4.01	2,7E-06	2	1876,022949	1876,022583	0,0004	0,21321705
4			DYVLQQTCGTFTPGSK	C8:+57.0215	-	-	4.01	5,2E-09	2	1801,840942	1801,842896	-0,002	-1,109974742
4			LYCCQELAEISQQCR	C3:+57.0215 - C4:+57.0215 - C14:+57.0215	-	-	4.01	6,9E-05	2	1957,854248	1957,856934	-0,0026	-1,327982664
4			SGNVGESGLIDLPGCPR	C15:+57.0215	-	-	4.01	1,4E-05	2	1727,837524	1727,838501	-0,001	-0,578757823
4			YFIALPVPSQPVDPR		-	-	4.01	2,7E-06	2	1698,919556	1698,921753	-0,0022	-1,294939041
1	**TC428590**	**S21**	TNVSPEVAASTR		-	-	1.02	0,0003	2	1231,627563	1231,628052	-0,0005	-0,405966759
1			VATPAQAQEVHANLR		2	2	1.01 1.02	0,00092	2	1604,851563	1604,85083	0,0008	0,498488694
1			IIYGGSVTGASCK	C12:+57.0215	2	2	1.01 1.02	2,2E-05	2	1312,656494	1312,656982	-0,0004	-0,304725468
1			IKDWTNVVVAYEPVWAIGTGK		2	2	1.01 1.02	2,4E-05	2	2346,250244	2346,249756	0,0006	0,255727261
2	**TC403071**		AGAAVGGQVVEK		2	2	2.01 2.02	7,6E-08	2	1085,594727	1085,595459	-0,0006	-0,552692115
2			VTYIQEGGSETSSLEVQR		2	2	2.01 2.02	1,6E-06	2	1982,966187	1982,967041	-0,0007	-0,353006363
2			IYAIFTSNAINSDDPSHPTSEAYSSVSNLLR		-	-	2.01	9,3E-06	3	3369,615967	3369,623779	-0,0078	-2,314798355
1	**TC428590**	**S22**	TNVSPEVAASTR		-	-	1.01	0,00049	2	1231,628052	1231,628052	0	0
1			EAGSTMEVVAEQTK	M6:+15.9949	-	-	1.01	5,4E-05	2	1495,694336	1495,694946	-0,0005	-0,334292799
1			VATPAQAQEVHANLR		3	3	1.01 1.02 1.03	7,8E-05	2	1604,849487	1604,85083	-0,0012	-0,747733057
1			IIYGGSVTGASCK	C12:+57.0215	3	3	1.01 1.02 1.03	3,1E-05	2	1312,657593	1312,656982	0,0007	0,533269525
1			SLMGESSEFVGEK	M3:+15.9949	2	3	1.01 1.02	0,00051	2	1415,636475	1415,636353	0,0002	0,141279221
1			LRPEIQVAAQNCWVK	C12:+57.0215	2	3	1.01 1.02	0,00012	2	1811,958618	1811,958862	-0,0003	-0,165566668
1			VIACVGETLEQR	C4:+57.0215	2	3	1.01 1.02	0,00084	2	1374,705444	1374,704956	0,0005	0,363714427
1			IKDWTNVVVAYEPVWAIGTGK		2	3	1.01 1.02	0,00011	3	2346,248047	2346,249756	-0,0016	-0,681939304
1			ELAGQPDVDGFLVGGASLKPEFIDIINAATVK		3	3	1.01 1.02 1.03	1,9E-06	3	3284,738525	3284,741699	-0,0032	-0,974201381
1			DWTNVVVAYEPVWAIGTGK		2	3	1.01 1.02	6,2E-05	2	2105,063232	2105,070557	-0,0074	-3,515321732
2	**TC374136**		YGEFSLAEVAPK		-	-	2.01	8,5E-05	2	1310,665283	1310,663086	0,0022	1,678539634
2			GLPYEYVEEDLMAGK	M12:+15.9949	-	-	2.01	2,5E-05	2	1729,798584	1729,799438	-0,0007	-0,404671162
2			VYDFIGLLK		2	2	2.01 2.02	0,00027	2	1067,614136	1067,614014	0,0002	0,187333614
7	**TC369624**		AAVGHPDTLGDCPFSQR	C12:+57.0215	-	-	7.01	0,00019	2	1827,846313	1827,844727	0,0017	0,930057108
7			YPTPSLVTPAEYASVGSK		-	-	7.01	1,6E-06	2	1866,948242	1866,948853	-0,0005	-0,267816663
7			LIDVSNKPDWFLK		-	-	7.01	0,00052	2	1574,856812	1574,858154	-0,0013	-0,825471163
7			WIADSDVITQVIEEK		-	-	7.01	7,5E-06	2	1745,896606	1745,895996	0,0006	0,343663096
7			ALVDELQALEEHLK		-	-	7.01	6,6E-05	2	1607,863403	1607,864258	-0,0009	-0,559748769

Group	Description	Sample	Sequence	Modifs	Used	on a total of	Sub-groups	E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**B7U6L4**	**S24**	AKDQQDEGFVAGPEQQQEHER		-	-	1.01	9,4E-07	2	2426,095947	2426,097168	-0,0011	-0,453403115
1			DQQDEGFVAGPEQQQEHER		-	-	1.01	1,8E-08	2	2226,965088	2226,965088	1E-04	0,044904158
1			AFVVPGLTDADGVGYVAQGEGVLTVIENGEKR		-	-	1.01	6,8E-05	3	3261,67041	3260,679932	0,99000001	303,6176453
2	**TC428590**		TNVSPEVAASTR		-	-	2.01	0,00018	2	1231,62793	1231,628052	-1E-04	-0,081193343
2			VATPAQAQEVHANLR		-	-	2.01	6,8E-05	2	1604,850952	1604,85083	0,0002	0,124622174
2			LRPEIQVAAQNCWVK	C12:+57.0215	-	-	2.01	0,00015	2	1811,966187	1811,958862	0,0073	4,028789043
1	**CK215488**	**S25**	YTIAMMGYAEEDK	M5:+15.9949 - M6:+15.9949	-	-	1.01	2,3E-05	2	1553,651367	1553,650146	0,0012	0,77237469
1			VVLVDHADFLK		-	-	1.01	8,8E-05	2	1255,70752	1255,704956	0,0026	2,070550203
1			GNAYAQVAIGTDDVYK		-	-	1.01	3E-07	2	1684,81665	1684,818115	-0,0014	-0,830950201
1			GPTPEPLCQVMLR	C8:+57.0215 - M11:+15.9949	-	-	1.01	0,00074	2	1513,749023	1513,75061	-0,0015	-0,990916193
1			QPGPLPGLNTK	Q1:-17.0265	-	-	1.01	7,6E-05	2	1104,605591	1104,605225	0,0004	0,36212033
1			AQDPDGYMFELIQR		-	-	1.01	3,3E-06	2	1682,785645	1682,784668	0,001	0,594253123
2	**CJ541649**		AKDQQDEGFVAGPEQQEQER		-	-	2.01	9,8E-05	3	2289,035889	2289,038086	-0,0024	-1,048475385
2			DQQDEGFVAGPEQQEQER		-	-	2.01	1,7E-05	2	2089,903564	2089,90625	-0,0026	-1,244074941
2			LDNPAQELTFGR		-	-	2.01	4,9E-05	2	1360,685059	1360,686035	-0,0009	-0,661431074
4	**Q41593**		YKAETQSVDFQTK		-	-	4.01	3,9E-07	2	1544,757568	1544,759521	-0,0019	-1,229964972
4			IKDILPPGSIDNTTK		-	-	4.01	0,00086	2	1611,8927	1611,89563	-0,0029	-1,799123883
4			AAEVTTQVNSWVEK		-	-	4.01	0,00018	2	1561,786377	1561,786133	0,0003	0,19208777
4			GAWTEQFDSYGTK		-	-	4.01	0,00058	2	1489,657837	1489,65979	-0,0019	-1,275459051
1	**CK215488**	**S26**	YTIAMMGYAEEDK	M5:+15.9949 - M6:+15.9949	-	-	1.01	2,3E-05	2	1553,651367	1553,650146	0,0012	0,77237469
1			VVLVDHADFLK		-	-	1.01	8,8E-05	2	1255,70752	1255,704956	0,0026	2,070550203
1			GNAYAQVAIGTDDVYK		-	-	1.01	3E-07	2	1684,81665	1684,818115	-0,0014	-0,830950201
1			GPTPEPLCQVMLR	C8:+57.0215 - M11:+15.9949	-	-	1.01	0,00074	2	1513,749023	1513,75061	-0,0015	-0,990916193
2	**CJ541649**		AKDQQDEGFVAGPEQQEQER		-	-	2.01	7E-08	2	2289,0354	2289,038086	-0,0028	-1,223221183
2			DQQDEGFVAGPEQQEQER		-	-	2.01	0,00018	2	2089,901855	2089,90625	-0,0043	-2,057508707
2			LDNPAQELTFGR		-	-	2.01	0,0009	2	1360,686523	1360,686035	0,0006	0,440954059
3	**Q9ST58**		IKDILPSGSVDNTTK		-	-	3.01	1,6E-05	2	1587,857666	1587,859253	-0,0015	-0,944668114
3			GAWTDQFDSSGTK		-	-	3.01	0,00021	2	1399,610352	1399,612915	-0,0025	-1,786208153
3			AAEVATQVNSWVEK		-	-	3.01	0,00033	2	1531,77417	1531,775513	-0,0013	-0,848688304
1	**TC374294**	**S31**	DQLVATLGEGEAER		-	-	1.01	0,00018	2	1487,738525	1487,734131	0,0045	3,024733782
1			TFVEVNETGTEAAAATIAK		-	-	1.01	4,6E-07	2	1922,969727	1922,970947	-0,0012	-0,624034464
1			HLGLQLPFSDEADLSEMVDSPMPQGLR	M17:+15.9949 - M22:+15.9949	-	-	1.01	5,8E-05	3	3014,421631	3014,423828	-0,0023	-0,762998223
1			EDTSGVVLFIGHVVNPLR		-	-	1.01	2,7E-06	2	1952,063599	1952,060425	0,0032	1,639293432
3	**TC369723**		KYGQDATNVGDEGGFAPNIQENK		-	-	3.01	0,00037	3	2452,134277	2452,137939	-0,0036	-1,468106627
3			YGQDATNVGDEGGFAPNIQENK		-	-	3.01	3,7E-05	3	2324,041016	2324,042969	-0,0019	-0,817540824
3			YGQDATNVGDEGGFAPN		-	-	3.01	0,00061	2	1711,717285	1711,719849	-0,0025	-1,460519314
3			VNQIGSVTESIEAVK		-	-	3.01	8,1E-06	2	1573,842285	1573,843628	-0,0013	-0,826003253

Group	Description	Sample	Sequence	Modifs	Used	on a total of	Sub-groups	E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**P93692**	**S32**	YKAEAQSVDFQTK		-	-	1.01	7,6E-06	2	1514,746826	1514,749023	-0,0021	-1,386368275
1			AAEVTAQVNSWVEK		-	-	1.01	1E-04	2	1531,7771	1531,775513	0,0016	1,044539452
1			VALSLITAGAGGATR		-	-	1.01	0,00035	2	1357,777832	1357,780151	-0,0023	-1,693941355
1			AFVEVNETGTEAAATTIAK		-	-	1.01	2,2E-05	2	1922,969238	1922,970947	-0,0017	-0,88404876
1			LSAEPEFLEQHIPR		-	-	1.01	3,3E-05	2	1665,858521	1665,859863	-0,0014	-0,840406835
2	**TC368907**		AAAAMVGHPEWEFPHDSGTYNDTPER	M5:+15.9949	-	-	2.03	1,1E-05	3	2901,247803	2901,253662	-0,0058	-1,999135733
2			DVGASDPDIFYTDQHGTR		-	-	2.03	3,7E-08	2	1993,885498	1993,889038	-0,0035	-1,755363584
2			VMLPLDAVSVNNR	M2:+15.9949	2	4	2.02 2.03	0,00033	2	1443,761108	1443,762817	-0,0017	-1,17747879
2			LSNQLVEGQNYVNFK		2	4	2.01 2.04	3,8E-05	2	1752,891479	1752,891968	-0,0004	-0,228194326
2			FFVDNGTYLTEQGR		3	4	2.01 2.03 2.04	6,2E-06	2	1646,781372	1646,78125	1E-04	0,060724515
2			SAVQMYTDYMASFR	M5:+15.9949	-	-	2.02	4E-06	2	1685,728394	1685,730225	-0,0018	-1,067786455
5	**TC383884**		KYGQDATNVGDEGGFAPNIQENK		-	-	5.01	0,00038	3	2452,141357	2452,137939	0,0035	1,427325964
5			YGQDATNVGDEGGFAPNIQENK		-	-	5.01	2,6E-07	2	2324,042236	2324,042969	-0,0007	-0,301199228
5			VNQIGSVTESIEAVK		-	-	5.01	4,6E-05	2	1573,842896	1573,843628	-0,0007	-0,444770992
5			IEEELGDAAVYAGLK		-	-	5.01	9,2E-05	2	1577,804321	1577,806152	-0,0018	-1,140824556
5			LAMQEFMILPTGAASFK	M3:+15.9949 - M7:+15.9949	-	-	5.01	4,3E-05	2	1886,938477	1886,939453	-0,001	-0,529958725
5			DPTAQTELDNFMVQQLDGTK		-	-	5.01	2,3E-08	2	2251,053955	2251,055176	-0,0011	-0,488659739
5			QLVLPVPAFNVINGGSHAGNK	Q1:-17.0265	-	-	5.01	5,7E-05	2	2115,126465	2115,13501	-0,0085	-4,018655777
6	**TC386312**		VPEGFDYELYNR		-	-	6.01	4,9E-06	2	1501,692749	1501,696167	-0,0034	-2,264106512
6			GTLFPMCGMNLAFDR	C7:+57.0215 - M6:+15.9949 - M9:+15.9949	-	-	6.01	0,00026	2	1761,775391	1761,776123	-0,0007	-0,397326291
6			QLIGPAMYFGLMGDGQPIGR	M7:+15.9949 - M12:+15.9949	-	-	6.01	8,1E-06	2	2153,052246	2153,052246	1E-04	0,04644569
1	**P93692**	**S33**	AEAQSVDFQTK		-	-	1.01	0,00087	2	1223,589478	1223,590698	-0,0012	-0,980720162
1			YKAEAQSVDFQTK		-	-	1.01	7,6E-06	2	1514,746826	1514,749023	-0,0021	-1,386368275
1			AAEVTAQVNSWVEK		-	-	1.01	2,5E-05	2	1531,77478	1531,775513	-0,0007	-0,45698601
1			AFVEVNETGTEAAATTIAK		-	-	1.01	2,6E-06	2	1922,969727	1922,970947	-0,0012	-0,624034464
1			VALSLITAGAGGATR		-	-	1.01	0,00035	2	1357,777832	1357,780151	-0,0023	-1,693941355
1			LSAEPEFLEQHIPR		-	-	1.01	2,2E-05	2	1665,858765	1665,859863	-0,0011	-0,660319626
2	**TC388221**		APSHAAELTAGYYNLHDR		-	-	2.04	1,2E-05	3	1985,945801	1985,946777	-0,0011	-0,553892016
2			VPSHAAEITAGYYNLHDR		-	-	2.01	2,3E-10	2	2013,974365	2013,978149	-0,0037	-1,837159991
2			MHANLPHDPCVDPVAPLQR	C10:+57.0215 - M1:+15.9949	2	4	2.01 2.04	7,5E-05	3	2183,046631	2183,048828	-0,0023	-1,053572416
2			NVGASDPDIFYTDQHGTR		-	-	2.03	6,1E-06	2	1992,897949	1992,905029	-0,007	-3,51246047
2			DVGASDPDIFYTDQHGTR		-	-	2.02	3,8E-06	2	1993,896484	1993,889038	0,0075	3,761493206
2			AAAAMVGHPEWEFPHDSGTYNDTPER	M5:+15.9949	-	-	2.02	5,3E-06	3	2901,252441	2901,253662	-0,0012	-0,413614303
4	**TC383884**		TYDLNFKEENNDGSQK		-	-	4.01	7,9E-06	2	1901,853027	1901,851563	0,0015	0,788705111
4			KYGQDATNVGDEGGFAPNIQENK		2	2	4.01 4.02	7,6E-05	3	2452,13916	2452,137939	0,0013	0,530149639
4			YGQDATNVGDEGGFAPNIQENK		2	2	4.01 4.02	2,8E-07	2	2324,041016	2324,042969	-0,0019	-0,817540824
4			IPLYQHIANLAGNK		2	2	4.01 4.02	0,00026	2	1551,862915	1551,864624	-0,0016	-1,03101778
4			VNQIGSVTESIEAVK		2	2	4.01 4.02	1,1E-06	2	1573,841675	1573,843628	-0,0019	-1,207235694
4			LAMQEFMILPTGAASFK	M3:+15.9949 - M7:+15.9949	-	-	4.01	4,2E-06	2	1886,937256	1886,939453	-0,0022	-1,165909052
4			DPTAQTELDNFMVQQLDGTK		-	-	4.01	4,9E-09	2	2251,054688	2251,055176	-0,0004	-0,17769444
4			QLVLPVPAFNVINGGSHAGNK	Q1:-17.0265	-	-	4.01	8,5E-06	2	2115,128906	2115,13501	-0,006	-2,836698294

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**TC389327**	**SG1**	AKDQQDEGFVAGPEQQQEHER		-	-	1.01	2,8E-05	3	2426,094971	2426,097168	-0,0023	-0,94802469
1			VLTAALKTSDER		-	-	1.01	7,9E-05	2	1303,720825	1303,722046	-0,0012	-0,920441687
1			DQQDEGFVAGPEQQQEHER		-	-	1.01	9,5E-07	2	2226,963867	2226,965088	-0,0011	-0,493945777
1			ILHTISVPGK		2	6	1.01 1.02	0,00043	2	1064,645264	1064,646729	-0,0014	-1,314990163
1			SFHALAQHDVR		4	6	1.01 1.03 1.04 1.05	6,3E-05	2	1280,648926	1280,649902	-0,0009	-0,702768147
1			DQQDEGFVAGPEQQQEHER		-	-	1.01	7,8E-08	2	2226,961426	2226,965088	-0,0036	-1,61654973
1			QGQEEESSISIVR		-	-	1.02	0,00069	2	1461,718628	1461,718384	0,0003	0,205237895
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	2	6	1.01 1.02	3,4E-08	2	2138,066162	2138,066406	-1E-04	-0,046771232
1			QASEGDQGHHWPLPPFR		-	-	1.01	0,00025	2	1958,923706	1958,926025	-0,0023	-1,174112797
1			QGQEEESSISIVR	Q1:-17.0265	-	-	1.02	0,00081	2	1444,687988	1444,691895	-0,0038	-2,630318642
1	**TC389327**	**SG2**	AKDQQDEGFVAGPEQQQEHER		-	-	1.01	4,4E-05	3	2426,0979	2426,097168	0,0007	0,288529247
1			DQQDEGFVAGPEQQQEHER		-	-	1.01	1,4E-06	2	2226,96167	2226,965088	-0,0033	-1,481837273
1			GSSNLQVVCFEINAER	C9:+57.0215	3	6	1.01 1.02 1.06	1,1E-05	2	1822,875	1822,87561	-0,0006	-0,329150289
1			LAVVLEGEGEVQIVCPHLGQDSER	C15:+57.0215	-	-	1.02	7,7E-05	3	2634,320557	2634,31958	0,001	0,379604667
1			QGDVIVAPAGSIMHLAN	Q1:-17.0265	-	-	1.01	2,5E-05	2	1675,847168	1675,847656	-0,0004	-0,238685176
1			SFVVPGLTDADGVGYVAQ		-	-	1.05	0,00069	2	1794,890625	1794,891357	-0,0006	-0,33428207
1	**TC399106**	**SG8**	ILHTISVPGK		3	6	1.01 1.02 1.03	0,00065	2	1064,645508	1064,646729	-0,0011	-1,033206582
1			SFHALAQHDVR		3	6	1.01 1.02 1.06	0,00069	2	1280,649536	1280,649902	-0,0003	-0,234256074
1			AKDQQDEGFVAGPEQQQEHER		2	6	1.01 1.05	2,5E-05	3	2426,091309	2426,097168	-0,0057	-2,349452496
1			QGQEEESSISIVR	Q1:-17.0265	-	-	1.03	0,00042	2	1444,688354	1444,691895	-0,0034	-2,353443146
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	2	6	1.01 1.03	1,4E-08	2	2138,062744	2138,066406	-0,0035	-1,63699317
1			VAQGEGVLTVIENGER		-	-	1.03	4,1E-05	2	1670,870972	1670,871216	-0,0002	-0,119698033
2	**TC425413**		GGGGSGSEKEDIQPR		-	-	2.01	0,00039	2	1473,692383	1473,693359	-0,0008	-0,542853713
2			SKGEGEIYEASEEQIR		-	-	2.01	9,7E-06	2	1824,861206	1824,86145	-0,0002	-0,109597355
2			DGYFEMACPHISSSGR	C8:+57.0215 - M6:+15.9949	-	-	2.01	6,6E-08	2	1829,758057	1829,758545	-0,0005	-0,273260087
2			VVMFINPVSTPGR	M3:+15.9949	-	-	2.01	0,00028	2	1432,760254	1432,762085	-0,0018	-1,256314635
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	2,7E-05	2	2098,093994	2098,093262	0,0009	0,42896089
2			AFLQPSHHDADEIAFVR		-	-	2.01	7,9E-06	2	1952,958862	1952,961792	-0,0029	-1,484924078
1	**TC382510**	**SG12**	IPSPMGESSVDCGR	C12:+57.0215 - M5:+15.9949	-	-	1.01	2,1E-06	2	1507,651245	1507,651978	-0,0007	-0,464298129
1			IGAPGVVSQECK	C11:+57.0215	-	-	1.01	0,00065	2	1244,630127	1244,630859	-0,0006	-0,482070684
1			LASMPDIAFSIGGK	M4:+15.9949	-	-	1.01	0,00013	2	1422,730225	1422,730103	1E-04	0,070287399
1			IGEGDATQCISGFTAMDIPRPR	C9:+57.0215 - M16:+15.9949	-	-	1.01	0,00012	3	2408,132324	2408,133789	-0,0013	-0,539837122
1			CAAIADSGTSLLSGPTAIITQINEK	C1:+57.0215	2	2	1.01 1.02	6E-06	2	2531,305176	2531,30249	0,0027	1,066644549
2	**TC421662**		DSVMGAAGGTADK		-	-	2.02	0,00068	2	1179,530151	1179,531494	-0,0012	-1,017353177
2			GKDVTVSTGGTAAEYAK		3	3	2.01 2.02 2.03	1,9E-06	2	1654,82666	1654,828735	-0,002	-1,208584309
2			TKDVTLSTAAQAAQK		2	3	2.01 2.02	1,4E-05	2	1532,829468	1532,828247	0,0012	0,782866597
2			DVTVSTGGTAAEYAK		3	3	2.01 2.02 2.03	2,5E-06	2	1469,713501	1469,71228	0,0013	0,884526849
2			DVTLSTAAQAAQK		2	3	2.01 2.02	0,00015	2	1303,686768	1303,685547	0,0012	0,920467436
2			GIVAGEEELIPVEGEAGK		2	3	2.01 2.02	0,00013	2	1796,92627	1796,928101	-0,0017	-0,946058989
5	**P93693**		YKAETQSVDFQTK		2	2	5.01 5.02	5,9E-07	2	1544,757813	1544,759521	-0,0017	-1,100494981
5			AETQSVDFQTK		2	2	5.01 5.02	1E-04	2	1253,599609	1253,601196	-0,0016	-1,276322961
5			AAEVAGQVNSWVEK		-	-	5.01	8,4E-06	2	1487,747437	1487,749268	-0,0018	-1,209881306

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**CD914053**	**SG17**	GGPAAVMQSAATLNAR	M7:+15.9949	-	-	1.01	0,00058	2	1530,769409	1530,769653	-0,0003	-0,195979849
1			GQLTGPVADAGVTVTEADLPGR		-	-	1.01	1,1E-09	2	2124,090088	2124,093506	-0,0034	-1,600682855
1			VVTESVAGQVVGR		2	2	1.01 1.02	0,00023	2	1300,720215	1300,722412	-0,0021	-1,61448741
1			DAAMLQSAENEVLGLGQTQK	M4:+15.9949	-	-	1.01	1,5E-05	2	2119,035645	2119,033936	0,0017	0,802252352
			YGDVFDVSGELAAQPVAPR		-	-	1.01	3,4E-07	2	1990,985229	1990,987305	-0,002	-1,004526734
1	**CD914053**	**SG18**	GGPAAVMQSAATLNAR	M7:+15.9949	-	-	1.01	0,00028	2	1530,768799	1530,769653	-0,0009	-0,58793956
1			VVTESVAGQVVGR		2	2	1.01 1.02	0,00068	2	1300,721924	1300,722412	-0,0004	-0,307521433
1			GQLTGPVADAGVTVTEADLPGR		-	-	1.01	3,4E-08	2	2124,092529	2124,093506	-0,001	-0,470789075
1			DAAMLQSAENEVLGLGQTQK	M4:+15.9949	-	-	1.01	1,1E-06	2	2119,02832	2119,033936	-0,0056	-2,642713547
1			YGDVFDVSGELAAQPVAPR		-	-	1.01	1,1E-05	2	1990,980713	1990,987305	-0,0065	-3,264711857
2	**BJ297680**		GVGVAEVTAH		-	-	2.01	0,00074	2	939,4889526	939,4898071	-0,0009	-0,957966805
2			VASYGVGVAEVTAH		-	-	2.01	0,00022	2	1359,686035	1359,690674	-0,0046	-3,383122444
2			GVKDEGLVVAPGEGPEGQTVGNIIAGDR		-	-	2.01	3,3E-07	3	2734,397705	2734,400879	-0,0032	-1,170274615
2			DEGLVVAPGEGPEGQTVGNIIAGDR		-	-	2.01	1,1E-10	2	2450,213379	2450,216309	-0,0028	-1,142756224
3	**TC368606**		AYYGAVEEFSGK		-	-	3.01	2,5E-05	2	1320,610596	1320,611084	-0,0004	-0,302890062
3			SAYYGAVEEFSGK		-	-	3.02	2,7E-07	2	1407,641968	1407,643066	-0,0011	-0,781448066
3			VVVADNIHDVVFK		-	-	3.02	9,4E-06	2	1454,800415	1454,800659	-0,0002	-0,137475878
3			EDQAPLILIQDSDSK		2	2	3.01 3.02	0,00024	2	1671,843262	1671,843994	-0,0007	-0,418699324
1	**B7U6L4**	**SG19**	AKDQQDEGFVAGPEQQQEHER		-	-	1.01	0,00041	3	2426,099365	2426,097168	0,0021	0,865587711
1			DQQDEGFVAGPEQQQEHER		-	-	1.01	1,8E-08	2	2226,965088	2226,965088	1E-04	0,044904158
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	-	-	1.01	0,00065	2	2138,0625	2138,066406	-0,0038	-1,777306795
1			QASEGDQGHHWPLPPFR	Q1:-17.0265	-	-	1.01	5,2E-06	2	1941,901245	1941,899536	0,0018	0,926927447
1			AFVVPGLTDADGVGYVAQGEGVLTVIENGEKR		-	-	1.01	6,8E-05	3	3261,67041	3260,679932	0,99000001	303,6176453
3	**TC373687**		CTLDGHGGYVSAVAVSPDGSLCASGGK	C1:+57.0215 - C22:+57.0215	2	2	3.01 3.02	0,00013	3	2622,188477	2622,192627	-0,0043	-1,639849067
3			YWLCAATQDSIK	C4:+57.0215	2	2	3.01 3.02	9E-05	2	1455,692261	1455,694092	-0,0018	-1,236523509
3			LWDLSTGVTTR		2	2	3.01 3.02	0,00043	2	1248,658203	1248,658691	-0,0005	-0,400429726

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**Q6W8Q2**	**SG23**	VTYPIMADPDR	M6:+15.9949	2	2	1.01 1.02	0,00025	2	1293,614258	1293,614746	-0,0005	-0,386513859
1			QLNMVDPDEKDAEGQLPSR		-	-	1.01	0,00049	2	2142,009521	2142,013672	-0,004	-1,867401838
1			KMFPQGFETADLPSK	M2:+15.9949	2	2	1.01 1.02	0,00076	2	1711,834106	1711,836426	-0,0022	-1,285169601
1			AVDSLLTAAK		2	2	1.01 1.02	0,00088	2	988,5669556	988,5678101	-0,0008	-0,809251487
1			VTYPIMADPDR		2	2	1.01 1.02	0,0008	2	1277,618774	1277,619873	-0,0011	-0,860975921
1			SHPGDFTPVCTTELAAMAN	C10:+57.0215 - M17:+15.9949	2	2	1.01 1.02	0,00028	2	2034,891602	2034,890015	0,0017	0,835426033
1			MFPQGFETADLPSK	M1:+15.9949	2	2	1.01 1.02	5,7E-08	2	1583,740967	1583,741455	-0,0004	-0,252566487
1			PGLTIGDTVPNLELDSTHGK		-	-	1.01	9,2E-06	2	2064,060791	2064,061279	-0,0004	-0,193792686
2	**TC394284**		QLNMVDPDEKDAEGQLPSR		-	-	1.01	0,00049	2	2142,009521	2142,013672	-0,004	-1,867401838
2			AAVGHPDTLGDCPFSQR	C12:+57.0215	2	2	2.01 2.02	0,00016	2	1827,843018	1827,844727	-0,0016	-0,875347853
2			YPTPSLVTPPEYASVGSK		-	-	2.01	0,00022	2	1892,967896	1892,964355	0,0035	1,848951936
2			LIDVSNKPDWFLK		2	2	2.01 2.02	0,00021	2	1574,8573	1574,858154	-0,0008	-0,507982254
2			WIADSDVITQVIEEK		2	2	2.01 2.02	2,1E-06	2	1745,894165	1745,895996	-0,0018	-1,03098917
2			ALVDELQALEEHLK		2	2	2.01 2.02	0,00018	2	1607,861694	1607,864258	-0,0026	-1,617051959
3	**TC417836**		HHATYVANYNK		2	2	3.01 3.02	0,00074	2	1317,63147	1317,633911	-0,0024	-1,821446896
3			NLKPISEGGGEAPHGK		-	-	3.02	3,7E-05	2	1590,823853	1590,823853	0	0
3			GDASAVVHLQSAIK		2	2	3.01 3.02	0,00019	2	1395,755615	1395,759521	-0,0038	-2,722532034
3			KLSVETTPNQDPLVTK		-	-	3.01	6,8E-06	2	1769,96228	1769,964844	-0,0025	-1,412457466
3			ALEQLDAAVSK		2	2	3.01 3.02	7,8E-05	2	1144,619873	1144,621338	-0,0013	-1,135746717
3			LSVETTPNQDPLVTK		2	2	3.01 3.02	0,00018	2	1641,869141	1641,869751	-0,0006	-0,365437061
3			LGWAIDEDFGSIEK		2	2	3.01 3.02	5,2E-05	2	1579,76355	1579,764282	-0,0007	-0,443104059
1	**TC389327**	**SG28**	SFHALAQHDVR		3	3	1.01 1.02 1.03	0,0008	2	1280,649902	1280,649902	1E-04	0,078085348
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	-	-	1.01	4,5E-05	2	2138,061035	2138,066406	-0,0052	-2,432104111
1			EASEGGQGHHWPLPPFR		-	-	1.03	1,5E-05	2	1901,899048	1901,904541	-0,0055	-2,891838074
1			QASEGDQGHHWPLPPFR	Q1:-17.0265	-	-	1.01	3,4E-05	2	1941,900269	1941,899536	0,0008	0,411967754
1			VAVANITPGSMTAPYLNTQSFK	M11:+15.9949	3	3	1.01 1.02 1.03	5,4E-06	2	2326,170166	2326,175293	-0,005	-2,149451017
1			DTFNLLEQRPK		2	3	1.01 1.03	0,0003	2	1360,721069	1360,722412	-0,0012	-0,881884575
2	**TC368657**		VNVGVLAACAPSK	C9:+57.0215	-	-	2.01	0,00025	2	1285,693237	1285,693726	-0,0004	-0,31111607
2			AVEAVAPFGVCYDTK	C11:+57.0215	-	-	2.01	5,1E-08	2	1626,783813	1626,783569	0,0002	0,122941986
2			GSTGVAGLANSGLALPAQVASAQK		-	-	2.01	1,9E-05	2	2168,163818	2168,16748	-0,0035	-1,614266515
1	**TC389327**	**SG29**	SIMHLANTDGR	M3:+15.9949	2	4	1.01 1.04	0,00026	2	1230,587402	1230,589966	-0,0025	-2,031545877
1			SFHALAQHDVR		2	4	1.01 1.04	0,00071	2	1280,649414	1280,649902	-0,0004	-0,312341392
1			ILHTISVPGK		4	4	1.01 1.02 1.03 1.04	0,00039	2	1064,645142	1064,646729	-0,0015	-1,408918023
1			VIVAPAGSIMHLANTDGR	M10:+15.9949	-	-	1.01	5,4E-07	2	1837,959229	1837,959351	0	0
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	2	4	1.01 1.02	4,6E-07	2	2138,062744	2138,066406	-0,0035	-1,63699317
1			DTFNLLEQRPK		3	4	1.01 1.03 1.04	0,00043	2	1360,721924	1360,722412	-0,0004	-0,293961465
2	**TC368657**		VNVGVLAACAPSK	C9:+57.0215	-	-	2.01	0,00025	2	1285,693237	1285,693726	-0,0004	-0,31111607
2			AVEAVAPFGVCYDTK	C11:+57.0215	-	-	2.01	5,1E-08	2	1626,783813	1626,783569	0,0002	0,122941986
2			GSTGVAGLANSGLALPAQVASAQK		-	-	2.01	1,9E-05	2	2168,163818	2168,16748	-0,0035	-1,614266515

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**TC380640**	**B1=BG34**	YKAEAQSVDFQTK		2	2	1.01 1.02	2,4E-05	2	1514,748779	1514,749023	-0,0002	-0,132035062
1			AAEVTAQVNSWVEK		2	2	1.01 1.02	7,4E-06	2	1531,776367	1531,775513	0,0009	0,587553501
1			AFVEVNETGTEAAATTIAK		-	-	1.01	7,2E-05	2	1922,971313	1922,970947	0,0004	0,208011463
1			DQLVATLGEGEAER		-	-	1.02	0,00011	2	1487,739746	1487,734131	0,0058	3,898545742
1			DILPAGSIDNTTR		-	-	1.01	0,00034	2	1372,706421	1372,707153	-0,0006	-0,437092513
1			LSAEPEFLEQHIPR		-	-	1.01	0,00047	2	1665,856567	1665,859863	-0,0033	-1,980959058
1			ISLGIEASDLLK		-	-	1.01	0,00074	2	1258,724243	1258,725708	-0,0014	-1,112236023
3	**TC386671**		NVGVSDPDIFYTDQHGTR		-	-	3.03	8,9E-06	2	2020,934937	2020,936279	-0,0014	-0,692748189
3			FFVDNGTYLTEQGR		3	3	3.01 3.02 3.03	8,4E-06	2	1646,78125	1646,78125	0	0
3			NIEYLTLGVDDQPLFHGR		2	3	3.02 3.03	4,1E-07	2	2087,056641	2087,056152	0,0006	0,287486315
5	**TC390079**		HADDEGEGGEIIFGGMDPK		-	-	5.01	1,1E-05	2	1973,856323	1973,85498	0,0014	0,709271967
5			NYMNAQYFGEIGVGTPPQK	M3:+15.9949	-	-	5.01	5,3E-06	2	2129,993652	2129,996582	-0,0028	-1,314556003
5			DQEFIEATKEPGVTFLVAK		-	-	5.01	0,00065	3	2122,104736	2122,107178	-0,0025	-1,178074241
5			GYWQFDMGDVLVGGK	M7:+15.9949	-	-	5.01	0,00017	2	1687,778687	1687,778931	-1E-04	-0,059249464
1	**TC380640**	**B2=B33**	YKAEAQSVDFQTK		-	-	1.01	3,7E-05	2	1514,748779	1514,749023	-0,0002	-0,132035062
1			AEAQSVDFQTK		-	-	1.01	0,00078	2	1223,59021	1223,590698	-0,0004	-0,326906711
1			YKAEAQSVDFQTK		-	-	1.01	4,9E-05	2	1514,748169	1514,749023	-0,0008	-0,528140247
1			AAEVTAQVNSWVEK		-	-	1.01	2,4E-05	2	1531,775391	1531,775513	-1E-04	-0,065283716
1			AFVEVNETGTEAAATTIAK		-	-	1.01	5,9E-05	2	1922,970459	1922,970947	-0,0005	-0,260014325
1	**TC380640**	**B3=BG32**	YKAEAQSVDFQTK		2	2	1.01 1.02	8,8E-05	2	1514,747681	1514,749023	-0,0013	-0,858227968
1			AFVEVNETGTEAAATTIAK		-	-	1.01	8,4E-06	2	1922,967285	1922,970947	-0,0037	-1,924106121
1			AAEVTAQVNSWVEK		2	2	1.01 1.02	7,4E-05	2	1531,775635	1531,775513	0,0002	0,130567431
1			LSAEPEFLEQHIPR		-	-	1.01	0,00032	2	1665,856812	1665,859863	-0,0031	-1,860900879
2	**TC401483**		GVTTIIGGGDSVAAVEK		2	2	2.01 2.02	6,1E-05	2	1573,841919	1573,843628	-0,0016	-1,016619444
2			LLLPTDVVVADKFAADAESK		-	-	2.01	1,8E-05	2	2102,140137	2102,138428	0,0018	0,856270909
2			SLVEEDKLELATSLIETAK		-	-	2.01	2,5E-07	2	2089,126465	2089,12793	-0,0014	-0,670136034
2			TVIWNGPMGVFEFEK	M8:+15.9949	2	2	2.01 2.02	0,00099	2	1769,853516	1769,857056	-0,0036	-2,034062624
2			MSHISTGGGASLELLEGKPLPGVLALDEA	M1:+15.9949	-	-	2.01	1,4E-05	3	2878,486572	2878,487061	-0,0005	-0,173702374
2			LSELLGLEVVMAPDCIGEEVEK	C15:+57.0215 - M11:+15.9949	-	-	2.01	0,00069	2	2446,209717	2446,209717	0,0002	0,08175914
2			LAAALPDGGVLLLENVR		2	2	2.01 2.02	2,4E-06	2	1720,994751	1720,995972	-0,0012	-0,697270691
1	**TC368820**	**B6=BG31**	LVSWYDNEWGYSNR		-	-	1.01	1,9E-05	2	1788,797729	1788,797974	-0,0003	-0,167710394
1			LKGIMGYVEEDLVSTDFVGDSR	M5:+15.9949	-	-	1.01	1,6E-06	2	2446,181152	2446,181152	0,0002	0,081760094
1			GIMGYVEEDLVSTDFVGDSR	M3:+15.9949	-	-	1.01	4,5E-07	3	2205,002686	2205,001953	0,0007	0,31746003
1			NPEEIPWGEAGADYVVESTGVFTDK		-	-	1.01	1,7E-07	3	2710,250244	2710,252197	-0,0021	-0,774835587
2	**TC372049**		GDATLAEGASESLHVK		-	-	2.03	0,00041	2	1584,789673	1584,786743	0,0029	1,829899192
2			VAPEVIAEYTVR		2	3	2.01 2.02	0,00026	2	1346,72998	1346,731934	-0,0018	-1,336568832
2			IGLTEPSQLSIDQNAQGLAR		-	-	2.02	5,6E-06	2	2111,109375	2111,109619	-1E-04	-0,047368452
2			CAYVTEMVLAACYK	C1:+57.0215 - C12:+57.0215 - M7:+15.9949 - C1:-17.0265	2	3	2.01 2.02	7,2E-06	2	1677,725708	1677,732544	-0,0068	-4,053089619
2			YAIICQENGLVPIVEPEILVDGPHDIDR	C5:+57.0215	3	3	2.01 2.02 2.03	0,0005	3	3175,602539	3174,614014	0,987999976	311,2189331

Group	Description	Sample	Sequence	Modifs	Used	on a total of	Sub-groups	E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**TC369987**	**B7=BG30**	GYVEEDLVSTDFVGDSR		-	-	1.01	0,00026	2	1887,862549	1887,861084	0,0015	0,794550002
1			LVSWYDNEWGYSNR		2	2	1.01 1.02	0,00046	2	1788,797607	1788,797974	-0,0004	-0,223613843
1			NPEEIPWGEAGADYVVESTGVFTDK		-	-	1.02	7,3E-05	3	2710,250244	2710,252197	-0,0021	-0,774835587
1			GIMGYVEEDLVSTDFVGDSR		2	2	1.01 1.02	2,2E-07	2	2189,007813	2189,00708	0,0007	0,319779664
2	**TC425413**		GGGGSGSEKEDIQPR		-	-	2.01	0,00012	2	1473,693237	1473,693359	0	0
2			FHQITGDQCHHLR	C9:+57.0215	3	3	2.01 2.02 2.03	0,00016	2	1648,779785	1648,776489	0,0033	2,001484156
2			SKGEGEIYEASEEQIR		-	-	2.01	1,2E-06	2	1824,859985	1824,86145	-0,0014	-0,767181516
2			GEGEIYEASEEQIR		-	-	2.01	0,00025	2	1609,733765	1609,734375	-0,0006	-0,372732341
2			AFLQPSHHDADEIAFVR		-	-	2.01	3,7E-05	2	1952,961304	1952,961792	-0,0004	-0,204817116
2			DGYFEMACPHISSSGR	C8:+57.0215	-	-	2.01	2E-07	2	1813,761597	1813,763672	-0,002	-1,10267961
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	4,7E-05	2	2098,091553	2098,093262	-0,0016	-0,762597144
1	**TC369987**	**B8=BG9**	YDTVHGQWK		-	-	1.02	0,00062	2	1133,53772	1133,537842	-1E-04	-0,088219374
1			VPTVDVSVVDLTVR		2	2	1.01 1.02	7,1E-05	2	1498,84668	1498,848022	-0,0012	-0,800614893
1			LVSWYDNEWGYSNR		-	-	1.01	2E-05	2	1788,798462	1788,797974	0,0005	0,279517323
1			GIMGYVEEDLVSTDFVGDSR	M3:+15.9949	-	-	1.01	3,8E-07	2	2205,000732	2205,001953	-0,0012	-0,544217229
1			LVSWYDNEWGYSTR		-	-	1.02	7,8E-05	2	1775,806396	1775,802856	0,0036	2,027252197
1			NPEEIPWGEAGADYVVESTGVFTDKDK		-	-	1.01	6E-05	3	2953,374023	2953,374268	-0,0002	-0,067719147
1	**TC432185**		GDATLGEGASESLHVK		2	2	1.01 1.02	0,00015	2	1570,770386	1570,77124	-0,0007	-0,445640951
1			GILAADESTGTIGK		2	2	1.01 1.02	2,1E-06	2	1332,700806	1332,70105	-1E-04	-0,07503558
1			GTVELAGTNGETTTQGFDDLGK		-	-	1.02	1,3E-05	2	2211,041748	2211,041504	0,0002	0,090455107
1	**TC369987**	**B9=BG29**	LVSWYDNEWGYSNR		-	-	1.01	0,00037	2	1788,795898	1788,797974	-0,0021	-1,173972726
1			VPTVDVSVVDLTVR		2	2	1.01 1.02	0,00021	2	1498,8479	1498,848022	0	0
1			GIMGYVEEDLVSTDFVGDSR	M3:+15.9949	-	-	1.01	6,8E-07	2	2205,002197	2205,001953	0,0002	0,090702862
1			GILGYVDEDLVSTDFQGDNR		-	-	1.02	8E-06	2	2213,02832	2213,036133	-0,0077	-3,479382992
1			NPEEIPWGEAGADYVVESTGVFTDK		-	-	1.01	9,3E-07	3	2710,251709	2710,252197	-0,0006	-0,22138162
2	**TC425413**		EQEQGRQEEEQGHGR		2	4	2.01 2.02	5E-05	2	1796,789185	1796,791138	-0,0018	-1,001785874
2			GGGGGSGSEKEDIQPR		3	4	2.01 2.03 2.04	1,6E-05	2	1530,714355	1530,714722	-0,0003	-0,195986882
2			FHQITGDQCHHLR	C9:+57.0215	4	4	01 2.02 2.03 2.(	4,9E-05	2	1648,784424	1648,776489	0,0079	4,791431427
2			SRGEGPISEGSEEQIR		3	4	2.01 2.03 2.04	0,00046	2	1730,829834	1730,830811	-0,0009	-0,519981503
2			AQPESVFVAGPQQQR		-	-	2.01	0,00014	2	1641,833862	1641,834839	-0,0009	-0,548167169
2			DGYFEMACPHISSSGR	C8:+57.0215	2	4	2.01 2.02	6E-05	2	1813,760132	1813,763672	-0,0035	-1,929689288
4	**TC373663**		VATVQCLSGTGSLR	C6:+57.0215	2	2	4.01 4.02	0,0004	2	1448,753052	1448,753052	1E-04	0,069024868
4			TEEGKPLVLNVVK		-	-	4.01	0,00013	2	1425,8302	1425,831543	-0,0013	-0,911748648
4			LIFGADSPAIQENR		2	2	4.01 4.02	8,7E-05	2	1530,790283	1530,791504	-0,0012	-0,783908188
4			MFVADGGELLMAQSYAK	M1:+15.9949 - M11:+15.9949	2	2	4.01 4.02	4,3E-09	2	1862,864624	1862,866699	-0,002	-1,07361412

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
2	**TC414845**	**B10=BG10**	GGGGSGSEKEDIQPR		-	-	2.01	0,00025	2	1473,693359	1473,693359	0,0002	0,135713428
2			SKGEGEIYEASEEQIR		-	-	2.01	0,00082	2	1824,862671	1824,86145	0,0013	0,712382853
2			DGYFEMACPHISSSGR	C8:+57.0215 - M6:+15.9949	-	-	2.01	4,8E-06	2	1829,759155	1829,758545	0,0006	0,327912152
3	**TC382425**		HVDTAAEYGVEK		-	-	3.01	0,00015	2	1318,627319	1318,627808	-0,0004	-0,30334565
3			GIHVTAYSPLGSSEK		-	-	3.01	1,9E-05	2	1545,791992	1545,79126	0,0009	0,582226098
3			AGSDTAHSVQTAITEAGYR		-	-	3.01	1,9E-06	2	1934,921387	1934,920654	0,0008	0,413453639
1	**TC422366**	**B15=BG15**	GPIQLSHNYNYGPAGR		2	2	1.01 1.02	0,00039	2	1743,852783	1743,856567	-0,0037	-2,121734142
1			GATSNYCTPSAQWPCAPGK	C7:+57.0215 - C15:+57.0215	2	2	1.01 1.02	1,2E-05	2	2052,889893	2052,890625	-0,0007	-0,340982616
1			NPDLVATDPTVSFK		2	2	1.01 1.02	0,00011	2	1503,767334	1503,769409	-0,002	-1,329991221
1			VGYGDNLDCYNQRPFA	C9:+57.0215	-	-	1.02	5,5E-05	2	1888,829224	1888,828735	0,0006	0,317657202
2	**TC422632**		DSVVVSGGPDYR		-	-	2.01	0,00016	2	1250,601929	1250,601563	0,0004	0,319846064
2			DFFEQFGVSMGK	M10:+15.9949	-	-	2.01	0,00042	2	1407,625488	1407,625244	0,0002	0,142083272
2			TPNVFDNQYYVDLVNR		-	-	2.01	1,3E-06	2	1956,942627	1956,945435	-0,0028	-1,430801272
3	**TC387479**		GGGGGSGSEKEDIQPR		-	-	3.01	0,00017	2	1530,714722	1530,714722	0	0
3			SRGEGPISEGSEEQIR		-	-	3.01	0,00089	2	1730,831665	1730,830811	0,0009	0,519981503
3			AFLQPSHYDADEIAFVR		-	-	3.01	5,7E-05	2	1978,964844	1978,966187	-0,0013	-0,656908631
1	**TC403178**	**B17=BG17**	GVKDEGLVVVPGEGPEGQTVGNIIAGDR		-	-	1.02	0,00084	3	2762,433594	2762,432373	0,0013	0,470599771
1			LVAGLLGVESAQDAVIR		2	2	1.01 1.02	9,7E-05	2	1710,974487	1710,975342	-0,0007	-0,409123331
1			DEGLVVVPGEGPEGQTVGNIIAGDR		-	-	1.02	1,8E-06	2	2478,246094	2478,247559	-0,0014	-0,564915299
1	**TC376693**	**B19=BG19**	HGGGLTMAPGHGR		-	-	1.01	1,1E-06	2	1247,609009	1247,606567	0,0024	1,923683405
1			YSGAEVHEYK		3	3	1.01 1.02 1.03	3,5E-05	2	1182,542603	1182,542969	-0,0004	-0,338254094
1			PPVHDTDGNELR		2	3	1.01 1.02	0,00041	2	1349,648193	1349,644775	0,0034	2,51918149
1			PPPVHDTDGNELR		2	3	1.01 1.02	2E-05	2	1446,697754	1446,697632	0,0002	0,138245881
1			IAPHGGGAPSDKIIR		-	-	1.02	0,00013	2	1488,828369	1488,828613	-1E-04	-0,067166895
1			IEKYSGAEVHEYK		3	3	1.01 1.02 1.03	9,3E-06	2	1552,764526	1552,764648	0	0
1			AHGGGLTMAPGHGR		2	3	1.01 1.02	5,7E-06	2	1318,643677	1318,643677	0	0
1			GDSCQDLGVFR	C4:+57.0215	-	-	1.03	0,00033	2	1253,557617	1253,55835	-0,0007	-0,558410347
1			CPLFVSQEADGQRDGLPVR	C1:+57.0215	2	3	1.01 1.02	1,4E-05	2	2144,053223	2144,055664	-0,0025	-1,166014552
1			PPPVHDTDGNELRADANYYVLPANR		2	3	1.01 1.02	0,00031	3	2794,350342	2794,354736	-0,0044	-1,5746032
1			LMACGDSCQDLGVFR	C4:+57.0215 - C8:+57.0215	3	3	1.01 1.02 1.03	1,2E-05	2	1728,746704	1728,75061	-0,0039	-2,255964518
1			AYTTCVQSTEWHIDSELVSGR	C5:+57.0215	2	3	1.01 1.02	9,8E-08	2	2439,121338	2439,125	-0,0036	-1,475939155
1			GGAWFLGATEPYHVVVFK		3	3	1.01 1.02 1.03	0,00044	2	1978,021729	1978,022583	-0,0008	-0,404444307
2	**TC378613**		TGCTFDGSGR	C3:+57.0215	-	-	2.01	0,00018	2	1057,435059	1057,437256	-0,0021	-1,985933304
2			FGGDTYCCR	C7:+57.0215 - C8:+57.0215	-	-	2.01	0,00035	2	1135,428589	1135,430054	-0,0013	-1,144940615
2			LDPGQSWALNMPAGTAGAR	M11:+15.9949	-	-	2.01	2,1E-05	2	1928,928833	1928,928711	1E-04	0,051842246
1	**A4GFQ9**	**B21=BG22**	EHGAQEGQAGTGAFPR		-	-	1.01	9,7E-06	2	1612,748047	1612,746704	0,0014	0,868084192
1			LPIVVDASGDGAYVCK	C15:+57.0215	-	-	1.01	2,7E-06	2	1663,835938	1663,836426	-0,0004	-0,240408242
1			DCCQQLADISEWCR	C2:+57.0215 - C3:+57.0215 - C13:+57.0215	2	2	1.01 1.02	1,7E-05	2	1840,742188	1840,741455	0,0007	0,380281538

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
2	**TC385921**	**B24=BG24**	RGSGSESESESEEQQDQQR		-	-	2.01	0,00014	2	2152,896484	2152,897705	-0,0013	-0,603837371
2			GSGSESESESEEQQDQQR		-	-	2.01	3,2E-05	2	1996,797607	1996,796753	0,001	0,5008021
2			WSEEEDDDQR		-	-	2.01	0,00051	2	1308,497314	1308,497925	-0,0005	-0,38211754
2			AKDQQDEGFVAGPEQQEQER		-	-	2.01	1,4E-05	2	2289,035889	2289,038086	-0,0023	-1,004788995
2			DQQDEGFVAGPEQQEQER		-	-	2.01	2,7E-05	2	2089,907471	2089,90625	0,0014	0,669886529
1	**TC387936**	**B25=BG25**	TIGDAGAAGGEERQTK		-	-	1.01	0,00014	2	1560,761963	1560,761719	0,0003	0,192213848
1			VVGIAGGDDSSAAKK		-	-	1.01	0,00024	2	1374,722046	1374,722778	-0,0007	-0,50919354
1			VVGIAGGDDSSAAK		-	-	1.01	7,5E-05	2	1246,626831	1246,627808	-0,0009	-0,72194761
1	**B7U6L4**		SFHALAQHDVR		3	4	1.01 1.02 1.04	0,0008	2	1280,64978	1280,649902	0	0
1			AKDQQDEGFVAGPEQQEQER		-	-	1.02	9,6E-06	3	2289,036133	2289,038086	-0,002	-0,873729527
1			DQQDEGFVAGPEQQEQERGDR		-	-	1.02	0,00052	3	2418,052734	2418,055664	-0,003	-1,24066627
1	**B7U6L4**	**B28=BG37**	SFHALAQHDVR		3	4	1.01 1.02 1.04	0,0008	2	1280,64978	1280,649902	0	0
1			AKDQQDEGFVAGPEQQEQER		-	-	1.02	4,8E-07	2	2289,03833	2289,038086	0,0002	0,087372944
1			ILHTISVPGK		3	4	1.01 1.02 1.03	0,00073	2	1064,646973	1064,646729	0,0003	0,28178364
1			DQQDEGFVAGPEQQEQER		-	-	1.02	3,5E-06	2	2089,903076	2089,90625	-0,003	-1,435471058
1			EASEGGQGHHWPLPPFR		-	-	1.02	0,00062	2	1901,905273	1901,904541	0,0007	0,368052095
1			GSSNLQVVCFEINAER	C9:+57.0215	3	4	1.01 1.02 1.04	1,3E-06	2	1822,874512	1822,87561	-0,0011	-0,603442192
1	**TC425413**	**B29=BG36**	EQEQGRQEEEQGHGREQEK		2	3	1.01 1.02	0,00058	2	2311,029297	2311,029785	-0,0004	-0,173083007
1			EQEQGRQEEEQGHGR	E1:-18.0106	2	3	1.01 1.02	0,00021	2	1778,779419	1778,780518	-0,001	-0,562182963
1			GGGGSGSEKEDIQPR		-	-	1.01	0,00079	2	1473,692505	1473,693359	-0,0007	-0,474997014
1			WQEGGDEGR		-	-	1.01	0,00071	2	1033,433228	1033,433838	-0,0005	-0,483823955
1			SKGEGEIYEASEEQIR		-	-	1.01	3,4E-06	2	1824,855469	1824,86145	-0,0059	-3,233122349
1			DGYFEMACPHISSSGR	C8:+57.0215 - M6:+15.9949	2	3	1.01 1.02	1,1E-08	2	1829,758911	1829,758545	0,0004	0,218608081
1			GEGEIYEASEEQIR		-	-	1.01	0,00073	2	1609,736694	1609,734375	0,0023	1,428807139
1			EGDVIVIPAGSIVYSANTHR	E1:-18.0106	-	-	1.01	0,00083	2	2080,087646	2080,08252	0,0051	2,451825857
2	**TC397230**		HGSGSESESEEEQDQQR		-	-	2.04	5,2E-07	2	1918,76355	1918,765015	-0,0014	-0,729636014
2			SFHALAQHDVR		5	5	2.02 2.03 2.04	0,0008	2	1280,649902	1280,649902	1E-04	0,078085348
2			EGDVIVAPAGSIMHLANTDGR	M13:+15.9949 - E1:-18.0106	3	5	2.02 2.03 2.04	8,2E-06	2	2121,034668	2121,039795	-0,005	-2,357334375
3	**TC421662**		GKDVTVSTGGTAAEYAK		2	2	3.01 3.02	1,8E-08	2	1654,828857	1654,828735	0,0002	0,120858431
3			TKDVTLSTAAQAAQK		2	2	3.01 3.02	2,7E-05	2	1532,827759	1532,828247	-0,0005	-0,326194435
3			DSVTGAVGGAVDK		-	-	3.02	0,00071	2	1175,588501	1175,590698	-0,0021	-1,786335945
3			DVTVSTGGTAAEYAK		2	2	3.01 3.02	1,3E-06	2	1469,713135	1469,71228	0,0009	0,612364769
3			DVTLSTAAQAAQK		2	2	3.01 3.02	3,2E-05	2	1303,683594	1303,685547	-0,002	-1,534112334
3			GIVAGEEELIPVEGEAGK		2	2	3.01 3.02	2,5E-06	2	1796,927246	1796,928101	-0,0008	-0,445204228

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**TC425413**	**B30=BG35**	EQEQGRQEEEQGHGR		2	3	1.01 1.02	2,4E-05	2	1796,791992	1796,791138	0,001	0,556547701
1			GGGGSGSEKEDIQPR		-	-	1.01	0,00037	2	1473,693604	1473,693359	0,0004	0,271426857
1			WQEGGDEGR		-	-	1.01	0,00075	2	1033,433105	1033,433838	-0,0006	-0,580588758
1			FHQITGDQCHHLR	C9:+57.0215	3	3	1.01 1.02 1.03	1E-04	2	1648,776245	1648,776489	-0,0003	-0,181953117
1			SKGEGEIYEASEEQIR		-	-	1.01	5,7E-06	2	1824,862915	1824,86145	0,0015	0,821980238
1			SKGEGEIYEASEEQIR		-	-	1.01	5,1E-05	2	1824,863403	1824,86145	0,002	1,095973611
1			GEGEIYEASEEQIR		-	-	1.01	0,00094	2	1609,737549	1609,734375	0,0032	1,987905502
2	**TC419611**		AKDQQDEGFVAGPEQQEQER		-	-	2.02	2,1E-06	3	2289,034424	2289,038086	-0,0038	-1,660086036
2			EASEGGQGHHWPLPPFR		-	-	2.02	0,00088	2	1901,910522	1901,904541	0,006	3,154732466
2			GSSNLQVVCFEINAER	C9:+57.0215	3	4	2.01 2.02 2.03	6E-05	2	1822,873901	1822,87561	-0,0017	-0,932592452
2			FQYFSAKPLLASLSK		2	4	2.01 2.02	3,7E-05	2	1699,94397	1699,942261	0,0018	1,058859468

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**TC408407**	**BG12**	VALVTGGDSGIGR		2	2	1.01 1.02	0,0006	2	1201,653198	1201,654053	-0,0007	-0,582530379
1			ALSGDLGYEENCR	C12:+57.0215	-	-	1.01	5,1E-06	2	1483,648804	1483,64856	0,0002	0,134802803
1			GHEDKDAEETLQALR		2	2	1.01 1.02	2,4E-07	2	1711,82251	1711,824951	-0,0024	-1,402012587
1			GNATLLDYTATK		2	2	1.01 1.02	0,00071	2	1267,653076	1267,65332	-0,0002	-0,157771841
2	**TC382451**		AELDHMAGTFGK	M6:+15.9949	2	2	2.01 2.02	5,2E-05	2	1292,59436	1292,59436	0	0
2			LNQDVLQCPVYDSDDK	C8:+57.0215	-	-	2.02	3,2E-07	2	1908,865723	1908,864746	0,001	0,523871601
2			LPLGAPALMVSPQGER	M9:+15.9949	2	2	2.01 2.02	5,1E-05	2	1651,884888	1651,884033	0,0009	0,544832408
1	**TC408407**	**BG41**	VVEEVANAHGGR		3	4	1.01 1.02 1.03	0,00034	2	1237,627319	1237,628784	-0,0014	-1,131195426
1			FPPQQQDCQPGK	C8:+57.0215	2	4	1.01 1.02	0,00041	2	1429,652588	1429,65332	-0,0007	-0,489629209
1			GHEDKDAEETLQALR		2	4	1.01 1.02	0,00097	3	1711,82605	1711,824951	0,0011	0,642589033
1			HMGPGSSIINTTSVNAYK	M2:+15.9949	3	4	1.01 1.02 1.04	2,7E-08	2	1892,91748	1892,91748	0	0
1			VALVTGGDSGIGR		2	4	1.01 1.02	0,00051	2	1201,654053	1201,654053	0,0002	0,166437253
1			ALAGDLGYEENCR	C12:+57.0215	-	-	1.02	1,1E-05	2	1467,654175	1467,653687	0,0005	0,340679824
1			HMGPGSSIINTTSVNAYK		3	4	1.01 1.02 1.04	5,5E-06	2	1877,92688	1876,922974	1,003999949	534,9180298
1			GNATLLDYTATK		4	4	01 1.02 1.03 1.(	0,00056	2	1267,653076	1267,65332	-0,0002	-0,157771841
2	**TC368832**		LVSWYDNEWGYSNR		-	-	2.02	1,6E-05	2	1788,798218	1788,797974	0,0002	0,111806922
2			VPTVDVSVVDLTVR		2	2	2.01 2.02	8,3E-05	2	1498,849854	1498,848022	0,0019	1,267640233
2			GIMGYVEEDLVSTDFVGDSR	M3:+15.9949	-	-	2.02	1,7E-05	2	2204,998535	2205,001953	-0,0034	-1,541948795
2			VALQSDDVELVAVNDPFITTEYMTYMFK	·15.9949 - M26:+15	-	-	2.02	0,00062	3	3271,546143	3271,542969	0,0032	0,978131771
3	**TC382451**		AELDHMAGTFGK	M6:+15.9949	2	2	3.01 3.02	8,4E-05	2	1292,593628	1292,59436	-0,0007	-0,541546524
3			LNQDVLQCPVYDSDDK	C8:+57.0215	-	-	3.02	2,3E-07	2	1908,865967	1908,864746	0,0012	0,628645897
3			LPLGAPALMVSPQGER	M9:+15.9949	2	2	3.01 3.02	5,2E-06	2	1652,879517	1651,884033	0,995999992	602,947876
3			LIGVEYIVSR		2	2	3.01 3.02	0,00017	2	1148,66748	1148,667847	-0,0003	-0,261172116
7	**TC433257**		GGPGKPLHLTATVR		-	-	7.01	3,1E-05	2	1403,810547	1403,812256	-0,0016	-1,13975358
7			NVYYGVAPVAQK		-	-	7.01	0,00093	2	1308,694702	1308,695068	-0,0003	-0,229235992
7			DNYGGIMLWDR	M7:+15.9949	-	-	7.01	0,00097	2	1355,604614	1355,605347	-0,0006	-0,442606717
2	**TC422632**	**BG13**	DSVVVSGGPDYR		-	-	2.01	0,00031	2	1250,601929	1250,601563	0,0004	0,319846064
2			GAVVSCADILALAAR	C6:+57.0215	-	-	2.01	2,7E-05	2	1486,807373	1486,805054	0,0024	1,614199638
2			TPNVFDNQYYVDLVNR		-	-	2.01	1,4E-07	2	1956,945068	1956,945435	-0,0003	-0,153300151
2			DFFEQFGVSMGK		-	-	2.01	7,4E-05	2	1391,630005	1391,630371	-0,0004	-0,287432641
1	**TC378868**	**BG20**	AHGGGLTMAPGHGR	M8:+15.9949	2	2	1.01 1.02	1,3E-06	2	1334,638062	1334,63855	-0,0005	-0,374633282
1			YSGAEVHEYK		2	2	1.01 1.02	3,7E-05	2	1182,542358	1182,542969	-0,0006	-0,507381201
1			IEKYSGAEVHEYK		2	2	1.01 1.02	3,7E-06	2	1552,765503	1552,764648	0,0009	0,579611301
1			IAPHGGAPSDKIIR		-	-	1.01	0,0005	2	1431,80835	1431,807129	0,0013	0,907943487
1			LMACGDSCQDLGVFR	·57.0215 - C8:+57.(	2	2	1.01 1.02	8,1E-06	2	1728,749512	1728,75061	-0,0011	-0,636297643
1	**TC371775**	**BG23**	VEVEDGNVLVVSGER		3	3	1.01 1.02 1.03	1,4E-06	2	1600,817505	1600,818115	-0,0006	-0,374808371
1			EEVKVEVEDGNVLVVSGER		3	3	1.01 1.02 1.03	7,2E-05	2	2086,065674	2086,06665	-0,001	-0,479371071
1			SIVPAISGGSSETAAFANAR		-	-	1.01	4E-07	2	1905,967041	1905,966919	0,0002	0,104933612
1			EEVKVEVEDGNVLVVSGER		3	3	1.01 1.02 1.03	3,6E-05	2	2086,071289	2086,06665	0,0047	2,25304389
1			SNVFDPFADLWADPFDTFR		3	3	1.01 1.02 1.03	6,8E-08	2	2260,0354	2260,034912	0,0005	0,221235529

1	**TC400242**	**BG25**	RGSGSESESESEEEQDQQR	-	-	1.01	3,6E-05	2	2153,881348	2153,881836	-0,0004	-0,185711205
1			GSGSESESESEEEQDQQR	-	-	1.01	3,7E-06	2	1997,779419	1997,78064	-0,0012	-0,600666583
1			AKDQQDEGFVAGPEQQSR	-	-	1.01	3,6E-05	2	1989,927124	1989,926514	0,0007	0,351771772
1			GSGSESESESEEEQDQQRYETVR	-	-	1.01	4,9E-05	3	2646,105469	2646,10376	0,0017	0,642454028
1			DQQDEGFVAGPEQQSR	-	-	1.01	4,3E-05	2	1790,796509	1790,794434	0,0021	1,172663808
2	**TC371775**		LPEDAKVEEVK	-	-	2.01	0,00066	2	1256,673462	1256,673706	-0,0002	-0,159150302
2			VEVEDGNVLVVSGER	-	-	2.01	0,00014	2	1600,81604	1600,818115	-0,002	-1,249361277
2			SIVPAISGGSSETAAFANAR	-	-	2.01	3,3E-05	2	1905,968628	1905,966919	0,0018	0,944402516
2			AGLENGVLTVTVPK	-	-	2.01	0,00027	2	1397,799438	1397,800293	-0,0008	-0,572327793

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**TC380640**	**B4**	AFVEVNETGTEAAATTIAK		-	-	1.01	7,2E-05	2	1922,97131	1922,97095	0,0004	0,20801146
1			DQLVATLGEGEAER		-	-	1.02	0,00031	2	1487,7356	1487,73413	0,0016	1,07546103
1			DILPAGSIDNTTR		-	-	1.01	0,00034	2	1372,70642	1372,70715	-0,0006	-0,4370925
1	**TC368832**	**B11**	SDIDIVSNASCTTNCLAPLAK	C11:+57.0215 - C15:+57.0215	-	-	1.01	7,5E-05	2	2250,06958	2250,07446	-0,0049	-2,1777058
1			TLLFGEKEVAVFGCR	C14:+57.0215	-	-	1.01	9,1E-05	2	1725,89868	1725,89966	-0,001	-0,579408
1			LVSWYDNEWGYSTR		-	-	1.01	5,1E-06	2	1775,80249	1775,80286	-0,0003	-0,1689377
1			GILGYVDEDLVSTDFQGDNR		-	-	1.01	1,7E-05	2	2213,0332	2213,03613	-0,0029	-1,3104169
1			VALQSPDVELVAVNDPFITTDYMTYMFK	M23:+15.9949 - M26:+15.9949	-	-	1.01	0,00011	3	3239,5542	3239,55298	0,0012	0,3704215
2	**TC382425**		HVDTAAEYGVEKEVGK		2	2	2.01 2.02	2E-05	2	1731,8562	1731,85522	0,001	0,57741553
2			DIGVCNYTVTK	C5:+57.0215	-	-	2.01	0,00023	2	1269,61499	1269,61475	0,0003	0,23629217
2			AGSDTAHSVQTAITEAGYR		2	2	2.01 2.02	1,1E-06	2	1934,92041	1934,92065	-0,0002	-0,1033634
2			DGAHKPPEAGEVLEFDMEGVWK		2	2	2.01 2.02	0,00015	3	2441,14404	2441,14453	-0,0005	-0,204822
1	**TC386688**	**B12**	GGGGGSGSEKEDIQPR		-	-	1.01	0,00035	2	1530,71326	1530,71472	-0,0014	-0,9146054
1			GEGPISEGSEEQIR		-	-	1.01	0,00074	2	1487,69751	1487,69775	-1E-04	-0,067218
1			DGYFEMACPHISSSGR	C8:+57.0215 - M6:+15.9949	-	-	1.01	0,00041	2	1829,76001	1829,75854	0,0015	0,81978035
2	**A4GFQ9**	**B20**	EHGAQEGQAGTGAFPR		-	-	2.01	4,7E-05	2	1612,74744	1612,7467	0,0008	0,49604812
2			LPIVVDASGDGAYVCK	C15:+57.0215	-	-	2.01	6,9E-07	2	1663,83826	1663,83643	0,0019	1,14193916
2			DCCQQLADISEWCR	C2:+57.0215 - C3:+57.0215 - C13:+57.0215	-	-	2.01	8,7E-07	2	1840,74133	1840,74146	-1E-04	-0,0543259
1	**TC374459**	**B23**	VEVEDGNVLVVSGER		2	2	1.01 1.02	0,00019	2	1600,8197	1600,81812	0,0016	0,99948889
1			SIVPAISGGNNETAAFANAR		-	-	1.02	0,00045	2	1959,98621	1959,98865	-0,0024	-1,224497
1			EEVKVEVEDGNVLVVSGER		2	2	1.01 1.02	3,8E-05	2	2086,06909	2086,06665	0,0025	1,19842768
1			AGLENGVLTVTVPK		2	2	1.01 1.02	5,8E-05	2	1397,80115	1397,80029	0,0009	0,6438688
2	**TC385921**		RGSGSESESESEEQQDQQR		-	-	2.01	0,00014	2	2152,896	2152,89771	-0,0017	-0,7896334
2			GSGSESESESEEQQDQQR		-	-	2.01	1,6E-05	2	1996,79663	1996,79675	0	0
2			AKDQQDEGFVAGPEQQEQER		-	-	2.01	8,7E-06	3	2289,03687	2289,03809	-0,0014	-0,6116106
1	**TC385921**	**B26**	GSGSESESESEEQQDQQR		-	-	1.01	1,7E-05	2	1996,79797	1996,79675	0,0014	0,70112294
1			AKDQQDEGFVAGPEQQEQER		2	2	1.01 1.02	4,6E-07	2	2289,03931	2289,03809	0,0011	0,48055118
1			DQQDEGFVAGPEQQEQER		2	2	1.01 1.02	0,00015	2	2089,90405	2089,90625	-0,0021	-1,0048298

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**TC397230**	**B27**	HGSGSESESEEEQDQQR		-	-	1.01	1,3E-08	2	1918,76587	1918,76501	0,001	0,52116859
1			RHGSGSESESEEEQDQQR		-	-	1.01	1,4E-05	2	2074,86499	2074,86621	-0,001	-0,4819588
1			HGSGSESESEEEQDQQRYETVR		-	-	1.01	0,00075	3	2567,08765	2567,08813	-0,0005	-0,1947732
1			AKDQQDEGFVAGPEQQSHEQEQER		-	-	1.01	7,8E-05	3	2770,23071	2770,23022	0,0005	0,18049042
1			AKDQQDEGFVAGPEQQSHEQEQERGDF		-	-	1.01	3,7E-05	3	3098,37891	3098,37988	-0,001	-0,3227493
1			ILHTISVPGK		2	2	1.01 1.02	0,00049	2	1064,64502	1064,64673	-0,0016	-1,5028459
1	**B7U6L4**		CVQECRDDQQQHGR	C1:+57.0215 - C5:+57.0215 - C1:-17.0265	-	-	1.02	0,00033	3	1798,73303	1798,73474	-0,0017	-0,9451088
1			AKDQQDEGFVAGPEQQQEHER		-	-	1.02	1,5E-05	3	2426,09741	2426,09717	0,0003	0,12365539
1			ILHTISVPGK		2	2	1.01 1.02	0,00049	2	1064,64502	1064,64673	-0,0016	-1,5028459
1	**TC425413**	**B31**	GGGGSGSEKEDIQPR		-	-	1.01	0,0003	2	1473,69299	1473,69336	-0,0002	-0,1357134
1			SGGSGRPYHFGQESYR		-	-	1.01	6,5E-05	2	1784,8103	1784,8103	0	0
1			EDVDRVFESK		-	-	1.01	0,00092	2	1223,58875	1223,5907	-0,0019	-1,5528069
1			SKGEGEIYEASEEQIR		-	-	1.01	2,9E-06	2	1824,86121	1824,86145	-0,0002	-0,1095974
1			DGYFEMACPHISSSGR	C8:+57.0215 - M6:+15.9949	2	4	1.01 1.02	1,9E-09	2	1829,75684	1829,75854	-0,0017	-0,9290844
1			GEGEIYEASEEQIR		-	-	1.01	0,00025	2	1609,73474	1609,73438	0,0003	0,18636617
1			AFLQPSHHDADEIAFVR		-	-	1.01	1,5E-05	2	1952,9613	1952,96179	-0,0004	-0,2048171
2	**TC383884**	**B32**	TYDLNFKEENNDGSQK		2	2	2.01 2.02	5,7E-07	2	1901,85303	1901,85156	0,0015	0,78870511
2			KYGQDATNVGDEGGFAPNIQENK		2	2	2.01 2.02	6,1E-05	3	2452,13818	2452,13794	0,0002	0,08156148
2			YGQDATNVGDEGGFAPNIQENK		2	2	2.01 2.02	1,7E-06	2	2324,04028	2324,04297	-0,0027	-1,1617686
2			GNPTVEVDVCCSDGTFAR	C10:+57.0215 - C11:+57.0215	2	2	2.01 2.02	4,4E-07	2	1983,85327	1983,85388	-0,0006	-0,3024416
2			VNQIGSVTESIEAVK		2	2	2.01 2.02	4,7E-06	2	1573,84326	1573,84363	-0,0003	-0,1906161
2			IEEELGDAAVYAGLK		-	-	2.01	0,00072	3	1577,80676	1577,80615	0,0007	0,443654
2			AAVPSGASTGVYEALELR		2	2	2.01 2.02	2,2E-06	2	1790,92688	1790,92871	-0,0018	-1,0050651
2			DPTAQTELDNFMVQQLDGTK	M12:+15.9949	2	2	2.01 2.02	1,9E-07	2	2267,04785	2267,05005	-0,0021	-0,9263139
2			LAMQEFMILPTGAASFK	M3:+15.9949 - M7:+15.9949	-	-	2.01	3E-06	2	1886,9397	1886,93945	0,0003	0,15898761
2			MTEECGVEVQIVGDDLLVTNPTR	C5:+57.0215 - M1:+15.9949	2	2	2.01 2.02	6,6E-08	2	2591,23218	2591,23315	-0,0009	-0,347325
2			DPTAQTELDNFMVQQLDGTK		2	2	2.01 2.02	4,4E-09	2	2251,0564	2251,05518	0,0013	0,57750696
4	**TC413770**		YKAETQSVDFQTK		2	2	4.01 4.02	5,4E-07	2	1544,75867	1544,75952	-0,0008	-0,5178799
4			IKNILPSGSVDNTTK		-	-	4.01	0,00048	3	1586,87439	1586,87524	-0,0009	-0,5671523
4			AAEVTTQVNSWVEK		2	2	4.01 4.02	0,00018	2	1561,78528	1561,78613	-0,0008	-0,512234
4			GAWTEQFDSYGTK		-	-	4.02	7,4E-06	2	1489,65881	1489,65979	-0,001	-0,6712942
1			DAGQYNDTPQR		2	4	1.01 1.04	0,0009	2	1264,5553	1264,55566	-0,0004	-0,3163166
1	**TC388221**	**B33**	MHANLPHDPCVDPVAPLQR	C10:+57.0215 - M1:+15.9949	**-**	**-**	**1.01**	**0,00018**	**3**	**2183,04541**	**2183,04883**	**-0,0034**	**-1,5574548**
1			EGLNMACENALPR	C7:+57.0215 - M5:+15.9949	-	-	1.01	0,00052	2	1490,67151	1490,67297	-0,0015	-1,0062569
1			AAAAMVGHPEWEFPR	M5:+15.9949	2	4	1.01 1.04	1,6E-05	2	1684,78967	1684,79053	-0,0007	-0,4154819
1			YDPTAYNTILR		-	-	1.01	0,00041	2	1326,66943	1326,66931	0,0002	0,15075347
1			LSNQLVEGQNYVNFK		-	-	1.01	0,00027	2	1752,8916	1752,89197	-0,0003	-0,1711458
1			FFVDNGTYLTEQGR		3	4	1.01 1.02 1.03	1,2E-05	2	1646,78064	1646,78125	-0,0007	-0,4250716
2	**Q41593**		AETQSVDFQTK		2	2	2.01 2.02	0,00012	2	1253,60022	1253,6012	-0,001	-0,7977019
2			YKAETQSVDFQTK		2	2	2.01 2.02	9,7E-05	2	1544,75928	1544,75952	-0,0002	-0,12947
2			GAWTEQFDSYGTK		-	-	2.01	0,00011	2	1489,6593	1489,65979	-0,0005	-0,3356471
5	**TC425761**		TYDLNFKEENNDGSQK		-	-	5.01	0,00086	2	1901,85352	1901,85156	0,002	1,05160689
5			GNPTVEVDVCCSDGTFAR	C10:+57.0215 - C11:+57.0215	-	-	5.01	5,2E-05	2	1983,85449	1983,85388	0,0006	0,30244163
5			IEEELGAAAVYAGLK		-	-	5.01	6,3E-06	2	1533,81738	1533,81628	0,0011	0,71716541

Chapter II

Immunochemical analyses for the assessment of the variation in the amount of allergenic polypeptides of GM genotypes in comparison to commercial durum and bread wheat.

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Abstract

Breeding has allowed developing numerous 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 allergenicity in a part of the population. More recently, the genetic modification (GM) technology for crop improvement has emerged and its impact on the allergenicity has to be evaluated. Although GM wheats are not available on the market at the moment, it is not excluded that they will be produced and commercialized. For this reason, our aim was to assess if the variation in the amount of allergenic polypeptides in GM wheats developed as research tools, in comparison to their untransformed genotypes, was equivalent to the variation observed among commercial cultivars, either durum or bread wheats. Because the most important factor is the amount of allergenic polypeptides, we performed Enzyme-Linked Immunoabsorbent Assay on subfractions of the soluble proteins by using sera of patients with documented wheat food or respiratory allergies. The results here reported show that, regardless the protein subfraction, there is a wide variation in the amount of allergenic polypeptides among durum and bread wheat cultivars, and that the differences observed between GM wheats and their untransformed counterparts are within such variation. Moreover, methods for the subfractionation of the soluble proteins, including Albumins/Globulins, metabolic proteins, chlorophorm-methanol soluble (CM) proteins, were compared by mass spectrometry analyses, in order to detect their efficiency in revealing immunogenic polypeptides.

1. Introduction

Breeding of cereals has been particularly active in the last hundred years, resulting in spectacular improvements in suitable traits such as: increased yields, biotic and abiotic resistance and technological performances. Safety had never been an issue of plant breeding, because it was assumed that crop varieties were safe based on their long history of use. Thus the typical standard seed certification only points on seed authenticity, genetic purity and stability over time.

The oncoming of transgenesis as an alternative breeding procedure has caused a general concern in consumers and, accordingly, new regulations have been implemented. Even if the transgenesis offers immense opportunities, there has been, and continues to be, a 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 safety of GM plants that are used for human consumption. The recommended approach is a comparison between the transgenic plant to its conventional counterpart at different levels. Few comparative studies have been reported in which transcriptomic, proteomic or metabolomic comparisons have been performed (Gregersen et al., 2005, Scossa et al., 2008, Baudo et al., 2006, Laino et al., 2010, Lovegrove et al., 2009, Balsamo et al., 2011, Barros et al., 2010, Zolla et al., 2008) but adverse effects or health risks studies remain unlikely (Domingo et al., 2011).

Endogenous allergen concentrations were shown to vary across crops cultivars, as LTP in soybean (Houston et al., 2011), maize, (Kuppannan et al., 2011), but also with environmental and growing conditions (Doerrer et al. 2010, Sancho et al. 2005), thus it is likely that, at this regard; variations will occur in transgenic plants as well.

The two most important cultivated wheat species are *Triticum aestivum* L. (bread wheat) and *T. durum* Desf. (durum wheat) which are mainly processed into a range of breads and other baked products, pasta, and other foods, most of which are typical of specific geographical regions. Most of allergen studies have been performed on *Triticum aestivum*, but little is known about the content of allergens and even less about their variability between and within cultivars.

Wheat is the most consumed staple food, and the wheat GM production is up to now still not accepted. This crop is known to present health risk for a fraction of population in which it triggers adverse IgE mediated reactions. Its estimated prevalence varied from 3 to 6% of the patients suffering from food allergies and 4 to 10% of bakers are affected by this occupational disease. (Sicherer et al., 2012, Salcedo et al., 2011).

Wheat proteins are classically typically classified into four fractions: albumins, soluble in water, globulins, soluble in salt/water solutions; gliadins, soluble in concentrate alcohol solutions, and glutenins, soluble in diluted acid or alkaline solutions (Osborne, 1924). Albumins and globulins (A/G) are generally considered soluble proteins, as opposed to gliadins and glutenins (that together make up the gluten) that are known as the insoluble protein fraction. Many proteins of the A/G fraction, such as α-amylase inhibitors (Rev. in Tatham and Shewry 2008), peroxidase, glyceraldehyde-3-phosphate deshydrogenase, LTP (Palacin et al., 2007), thaumatin-like, protein associated with the starch granule, and serine protease inhibitor were described as allergens in the baker's asthma (Letho et al., 2010); some of them were also associated to food allergy (mainly in children patients) (Pastorello et al., 2007; Šotkovský et al., 2008, Constantin et al., 2008 ; Larré et al 2011). Gliadins and, at a lower extent, glutenins, are implicated in food allergy (Matsuo et al., 2004, Simonato et al., 2001), although some gliadins may bind with IgE from patients with baker's asthma.

The content of allergenic polypeptides of GM plants is now one of the issues requested by the Codex Alimentarius for the assessment of their safety when the recipient is known to be an allergen. Generally 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). Focusing on allergy, it is necessary to establish if the genetic modifications introduced have potentially increased the intrinsic allergenicity, as an unintended effect. A recent paper suggested making this comparison with appropriate comparator(s) in order to take into account the natural variability (EFSA journal 2010, http://www.efsa.europa.eu/en/efsajournal/pub/1700.htm). As the allergenicity is the result of an abnormal response of individuals, the choice of the patients is essential for the risk assessment.

In this paper, we focused on wheat seed soluble proteins in which many allergens are found. Using these proteins we compare GM lines with their untransformed counterparts and also with other varieties. In order to reach enough sensitivity in the IgE response, a total A/G fraction and compared with subfractions, their composition was examined by electrophoresis, mass spectrometry and finally their ability to bind IgE. Subfractions were then prepared for each of the 29 varieties and tested in ELISA with IgE of twenty-four 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 *Triticum aestivum* and ten of *Triticum durum* were used in this study. All these cultivars were kindly provided by APSOVSEMENTI S.p.A. (Voghera, Pavia, Italy). These varieties were cultivated under the same condition (bread wheats were grown in Voghera and durum wheats in Grosseto, Italy) and certified for their homogeneity. Three GM genotypes of durum wheat (*cvs*. Svevo and Ofanto), and two GM lines of bread wheat *cv* Bobwhite, along with their corresponding *wt* and null-segregant genotypes (where available) were grown in greenhouse at University of Tuscia (Italy) and were also included in this study (**Table 1**).

2.1.1. GM wheat genotypes

Five GM wheat lines (two bread wheats and three durum wheats) were used in this study. The bread wheat *cv* Bobwhite was transformed with a LMW-GS in order to increase the amount of these polypeptides (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 showed a drastic decrease in the amount of all endogenous proteins, including CM-like proteins, with respect to the *wt* genotype, as an unintended effect of genetic transformation (Scossa et al., 2008).

Bread wheat *cv* Bobwhite was transformed with bean PolyGalacturonase Inhibitor Protein (PvGIP2 gene) in order to investigate whether the PGIP may protect wheat tissue from PG degradation (Janny et al., 2008). The authors have tested the activity of the transgene protein (PvPGIP2) against *Fusarium moniliforme* endopolygalacturonase (FmPG). In fact this enzyme is inhibited by PvPGIP2 but is resistant to the inhibition by the endogenous wheat PGIP. The test showed that GM wheat line inhibited FmPG, in opposition, the null sengregant and wt genotype did not affect the activity of the enzyme (Janni et al., 2008). Also null-segregant line (control materials that lacks for segregation the transgene) was included in our study.

Sestili et al. (2010) has transformed two genotype of *Triticum durum cv* Svevo and *cv* Ofanto. This GM wheats were silenced for gene encoding starch branching enzymes of class II (SBEIIa) using the RNA interference (RNAi) approach, in order to increase amylose content. In particular Svevo GM line was transformed by biolistic method and Ofanto GM line was transformed with *Agrobacterium* technique. The authors showed that the silencing of *SBEIIa* genes in the two GM

wheat lines causes alterations in granule morphology and starch composition, and and increase amylose content. Moreover the results obtained with two different methods of transformation in the two GM lines were comparable (Sestili et al., 2010).

Durum wheat *cv* Svevo was transformed with Wx-B1 gene by biolistic method in order to investigate the effect on overexpression of this gene on amylose content. In fact Wx-B1 gene in durum wheat has a strong effect on amylose content and starch properties (Sestili et al. submitted). This GM wheat line not show an increase of amylose content and RVA analysis showed viscosity properties comparable to wt genotype (Sestili et al., submitted).

2.2.Human sera

Sera of twenty-four patients with wheat allergy (food allergy and baker's asthma) were used for the study. Clinical data of the patients (symptoms, age, and wheat-specific IgE reactivity against the A/G fraction of the reference cultivar Récital) are summarized in **Table 2**. Sera were obtained from the Service of Clinical Immunology and Allergology at the University Hospital (CHU) of Nancy, and University of Udine with the informed consent of the patient. Control sera were obtained from healthy volunteers.

Table 1: Wheat genotypes included in this study

Sample	Wheat lines	Genotype
1	Bobwhite *wt*	Bread wheat
2	Bobwhite Null-segregant line of GM PGIP-GS	Bread wheat
3	Bobwhite over-expressing LMW-GS	GM Bread wheat
4	Bobwhite (over-expressing PGIP-GS)	GM Bread wheat
5	Svevo *wt*	Durum wheat
6	Svevo (over-expressing *Wx-B1*)	GM Durum wheat
7	Svevo (RNAi silencing SBEIIa)	GM Durum wheat
8	Ofanto Null-Segregant	Durum wheat
9	Ofanto (SBEIIa silencing with *Agrobacterium*)	GM Durum wheat
10	Casanova	Durum wheat
11	Claudio	Durum wheat
12	Creso	Durum wheat
13	Dorato	Durum wheat
14	Minosse	Durum wheat
15	Neodur	Durum wheat
16	Pitagora	Durum wheat
17	Simeto	Durum wheat
18	Tripudio	Durum wheat
19	Vinci	Bread wheat
20	Antille	Bread wheat
21	Apache	Bread wheat
22	Avorio	Bread wheat
23	Colledoro	Bread wheat
24	Exotic	Bread wheat
25	Feria	Bread wheat
26	Genesi	Bread wheat
27	Lilliput	Bread wheat
28	Masaccio	Bread wheat
29	Valbona	Bread wheat

Table 2: Clinical characteristics of patients and their IgE reactivity against A/G in ELISA or immunoblotting. AEDS: atopic eczema dermatitis syndrome, GI=Gastro-intestinal symptoms AS: anaphylactic shock, R=Rhinitis, AT= Asthma and nd: not done, +: = response at the lower limit of quantification, neg: not reactivity.

Patient serum n°	Age	Symptoms	Wheat IgE specific for A/G in ELISA
9	1,5	AEDS	9
22	8	AEDS	28
43	6	AEDS	33
44	37	AEDS	20
269	6	AEDS+GI	127
398	2	AEDS	+
403	2	AEDS+AT	29
646	5	AEDS	121
781	6	AEDS	84
476	37	nd	0
485	9	nd	29
684	16	nd	22
458	55	R+AT	+
633	66	AT	49
857	20	R	6
858	47	AT	75
859	34	AT	+
860	35	AT	2
863	41	AT	12
864	30	R+AT	neg
865	23	AT	12
1020	49	R+AT	18
1021	30	R+AT	19
1028	20	AT	13

2.3. Extraction of Albumins/Globulins (A/G) proteins fraction

The A/G fraction was extracted from Bobwhite's flour and from the reference cultivar Récital with 0.05 M phosphate buffer, pH7.8, 0.1 M NaCl for 2 h at 4°C with constant stirring, according to the method of Nicolas et al (1998). After centrifugation (8000 rpm for 15 min at 4 °C), the supernatant was collected, dialyzed against water and freeze-dried. The protein content was measured according to the Kejdahl's method as described in Larré et al. (2011).

2.4. Extraction of Metabolic and CM-like proteins fraction

Bobwhite's seeds were milled and treated for 30 min with 2% PVPP and centrifuged at 8500 rpm for 1h. The pellet was suspended in cold (4°C) KCl buffer (containing 50mM Tris–HCl, 100mM KCl, 5mM EDTA, pH 7.8) as described by Hurkman et al (2007), and incubated on ice for 1 h with intermittent mixing and centrifuged at 8500 rpm for 40 min at 4°C. The supernatant (or KCl soluble fraction) was collected and 5 vol. of 0.1M ammonium acetate in methanol were added at room temperature. Following incubation overnight at -20 °C, the methanol-insoluble fraction was pelleted by centrifugation at 8500 rpm for 45 min at 4 °C. The pellet (metabolic fraction) was rinsed with cold acetone. The proteins in the methanol soluble fraction were precipitated with cold acetone, and the pellet (CM-like proteins, namely the proteins that are soluble in Chloroform and Methanol) was rinsed 3 times with cold acetone. The metabolic and CM-like fractions obtained were solubilized in Tris-HCl buffer pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 4% β-mercaptoethanol, and bromophenol blue. The protein concentration was determined by BC Assay protein quantification Kit (*Uptima*).

2.5. SDS-PAGE, Western and Immunoblotting

The A/G, metabolic and CM-like fractions of the bread wheat *cv* Bobwhite were solubilized in Tris–HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 4% β-mercaptoethanol, and bromophenol blue; these were separated by 1D SDS-PAGE on 15% acrylamide gels (18x16 cm gel with SE600X-Hoefer). After 1DE the protein fractions were transferred to nitrocellulose membranes (0.2 μm, Sartorius, Germany) in 25mM Tris, 192 mM glycine, 0.1% SDS and 20% ethanol. Gels were either stained with Coomassie Brilliant Blue G250 (Sigma-Aldrich) according to Devouge et al (2007) or used for blotting. In this latter case, semidry transfer was achieved at 300 mA for 40 min.

After transfer of the gels, the sheet was washed in PBS and blocked over night at room temperature in 5%-milk/PBS. After three washes with PBS-0.05%/Tween 20, nitrocellulose membrane was incubated for 1 h with anti-LTP IgG antibody diluted at 1/500 in milk 2%-PBS. The sheet was washed 3 times with washing buffer and incubated for 1 h with alkaline phosphatase conjugate anti-rabbit diluted at 1/3000 in the 2% milk/PBS. After further washing with PBS and 0.1 M Tris pH 9.5, the sheet 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 a camera (Luminescent ImageAnalyzer LAS 3000; Fujifilm).

In case of immunoblotting with IgE, nitrocellulose sheets were washed in PBS and blocked for 4 hours at room temperature in 4% PVP 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 sera at the appropriate dilution (ranging from1/20 to 1/50) in the washing buffer. Membranes were then washed 3 times and incubated for 1 h with peroxidase-labelled rabbit anti-human IgE (P0295, Dako, Denmark) diluted at 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 ImageAnalyzer LAS 3000; Fujifilm).
The metabolic and CM-like protein profiles of each wheat genotype (29 lines) were obtained by extracting as described in paragraph 2.2 and by using 1D gradient gels (18x16 cm gel) . Thirty-five µg were loaded on 10-15% acrylamide gradient SDS-PAGE gel under reducing conditions (**Figs. 1, 2, and 3**). After electrophoretic run (200V for about 3:30h at 10°C with SE600X-Hoefer) gels were stained with Coomassie Brilliant Blue G-250 (Neuhoff et al., 1988).

2.6. Protein identification by mass spectrometry

Protein bands were cut manually and prepared for mass spectrometry. In-gel digestion was performed using trypsin hydrolysis following the protocol described in Larré et al (2010).
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 2µm 100A, 75-µm i.d. x 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. 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 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.

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/), using 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 Viridiplantae (release 2010_029, August 2010). Another databank 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 methionins 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 having 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. Protein identifications were compared in the two databanks (Uniprot and Wheat TIGR): best matches were validated and when the results were identical in the two databanks, the identification in UniProt was chosen.

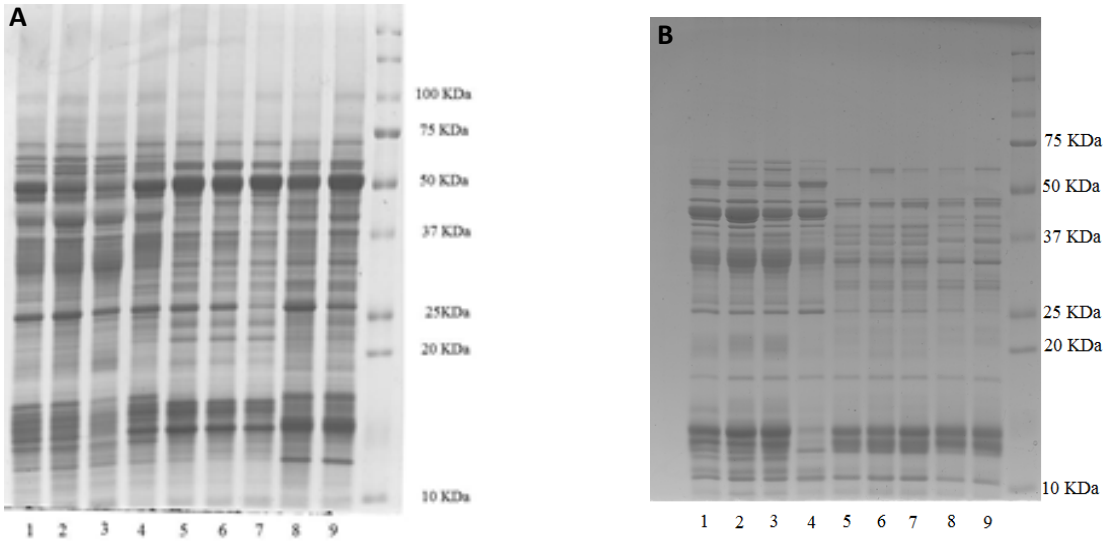

Fig 1: SDS-PAGE gradient gels (10-15% acrylamide) performed on metabolic fraction (A) and CM-like fraction (B) of 2 bread GM lines (lines 3 and 4), null-segregant line (line 2), its corresponding wt genotype (line 1), 3 durum GM lines (lines 6, 7, 9), and its control genotypes (lines 5 and 8).

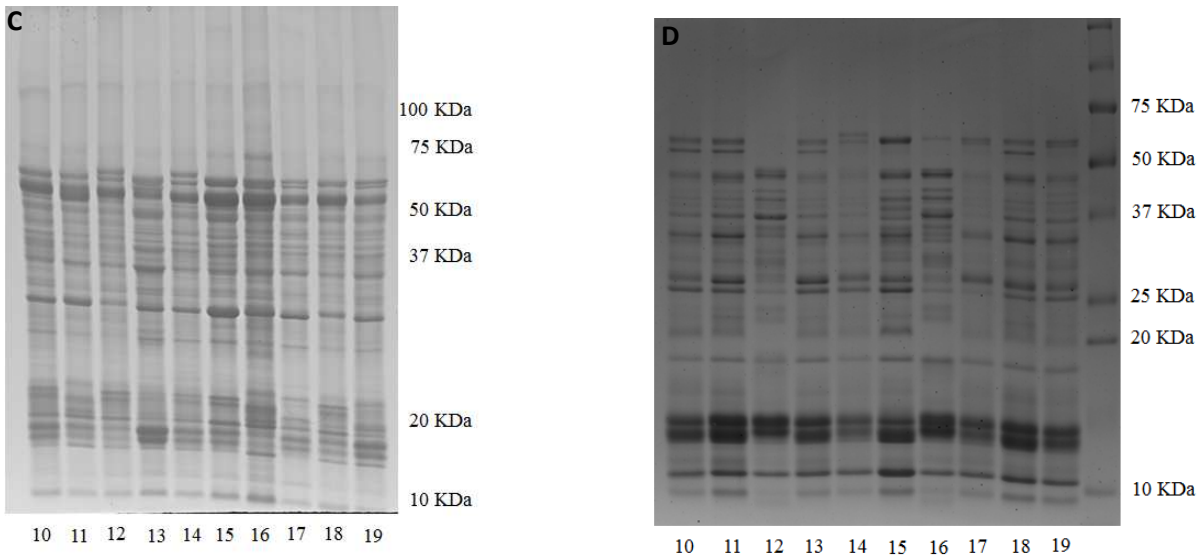

Fig 2: SDS-PAGE gradient gels (10-15% acrylamide) performed on metabolic fraction (C) and CM-like fraction (D) of ten genotypes of durum wheat.

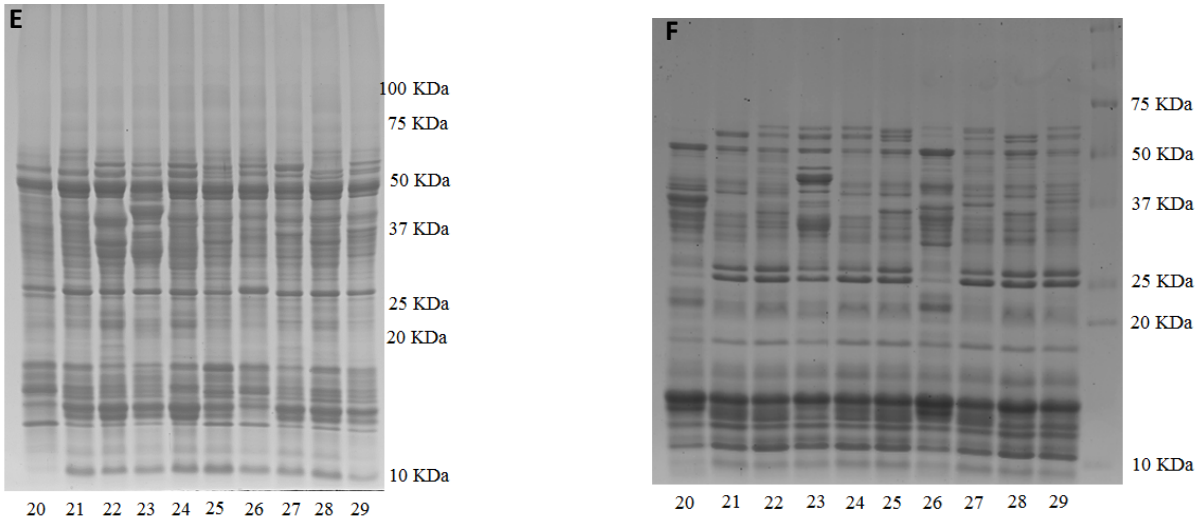

Fig 3: SDS-PAGE gradient gels (10-15% acrylamide) performed on metabolic fraction (E) and CM-like fraction (F) of ten cv of bread wheat.

2.7. Enzyme Linked ImmunoSorbent Assay (ELISA)

The ELISA test was performed by Biomek® NXP Laboratory Automation Workstation. The wells on microtiter plates (Nunc MaxiSorp 384 well) were coated with 20µL of each antigen diluted at 5µg/mL in 100mM carbonate buffer (pH 9.6) for 2h at room temperature. A standard curve was made with a serial dilution from160 ng/mL to 0.07 ng/mL of IgE standard (Bodinier et al 2008). The plates were blocked with PBS-0.1%Tween 20 and 0.5% porcine gelatin (G2500 SIGMA) for 1 hour at 37°C. After three washes with PBS-0.1% Tween 20, the microplates were incubated with patient's sera diluted at 1:10 with 0.5% gelatin/PBS/Tween for 15h at 37°C. Goat anti-human IgE antibodies (ε-chain specific-Alkaline phosphatase developed in goat - Affinity isolated antigen Specific antibody - A3525-SIGMA) diluted at 1:500 in 0.5% G–PBST was incubated for 2h at 37°C. Finally, the fluorescent substrate (4-Methylumbelliferyl phosphate M3168-SIGMA) diluted at 1:5 in 1M 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) and the concentration of specific IgE binding to the antigen was calculated by reference to the standard curve of the plate. The fluorescence data for each antigen was corrected by subtracting the fluorescence of the control with no antigen. For each antigen and serum, three replicates and three controls wells with 10mM ethanolamine in carbonate were performed. The corresponding specific IgE concentrations were calculated from the adjusted standard curve provided that the fluorescence was between the quantification and the saturation limits which were set at 1.2 and 320 ng/mL respectively. When necessary, IgE reactive concentration was corrected by the dilution.

2.7.1. Statistical analysis

The reactivity of the twenty two patient's sera was measured on the Metabolic and CM-like fractions extracted from the twenty nine wheat varieties. Two subgroups were created, corresponding to durum and bread wheat. Statistical differences determined by cultivar, serum and cultivar × serum interaction were assessed by two-way ANOVA with the F test for assessing IgE concentration with 95% confidence intervals with Statgraphics software. The IgE responses were measured in triplicate.

3. Results and discussion

3.1.Choice of the soluble fraction extraction method by Immunoblotting

The two extraction methods: the first for A/G, and the second for metabolic and CM-like fractions were compared by SDS-PAGE and Immunoblotting, in order to choose the fraction containing most of the reactive polypeptides. The fraction A/G, in fact, should contain both metabolic and CM-proteins, but the high number of polypeptides might hide the less abundant ones. For this reason, we used an additional fractionation procedure (Hurkman et al, 2007) that allowed separating further these groups of proteins. Moreover, because LTP are not easily detectable in the A/G fraction, we performed immunoblotting analyses on the three protein type extracts, by using antibodies against this important allergen. This analysis showed that the antibody reacted only against the CM-like fraction with a specific band with molecular weight around 10 KDa, while there was no reaction against the A/G and metabolic fractions (**Fig. 4 right**).

The IgE-binding potential of the three protein fractions were investigated also by immunoblotting (**Fig. 5**) with three patient's allergic sera (two affected by baker's asthma and one patient affected by food allergy). Several IgE-binding components between were detected by serum 68 in the metabolic fraction as well as one stongly reactive band about 10 KDa (probably LTP) in the CM-like extract, whereas only a few of these components where bound by IgE in the A/G extract. The same response was observed for serum 458 but with a weak intensity and the low MW band was not detected. Serum 858 shows strongly bound to several proteins of low MW between 10 and 15 KDa in the CM-like extract and slitghly less of these components in the A/G extract. The extracts A/G

and CM-like showed the same high reactivity for the proteins with molecular weights between 10 KDa and 15 KDa for the serum 858.

All these data gave indications that the fractionation procedure that allowed obtaining metabolic proteins separated from CM-proteins was the best for our analyses.

Some of the main IgE reactive bands (**Fig. 4 left**) were analyzed by mass spectrometry (Q-TOF) revealing the presence of mostly known allergens in these fractions (**Table 3**).

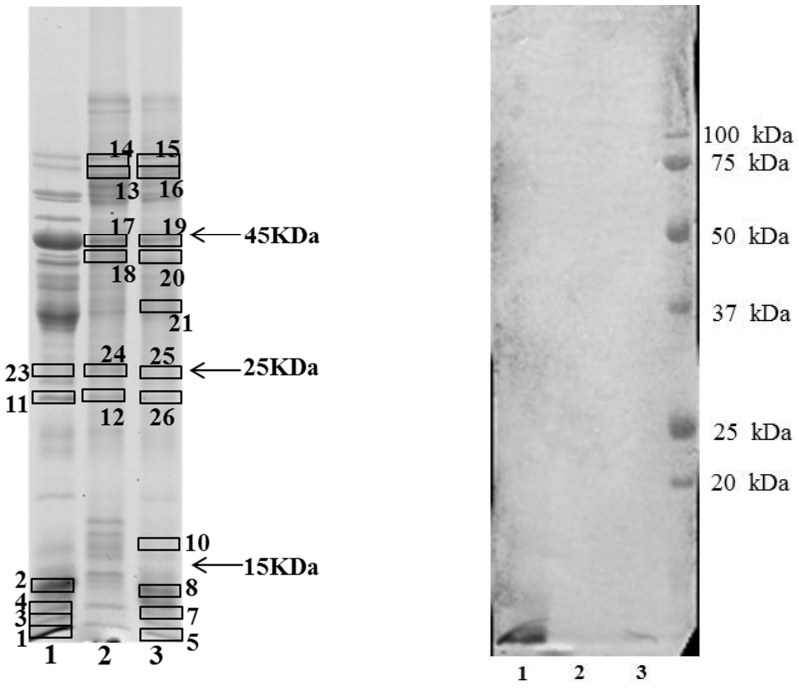

Fig. 4: SDS-PAGE (left) and Western Blotting obtained after incubation of the membrane with anti-LTP antibodies (right) of different soluble proteins extraction of bread wheat cv Bobwhite. Line 1: CM-like fraction; line 2: Metabolic fraction; line 3: A/G fraction.

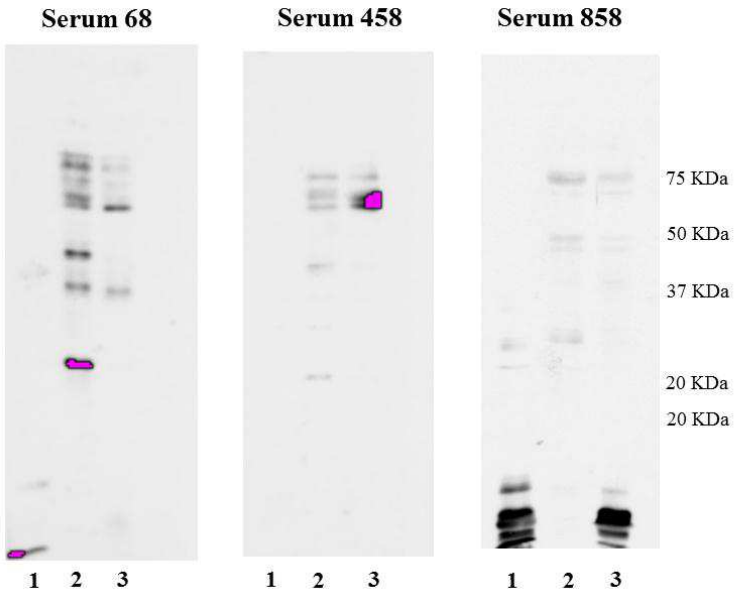

Fig. 5: Immunoblotting obtained after incubation of the sheets with human sera from allergic patient to wheat (sera 458 and 858 from patients affected by backer's asthma and serum 68 from patient suffering of food allergy). Line 1: CM-like proteinic extract; line 2: Metabolic proteinic extract; line 3: A/G extract (Lines 1, 2, 3: soluble proteins extract from Bobwhite).

3.2. ELISA tests on the metabolic and CM-proteins of the twenty nine wheat genotypes

The concentration in IgE specific in twenty-four sera tested on metabolic and CM-like fractions are shown in **Figs. 6** and **7**, respectively. Five GM wheat lines, their corresponding wt and null-segregant genotypes (where available) and 20 wheat cultivars (10 durum and 10 bread) have been compared.

Fig. 6 shows the concentration in IgE specific as means values (with their standard errors) for all sera tested. GM Bobwhite-over expressing LMW-GS (3) and its commercial *cv* (1) did not show difference in amount of IgE specific, while GM Bobwhite-over-expressing PGIP-GS (4) shows a lower reactivity (35 ng/mL) in comparison to the null-segregant line (2) and its *wt* genotype (1) (38 and 41 ng/mL respectively). The same lower concentration of specific IgE (31 ng/mL) is showed for the durum GM line Svevo-RNAi silencing SBEIIa (7) compared to its parental line (35 ng/mL) (5), while Ofanto-SBEIIa silenced with Agrobacterium (9) shows higher (32 ng/mL) reactivity than its null segregant line (24 ng/mL) (8). In this latter case, the corresponding untransformed wheat cultivar, grown in the same conditions, was not available. Finally, the concentration of specific IgE between GM Svevo-over-expressing *Wx-B1*-GS (6) and Svevo *wt* (5) was comparable.

Among the cultivated durum wheats, Casanova (10) and Vinci (19) showed the highest reactivity (46 and 49 ng/mL respectively), whereas the lowest was for Creso (12) and Pitagora (16) (32 and 30 ng/mL respectively). It is interesting to note that there is a wide variation of reactivity in durum wheats, whereas such variation is restricted in bread wheats. In fact, all varieties range between 44 and 49 ng/mL, with cultivar Genesi (26) showing the lower reactivity (38 ng/mL) and cultivar Masaccio (28), showing the highest (50 ng/mL).

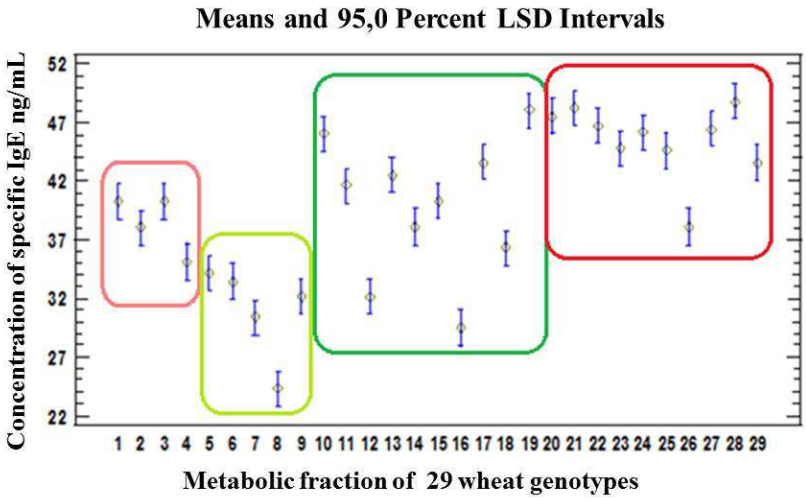

Fig. 6: Concentration of specific IgE for twenty-four sera from patients affected by food and respiratory allergy to wheat. The pink rectangle contains bread GM lines (3, 4), nulle-segregant line (2) and its corresponding genotype (1).The clear green rectangle contains durum GM lines (6, 7, 9) and its corresponding genotypes (5, 8).
The green rectangle contains ten *cv* of durum wheat (10-19) while the red rectangle contains ten *cv* of bread wheat (20-29).

Fig. 7 shows the reactivity of the 24 sera tested on the CM-like fraction. The concentrations, as for metabolic fraction, are represented as means values (with their standard errors) for all sera tested. GM Bobwhite-over expressing LMW-GS (3) shows a lower reactivity (44 ng/mL) than its parental line (1) (57 ng/mL), while GM Bobwhite-over-expressing PGIP-GS (4) has higher response (65 ng/mL) compared to null-segregant line (2) and *wt* genotype (1) (55 and 57 ng/mL). GM Svevo-over-expressing *Wx-B1*-GS (6), GM Svevo-RNAi silencing SBEIIa (7) and Svevo *wt* (5) did not show significative differences. Nevertheless for GM Ofanto-SBEIIa silenced with Agrobacterium (9) the reactivity is higher (65 ng/mL) than the control line (55 ng/mL) (8). In the case of CM-proteins, most of the varieties present the same range of reactivity, although, in case of durum wheats, there are a few outliers, such as Minosse (14) that exhibit the lower value (41 ng/mL), and

Casanova (10) and Claudio (11) that show the highest (64 and 70 ng/mL). The wider range of reactivity shown in durum wheat both in metabolic and CM-proteins is likely caused by its generally highest protein content with respect to bread wheat. Moreover, the specific IgE of patients show a higher reactivity against the CM-like fraction (up to about 80 µg/ml) with respect to the metabolic proteins (up to about 50 µg/ml). This confirmed that the CM-like fraction contains most of the major wheat allergens (Rev. in Tatham and Shewry 2008).

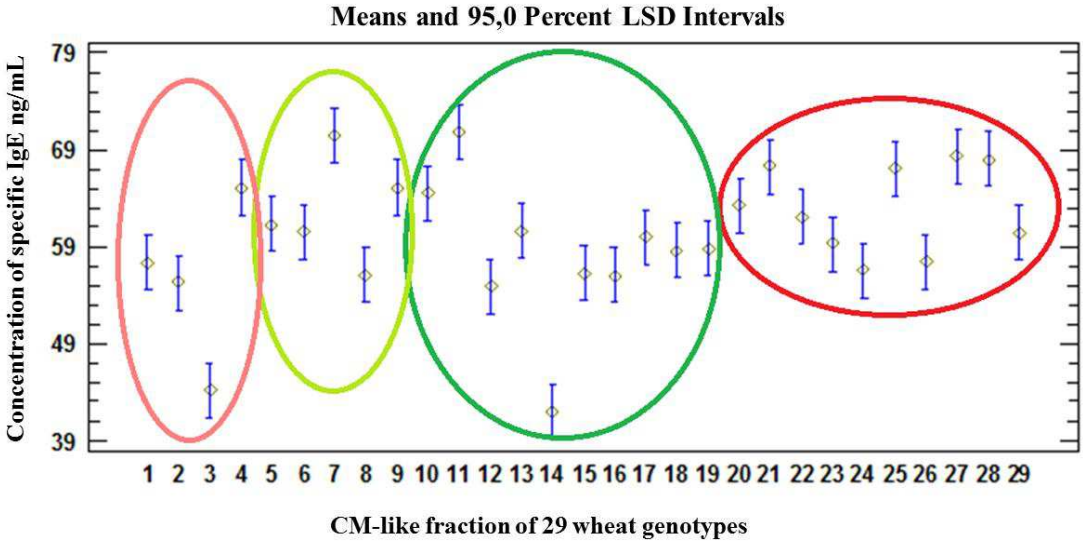

Fig. 7: Concentration of specific IgE for twenty-four sera from patients affected by food and respiratory allergy to wheat.
The pink circle contains bread GM lines (3, 4), nulle-segregant line (2) and its corresponding genotype (1).
The clear green circle contains durum GM lines (6, 7, 9) and its corresponding genotypes (5, 8).
The green circle contains ten *cv* of durum wheat (10-19) while the red circle contains ten *cv* of bread wheat (20-29) used for the study.

4. Conclusion

Wheat is one of the most cultivated crops in the world, but is also the main cause of some food and respiratory allergies, that are due to proteins contained in the wheat kernel. The overwhelming majority of wheat seed proteins consist of about 80% of storage proteins. The remining 20% correspond to water and salt soluble proteins also described as structural and metabolic proteins. Part of these proteins is responsible for triggering food allergy and also baker's asthma, the most common of professional respiratory allergy (Baur et al., 1998, Ameille et al., 2003, Salcedo et al., 2011).

Although, at the moment, GM wheat varieties are not commercial yet, it is possible that they will be available in the next years, and assessment of their safety would be mandatory. Because the content of allergenic polypeptides of GM plants is one of the main issues, here we have proposed a comparison based on ELISA test between the different salt soluble fractions extracted from seeds obtained from GM wheat lines with their control lines and a range of commercial varieties.

Even if some differences between GM wheats and control lines were found, the range of such variation was lower than that observed in wheat cultivars and, in general, the highest values were observed in commercial cultivars and not in the GM lines.

Our results thus suggest that the assessment of food safety in GM plants, at least in regard to allergenicity of wheat, should be evaluated case by case, and that it is not possible to leave out of consideration the comparison with the natural variation of parameters under evaluation.

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Annexe-Chapter II

Table 3: List of identified proteins from 1D gel of Bobwhite *wt* (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 Wheat TIGR databank, Uniprot 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. Bande	Sub-group	Prot Id TC or Uniprot	Best homologue protein name	log (E-value)	Coverage	MW	Total Unique Peptides
1 (CM-like)	1.01	A4ZIW9	Monomeric alpha-amylase inhibitor OS=*Triticum aestivum* PE=4 SV=1	-20,636467	40	13,10	4
2 (CM-like)	1.01	TC402211	WHEAT Alpha-amylase/trypsin inhibitor CM3 OS=*Triticum aestivum* PE=1 SV=1	-36,725636	50	18,10	5
3 (CM-like)	1.01	A4ZIW9	WHEAT Monomeric alpha-amylase inhibitor OS=*Triticum aestivum* PE=4 SV=1	-21,595882	40	13,10	4
4 (CM-like)	1.01	A4GFN8	Dimeric alpha-amylase inhibitor OS=*Triticum turgidum* subsp. *dicoccoides* PE=4 SV=1	-34,129745	70	13,10	6
5 (A/G)	1.01	A4ZIW9	WHEAT Monomeric alpha-amylase inhibitor OS=*Triticum aestivum* PE=4 SV=1	-13,291083	28	13,10	3
7 (A/G)	1.01	A4GFN8	Dimeric alpha-amylase inhibitor OS=*Triticum turgidum* subsp. *dicoccoides* PE=4	-38,641449	70	13,10	6
8 (A/G)	1.01	TC402211	WHEAT Alpha-amylase/trypsin inhibitor CM3 OS=*Triticum aestivum* PE=1 SV=1	-39,970905	50	18,10	5
9 (CM-like)	1.01	TC402211	WHEAT Alpha-amylase/trypsin inhibitor CM3 OS=*Triticum aestivum* PE=1 SV=1	-16,728159	28	18,10	3

10 (A/G)	1.01	CD921608	Embryo globulin; n=2; Triticeae	-7,8745189	13	15,89	2
11 (CM-like)	1.01	TC382793	Class II chitinase; n=4; Triticeae	-17,094139	25	23,10	3
12 (Met)	1.01	TC377918	Cupin family protein, expressed [*Oryza sativa Japonica* Group].	-17,328352	21	32,79	4
	2.01	CA613733	Triosephosphate isomerase OS=*Triticum aestivum* GN=tpis PE=2 SV=1	-11,616328	16	26,70	3
	6.01	TC450362	Cupin family protein, expressed; n=2; Oryza sativa Japonica	-12,095279	12	28,39	3
13 (Met)	1.01	TC388221	beta amylase [*Triticum aestivum*]	-41,661324	29	37,90	7
	3.02	TC387479	Globulin-like protein; n=1; *Oryza sativa Japonica*	-22,490707	17	45,29	5
	4.01	TC425413	Globulin-2 precursor; n=1; *Zea mays*	-25,216774	18	49,29	5
14 (Met)	1.01	TC388221	beta amylase [*Triticum aestivum*]	-41,031673	34	37,90	7
	2.01	TC425413	Globulin-2 precursor; n=1; *Zea mays*	-42,585594	27	49,29	9
15 (A/G)	1.01	TC368907	Beta-amylase precursor; n=1; *Hordeum vulgare* subsp.	-49,49202	40	37,00	8
	5.01	D1KFM6	Granule bound starch synthase (Fragment) OS=*Triticum turgidum* subsp. *dicoccon* GN=GBSSI PE=3 SV=1	-15,006124	8	63,59	3
16(A/G)	1.01	TC388221	beta amylase [*Triticum aestivum*]	-46,499233	33	37,90	8
	2.01	TC425413	Globulin-2 precursor; n=1; *Zea mays*	-37,274471	24	49,29	8
17(Met)	1.01	TC388221	beta amylase [*Triticum aestivum*]	-42,502995	37	37,90	8
	2.02	TC425413	Globulin-2 precursor; n=1; *Zea mays*	-22,522726	18	49,29	5
	4.01	A7UME2	Xylanase inhibitor 725ACCN OS=*Triticum aestivum* PE=4 SV=1	-18,931458	17	41,09	4
	5.01	P93693	Serpin-Z1B OS=*Triticum aestivum* PE=1	-20,115255	20	42,90	5

			SV=1				
	6.01	TC383884	Enolase; n=2; *Oryza sativa Japonica* Group	-25,064291	17	52,00	5
18(Met)	1.01	TC388221	beta amylase [*Triticum aestivum*]	-29,458488	23	37,90	5
	2.01	TC387479	Globulin-like protein; n=1; *Oryza sativa Japonica* Group	-20,967665	17	45,29	4
	3.01	TC368656	Xylanase inhibitor 725ACC OS=*Triticum aestivum* PE=4 SV=1	-13,00596	13	41,09	3
	5.01	TC383884	Enolase; n=2; *Oryza sativa Japonica* Group|Rep:	-22,299124	14	52,00	4
	6.01	TC380640	Serpin; n=2; *Triticum aestivum*	-13,542846	10	44,59	3
19(A/G)	1.01	TC388221	beta amylase [*Triticum aestivum*]	-19,360291	17	37,90	4
	5.01	TC378369	Globulin-like protein; n=1; *Oryza sativa Japo*	-18,613323	16	46,50	4
	6.01	Q40345	Isocitrate dehydrogenase [NADP], chloroplastic (Fragment) OS=*Medicago sativa* PE=2 SV=1	-15,8642	8	48,29	3
	7.01	TC380640	Serpin-Z2B OS=*Triticum aestivum* PE=1 SV=1	-12,250573	11	42,90	3
20(A/G)	1.01	A7UME2	Xylanase inhibitor 725ACCN OS=*Triticum aestivum* PE=4 SV=1	-21,596125	17	41,09	5
	3.01	TC388221	beta amylase [*Triticum aestivum*]	-19,127186	17	37,90	4
	4.01	Q41593	Serpin-Z1A OS=*Triticum aestivum* GN=WZCI PE=1 SV=1	-22,302813	17	43,00	5
	5.01	TC387479	Globulin-like protein; n=1; *Oryza sativa Japonica*	-18,307329	13	45,29	3
	6.01	TC401483	Phosphoglycerate kinase, cytosolic OS=*Triticum aestivum* PE=2 SV=1	-20,702953	21	42,00	5
21(A/G)	1.01	B7U6L4	Globulin 3 OS=*Triticum aestivum* GN=glo-3A PE=4 SV=1	-20,867655	10	66,19	4
	2.01	Q8LK23	Peroxidase OS=*Triticum aestivum* GN=WSP1 PE=2 SV=1	-21,369686	15	38,70	4

23(CM-like)		TC433257	Xylanase inhibitor protein 1 OS=*Triticum aestivum* GN=XIPI PE=1 SV=2	-25,258522	21	33,20	4
24(Met)	1.01	Q07810	Tritin OS=*Triticum aestivum* GN=trig7 PE=4 SV=1	-63,970821	53	29,50	14
	2.01	TC374564	Globulin 1 OS=*Triticum aestivum* PE=4 SV=1	-22,647966	45	24,5	6
	5.01	TC425413	Globulin-2 precursor; n=1; *Zea mays*	-19,348436	15	49,29	4
25(A/G)	1.01	TC414102	Tritin OS=*Triticum aestivum* GN=trig7 PE=4 SV=1	-50,42556	46	29,5	11
	2.01	TC400752	Globulin 1 OS=*Triticum aestivum* PE=4 SV=1	-20,277489	34	24,5	4
	7.01	TC425413	Globulin-2 precursor; n=1; *Zea mays*	-12,900319	11	49,29	3
	8.01	CV065553	Alpha-amylase/trypsin inhibitor CM3 OS=*Triticum aestivum* PE=1 SV=1	-16,602755	28	18,10	3
26(A/G)	1.01	Q07810	Tritin OS=*Triticum aestivum* GN=trig7 PE=4 SV=1	-49,414116	46	29,50	11
	2.01	TC400752	Globulin 1 OS=*Triticum aestivum* PE=4 SV=1	-14,897979	29	24,50	3
	3.01	A7UME2	Xylanase inhibitor 725ACCN OS=*Triticum aestivum* PE=4 SV=1	-18,833675	13	41,09	3

Group	Description	Sample	Sequence	Modifs	Used	on a total of Sub-groups		E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**A4ZIT6**	**1 (CM-like)**	LTAASVPEVCK	C10:+57.0215	-	-	1.01	0,00084	2	1174,59875	1174,61401	-0,015	-12,770152
1			LTAASVPEVCKVPIPNPSGDR	C10:+57.0215	-	-	1.01	4,5E-09	3	2207,15576	2207,14941	0,0064	2,8996675
1			LQCVGSQVPEAVLR	C3:+57.0215	-	-	1.01	4,9E-05	2	1555,83557	1555,82654	0,009	5,78470659
1			SGPWSWCDPATGYK	C7:+57.0215	-	-	1.01	1,3E-05	2	1611,72375	1611,68994	0,034	21,0958691
1	**TC402211**	**2 (CM-like)**	LLVAPGQCNLATIHNVR	C8:+57.0215	-	-	1.01	6,7E-06	3	1875,94519	1876,02295	-0,077	-41,044273
1			DYVLQQTCGTFTPGSK	C8:+57.0215	-	-	1.01	2,2E-10	2	1801,80896	1801,84302	-0,034	-18,869568
1			SGNVGESGLIDLPGCPR	C15:+57.0215	-	-	1.01	2,7E-08	2	1727,77808	1727,83899	-0,06	-34,725456
1			LPEWMTSASIYSPGKPYLAK		-	-	1.01	7,3E-10	3	2239,18188	2239,14697	0,035	15,6309528
1			SGNVGESGLIDLPGCPR	C15:+57.0215	-	-	1.01	7,6E-10	2	1727,81335	1727,83899	-0,025	-14,468941
1	**A4ZIW9**	**3 (CM-like)**	LTAASVPEVCK	C10:+57.0215	-	-	1.01	0,00067	2	1174,62256	1174,61414	0,0085	7,2364192
1			LQCVGSQVPEAVLR	C3:+57.0215	-	-	1.01	2,4E-05	2	1555,81091	1555,82703	-0,016	-10,28392
1			LTAASVPEVCKVPIPNPSGDR	C10:+57.0215	-	-	1.01	1,9E-09	3	2207,17896	2207,14893	0,03	13,5921955
1			SGPWSWCDPATGYK	C7:+57.0215	-	-	1.01	8,3E-06	2	1611,70154	1611,68994	0,011	6,82513428
1	**A4GFN8**	**4 (CM-like)**	EHGVQEGQAGTGAFPSCR	C17:+57.0215	-	-	1.01	3,7E-07	3	1887,85889	1887,84094	0,018	9,53470135
1			LQCNGSQVPEAVLR	C3:+57.0215	2	2	1.01 1.02	7,2E-05	2	1570,82434	1570,80103	0,023	14,642211
1			LPIVVDASGDGAYVCK	C15:+57.0215	-	-	1.02	8,5E-09	2	1663,8623	1663,83606	0,026	15,6265392
1			SGPWMCYPGYAFK	C6:+57.0215 - M5:+15.9949	-	-	1.01	1,9E-06	2	1579,69153	1579,67102	0,02	12,6608639
1			CGALYSMLDSMYK	C1:+57.0215 - M11:+15.9949	2	2	1.01 1.02	0,00045	2	1554,68616	1554,66394	0,022	14,1509686
1			DCCQQLADISEWCR	C2:+57.0215 - C3:+57.0215 - C13:+57.0215	2	2	1.01 1.02	7,1E-07	2	1840,75891	1840,74194	0,017	9,23540688
1	**A4ZIT6**	**5 (CM-like)**	LTAASVPEVCKVPIPNPSGDR	C10:+57.0215	-	-	1.01	4,5E-09	3	2207,15576	2207,14941	0,0064	2,8996675
1			LQCVGSQVPEAVLR	C3:+57.0215	-	-	1.01	4,9E-05	2	1555,83557	1555,82654	0,009	5,78470659
1			SGPWSWCDPATGYK	C7:+57.0215	-	-	1.01	1,3E-05	2	1611,72375	1611,68994	0,034	21,0958691
1	**A4GFN8**	**7 (A/G)**	EHGVQEGQAGTGAFPSCR	C17:+57.0215	-	-	1.01	7,8E-08	3	1887,89636	1887,84094	0,056	29,663517
1			LQCNGSQVPEAVLR	C3:+57.0215	2	2	1.01 1.02	0,00015	2	1570,81531	1570,80103	0,014	8,91265106
1			LPIVVDASGDGAYVCK	C15:+57.0215	-	-	1.02	4,7E-09	2	1663,88098	1663,83606	0,045	27,0459347
1			CGALYSMLDSMYK	C1:+57.0215 - M11:+15.9949	2	2	1.01 1.02	0,00032	2	1554,72327	1554,66394	0,059	37,950325
1			DCCQQLADISEWCR	C2:+57.0215 - C3:+57.0215 - C13:+57.0215	2	2	1.01 1.02	1,1E-06	2	1840,78357	1840,74194	0,042	22,8168869
1			SGPWMCYPGYAFK	C6:+57.0215 - M5:+15.9949	-	-	1.01	3,3E-07	2	1579,7229	1579,67102	0,052	32,9182472
1	**TC402211**	**8 (A/G)**	DYVLQQTCGTFTPGSK	C8:+57.0215	-	-	1.01	7,8E-11	2	1801,81433	1801,84302	-0,029	-16,094631
1			SGNVGESGLIDLPGCPR	C15:+57.0215	-	-	1.01	8,4E-09	2	1727,76111	1727,83899	-0,077	-44,564339
1			LPEWMTSASIYSPGKPYLAK		-	-	1.01	1,6E-11	3	2239,13281	2239,14697	-0,014	-6,2523813
1			YFIALPVPSQPVDPR		-	-	1.01	8,5E-05	2	1698,95789	1698,922	0,036	21,1899052
1			SGNVGESGLIDLPGCPR	C15:+57.0215	-	-	1.01	2,2E-05	2	1727,86316	1727,83899	0,025	14,4689407
1	**TC402211**	**9 (CM-like)**	LLVAPGQCNLATIHNVR	C8:+57.0215	-	-	1.01	1,7E-05	3	1876,05957	1876,02295	0,037	19,7225723
1			SGNVGESGLIDLPGCPR	C15:+57.0215	-	-	1.01	4,4E-09	2	1727,85791	1727,83899	0,019	10,9963951
1			YFIALPVPSQPVDPR		-	-	1.01	0,00025	2	1698,95593	1698,922	0,034	20,0126915
1	**CD921608**	**10 (A/G)**	GSAFVVPPGHPVVEIASSR		-	-	1.01	1,5E-05	3	1906,04041	1906,01904	0,022	11,5423822
1			PPGHPVVEIASSR		-	-	1.01	0,00089	2	1345,74695	1345,72302	0,024	17,83428

Group	Description	Sample	Sequence	Modifs	Used	on a total of	Sub-groups	E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**TC382793**	**11(CM-like)**	GPIQLSHNYNYGPAGR		-	-	1.01	9,7E-07	2	1743,8573	1743,856567	0,0008	0,458753318
1			NPDLVATDPTVSFK		-	-	1.01	9,1E-05	2	1503,766479	1503,769409	-0,0029	-1,928487062
1			GFYTYDAFVAAANAFPGFGATGSNDAR		-	-	1.01	3,7E-05	3	2758,253418	2758,253662	-0,0003	-0,108764477
1	**TC377918**	**12(Met)**	TGGTGGPFVSYTTESGSGGK		-	-	1.02	0,0003	2	1846,844849	1846,845825	-0,0009	-0,487317324
1			NKPQFLTGPTSVFR		-	-	1.01	0,00076	3	1591,860107	1591,859497	0,0006	0,37691772
1			MMLGPELAAGLGVPEK	M1:+15.9949 - M2:+15.9949	-	-	1.02	3,2E-06	2	1644,831421	1644,833984	-0,0025	-1,519910097
1			APEPYNLFDHEPSFR		3	3	1.01 1.02 1.03	7,9E-05	2	1818,844238	1818,844971	-0,0007	-0,384859622
2	**CA613733**		VASPAQAQEVHANLR		-	-	2.01	1,6E-05	2	1590,833496	1590,835083	-0,0016	-1,005761027
2			EAGSTMAVVAEQTK		-	-	2.01	0,00028	2	1421,693237	1421,694458	-0,0012	-0,844063282
2			SLMGESSEFVGEK	M3:+15.9949	-	-	2.01	0,00054	2	1415,636597	1415,636353	0,0003	0,211918846
6	**TC450362**		SPQLIIMYNPDQEKK	M7:+15.9949	-	-	6.01	3,9E-05	2	1819,924438	1819,92627	-0,0018	-0,989051044
6			SPQLIIMYNPDQEK	M7:+15.9949	-	-	6.01	2,9E-05	2	1691,82959	1691,831299	-0,0017	-1,004828334
6			QGFGVSGEVVEAIQSAK		-	-	6.01	0,00071	2	1705,875366	1705,875977	-0,0005	-0,293104559
1	**TC388221**	**13(Met)**	PDIFYTDQHGTR		-	-	1.03	2,1E-06	2	1449,675415	1449,676147	-0,0007	-0,482866436
1			SHAAEITAGYYNLHDR		-	-	1.04	1,6E-05	3	1817,855835	1817,857056	-0,0011	-0,605108082
1			VPSHAAEITAGYYNLHDR		-	-	1.01	6,5E-05	3	2013,974976	2013,978149	-0,0031	-1,539242148
1			NVGASDPDIFYTDQHGTR		-	-	1.05	3E-09	2	1992,90271	1992,905029	-0,0023	-1,154094219
1			APSHAAELTAGYYNLHDR		-	-	1.03	9,9E-10	2	1985,946411	1985,946777	-0,0004	-0,201415256
1			YPSYPQSHGW		-	-	1.01	0,00045	2	1221,531982	1221,532837	-0,0007	-0,573050499
1			AAAAMVGHPEWEFPR	M5:+15.9949	2	5	1.01 1.05	8,5E-05	2	1684,788452	1684,790527	-0,002	-1,187091231
3	**TC387479**		GGGGGSGSEKEDIQPR		2	2	3.01 3.02	1,6E-05	2	1530,713501	1530,714722	-0,0012	-0,783947527
3			SRGEGPISEGSEEQIR		2	2	3.01 3.02	0,00024	2	1730,823364	1730,830811	-0,0074	-4,2754035
3			GEGPISEGSEEQIR		2	2	3.01 3.02	0,00057	2	1487,696777	1487,697754	-0,0008	-0,537743628
3			AFLQPSHYDADEIAFVR		-	-	3.02	3,6E-07	2	1978,966187	1978,966187	1E-04	0,050531432
3			EGDVFVIPAGSIVYSANTHR		-	-	3.02	4,1E-05	2	2132,077393	2132,077393	-1E-04	-0,046902608
4	**TC425413**		GGGGSGSEKEDIQPR		-	-	4.01	0,0003	2	1473,692505	1473,693359	-0,0007	-0,474997014
4			SKGEGEIYEASEEQIR		-	-	4.01	1,5E-06	2	1824,864502	1824,86145	0,0031	1,698759198
4			AFLQPSHHDADEIAFVR		-	-	4.01	7,1E-07	2	1952,959839	1952,961792	-0,0019	-0,972881377
4			EGDVIVIPAGSIVYSANTHR		-	-	4.01	3,8E-07	2	2098,091797	2098,093262	-0,0013	-0,61961019
4			VVMFINPVSTPGR		-	-	4.01	0,0005	2	1416,768433	1416,767212	0,0013	0,917581916
1	**TC388221**	**14(Met)**	PDIFYTDQHGTR		-	-	1.02	0,00063	2	1449,674561	1449,676147	-0,0015	-1,034713864
1			APSHAAELTAGYYNLHDR		-	-	1.02	1,5E-08	2	1985,942993	1985,946777	-0,0038	-1,913444996
1			VPSHAAEITAGYYNLHDR		-	-	1.01	8,9E-06	3	2013,975342	2013,978149	-0,0028	-1,390283108
1			AAAAMVGHPEWEFPHDSGTYNDTPER	M5:+15.9949	-	-	1.03	2,9E-05	3	2901,251465	2901,253662	-0,0022	-0,758292854
1			NVGVSDPDIFYTDQHGTR		-	-	1.02	8,6E-08	2	2020,934448	2020,936279	-0,0018	-0,89067626
1			NIEYLTLGVDDQPLFH		-	-	1.02	1,5E-06	2	1873,936157	1873,933472	0,0027	1,440819621
1			FFVDNGTYLTEQGR		3	4	1.01 1.02 1.03	0,00019	2	1646,780762	1646,78125	-0,0005	-0,303622603
2	**TC425413**		WQEGGDEGR		-	-	2.01	0,00035	2	1033,43335	1033,433838	-0,0004	-0,387059122
2			SRGEGPISEGSEEQIR		-	-	2.03	0,00061	2	1730,828979	1730,830811	-0,0018	-1,039963007
2			SGGSGRPYHFGQESYR		-	-	2.01	0,00028	2	1784,807251	1784,810303	-0,003	-1,680850863
2			GEGPISEGSEEQIR		-	-	2.03	0,00043	2	1487,696045	1487,697754	-0,0016	-1,075487256
2			SKGEGEIYEASEEQIR		2	3	2.01 2.02	5,6E-06	2	1824,860107	1824,86145	-0,0013	-0,712382853
2			DGYFEMACPHISSSGR	C8:+57.0215 - M6:+15.9949	2	3	2.01 2.02	6,6E-08	2	1829,758179	1829,758545	-0,0003	-0,163956076
2			AFLQPSHHDADEIAFVR		-	-	2.01	1E-07	2	1952,960205	1952,961792	-0,0015	-0,768064201
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	6,6E-05	2	2098,091797	2098,093262	-0,0013	-0,61961019
2			FQEFFLIGSGDERPQSF		-	-	2.02	5,6E-05	2	2003,952271	2003,950195	0,0021	1,047930241

Group	*Description*	*Sample*	*Sequence*	*Modifs*	*Used*	*on a total of*	*Sub-groups*	*E-value*	*Charge*	*MH+ Obs*	*MH+ theo*	*DeltaMH+*	*Delta-ppm*
1	**TC368907**	**15(A/G)**	AAAAMVGHPEWEFPHDSGTYNDTPER		-	-	1.01	1,5E-05	3	2885,257568	2885,258789	-0,0013	-0,450566173
1			SAVQMYADYMASFR	M5:+15.9949 - M10:+15.9949	3	6	1.01 1.02 1.03	5,4E-07	2	1671,714111	1671,7146	-0,0004	-0,239275292
1			NVGVSDPDIFYTDQHGTR		2	6	1.02 1.03	3E-08	2	2020,933105	2020,936279	-0,0032	-1,583424449
1			FFVDNGTYLTEQGR		3	6	1.01 1.02 1.03	3,3E-07	2	1646,779053	1646,78125	-0,0022	-1,335939407
1			DVGASDPDIFYTDQHGTR		-	-	1.01	5,2E-06	2	1993,890625	1993,889038	0,0016	0,802451849
1			SGPELTIEMILQAAQPK		2	6	1.04 1.05	7E-05	2	1825,973022	1825,973145	-0,0002	-0,109530635
1			DSEQSSQAMSAPEELVQQVLSAGWR	M9:+15.9949	2	6	1.04 1.05	0,00086	3	2749,272705	2749,273682	-0,0011	-0,400105685
1			LVDAGVDGVMVDVWWGLVEAK		-	-	1.01	4,6E-09	2	2258,152832	2258,153076	-1E-04	-0,044283975
5	**D1KFM6**		IYGPDAGTDYEDNQLR		-	-	5.01	5E-05	2	1826,817627	1826,81958	-0,0019	-1,04005897
5			FLAANYDVTTALEGK		-	-	5.01	3,4E-06	2	1612,821045	1612,822144	-0,0011	-0,682034254
5			VLTVSPYYAEELISGEAR		-	-	5.01	5,8E-06	2	1997,022827	1997,022949	-1E-04	-0,050074536
1	**TC388221**	**16(A/G)**	EGLNMACENALPR	C7:+57.0215 - M5:+15.9949	-	-	1.01	0,00076	2	1490,671631	1490,672974	-0,0014	-0,939173102
1			VPSHAAEITAGYYNLHDR		-	-	1.01	8,3E-12	2	2013,979736	2013,978149	0,0016	0,794447541
1			LSNQLVEGQNYVNFK		-	-	1.01	7,1E-06	2	1752,890381	1752,891968	-0,0015	-0,855728745
1			AAAAMVGHPEWEFPR		2	4	1.01 1.04	3,1E-07	2	1668,794067	1668,795532	-0,0014	-0,838928401
1			FFVDNGTYLTEQGR		3	4	1.01 1.02 1.03	1,6E-07	2	1646,778198	1646,78125	-0,0031	-1,882460237
1			SAVQMYADYMASFR	M5:+15.9949	2	4	1.02 1.03	1,2E-06	2	1655,718018	1655,719727	-0,0016	-0,966347098
1			YDPTAYNTILR		-	-	1.01	1E-04	2	1326,668457	1326,669312	-0,0008	-0,603013873
			SGPELTIEMILQAAQPK	M9:+15.9949	-	-	1.01	4,4E-06	2	1841,967163	1841,96814	-0,0009	-0,488607794
2	**TC425413**		WQEGGDEGR		-	-	2.01	0,0002	2	1033,433472	1033,433838	-0,0003	-0,290294379
2			SKGEGEIYEASEEQIR		2	2	2.01 2.02	2,2E-05	2	1824,860718	1824,86145	-0,0007	-0,383590758
2			GEGEIYEASEEQIR		2	2	2.01 2.02	0,0009	2	1609,735107	1609,734375	0,0007	0,434854329
2			DGYFEMACPHISSSGR	C8:+57.0215 - M6:+15.9949	2	2	2.01 2.02	3,1E-07	2	1829,759033	1829,758545	0,0005	0,273260087
2			VVMFINPVSTPGR	M3:+15.9949	2	2	2.01 2.02	0,00014	2	1432,761353	1432,762085	-0,0007	-0,488566786
2			AFLQPSHHDADEIAFVR		-	-	2.01	6,4E-08	2	1952,9552	1952,961792	-0,0065	-3,328278065
2			EGDVIVIPAGSIVYSANTHR		-	-	2.01	4,7E-05	2	2098,092773	2098,093262	-0,0003	-0,142986983
2			FQEFFLIGSGDERPQSF		-	-	2.02	4,6E-05	2	2003,954712	2003,950195	0,0046	2,295466185

Group	Description	Sample	Sequence	Modifs	Used	on a total of	Sub-groups	E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**TC388221**	**17(Met)**	VPSHAAEITAGYYNLHDR		-	-	1.01	1,5E-09	2	2013,977661	2013,978149	-0,0004	-0,198611885
1			EGLNMACENALPR	C7:+57.0215	-	-	1.01	0,00088	2	1474,678833	1474,678101	0,0008	0,542491257
1			AAAAMVGHPEWEFPHDSGTYNDTPER	M5:+15.9949	-	-	1.03	1,1E-05	3	2901,247559	2901,253662	-0,0062	-2,137007236
1			LSNQLVEGQNYVNFK		2	4	1.01 1.02	3,7E-05	2	1752,890137	1752,891968	-0,0018	-1,026874423
1			SAVQMYTDYMASFR	M5:+15.9949	-	-	1.04	9,4E-06	2	1685,72998	1685,730225	-0,0002	-0,118642941
1			SGPELTIEMILQAAQPK	M9:+15.9949	2	4	1.01 1.02	5,4E-08	2	1841,969116	1841,96814	0,001	0,542897582
1			DSEQSSQAMSAPEELVQQVLSAGWR	M9:+15.9949	-	-	1.01	2,1E-05	3	2749,272461	2749,273682	-0,0012	-0,436478913
1			FFVDNGTYLTEQGR		3	4	1.01 1.02 1.03	2,1E-05	2	1647,779297	1646,781006	0,998000026	606,0308228
2	**TC425413**		SKGEGEIYEASEEQIR		2	4	2.02 2.03	9E-07	2	1824,859253	1824,86145	-0,0021	-1,150772333
2			AFLQPSHHDADEIAFVR		-	-	2.02	1,9E-05	2	1952,959595	1952,961792	-0,0021	-1,075289845
2			VVMFINPVSTPGR	M3:+15.9949	2	4	2.02 2.03	0,00018	2	1432,761719	1432,762085	-0,0004	-0,279181033
2			DGYFEMACPHVSSFGR	C8:+57.0215	2	4	2.01 2.04	2,1E-06	2	1859,786011	1859,784424	0,0017	0,914084494
2			EGDVIVIPAGSIVYSANTHR		-	-	2.02	0,00015	2	2098,091553	2098,093262	-0,0016	-0,762597144
4	**A7UME2|**		ALAAQHANGAPVAR		2	2	4.01 4.02	0,00082	2	1346,727051	1346,729248	-0,0021	-1,559333444
4			VNVGVLAACAPSK	C9:+57.0215	2	2	4.01 4.02	0,0002	2	1285,693604	1285,693726	-1E-04	-0,077779017
4			VPVSEGALATGGVMLSTR	M14:+15.9949	-	-	4.01	0,00036	2	1760,919189	1760,921509	-0,0023	-1,306134343
4			GSTGVAGLADSGLALPAQVASAQK		-	-	4.01	4,2E-08	2	2169,149902	2169,151367	-0,0015	-0,691514671
5	**P93693**		YKAETQSVDFQTK		-	-	5.01	1,2E-05	2	1544,759155	1544,759521	-0,0003	-0,194205001
5			AAEVTTQVNSWVEK		-	-	5.01	0,00051	2	1561,784424	1561,786133	-0,0016	-1,024468064
5			SALSLLAAGAGSATR		-	-	5.01	4,4E-06	2	1345,748169	1345,743774	0,0044	3,26956749
5			VSSVFHQAFVEVNEQGTEAAASTAIK		-	-	5.01	0,00089	3	2720,360596	2720,353027	0,0075	2,756994963
5			SAASNAAFSPVSLHSALSLLAAGAGSATR		-	-	5.01	0,00032	3	2685,39624	2685,395996	0,0003	0,111715376
6	**TC383884**		YGQDATNVGDEGGFAPNIQENK		-	-	6.01	2E-07	2	2324,040039	2324,042969	-0,0029	-1,247825503
6			VNQIGSVTESIEAVK		-	-	6.01	3,5E-06	2	1573,842163	1573,843628	-0,0014	-0,889541984
6			VVIGMDVAASEFYNDK	M5:+15.9949	-	-	6.01	0,00044	2	1773,840332	1773,836792	0,0036	2,029499054
6			IEEELGDAAVYAGLK		-	-	6.01	5E-05	2	1577,803711	1577,806152	-0,0024	-1,521099567
6			LAMQEFMILPTGAASFK	M7:+15.9949	-	-	6.01	5,6E-06	2	1870,944946	1870,94458	0,0004	0,213795736
1	**TC388221**	**18(Met)**	VPSHAAEITAGYYNLHDR		-	-	1.01	1E-05	3	2013,97998	2013,97815	0,0018	0,89375347
1			EGLNMACENALPR	C7:+57.0215	-	-	1.01	0,00088	2	1474,678833	1474,678101	0,0008	0,542491257
1			SGPELTIEMILQAAQPK		2	4	1.01 1.02	3,7E-05	2	1825,974854	1825,973145	0,0017	0,931010425
1			DSEQSSQAMSAPEELVQQVLSAGWR	M9:+15.9949	-	-	1.01	2,1E-05	3	2749,272461	2749,273682	-0,0012	-0,436478913
1			FFVDNGTYLTEQGR		3	4	1.01 1.02 1.03	0,00021	2	1646,782959	1646,78125	0,0017	1,032316804
2	**TC387479**		GGGGGSGSEKEDIQPR		-	-	2.01	9,5E-06	2	1530,713501	1530,714722	-0,0012	-0,783947527
2			SRGEGPISEGSEEQIR		-	-	2.01	3,5E-05	2	1730,828979	1730,830811	-0,0018	-1,039963007
2			AFLQPSHYDADEIAFVR		-	-	2.01	6,4E-07	2	1978,966064	1978,966187	-1E-04	-0,050531432
2			EGDVFVIPAGSIVYSANTHR	E1:-18.0106	-	-	2.01	6,9E-06	2	2114,064697	2114,066895	-0,0022	-1,040648222
3	**TC368656**		ALAAQPANGAPVAR		-	-	3.03	7,2E-05	2	1306,722046	1306,723022	-0,0009	-0,688745797
3			VPVPEGALATGGVMLSTR	M14:+15.9949	-	-	3.02	2,4E-06	2	1770,942261	1770,942261	0	0
3			GSTGVAGLADSGLALPAQVASAQK		2	3	3.01 3.03	7,7E-07	2	2169,154541	2169,151367	0,0032	1,475231171
5	**TC383884**		YGQDATNVGDEGGFAPNIQENK		2	2	5.01 5.02	9,3E-08	2	2324,037842	2324,042969	-0,0051	-2,194451809
5			VNQIGSVTESIEAVK		2	2	5.01 5.02	4,8E-07	2	1573,842163	1573,843628	-0,0014	-0,889541984
5			IEEELGDAAVYAGLK		-	-	5.01	1,5E-05	2	1577,803345	1577,806152	-0,0028	-1,774616003
5			LAMQEFMILPTGAASFK	M3:+15.9949 - M7:+15.9949	-	-	5.01	7,5E-05	2	1886,939575	1886,939453	1E-04	0,052995868
6	**TC380640**		AEAQSVDFQTK		-	-	6.01	3,8E-05	2	1223,589355	1223,590698	-0,0013	-1,062446833
6			AFVEVNETGTEAAATTIAK		-	-	6.01	2,9E-05	2	1922,969849	1922,970947	-0,0011	-0,572031498
6			LSAEPEFLEQHIPR		-	-	6.01	2,6E-05	2	1665,859131	1665,859863	-0,0007	-0,420203418

Group	Description	Sample	Sequence	Modifs	Used	on a total of	Sub-groups	E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**TC388221**	**19(A/G)**	LSNQLVEGQNYVNFK		-	-	1.01	7,1E-05	2	1752,890381	1752,891968	-0,0015	-0,855728745
1			FFVDNGTYLTEQGR		3	4	1.01 1.02 1.03	6,4E-06	2	1646,776611	1646,78125	-0,0047	-2,854052305
1			SAVQMYADYMASFR	M5:+15.9949	2	4	1.02 1.03	1E-04	2	1655,716187	1655,719727	-0,0034	-2,053487539
1			SGPELTIEMILQAAQPK	M9:+15.9949	-	-	1.01	6,4E-06	2	1841,970215	1841,96814	0,0021	1,140084863
5	**TC378369**		GGGGGSGSEKEDIQPR		2	2	5.01 5.02	2,9E-05	2	1530,713135	1530,714722	-0,0015	-0,979934394
5			SGSGSGRPYHFGEESFR		-	-	5.01	0,00012	2	1856,83313	1856,831543	0,0017	0,915538132
5			AFLQPSHYDADEIAFVR		-	-	5.02	1,1E-05	2	1978,964966	1978,966187	-0,0012	-0,606377244
5			EGDVFVIPAGSIVYSANTHR		2	2	5.01 5.02	2,5E-06	2	2132,075928	2132,077393	-0,0015	-0,703539193
6	**Q40345**		TIEAEAAHGTVTR		-	-	6.01	9,3E-06	2	1355,68811	1355,691772	-0,0036	-2,655470848
6			VANPIVEMDGDEMTR	M8:+15.9949 - M13:+15.9949	2	2	6.01 6.02	3,5E-07	2	1708,751587	1708,752075	-0,0004	-0,234088957
6			YEAAGIWYEHR		2	2	6.01 6.02	4,2E-05	2	1394,648438	1394,64917	-0,0007	-0,501918375
7	**TC380640**		AEAQSVDFQTK		-	-	7.01	0,00013	2	1223,590332	1223,590698	-0,0003	-0,245180041
7			AFVEVNETGTEAAATTIAK		-	-	7.01	2,7E-05	2	1922,97229	1922,970947	0,0014	0,728040099
7			LSAEPEFLEQHIPR		-	-	7.01	0,00016	2	1665,861328	1665,859863	0,0015	0,900435925
1	**TC368656**	**20(A/G)**	ALAAQPANGAPVAR		-	-	1.03	0,00021	2	1306,722534	1306,723022	-0,0005	-0,382636577
1			ALATGGVMLSTR		-	-	1.03	0,00055	2	1176,641235	1176,640869	0,0003	0,2549631
1			VNVGVLAACAPSK	C9:+57.0215	2	3	1.01 1.02	1,2E-05	2	1285,694336	1285,693726	0,0007	0,544453144
1			VPVPEGALATGGVMLSTR	M14:+15.9949	-	-	1.02	8,2E-07	2	1770,939697	1770,942261	-0,0025	-1,411677837
1			GSTGVAGLANSGLALPAQVASAQK		-	-	1.02	5,1E-07	2	2168,164551	2168,16748	-0,0028	-1,291413069
3	**TC388221**		NVGVSDPDIFYTDQHGTR		-	-	3.02	1,7E-07	2	2020,927612	2020,936279	-0,0087	-4,304935455
3			LSNQLVEGQNYVNFK		-	-	3.01	9,9E-06	2	1752,894165	1752,891968	0,0023	1,312117457
3			FFVDNGTYLTEQGR		3	4	3.01 3.02 3.04	2,1E-06	2	1646,777222	1646,78125	-0,0041	-2,489705324
3			SGPELTIEMILQAAQPK	M9:+15.9949	-	-	3.01	3,4E-05	2	1841,966919	1841,96814	-0,0012	-0,651477098
4	**Q41593**		YKAETQSVDFQTK		-	-	4.01	2,6E-06	2	1544,759399	1544,759521	-1E-04	-0,064734988
4			IKDILPPGSIDNTTK		-	-	4.01	0,00057	3	1611,89624	1611,89563	0,0006	0,372232556
4			AAEVTTQVNSWVEK		-	-	4.01	5E-05	2	1561,781494	1561,786133	-0,0045	-2,881316185
4			GAWTEQFDSYGTK		-	-	4.01	1,4E-05	2	1489,659302	1489,65979	-0,0005	-0,335647106
4			SALSLLAAGAGSATR		-	-	4.01	4,8E-05	2	1345,74231	1345,743774	-0,0015	-1,114625335
5	**TC387479**		DGYFEMACPHVSSFGR	C8:+57.0215 - M6:+15.9949	-	-	5.01	1,1E-08	2	1875,775146	1875,779297	-0,0041	-2,185758114
5			EGDVFVIPAGSIVYSANTHR		-	-	5.01	0,00016	2	2132,075684	2132,077393	-0,0018	-0,844247043
5			AFLQPSHYDADEIAFVR		-	-	5.01	9,7E-07	2	1978,965332	1978,966187	-0,0008	-0,404251456
6	**TC401483**		GVTTIIGGGDSVAAVEK		-	-	6.01	7,3E-06	2	1573,841431	1573,843628	-0,0021	-1,334312916
6			LASVADLYVNDAFGTAHR		-	-	6.01	0,00019	2	1919,966675	1919,961426	0,0053	2,760472059
6			FLRPSVAGFLMQK		-	-	6.01	0,0008	3	1493,830444	1493,830078	0,0003	0,200826064
6			TVIWNGPMGVFEFEK	M8:+15.9949	-	-	6.01	0,00047	2	1769,851074	1769,857056	-0,006	-3,390104294
6			IVPATAIPDGWMGLDVGPDSIK	M12:+15.9949	-	-	6.01	3,8E-06	2	2268,15625	2268,158447	-0,0022	-0,969949841

Group	Description	Sample	Sequence	Modifs	Used	on a total of	Sub-groups	E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**TC389327**	**21(A/G)**	lLHTISVPGK		2	2	1.01 1.02	0,00054	2	1064,64502	1064,646729	-0,0016	-1,502845883
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	2	2	1.01 1.02	4,4E-07	2	2138,065186	2138,066406	-0,0011	-0,514483511
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	2	2	1.01 1.02	6,9E-09	2	2138,063232	2138,066406	-0,003	-1,403136969
1			QASEGGQGHHWPLPPFR		-	-	1.01	6,7E-06	2	1900,919556	1900,920654	-0,001	-0,526060879
1			QASEGGQGHHWPLPPFR		-	-	1.01	0,00054	3	1900,920898	1900,920654	0,0004	0,210424349
1			QASEGGQGHHWPLPPFR	Q1:-17.0265	-	-	1.01	0,00011	2	1883,890381	1883,894043	-0,0036	-1,910935402
1			QGDVIVAPAGSIMHLANTDGR		2	2	1.01 1.02	2,3E-07	2	2122,069092	2122,071289	-0,0022	-1,036722898
1			QASEGDQGHHWPLPPFR	Q1:-17.0265	-	-	1.02	0,0004	2	1941,896606	1941,899536	-0,0028	-1,44188714
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949 - Q1:-17.0265	2	2	1.01 1.02	4,6E-06	2	2121,040039	2121,039795	0,0003	0,141440064
1			FQYFSAKPLLASLSK		2	2	1.01 1.02	9,1E-07	2	1699,942505	1699,942261	0,0003	0,176476583
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949 - Q1:-17.0265	2	2	1.01 1.02	7E-06	2	2121,032715	2121,039795	-0,007	-3,300268173
1			QGDVIVAPAGSIMHLANTDGR	M13:+15.9949	2	2	1.01 1.02	9,5E-05	3	2138,062988	2138,066406	-0,0033	-1,543450594
2	**TC422632**		DSVVVSGGPDYR		-	-	2.01	4,2E-05	2	1250,60083	1250,601563	-0,0007	-0,559730589
2			GAVVSCADILALAAR	C6:+57.0215	-	-	2.01	1,1E-05	2	1486,806152	1486,805054	0,0012	0,807099819
2			TPNVFDNQYYVDLVNR		-	-	2.01	8,5E-08	2	1956,943481	1956,945435	-0,0019	-0,970900834
2			DFFEQFGVSMGK	M10:+15.9949	-	-	2.01	2,1E-05	2	1407,626099	1407,625244	0,0008	0,568333089
1	**TC433257**	**23(CM-like)**	GGPGKPLHLTATVR		-	-	1.01	2,7E-06	2	1403,811157	1403,812256	-0,001	-0,712345958
1			NVYYGVAPVAQK		-	-	1.01	0,00062	2	1308,694336	1308,695068	-0,0007	-0,534883976
1			YHLDLSGHDLSSVGADIK		-	-	1.01	5,4E-08	2	1926,953125	1926,956055	-0,0028	-1,453068852
1			GVPVSLSIGGYGTGYSLPSNR		-	-	1.01	6,1E-10	2	2081,07251	2081,06665	0,0059	2,835084677
1	**Q07810**	**24(Met)**	TSPASTGLTLATR		3	5	1.01 1.02 1.03	7,2E-05	2	1275,689941	1275,690674	-0,0007	-0,548722386
1			SSHNRPVLPPIEPNVPPSR		-	-	1.01	8,7E-06	2	2093,124023	2093,125488	-0,0014	-0,668856204
1			QQMADAVTALYGR	M3:+15.9949	5	5	1.02 1.03 1.04	7,3E-05	2	1439,694214	1439,69519	-0,0009	-0,625132322
1			PVLPPIEPNVPPSR		-	-	1.01	0,00043	2	1511,856567	1511,858521	-0,0018	-1,190587521
1			ADNLYWEGFK		5	5	1.02 1.03 1.04	0,00069	2	1242,578979	1242,579346	-0,0004	-0,321911007
1			EAVTTLLLMVHEATR	M9:+15.9949	3	5	1.01 1.02 1.03	4,6E-05	2	1699,903687	1699,905151	-0,0014	-0,823575318
1			DLLGDTDKLTNVALGR		5	5	1.02 1.03 1.04	8,6E-05	2	1700,915283	1700,918213	-0,0028	-1,646169662
1			FQTVSGFVAGVLHPK		2	5	1.01 1.02	3,5E-07	2	1586,865479	1586,869385	-0,0038	-2,394652128
1			VQASSADYVTFINGIR		-	-	1.01	5,3E-05	2	1740,889526	1740,891968	-0,0024	-1,378603697
1			NVQASSADYVTFINGIR		-	-	1.01	1,6E-05	2	1854,932251	1854,934937	-0,0026	-1,401666403
1			SADYVTFITGIR		2	5	1.03 1.04	7,3E-05	2	1342,700195	1342,700562	-0,0003	-0,223430321
1			SSSADYVTFITGIR		2	5	1.03 1.04	7,7E-05	2	1516,764526	1516,764648	0	0
1			QQMADAVTALYGR	Q1:-17.0265	5	5	1.02 1.03 1.04	0,00011	2	1406,671875	1406,673706	-0,0018	-1,279614449
1			AQVNGWQDLSEALLK		5	5	1.02 1.03 1.04	0,00013	2	1671,870361	1671,870483	-1E-04	-0,059813246
2	**TC374564**		QQEQGCSGESTEPEQR	C6:+57.0215	2	3	2.01 2.02	0,00037	2	1849,760864	1849,762085	-0,0012	-0,648732126
2			QQGEGFSGEGAQQKPQAGR	Q1:-17.0265	3	3	2.01 2.02 2.03	6,6E-05	2	1942,899902	1942,900635	-0,0007	-0,360286027
2			GEVQEKPLLACR	C11:+57.0215	-	-	2.01	0,00043	2	1399,736084	1399,736572	-0,0005	-0,3572101
2			QEVQGGQYGSETGGSQQQQQGGGYHGVTVGR		-	-	2.02	2,5E-07	3	3164,439697	3164,437988	0,0016	0,505618989
2			DYEQSMPPLGEGR	M6:+15.9949	3	3	2.01 2.02 2.03	0,00034	2	1494,652954	1494,653442	-0,0004	-0,267620564
2			AGEGAFGVPLFQAQSDAR		-	-	2.03	2,1E-05	2	1820,890869	1820,892944	-0,0021	-1,153280377
5	**TC425413**		SKGEGEIYEASEEQIR		2	2	5.01 5.02	1E-05	2	1824,857788	1824,86145	-0,0036	-1,972752452
5			AFLQPSHHDADEIAFVR		-	-	5.01	7,7E-06	2	1952,959961	1952,961792	-0,0018	-0,921676993
5			EGDVIVIPAGSIVYSANTHR		-	-	5.01	7,1E-07	2	2098,092285	2098,093262	-0,0008	-0,381298572
5			FQEFFLIGSGDERPQSF		-	-	5.02	0,00013	2	2003,947021	2003,950195	-0,0031	-1,546944618

Group	Description	Sample	Sequence	Modifs	Used	on a total of	Sub-groups	E-value	Charge	MH+ Obs	MH+ theo	DeltaMH+	Delta-ppm
1	**TC414102**	**25(A/G)**	NPGQSSHNRPVLPPIEPNVPPSR		-	-	1.02	0,00043	3	2489,301025	2489,30127	-1E-04	-0,040171914
1			TSPASTGLTLATR		2	4	1.01 1.03	0,0002	2	1275,691162	1275,690674	0,0005	0,391944557
1			GQSSHNRPVLPPIEPNVPPSR		-	-	1.02	0,00027	3	2278,202148	2278,205566	-0,0033	-1,448508501
1			SSHNRPVLPPIEPNVPPSR		-	-	1.01	6,2E-07	2	2093,125244	2093,125488	-0,0002	-0,095550887
1			QQMADAVTALYGR	M3:+15.9949	4	4	01 1.02 1.03 1.(	0,00036	2	1439,694946	1439,69519	-0,0002	-0,138918296
1			DLLGDTDKLTNVALGR		4	4	01 1.02 1.03 1.(	3,8E-05	2	1700,915527	1700,918213	-0,0026	-1,528586149
1			FQTVSGFVAGVLHPK		-	-	1.01	1E-07	2	1586,86731	1586,869385	-0,002	-1,260343194
1			NVQASSADYVTFINGIR		-	-	1.01	8,6E-05	2	1854,933838	1854,934937	-0,001	-0,539102495
1			VQASSADYVTFINGIR		-	-	1.01	1,3E-05	2	1741,893188	1740,891968	1,001000047	574,9926147
1			SSSADYVTFITGIR		2	4	1.02 1.03	0,00039	2	1516,766113	1516,764648	0,0015	0,988947153
1			AQVNGWQDLSEALLK		4	4	01 1.02 1.03 1.(	6,8E-05	2	1671,869629	1671,870483	-0,0008	-0,478505969
2	**TC400752**		QQGEGFSGEGAQQKPQAGR	Q1:-17.0265	3	3	2.01 2.02 2.03	9,6E-05	2	1942,901489	1942,900635	0,0009	0,463224918
2			QEVQGGQYGSETGGSQQQGGGYHGVTVGR		-	-	2.03	6,4E-06	3	2908,319824	2908,320801	-0,0011	-0,378225118
2			DYEQSMPPLGEGR	M6:+15.9949	3	3	2.01 2.02 2.03	0,00027	2	1494,653931	1494,653442	0,0006	0,401430875
2			AGEGAVGVPLFHAQWGAR		-	-	2.01	4,6E-06	2	1822,934204	1822,935181	-0,0009	-0,493709236
7	**TC425413**		GGGGSGSEKEDIQPR		-	-	7.01	0,0002	2	1473,691406	1473,693359	-0,0018	-1,221420884
7			AFLQPSHHDADEIAFVR		-	-	7.01	3,7E-05	2	1952,961792	1952,961792	1E-04	0,051204279
7			EGDVIVIPAGSIVYSANTHR		-	-	7.01	1,7E-05	2	2098,090576	2098,093262	-0,0025	-1,191558123
8	**CV065553**		DYVLQQTCGTFTPGSK	C8:+57.0215	-	-	8.01	1,3E-08	2	1801,84314	1801,842896	0,0002	0,110997468
8			SGNVGESGLIDLPGCPR	C15:+57.0215	-	-	8.01	0,00012	2	1727,837036	1727,838501	-0,0014	-0,810260892
8			YFIALPVPSQPVDPR		-	-	8.01	1,6E-05	2	1698,920044	1698,921753	-0,0017	-1,00063467
1	**Q07810**	**26(A/G)**	NPGHSSHNRPVLPPIEPNVPPSR		-	-	1.01	0,00042	3	2498,300781	2498,301514	-0,0007	-0,280190349
1			SSHNRPVLPPIEPNVPPSR		-	-	1.01	0,00017	3	2093,125488	2093,125488	0	0
1			NRPVLPPIEPNVPPSR		-	-	1.01	0,00028	3	1782,002319	1782,002441	-0,0002	-0,112233289
1			QQMADAVTALYGR	M3:+15.9949	-	-	1.01	3,2E-05	2	1439,693726	1439,69519	-0,0014	-0,972427964
1			TSPASTGLTLATR		-	-	1.01	0,00059	2	1275,690796	1275,690674	1E-04	0,078388907
1			EAVTTLLLMVHEATR	M9:+15.9949	-	-	1.01	2,2E-05	2	1699,904419	1699,905151	-0,0007	-0,411787659
1			VQASSADYVTFINGIR		-	-	1.01	9,9E-06	2	1740,893188	1740,891968	0,0013	0,746743619
1			FQTVSGFVAGVLHPK		-	-	1.01	2,3E-06	2	1586,868286	1586,869385	-0,001	-0,630171597
1			NVQASSADYVTFINGIR		-	-	1.01	3,3E-06	2	1854,933838	1854,934937	-0,001	-0,539102495
1			DLLGDTDKLTNVALGR		-	-	1.01	1,9E-05	2	1700,914429	1700,918213	-0,0037	-2,175295591
1			AQVNGWQDLSEALLK		-	-	1.01	0,00025	2	1671,87207	1671,870483	0,0016	0,957011938
2	**TC400752**		QQGEGFSGEGAQQKPQAGR	Q1:-17.0265	2	2	2.01 2.02	4,1E-05	3	1942,896973	1942,900635	-0,0037	-1,904369116
2			QEVQGGQYGSETGGSQQQGGGYHGVTVGR		-	-	2.01	3,1E-05	3	2908,324951	2908,320801	0,004	1,375364184
2			AGEGAVGVPLFQAQWGAR		-	-	2.02	5,9E-05	2	1813,932983	1813,934814	-0,0018	-0,992317855
3	**A7UME2**		VNVGVLAACAPSK	C9:+57.0215	2	2	3.01 3.02	0,00084	2	1285,692017	1285,693726	-0,0016	-1,244464278
3			VPVSEGALATGGVMLSTR		-	-	3.01	9,7E-09	2	1744,924561	1744,926636	-0,002	-1,146180034
3			GSTGVAGLADSGLALPAQVASAQK		-	-	3.01	1,8E-08	2	2169,14917	2169,151367	-0,0022	-1,014221549

Annex II

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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.

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 (ω5-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 Kejdahl 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 μg/ml in 100 m M 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 μl/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 μl/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 μl 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 μg) 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 10×10 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 7×7 cm 2D gels immunoblotted with some patient sera revealed a large amount of IgE-binding spots weakly resoluted in the pI range of 4–9 and no IgE-binding spots at extreme pIs ranging between 3and 4 and 9and 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 10×10 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 μm 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 μm, 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×10 cm×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 μm 100A, 75-μm i.d. ×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 (FWMH 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 αβ- 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 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 ± 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±6	28	34	8±2	8	60	30±1	12	98	16±4	85	39±5	62	105	11	120	56±2
Engrain	21	11±5	36	19	<Q	6	11	<Q	<Q	79±2	15 ± 4	36	15±3	31	30	8	66	9±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 adenylyltransferase (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.

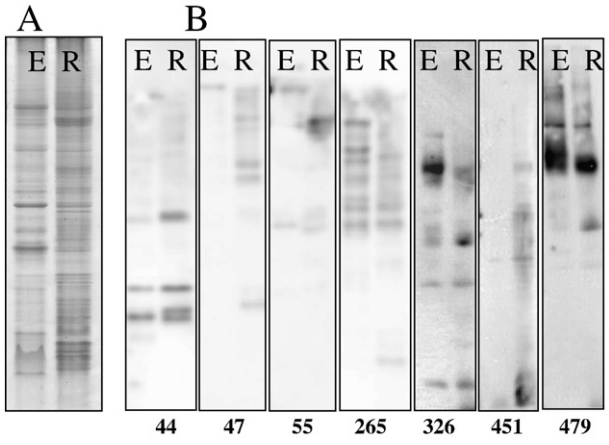

Fig. 1 – A/ SDS electrophoresis of A/G fractions extracted from Engrain (E) and Récital (R), 10 μg of proteins loaded on each lane. B/ Reactivity in immunoblotting of IgE from patients suffering from wheat allergy with A/G fractions from Engrain (E) and Récital (R).

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.

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

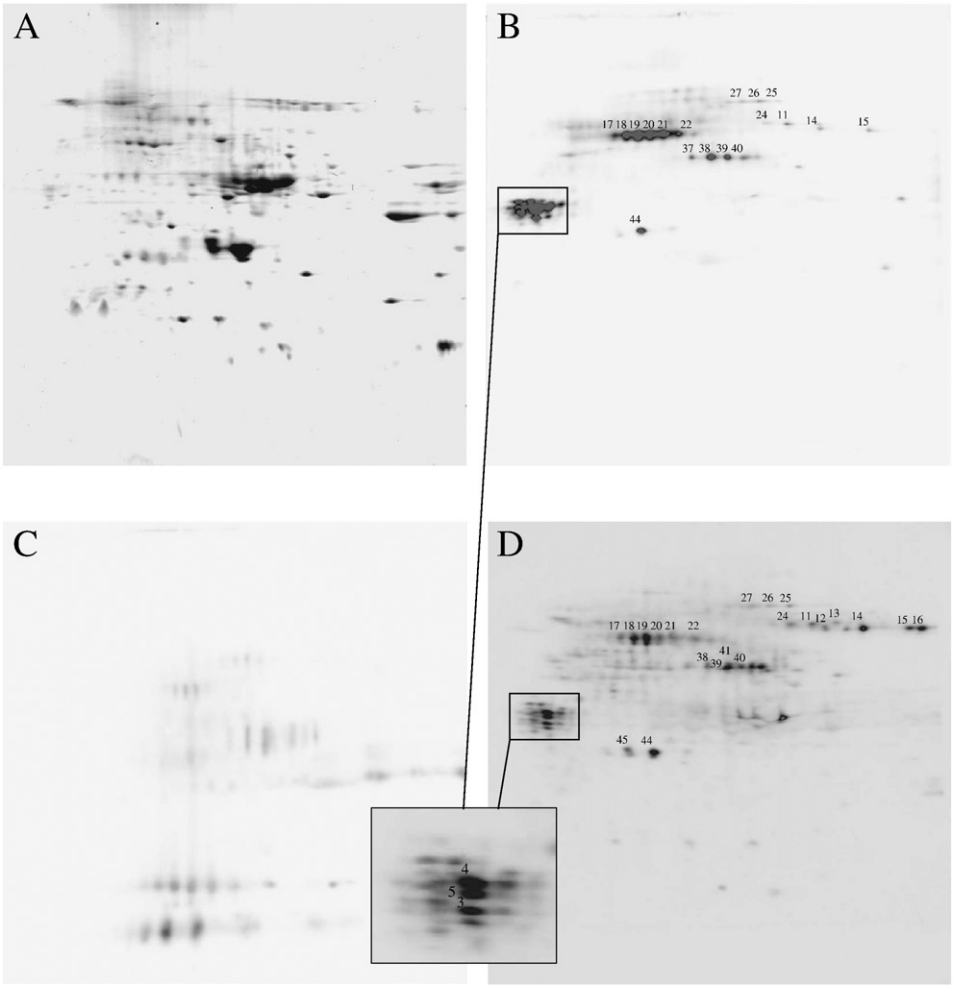

Fig. 2 – Two dimensional map of A/G extractable fraction of Engrain and immunoblotting with sera from wheat allergic patient. A/ Proteins spots were visualized by Coomassie staining. B/ Immunoblotting with serum 38. C/ Immunoblotting with serum 44. D/ Immunoblotting with serum 265. The identified and 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 Sotkowsky 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

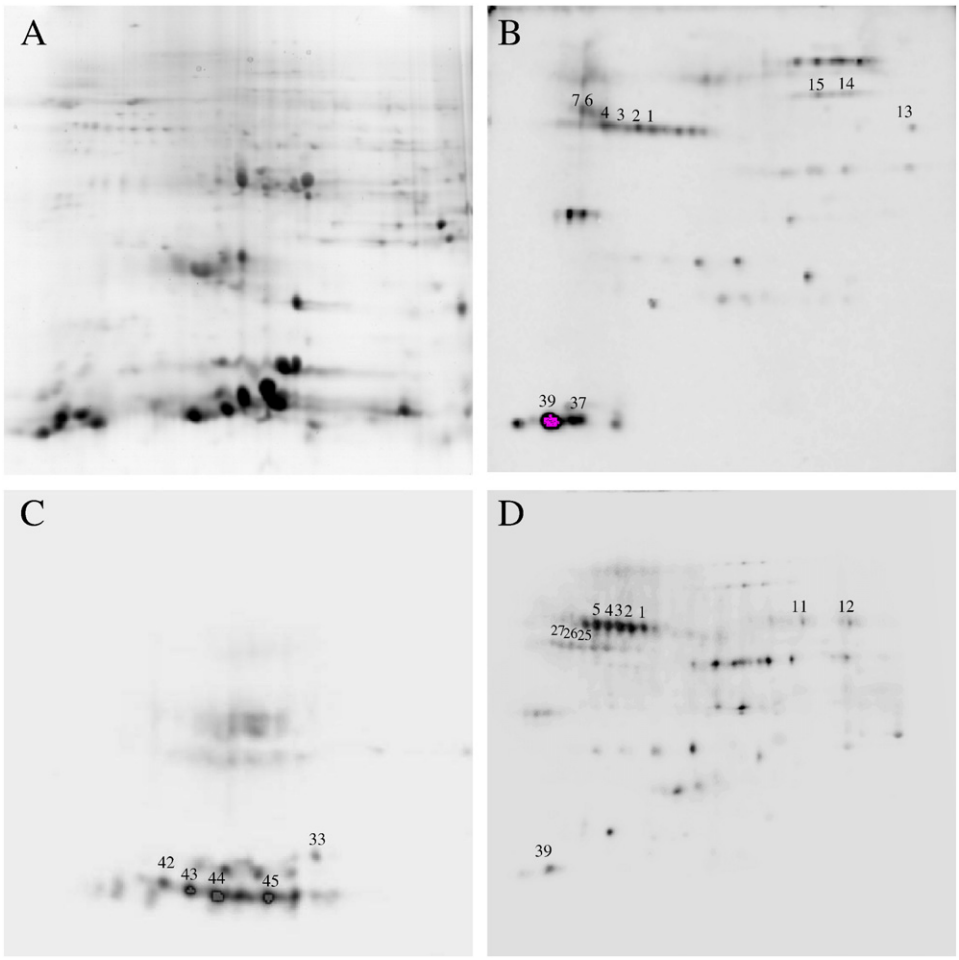

Fig. 3 – 2D gel of A/G fraction extracted from Récital and immunoblotting with sera from wheat allergic patient. A/ Proteins spots were visualized by Coomassie staining. B/ Immunoblotting with serum 38. C/ Immunoblotting with serum 44. D/Immunoblotting with serum 265. The identified and labeled spots 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
A. Engrain corresponding to Fig. 2								
3	TC407122	OSJNBb0118P14.11 protein; *Oryza sativa* Japonica Group. 85% Id	19,139	5.21	58.9	7	39	499
	CD914053	Putative embryonic cell protein; *Oryza sativa*. Partial (48%). 88% Id	18,353	5.73	40	6	6	337
	Q08G36	14-3-3 protein — *Triticum aestivum*	29,388	4.83	43.3	10	11	328
4	TC407122	OSJNBb0118P14.11 protein; *Oryza sativa* Japonica Group. 85% Id	19,139	5.21	54.4	6	12	457
5	Q0JDG9	Os04g0404400 protein — *Oryza sativa*	31,329	4.9	5.8		12	713
	TC407122	OSJNBb0118P14.11 protein; *Oryza sativa* Japonica Group. 85% Id	19,139	5.21	56.1	7	38	521
	TC387595	14-3-3 protein — *Hordeum vulgare*. 99% Id	30,371	4.73	28.9	9	13	452
11	TC424895	Beta-glucosidase; *Hordeum vulgare*. Partial (95%). 97% Id	56,829	8.58	27.8	11	24	657
	TC425413	Globulin-2 precursor — *Zea mays* (Maize). Partial (47%). 72% Id	49,650	8.54	27.3	9	16	434
	TC404101	Endoglucanase 11 precursor *Oryza sativa* Japonica. Partial (91%). 80% Id	54,717	8.39	29	9	13	352
	B7U6L4	Globulin 3 —*Triticum aestivum*	66,652	7.78	8.2	4	5	119
12	TC424895	Beta-glucosidase; *Hordeum vulgare*. Partial (95%). 97% Id	56,829	8.58	20.5	10	33	522
	TC404101	Endoglucanase 11 precursor; *Oryza sativa*. Partial (91%). 80% Id	54,717	8.39	30.8	9	15	482
	TC425413	Globulin-2 precursor — *Zea mays*. Partial (47%). 72% Id	49,650	8.54	32.6	12	19	479
	B7U6L4	Globulin 3 — *Triticum aestivum*	66,652	7.78	18.2	9	12	253
13	TC425413	Globulin-2 precursor — *Zea mays*. Partial (47%). 72% Id	49,650	8.54	25.5	8	14	518
	B7U6L4	Globulin 3 — *Triticum aestivum*	66,652	7.78	24.1	12	17	329
14	B7U6L3	Globulin 3C (Fragment) — *Triticum aestivum*	38,634	9.15	16.6	4	8	254
	Q7DMU0	Storage protein — *Triticum aestivum*	72,551	6.8	11.5	8	10	223
	B7U6L5	Globulin 3B — *Triticum aestivum*	57,068	7.36	12.9	7	8	166
	B5A496	Beta-glucosidase — *Hordeum vulgare*	57,674	7.64	9.6	5	5	133
15	B7U6L4	Globulin 3 — *Triticum aestivum*	66,652	7.78	24.1	17	19	339
	B7U6L3	Globulin 3C (Fragment) — *Triticum aestivum*	38,634	9.15	16.6	8	8	201
	Q7DMU0	Storage protein — *Triticum aestivum*	72,551	6.8	11.5	8	9	170
16	B7U6L4	Globulin 3 — *Triticum aestivum*	66,652	7.78	23	11	16	335
	B7U6L3	Globulin 3C (Fragment) — *Triticum aestivum*	38,634	9.15	16.6	4	8	212
	Q7DMU0	Storage protein — *Triticum aestivum*	72,551	6.8	10.4	6	8	148
	B7U6L5	Globulin 3B — *Triticum aestivum*	57,068	7.36	11.5	6	7	146
	TC383884	Enolase — *Oryza sativa*. Complete	52,518	6.15	48.2	19	21	964
17	Q9ST58	Serpin-Z1C — *Triticum aestivum*	42,969	5.62	43.2	12	37	968
	TC383884	Enolase — *Oryza sativa*. Complete. 96% Id	52,518	6.15	48.2	14	21	964
	C0LF32	Serpin 3 — *Triticum aestivum*	43,227	5.56	41.6	12	25	679
18	Q0PG36	Glucose-1-phosphate adenylyltransferase — *Triticum aestivum*	58,399	6.12	44.1	18	19	611
	C0LF32	Serpin 3 — *Triticum aestivum*	43,227	5.56	38.3	11	20	523
	Q9ST58	Serpin-Z1C — *Triticum aestivum*	42,969	5.62	36.9	11	18	446
	TC383884	Enolase — *Oryza sativa*. Complete. 96% Id	52,518	6.15	33.3	14	15	485
	P08819	Serine carboxypeptidase 2 —*Triticum aestivum*	49,817	5.96	25.5	8	11	346
19	TC382510	Phytepsin precursor (Aspartic proteinase) — *Hordeum vulgare*. Partial (53%). 82% Id	29,405	5.48	41.3	10	26	853
	P93693	Serpin-Z1B — *Triticum aestivum*	43,120	5.44	43.6	14	20	560
	C0LF30	Serpin 1 — *Triticum aestivum*	43,261	5.44	41.4	13	18	536
20	P93692	Serpin-Z2B — *Triticum aestivum*	43,011	5.18	26.6	7	10	438
	C0LF30	Serpin 1 — *Triticum aestivum*	43,261	5.44	30.8	10	12	365
	TC382510	Phytepsin precursor (Aspartic proteinase — *Hordeum vulgare*. Partial (53%). 82% Id	29,405	5.48	42.8	11	24	753
	TC387159	Aspartic proteinase — *Triticum aestivum*. Partial (58%). 79% Id	36,023	8.24	41.8	9	33	709
21	Q0PG36	Glucose-1-phosphate adenylyltransferase — *Triticum aestivum*	58,399	6.12	34.3	17	19	545
	Q9ST58	Serpin-Z1C — *Triticum aestivum*	42,969	5.62	29.6	13	9	264
	TC382510	Aspartic proteinase — *Triticum aestivum*. Partial (58%). 79% Id	29,405	5.48	49.1	12	42	596
24	TC424895	Beta-glucosidase — *Hordeum vulgare*. Partial (95%). 96% Id	56,829	8.58	29.8	14	35	1056
	TC425413	Globulin-2 precursor — *Zea mays*. Partial (47%). 72% Id	49,650	8.54	33.9	13	43	1020
	TC387479	Globulin-like protein — *Oryza sativa*. Partial (41%). 78% Id	45,770	7.26	23.8	8	18	409
25	Q8RWR5	Beta-D-glucan exohydrolase — *Triticum aestivum*	67,714	6.86	37.3	19	33	940
	B2CGM5	Triticin — *Triticum aestivum*	65,241	6.37	12.5	6	7	187
26	Q8RWR5	Beta-D-glucan exohydrolase — *Triticum aestivum*	67,714	6.86	38	21	27	834
27	Q8RWR5	Beta-D-glucan exohydrolase — *Triticum aestivum*	67,714	6.86	42.3	22	30	944
	B2CGM5	Triticin — *Triticum aestivum*	65,241	6.37	16.3	7	11	502
37	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase — *Triticum aestivum*	36,619	6.67	27	7	11	352
38	B0FRH4	Aspartate aminotransferase (Fragment) — *Triticum aestivum*	42,060	6.77	48.2	17	22	615
	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase — *Triticum aestivum*	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) — *Triticum aestivum*	42,060	6.77	55.8	23	33	859
	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase — *Triticum aestivum*	36,619	6.67	43	12	13	340
40	B0FRH4	Aspartate aminotransferase (Fragment) — *Triticum aestivum*	42,060	6.77	52.1	21	35	957
	P93692	Serpin-Z2B — *Triticum aestivum*	43,011	5.18	26.6	8	12	458
	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase — *Triticum aestivum*	36,619	6.67	32.6	9	10	304
41	B0FRH4	Aspartate aminotransferase (Fragment) — *Triticum aestivum*	42,060	6.77	23.4	11	13	330
	C7C4X1	Glyceraldehyde-3-phosphate dehydrogenase — *Triticum aestivum*	36,619	6.67	33.8	7	8	215
44	Q9FS79	Triosephosphate isomerase — *Triticum aestivum*	27,014	5.38	38.7	9	10	374
	C9EF64	Dehydroascorbate reductase — *Triticum aestivum*	23,457	5.88	50	8	13	309
45	O23983	Ascorbate peroxidase — *Hordeum vulgare*	27,532	5.85	38	8	11	330
	C9EF64	Dehydroascorbate reductase — *Triticum aestivum*	23,457	5.88	50	8	11	306
	Q9FS79	Triosephosphate isomerase — *Triticum aestivum*	27,014	5.38	32.4	7	8	250
B. Récital corresponding to Fig. 3								
R1	TC388221	Beta-amylase precursor; *Hordeum vulgare* 89% Id	38,254	5.86	41.2	13	36	571
	TC383884	Enolase; *Oryza sativa* 96% Id	52,518	6.15	20.7	7	9	425
R2	TC388221	Beta-amylase precursor; *Hordeum vulgare* 89% Id	38,254	5.86	35.9	11	22	589
	TC425761	Enolase; *Oryza sativa* 96% Id	52,758	5.8	10.2	4	4	227
R3	TC374294	Serpin; *Triticum aestivum*	45,523	5.81	26.6	9	9	533
	TC388221	Beta-amylase precursor; *Hordeum vulgare* 89% Id	38,254	5.86	33.8	10	34	476
	TC383884	Enolase; *Oryza sativa* Japonica 96% Id	52,518	6.15	13.9	5	6	297
R4	TC380640	Serpin; *Triticum aestivum*	44,790	5.37	26.8	9	13	605
	TC388221	Beta-amylase precursor; *Hordeum vulgare* 89% Id	38,254	5.86	39.1	12	41	556
	TC374294	Serpin; *Triticum aestivum*	45,523	5.81	26.6	9	10	540
R5	TC388221	Beta-amylase precursor; *Hordeum vulgare* 89% Id	38,254	5.86	35	10	37	528
	TC380640	Serpin; *Triticum aestivum*	44,790	5.37	24.4	7	12	478
R11	B7U6L4	Globulin 3 — *Triticum aestivum*	66,652	7.78	23.3	14	25	410
	B7U6L3	Globulin 3C (Fragment) — *Triticum aestivum*	38,634	9.15	20.1	7	13	248
	Q8LK23	Peroxidase — *Triticum aestivum*	39,255	8.14	11.7	4	4	109
R12	B7U6L4	Globulin 3 — *Triticum aestivum*	66,652	7.78	21.9	12	21	399
	B7U6L3	Globulin 3C (Fragment) — *Triticum aestivum*	38,634	9.15	16.9	6	9	213
R13	B7U6L4	Globulin 3 — *Triticum aestivum*	66,652	7.78	22.1	13	24	435
	B7U6L3	Globulin 3C (Fragment) — *Triticum aestivum*	38,634	9.15	14.6	6	10	271
	B7U6L5	Globulin 3B — *Triticum aestivum*	57,068	7.36	10.5	7	10	151
R14	B7U6L4	Globulin 3 — *Triticum aestivum*	66,652	7.78	17.5	12	12	415
	TC424895	Beta-glucosidase — *Hordeum vulgare*	56,829	8.58	15	9	9	266
	Q8LK23	Peroxidase — *Triticum aestivum*	39,255	8.14	6.1	2	2	84
R15	B7U6L4	Globulin 3 — *Triticum aestivum*	66,652	7.78	18.7	17	17	364
	B7U6L3	Globulin 3C (Fragment) — *Triticum aestivum*	38,634	9.15	14.6	6	6	181
	Q8LK23	Peroxidase — *Triticum aestivum*	39,255	8.14	9.5	4	4	108
R25	P93692	Serpin-Z2B — *Triticum aestivum*	43,011	5.18	30.7	10	32	1041
	Q9ST57	Serpin-Z2A — *Triticum aestivum*	43,341	5.46	33.2	11	30	749
	C0LF30	Serpin 1 — *Triticum aestivum*	43,261	5.44	22.3	9	12	382
R26	P93692	Serpin-Z2B — *Triticum aestivum*	43,011	5.18	28.4	9	42	1574
	Q9ST57	Serpin-Z2A — *Triticum aestivum*	43,341	5.46	30.9	9	25	552
	C0LF30	Serpin 1 — *Triticum aestivum*	43,261	5.44	20.1	8	10	316
R27	P93692	Serpin-Z2B — *Triticum aestivum*	43,011	5.18	39.4	11	48	2132
	Q9ST57	Serpin-Z2A — *Triticum aestivum*	43,341	5.46	33.2	10	21	489
	C0LF31	Serpin 2 — *Triticum aestivum*	43,518	5.11	24.2	11	14	346
	Q401N7	Aspartic proteinase — *Triticum aestivum*	54,965	5.14	4.9	2	3	130
R33	Q6S5B1	Alpha amylase inhibitor CM3 — *Triticum turgidum*	18,893	7.44	57.1	8	62	2401
R37	C3VWA4	Dimeric alpha amylase inhibitor — *Triticum turgidum*	15,605	5.01	75.2	11	18	651
R39	B5B0D5	Major allergen CM16 — *Triticum aestivum*	16,400	4.86	41.3	14	14	288
R42	C3VW80	Dimeric alpha amylase inhibitor — *Triticum turgidum*	13,743	6.49	89.5	10	19	793
	Q6S5B1	Alpha amylase inhibitor CM3 — *Triticum turgidum*	18,893	7.44	42.3	5	6	225
R43	C3VW80	Dimeric alpha amylase inhibitor — *Triticum turgidum*	15,588	5.28	69.5	10	11	442
	A4ZIU3	Monomeric alpha amylase inhibitor (Fragment) — *Triticum monococcum*	13,717	6.18	62	6	7	183
R44	A4ZIU3	Monomeric alpha amylase inhibitor (Fragment) — *Triticum monococcum*	13,717	6.18	92.6	13	98	2499
	TC435176	Alpha amylase inhibitor 0.28 precursor; *Triticum aestivum* 99% Id	17,530	6.73	63.6	12	84	608
R45	A4ZIU3	Monomeric alpha amylase inhibitor (Fragment) — *Triticum monococcum*	13,717	6.18	84.3	10	24	514
	C4P5B9	Monomeric alpha amylase inhibitor — *Triticum turgidum*	17,190	6.55	45	6	19	399
	TC431321	Dimeric alpha amylase inhibitor; *Triticum aestivum* 99% Id	18,467	8.03	52.1	6	7	498
	TC435176	Alpha amylase inhibitor 0.28 precursor; *Triticum aestivum* 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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Conclusion and perspectives

Conclusion and perspectives

The genetic transformation of crops offers indisputable advantages, but in Europe the use of GM plants remains controversial. Since the first generation of GM crops, two main concerns have emerged: environmental and health risks. Considering health risks, the major problem is represented by possible unintended effects caused by the site of the transgene insertion or modification of metabolic pathways, or in general pleiotropic effects that could to have an effect on the safety (e.g. increases in toxicity and allergy).

The aim of this work was to investigate the allergenicity of GM wheats in comparison to their untransformed genotypes (*wt*), and also to increase the knowledge of allergenic proteins of wheat.

We considered five GM wheat genotypes (including durum and bread wheats) transformed with different methods (biolistic and *Agrobacterium*) and genes (involved in technological properties, defense and starch biosynthesis). In particular, a detailed proteomic comparison was performed on a bread wheat line over-expressing a transgenic LMW-GS and a durum wheat genotype over-expressing the *Wx-B1* transgene, along with their parental lines. The main results obtained are that the concentration of A/G specific IgE measured by ELISA for the GM lines are highly similar to their *wt* genotypes. Second, and most important, the proteomic profiles of IgE binding polypeptides were very similar between the GM and their parental lines. Moreover, we did not detect any new allergenic polypeptide in GM wheats. Numerous allergenic polypeptides were identified by mass spectrometry in each genotype, mostly of them already known for their allergenicity. No many new potential allergenic polypeptides were identified, caused probably, by the so-called "déjà vu" phenomenon for which the same proteins seem to predominate regardless of the experiment, tissue, and species (Petrak et al., 2008).

Although our study focused only on few transgenic wheat lines and thus they do not allow drawing general conclusion on the substantial equivalence of transgenic wheats, they clearly show the need to perform risk assessments by a case by case approach. In fact, we have demonstrated that the allergenicity may be either increased or decreased depending on the transformation, meaning that each case is different and that individual characterization is required. One difficulty with the assessment of allergenicity is the absence of defined threshold for the concentration of allergens, as no correlation has

been established between the amount of allergens (dose effect) and the symptoms. Moreover, only a few studies about the molecular characterization of new allergens are present, but in this case patients' sera would be strictly necessary.

Patient sera can only be obtained in very limited amounts, and this is a strong limiting factor; tests could be performed with pool of patients' sera in order to decrease the amounts needed and the cost of experiments.

The possibility to have a large collection of sera would allow having a better representation of individual variability, which cannot be investigated using sera's pools.

In any case, it is important to perform all the necessary evaluations before commercializing any GM variety, in order to contribute to public acceptability. Of course this should be performed by public, independent research institutions, and not by biotechnology seed companies, since they might have a conflict of interest.

Today, the recommended safety assessment procedures can only reveal known or already present allergens in food, even when tested with patient's sera in the specific case of allergy. The possibility that a novel allergen is developed, however, cannot be assessed, since it is not possible that GM food is ingested before safety assessment has been performed. Today, there are no direct methods that can reliably predict whether a protein will become a food allergen *de novo*. However, the use of animal models for predicting food allergy is an interesting alternative also recommended by FAO/WHO (FAO/WHO, 2001). The development of allergic animal models for predicting food allergenicity is still challenging. Following the work described by Bodinier et al. (2009) a model of Balb/c mice sensitized with the A/G fraction can be developed. The authors developed a mouse model of allergy to wheat gliadins by testing different doses of sensitization by a whole gliadin extract and by comparing different strains of Th2-biased mice. Many allergens or potential allergens were identified in our study, some of them having been already described in similar studies; further studies are required to assess their allergenicity. We propose to address this question to the specific case of serpins that were often suggested as potential allergens in food and respiratory allergies. Serpin would be expressed as a recombinant protein, its molecular and structural characterizations controlled and then used to test the IgE reactivity of allergic patients. This methodology was already successfully used for assessing the allergenicity of other proteins from the albumin/globulin fraction.

An alternative for the production of hypoallergenic wheats, as already occurred in soybean and peanut. In the specific case of wheat, the major allergens are well- known.

For example in the case of bakers ‘asthma, some CM-like proteins can represent good target for producing a hypoallergenic wheat. In this specific case, several approaches are possible: e first, wheat germplasm lines could be screened for the absence or reduced content of specific allergenic protein(s), or using genotypes with low amount of CM-like proteins, such as *T. monoccocum* (Larré et al., 2011). Another possible approach, if GM approach becomes acceptable, is the genetic transformation for silencing native genes encoding allergenic proteins.