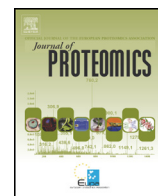




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Comparative proteomic analysis of two transgenic low-gliadin wheat lines and non-transgenic wheat control

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ABSTRACT

Gluten proteins are major determinants of the bread making quality of wheat, but also of important wheat-related disorders, including coeliac disease (CD), and allergies. We carried out a proteomic study using the total grain proteins from two low-gliadin wheat lines, obtained by RNAi, and the untransformed wild type as reference. The impact of silencing on both target and on non-target proteins was evaluated. Because of the great protein complexity, we performed separate analyses of four kernel protein fractions: gliadins and glutenin subunits, and metabolic and CM-like proteins, by using a classical 2D electrophoresis gel based approach followed by RP-HPLC/nESI-MS/MS.

As a result of the strong down-regulation of gliadins, the HMW-GS, metabolic and chloroform/methanol soluble proteins were over-accumulated in the transgenic lines, especially in the line D793, which showed the highest silencing of gliadins. Basing on these data, and considering that metabolic proteins and chloroform/methanol soluble proteins (CM-like), such as the α -amylase/trypsin inhibitor family, β -amylase and serpins, were related to wheat allergens, further *in vivo* analysis will be needed, especially those related to clinical trials in controlled patients, to determine if these lines could be used for food preparation for celiac or other gluten intolerant groups. **Biological significance:** Several enteropathies and allergies are related to wheat proteins. Biotechnological techniques such as genetic transformation and RNA interference have allowed the silencing of gliadin genes, providing lines with very low gliadin content in the grains. We report a proteomic-based approach to characterize two low-gliadin transgenic wheat lines obtained by RNAi technology. These lines harbor the same silencing fragment, but driven by two different endosperm specific promoters (γ -gliadin and D-hordein). The comprehensive proteome analysis of these transgenic lines, by combining two-dimensional electrophoresis and RP-HPLC/nESI-MS/MS, provided a large number of spots differentially expressed between the control and the transgenic lines. Hence, the results of this study will facilitate further safety evaluation of these transgenic lines.

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1. Introduction

Wheat is one of the most widespread crops being used for human consumption due to its technological and nutritional properties, as well as its versatility in giving different products. Although the major component in wheat grain is the starch (60–75%), proteins (9–18%) have an essential role in the dough functionality. There are two major groups of proteins in wheat grains: gluten proteins (80%), responsible for rheological quality; and non-gluten proteins (20%), comprising albumins and globulins with mainly structural and metabolic functions and a minor role in wheat quality, although the albumin/globulin ratio has been correlated with bread-making quality [1]. Osborne [2] developed

a classification of wheat proteins based on their solubility, thus these groups of proteins could be separated in the alcohol-soluble gliadins, clustered in ω -, γ - and α -gliadins, monomeric proteins contributing to viscosity and extensibility of wheat dough, and the alcohol-insoluble glutenins, responsible of the elasticity and dough strength, comprising high (HMW-GS) and low (LMW-GS) molecular weight glutenin subunits, that interact by covalent and hydrogen bonds, by forming glutenin polymers. The non-gluten fraction is composed by the water-soluble albumins and saline solutions-soluble globulin proteins, which can also be separated in metabolic and chloroform/methanol-soluble (CM) proteins fractions [3] described above.

Wheat flour mixed with water produce a protein network responsible for the viscoelastic properties and gas holding capacity during formation of the bread dough. The protein composition of wheat flour influences the properties of dough, leading to the development of

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different end products such as bread, biscuits, cakes and noodles. Gluten proteins, also known as prolamins because of their high content in proline and glutamine [4,5], are related with a widespread human enteropathy, that is the coeliac disease (CD) [6], which affects both children and adults at a frequency of about 1% worldwide, and is caused mostly by α -gliadins [7,8], although all gluten proteins are involved. Gliadins are responsible also of other adverse reactions to wheat, among which Wheat-Dependent Exercise-Induced Anaphylaxis (WDEIA) caused by the major allergens ω -5 gliadin [9,10]. Another widespread pathology, named as Non Celiac Wheat Sensitivity (NCWS), might be related to gluten proteins as well, but actually this is far to be clear since other proteins, such as α -amylase/trypsin inhibitors (ATI) [11], that are metabolic proteins, or the fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPS) seem likely candidates. ATI, as well as other metabolic proteins, especially those present in the CM-fraction, have also a role in the human allergies, in particular respiratory allergies. ATI are considered the main wheat proteins related to Baker's asthma, an allergy caused by the inhalation of wheat and cereal flours [12] that is considered the most common form of professional asthma [13].

Because both gluten and non-gluten proteins belong to the same prolamin-superfamily [14], having the same origin, very often allergies to a specific polypeptide is associated with cross-reactivity to other wheat proteins, and they can be identified in sera of patients with food allergy to wheat by using immunoblotting procedures [15].

Coeliac disease (CD) is an autoimmune disease caused by several immunogenic peptides present in gluten proteins of wheat, barley, rye and, presumably, in some oat varieties which trigger the small bowel damage and, therefore, gastrointestinal and malabsorption symptoms. Individuals with CD are genetically predisposed because of the presence of genes encoding the human leukocyte antigen (HLA) DQ2 or DQ8, that are present in 95% and 5% of coeliac patients, respectively. Moreover, a relationship between the incidence of CD and the amount and timing of exposure to gluten has been suggested [16,17].

People suffering this pathology are recommended a life-long gluten-free diet (GFD) [16,17]. However, GFD is characterized by an unbalanced intake of different nutrients, with an excess of saturated and hydrogenated fatty acids, poor in alimentary fibers, low contribution to the recommended daily protein intake, and high carbohydrate, providing an increase in the glycemic index [18,19] and a detriment to gut bacterial microbiota [20].

The development of wheat lines suitable for CD is a desirable target, though at the moment, still difficult to be reached, because of the high complexity of gluten proteins. Nevertheless, to achieve this goal, it is necessary to proceed, first by eliminating the most important protein types triggering this pathology, and then evaluating their immunogenic potential, and also the effects on dough technological properties, since this is an important factor, both for the industry and wheat-based foods palatability. RNAi technology is a very promising tool previously used to down-regulate the expression of gliadin fractions, that contain most of the immunodominant epitopes responsible for CD, that provided wheat lines with partial [21–23] or total silencing [24] of gliadins. These lines showed reduced capacity to stimulate DQ2- and DQ8- restricted T-cell clones from patients with CD and, in regard to dough technological properties, they had also an increased stability and tolerance to over-mixing [25]. Despite the strong silencing of all three gliadin groups, total grain protein was not significantly decreased, or even increased in the case of some lines [26]. Therefore, there is a compensation or over-compensation of total protein, first with HMW-GS as those lines showed significant increment in the HMW-GS; and second with non-gluten proteins such as albumins and globulins, as reported previously [27,28].

Proteomic characterization of these compensation effects is particularly important for the low-gliadin RNAi lines. Although albumins and globulins are not related to CD, they play an important role in allergies, and therefore needs to be evaluated in these GM lines [29]. Proteome analysis allows evaluating not only the actual expression or silencing

of target genes, but also possible effects on the expression of other kernel proteins [30–33] [34–36]. For this reason, we performed a detailed proteomic analysis of the most represented wheat kernel protein fractions, that are gliadins, glutenins (both forming the gluten), metabolic and CM-proteins, in two RNAi-silenced lines obtained from the *T. aestivum* cv Bobwhite (used as a control), targeted to silence α/β -, γ -, ω -gliadins by using constructs containing the same silencing fragment, but driven by two different endosperm specific promoters: a D-hordein promoter [37] and a γ -gliadin promoter [38]. These lines have been the subject of previous characterizations [24–26,39], but still a comprehensive proteomic characterization able to evaluate the effects on the accumulation of other seed proteins was missing. Because of the great protein complexity, we performed separate analyses of four kernel protein fractions: gliadins, glutenin subunits, metabolic proteins and CM-like proteins, by using a classical gel based approach (2D electrophoresis) followed by RP-HPLC/nESI-MS/MS.

2. Material and methods

2.1. Plant material

Two transgenic lines obtained from the *T. aestivum* cv Bobwhite (denoted as Bobwhite 208, and here used as a control) were studied. These transgenic lines are named as D783 and D793. Table 1 summarizes the characteristics of these lines. These lines are homozygous since they are at least in the T₄ generation.

The two transgenic lines as well as the control line were pre-germinated into a growing chamber at 4 °C and later grown in a greenhouse under controlled conditions of temperature and humidity, according to Pistón et al. [38]. At tillering, wheat plants were field transferred in a dedicated greenhouse under controlled environmental conditions. The two transgenic lines were grown in three randomized blocks, each comprising five plants.

2.2. Isolation and quantification of protein fractions

Mature kernels from the five plants from each block were pooled and three technical replicates, corresponding to three different flour extracts, were performed by crushing with mortar and pestle and extracting the different protein fractions. Four protein fractions were isolated, that should include most of the wheat proteins present in mature kernels. They were gliadins and glutenin subunits (that together make the gluten proteins), metabolic proteins, and the CM-like proteins (these two latter together make up most of the albumin/globulin protein fraction). These protein fractions were quantified with 2-D quant Kit Assay (GeHealthcare). Differently from Scossa et al. [31], who used the same volumes of extracts to detect quantitative differences between transgenic lines and their control, we used the same concentration of protein in both control and transgenic lines within each protein fraction. This was decided with the objective of specifically quantify the variation in the accumulation of proteins related to adverse reactions to wheat among lines.

Gliadins and glutenins were quantified by means of Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) as described by Pistón et al. [40]. Three independent repetitions were carried out for each transgenic line and control.

Table 1
Characteristics of lines used for proteomic analysis.

Genotype	Line	Constructs	Promoter	Prolamin target
BW208	Wild type	NA	NA	NA
	D783	pDhp- ω / α	D-hordein	α/β -, γ -, ω -
	D793	pGhp- ω / α	γ -Gliadin	α/β -, γ -, ω -

NA, non-applicable.

The protein content of flour was calculated from the Kjeldahl nitrogen content (%N $\times$ 5.7) according to the standard ICC method no. 105/2 (ICC, 1994) [41]. The non-gluten proteins, expressed in percentage of dried weight (% DW), were calculated as follow: [Total protein in %DW – (Prolamin content in $\mu\text{g}/\text{mg} \times 10) / (100 - \text{moisture in } \%)$].

2.2.1. Gliadin

Gliadins were extracted from 100 mg of flour with ethanol 70% in a 1/10 (mg/ μl) ratio for 45 min at room temperature. The supernatants obtained after a centrifugation at 13000 rpm for 15 min, containing the gliadin fraction, were dried down in a Savant™ Speed Vac™ evaporator.

2.2.2. Glutenin

Glutenin proteins were extracted from pellet remaining after gliadin extractions as described in Muccilli et al. [42], but amounts were extrapolated to 100 mg of flour.

2.2.3. Metabolic and CM-like proteins

Because non-gluten proteins are soluble in salt solutions, a KCl extraction was performed. The extraction method used is based on the method described by Vensel et al. [43], but amounts were scaled up to perform large 2D gels (18 cm). Protein amounts were assessed by 2-D quant Kit Assay (GE Healthcare).

2.3. Two-dimensional gel electrophoresis

In all the following cases, the samples were run in triplicate, thus, twenty seven gels were performed (3 genotypes $\times$ 3 biological replicates $\times$ 3 technical replicates) for each of the four protein fractions here analyzed.

2.3.1. Gliadins

In case of gliadins, A-PAGE vs SDS-PAGE were performed. The dried protein samples were dissolved in a rehydration buffer (7 M Urea, 2 M Thiourea, 2% (w/v) CHAPS, 2% (v/v) Triton $\times$ 100).

Aliquots of 70 μg of protein dissolved in 10 μl of loading buffer (6 M Urea, dimethyl violet in traces, 20% sucrose and 0.14% acetic acid) were analyzed by Acid-PAGE in acetic acid buffer using 13 cm gels ($T = 6\%$, $C = 2.67\%$), as described by Masci et al. [44]. Gels were stained according to Neuhoﬀ et al. [45]. Second dimension (SDS-PAGE) was performed according to Masci et al. [44]. In this latter case, the main gel was $T = 15\%$, $C = 0.5\%$. The run was performed in a Hoefer apparatus at 40 mA per gel for 3–4 h at 12–14 °C and stopped one hour after the dye front left the gel. Gels were stained with Coomassie Blue G250 according to Neuhoﬀ et al. [45].

2.3.2. Glutenins

Glutenin subunits were analyzed by 2D gel by using IEF vs SDS-PAGE. 250 μg proteins were suspended in 340 μl of rehydration buffer as described in Egidi et al. [46], but using 0.5% (v/v) IPG buffer pH 3–10. We used IEF linear IPG strips 18 cm, pH 3–10 (GE Healthcare) that were rehydrated in 12–20 h at room temperature. IEF and SDS-PAGE was carried out according to Egidi et al. [46], but, for the second dimension, the strips were placed on 18 $\times$ 20 cm polyacrylamide gels ($T = 11\%$, $C = 2.67\%$) of 1 mm thickness (Protean Plus multi-casting chamber, Bio-Rad; Protean Plus Dodeca Cell, Bio-Rad) and run at 40 mA per gel for 5–6 h at 10 °C until the dye front left the gel.

2.3.3. Metabolic proteins

350 μg of metabolic proteins were dissolved in 300 μl of strip rehydration buffer (as described in Egidi et al. [46]) but, in this case, by adding 0.3% IPG buffer pH 3–10 and 0.2% IPG buffer pH 6–11 at the time of use. The proceedings to accomplish the first dimension (IEF) were the same as described for glutenin subunits. After 12–20 h of rehydration, isoelectric focusing was performed at 20 °C for 80 kVh (200 V

8 h, 300 V 3 h, 1000 V 2 h, 3500 V 30 min gradient, 3500 V 3.5 h, 8000 V 8 h) using the IPGphor™ Isoelectric Focusing System (GE Healthcare) with Manifold. The gel strips were equilibrated and the second dimension (SDS-PAGE) was carried out according to Laino et al. [47].

2.3.4. Chloroform/methanol proteins (CM-like)

In this case, 200 μl of rehydration buffer were added to protein pellets previously obtained, and 250 μg were dissolved in 340 μl of rehydration buffer. The IEF was developed as described for metabolic proteins, but the focusing was performed at 20 °C for 80 kVh with the following program: 200 V 4 h, 500 V 2.5 h, 1000 V 2 h, 1000 V 3 h, 5000 V 2 h, 8000 V 30 min gradient, 8000 V 8.5 h. The equilibration and second dimension steps were the same as those used for metabolic proteins.

2.4. Protein staining, image acquisition and gel spots excision

In order to quantitatively compare the proteins present in each of the gels, these were stained overnight with Coomassie Brilliant Blue G-250 (Sigma-Aldrich) according to Neuhoﬀ et al. [45]. Gels were digitalized by using a scanner to acquire images with the same conditions for each gel (300 dots per inch and 16-bit grayscale pixel depth), according to Scossa et al. [31]. Comparisons were made between triplicate gels from each of the three biological replicates for non-transgenic and transgenic lines D783 and D793. To facilitate these comparisons between data obtained from different gels, the volumes of the identified spots were normalized as described in Scossa et al. [31]. This pre-processing step is used to correct the differences between images. The protein spots that had p value (obtained by ANOVA) lower than 0.05 and fold value equal to or >1.2 , were considered as significant differentially expressed using the software SameSpots Progenesis (version 4.6.1.218, Nonlinear Dynamics, UK). Moreover, Principal Components Analysis (PCA) was calculated according to O’Gorman et al. [48].

Differential spots were picked from those gels in which the spot color was more intense, (where the protein concentration was supposed to be higher) and transferred into 1.5 ml tubes. In case of low-abundance protein spots, they were picked from replicate gels and pooled. The excision of the spots was carried out by using the instrument EXQuest™ Spot Cutter by Bio-Rad®.

2.5. In-gel digestion and mass spectrometric analysis

The spots were washed and subjected to *in-gel* trypsin digestion and subsequently mass spectrometric analysis according to Muccilli et al. [42] and Sestili et al. [32]. After soaking trypsin (Modified porcine trypsin, Promega,) into the gel pieces, the supernatant containing excess trypsin was removed and the gel pieces were covered with 50 μl of 50 mM NH_4HCO_3 and incubated at 37 °C overnight. The enzymatic reaction was stopped by cooling the gel pieces and the supernatant solution at -20 °C. After *in-gel* digestion, the peptides solutions were transferred into clean 0.5 ml tubes. The peptides were extracted from gel pieces with 40 μl 0.1% TFA and subsequently with the same volume of acetonitrile. This extraction procedure was repeated three times. The total extracts were pooled, combined with the first supernatant, lyophilized and dissolved in 15 μl of 0.1% TFA. Capillary RP-HPLC/nESI-MS/MS was performed using an Ultimate 3000 LC system combined with an autosampler and a flow splitter 1:100 (Dionex Corporation, USA) coupled on-line with a linear ion trap nano-electrospray mass spectrometer (LTQ, Thermo Fischer Scientific, USA). Ionization was performed with liquid junction using a no coated capillary probe (30 ± 2 μm i.d.; New Objective, USA).

The peptide solution was loaded onto a C18 μ -pre-column cartridge (0.3×5 mm, 100 Å, 5 μm , PepMap, Dionex) equilibrated with 0.5% aqueous formic acid (FA) at a flow rate of 20 $\mu\text{l}/\text{min}$ for 4 min. Peptides were then applied onto a C18 capillary column (0.18 mm $\times$ 150 mm, 300 Å, 5 μm , Thermo Electron) and eluted at room temperature with a linear

gradient of CH₃CN-0.5% FA/H₂O-0.5% FA from 10 to 50% in 50 min at a flow rate of 1.5 µl/min. Repetitive mass spectra were scanned using the following electrospray ion source parameters: capillary temperature, 220 °C; spray voltage, 1.9 kV. Peptide ions were analyzed by the data-dependent method as follows: (1) full MS scan (mass-to-charge ratio 350–1900); (2) ZoomScan (scan three major ions with higher resolution; isolation width 2 Da); and (3) MS/MS of the three major ions (Q 0.250, collision energy 29 a.u.). Mass calibration was made using a standard mixture of caffeine (Mr 194.1 Da), MRFA peptide (Mr 523.6 Da) and Ultramark (Mr 1621 Da). Data acquisition was performed using the Excalibur v. 1.4 software (Thermo Fisher Scientific).

2.6. Database search and protein identification

LC/MS/MS data were processed by Proteome Discoverer v. 1.4.1.14 (Thermo Fisher Scientific). Data were searched against the “*Viridiplantae*” UniProt database, 36021 sequences, and trEMBL, 2836128 sequences (release July 2015), using the Mascot algorithm (Matrix Science, London, UK, version 2.5.1). Full tryptic peptides with a maximum of 3 missed cleavage sites were subjected to bioinformatics search. Cysteine carbamidomethylation was set as fixed modification, whereas oxidation of methionine, transformation of N-terminal glutamine and N-terminal glutamic acid residue in the pyroglutamic acid form were included as variable modifications. The precursor mass tolerance threshold was 1.2 Da and the max fragment mass error was set to 0.6 Da. Peptide spectral matches (PSMs) were validated using Target Decoy PSM Validator node based on q-values at a 1% FDR. A protein was considered identified with: minimum of 2 peptides with a Mascot score >20 and Expect <0.05; proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. In the case of gliadins and some LMW-GS, they were considered identified with a single peptide and its MS/MS spectrum was manually verified for the purpose of supporting this identification. This derogation from the number of peptides for the identifications is applied given the peculiarity of the amino acid sequence.

3. Results

Table 2 recapitulates the whole grain protein characteristics of the lines used in this study. Although total protein contents are not different, the transgenic lines have significantly lower contents of gliadins (both total and of each different fraction) than the control [24]. On the opposite, HMW-GS show a significant increase in the transgenic lines with respect to the control; however, for LMW glutenin subunits, the line D783 showed a significant increment while the line D793 had a significant decrease compared to the control, as also reported by Gil-

Table 2
Gliadins and glutenins fractions and total protein contents of wild-type (BW208) and the two transgenic lines (D783 and D793). The values are the averages of the years 2010, 2011, 2012, and 2014.

Protein fraction (µg/mg flour)	Line		
	BW208	D783	D793
ω gliadins	10.5a	6.0b	6.6b
α gliadins	27.5a	11.6b	6.5b
γ gliadins	22.8a	3.7b	0.50b
Total gliadins	60.9a	21.3b	13.7b
HMW	8.3b	19.4a	18.7a
LMW	13.7b	19.2a	10.5c
Total glutenins	22.0c	38.6a	29.20b
Total prolamins	82.8a	59.9b	42.9c
Non gluten proteins (%DW)	4.4c	7.1b	9.2a
Total protein (%DW)	13.5a	13.6a	13.9a

HMW, high molecular weight; LMW, low molecular weight; DW, dried weight.

Means with the same letter for each line and protein fraction are not significantly different as determined by Tukey multiple comparisons at $p < 0.05$.

Humanes et al. [25]. Therefore, with the silencing of gliadins, there has been a protein compensation resulting in an increase of the glutenins and the non-gluten proteins, thus ensuring an adequate supply of proteins for the correct development of the grain.

Results from comparative proteome analysis are presented relative to each of the four protein fractions here studied. In each case, comparisons were performed among the three genotypes and also between pairs corresponding to the three combinations of the three genotypes (namely BW208 vs D783, BW208 vs D793, and D783 vs D793). Thus, each comparison is independent and we realized a new normalization for each comparison obtaining a different ANOVA and fold change for each sample; for that, the number of spots identified in the comparisons among the three genotypes present more spots differentially accumulated than comparisons between pairs. PCA analysis indicates if the groups of each protein fraction studied are properly clustered on the basis of 2D electrophoretic patterns.

3.1. Prolamins

3.1.1. Comparison among the three genotypes BW208 vs D783 vs D793

Fig. 1 reports the 2D gels of gliadins with indication of different abundance in the protein spots, whereas Supplementary Fig. S1A reports the PCA analysis relative to such comparison and indicates that the groups are properly clustered on the basis of 2D electrophoretic patterns.

As regards gliadins, sixteen spots were identified by mass spectrometry (Table 3). Nine spots were up-accumulated in BW208, five of which were identified as γ-gliadins (149, 144, 128, 273 and 123), two as α/β-gliadins (243 and 142) and the spots 217 and 279 were heterogeneous, since more than one protein was identified (γ-gliadin and LMW-GS). When the pairwise comparison was carried out, the number of spots identified was lower, as explained above. Thus, we obtained that only the spots 273, 243, 217 and 279 had differentially significant abundance in the wild-type (Supplementary Table S1 and Supplementary Fig. S2). The remaining seven spots had higher values for normalized spot volumes in the line D783 (88, 87, 226, 245, 185, 107 and 108) and they belonged to LMW-GS family. Of these seven spots, only two (226 and 245) were identified as mostly expressed in the pairwise comparison between BW208 and D783 (Table 3 and Supplementary Table S1A). None spot was significantly increased on the line D793 when compared to both the control and the other transgenic line D783 (Table 3 and Supplementary Table S1B and C). Table 3 shows that the spots 217, 273 and 243 had a significant increase in the line D783 compared to the line D793.

Comparative analysis of the glutenins fractions extracted from transgenic lines with respect to wild type highlighted nine differentially spots. Among which, six were up-accumulated in BW208 (Table 4 and Fig. 2). Spots 1925, 4602, 4608 and 2690 corresponded to γ-gliadins and were at higher amount in BW208 wild type; 2716 was identified like “Uncharacterized protein” and spot 4248 was a Heat shock protein class I, and both were also up-accumulated in the wild type in comparison to that of the transgenic lines. Spots with higher expression in both transgenic lines (359, 397 and 4613) were identified as HMW-GS DY10 (Table 4 and Fig. 2). The corresponding PCA analysis is shown in the Supplementary Fig. S1E and it shows that cluster spots from D783 and D793 are mixed to form a single cluster, indicating that they are not separated on the basis of the 2D patterns of glutenin subunits.

The different abundances of spots from glutenins for comparisons between pairs is shown in the Supplementary Fig. S3; the identification of those spots are represented in the Supplementary Table S3. Full details of the gliadins and glutenins identifications are reported in Supplementary data Tables S2 and S4, respectively.

3.2. Metabolic proteins

3.2.1. Comparison among the three genotypes BW208 vs D783 and D793

The 2-DE analysis of metabolic proteins of wheat kernels revealed significant differences for fifteen spots between the control and its

A-PAGE vs SDS-PAGE

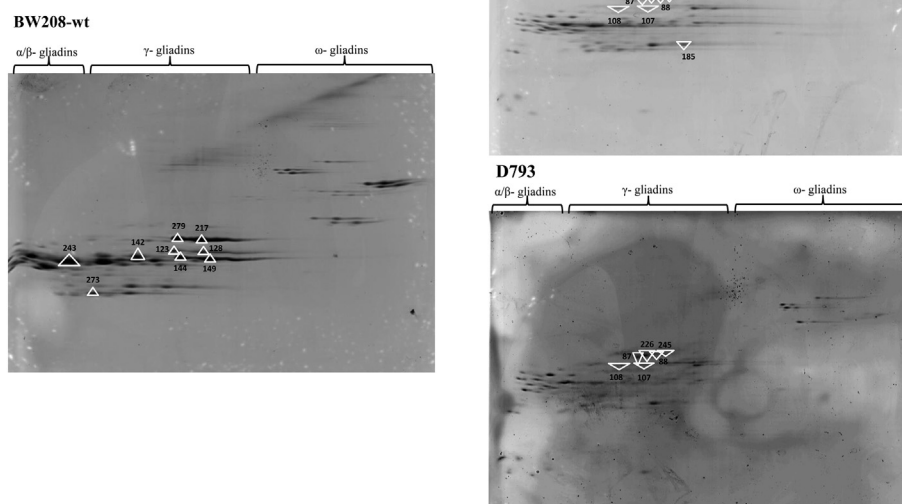


Fig. 1. 2-DE map of the comparative gliadins analysis from wild-type BW208 and transgenic lines D783 and D793. Differentially accumulated spots are marked with up (for over-accumulated) and down (for down-accumulated) triangles using the control line as reference.

derived transgenic lines (Table 5 and Fig. 3). PCA analysis was carried out and it showed that D783 and D793 are not well separated (Supplementary Fig. S4A and D). Details of these identifications are reported in Supplementary Table S5. Among these, taken the control as reference, five increased (2646, 6223, 2172, 2096 and 2565) and ten decreased (4305, 6219, 5506, 2879, 2931, 2882, 6228, 2936, 6218 and 2754) in relative abundance. Of these fifteen spots, four (4305, 5506, 6218 and 2754) had not significant differences in the pairwise comparisons

(Supplementary Table S6). Within the spots with more abundance in the transgenic lines (6219, 2879, 2931, 2882, 6228, 2936), the spots 6219, 2879 and 2882 had more abundance in both transgenic lines in comparison to the BW208, while the spots 2931, 6228 and 2936 present significant differences between BW208 and D793 but no between BW208 and D783. Six spots showed unique peptides related to more than one protein in each spot. Thus, the spot 2646 was described as Serpin-Z2A and Serpin-Z1B; spot 6223 as β -amylase and

Table 3

List of differentially expressed gliadin spots comparing the three lines used in this study.

Spot	ANOVA	Fold change	Protein	Accession	Species	Score/%Cov	Average normalized volumes		
							BW208	D783	D793
149	4.39E−12	(+) 4.8	Gamma-gliadin	P08453*	<i>T. aestivum</i>	1302/13	2.92E+07	1.04E+07	6.08E+06
217	1.84E−13	(+) 4.6	Gamma-gliadin	R9XV62#	<i>T. aestivum</i>	96/12	3.00E+07a	1.26E+07b	6.51E+06c
			LMW-GS	P10385#	<i>T. aestivum</i>	192/9			
			LMW-GS	R4JAQ1#	<i>T. aestivum</i>	72/10			
88	1.98E−09	(−) 4.4	LMW-GS B3-2	D3UAL7#	<i>T. aestivum</i>	58/11	1.10E+06	4.89E+06	3.28E+06
87	1.36E−08	(−) 4.4	LMW-GS	Q84U14#	<i>T. aestivum</i>	58/12	1.15E+06	5.08E+06	4.52E+06
			LMW-GS B3-2	D3UAL7#	<i>T. aestivum</i>	109/11			
144	3.99E−09	(+) 4.1	Gamma-gliadin	P08453*	<i>T. aestivum</i>	221/9	1.94E+07	9.61E+06	4.77E+06
128	1.77E−11	(+) 3.4	Gamma-gliadin	Q94G97#	<i>T. aestivum</i>	110/5	5.28E+06	2.07E+06	1.54E+06
226	2.07E−08	(−) 3.3	LMW-GS B3-2	D3UAL7#	<i>T. aestivum</i>	155/28	1.03E+06a	3.40E+06b	3.00E+06ab
243	1.97E−07	(+) 3.1	Alpha/beta-gliadin A-I	P04721*	<i>T. aestivum</i>	93/10	3.43E+07a	2.62E+07a	1.10E+07b
245	9.98E−08	(−) 3	LMW-GS	B8XU58#	<i>T. monococcum</i>	123/12	1.01E+06a	3.02E+06b	2.09E+06ab
142	1.06E−06	(+) 2.9	Alpha/beta-gliadin MM1	P18573*	<i>T. aestivum</i>	121/16	1.32E+07	6.53E+06	4.55E+06
185	1.97E−11	(−) 2.6	Gamma-gliadin	B6DQD9#	<i>T. aestivum</i>	58/5	1.09E+07	1.34E+07	5.12E+06
279	0.001	(+) 2.3	Gamma-gliadin	P08453*	<i>T. aestivum</i>	164/4	3.84E+06a	2.24E+06b	1.67E+06b
			LMW-GS	P10385*	<i>T. aestivum</i>	54/4			
273	6.41E−05	(+) 2	Gamma-gliadin	B8XU48#	<i>T. monococcum</i>	73/12	1.18E+07a	9.70E+06a	6.06E+06b
107	0.002	(−) 2	LMW-GS	P10385*	<i>T. aestivum</i>	149/9	1.98E+06	4.01E+06	3.68E+06
108	0.002	(−) 1.9	LMW-GS B3-2	D3UAL7#	<i>T. aestivum</i>	50/11	3.66E+06	6.92E+06	4.97E+06
123	0.007	(+) 1.9	Gamma-gliadin	Q94G97#	<i>T. aestivum</i>	54/5	5.73E+06	3.73E+06	3.08E+06

The criteria used was Mascot score >20 and Expect <0.05; the columns correspond to: Spot, number assigned to each identified protein; ANOVA, the ANOVA value for each spot; Fold Change of the normalized volumes among the spots in the control and in both transgenic lines; Protein, protein names corresponding to each identification; Accession, protein identify as referred to in UniProt database (asterisks indicate that sequences have been reviewed with Swiss Prot; pads indicate unreviewed sequences TrEMBL); Species, species to which the identified protein belongs; Score, the Mascot total ion score; %Cov, the per cent sequence coverage. (−) and (+) symbols in the fold change indicate whether the protein expression is up (+) or down (−) regulated in the control with respect to the transgenic(s). The letters indicate the significant differences between the samples for each spot according to the data obtaining with the pairwise comparisons. The spots without letters mean that no significant differences were found in the comparison by pairs.

Table 4

List of differentially expressed glutenin spots comparing the three lines used in this study.

Spot	ANOVA	Fold change	Protein	Accession	Species	Score/%Cov	Average normalized volumes		
							BW208	D783	D793
359	1.11E−08	(−) 4.9	HMW-GS DY10	P10387*	<i>T. aestivum</i>	455/15	1.03E+06a	4.64E+06b	5.08E+06b
4613	5.82E−08	(−) 4.4	HMW-GS DY10	P10387*	<i>T. aestivum</i>	515/16	2.25E+06a	9.97E+06b	9.68E+06b
397	1.52E−06	(−) 3.8	HMW-GS DY10	P10387*	<i>T. aestivum</i>	969/26	3.92E+06a	1.44E+07b	1.49E+07b
1925	2.75E−08	(+) 3.7	Gamma-gliadin	P08453*	<i>T. aestivum</i>	83/4	1.09E+06a	3.28E+05b	2.92E+05b
4602	8.59E−04	(+) 3.2	Gamma-gliadin	Q94C97C	<i>T. aestivum</i>	83/5	1.48E+06a	7.56E+05b	4.60E+05b
4608	1.05E−05	(+) 3	Gamma-gliadin	P08453*	<i>T. aestivum</i>	233/4	5.21E+05a	2.14E+05b	1.76E+05b
2716	0.009	(+) 2.3	Uncharacterized protein	W5E3J3#	<i>T. aestivum</i>	836/60	2.71E+05a	1.47E+05b	1.20E+05b
4248	0.009	(+) 2.1	16.9 kDa class I Heat shock protein	P12810*	<i>T. aestivum</i>	153/36	5.54E+05a	3.08E+05b	2.67E+05b
2690	0.008	(+) 2	Gamma-gliadin (fragment)	B6DQD9V	<i>Triticum aestivum</i> × <i>Lophopyrum elongatum</i>	86/5	3.05E+06a	2.48E+06ab	1.53E+06b

The criteria used was Mascot score >20 and Expect <0.05; the columns correspond to: Spot, number assigned to each identified protein; ANOVA, the ANOVA value for each spot; Fold Change of the normalized volumes among the spots in the control and in both transgenic lines; Protein, protein names corresponding to each identification; Accession, protein identify as referred to in UniProt database (asterisks indicate that sequences have been reviewed with Swiss Prot; pads indicate unreviewed sequences TrEMBL); Species, species to which the identified protein belongs; Score, the Mascot total ion score; %Cov, the per cent sequence coverage. (−) and (+) symbols in the fold change indicate whether the protein expression is up (+) or down (−) regulated in the control with respect to the transgenic(s). The letters indicate the significant differences between the samples for each spot according to the data obtaining with the pairwise comparisons.

Enolase; spot 4305 as α -amylase inhibitor 0.53 and Serpin-Z1B; spot 6219 presented three types of serpins (Z1A, Z2A); α -amylase inhibitors 0.19 and CM3 were found in the spot 5506 and Serpins Z1A and Z2B were obtained in the spot 6218. The remaining spots were referred to two wheat protein types correlated with human allergies; β -amylase (2096) and Serpin-Z1A (2879, 2931, 6228 and 2936), Serpin 1 (2172, 2882 and 2565) and Serpin-Z2B (2754). The different abundance of spots for comparisons between pairs is shown in the Supplementary Fig. S5.

3.3. CM-like proteins

3.3.1. Comparison among the three genotypes BW208 vs D783 vs D793

The results from 2-DE and mass spectrometry showed 19 differentially expressed spots (Table 6 and Fig. 4). Details of these identifications

are reported in Supplementary data Table S7. Among these 19 spots, 8 were up-accumulated in both transgenic lines and 9 were differentially accumulated in the line D793 comparing with the control. Only two spots were down-regulated in transgenic lines (824 and 813), which were identified as γ -gliadin and Granule-bound starch synthase 1, respectively. The spot 813 was not differentially accumulated in the pairwise comparisons (Supplementary Table S8 and Supplementary Fig. S6). The only spot with differentially significant abundance between the two transgenic lines was the spot 824. Twelve of the spots up-regulated in transgenic lines corresponded to α -amylase/trypsin inhibitor (2226, 2325, 2257, 1722, 2191, 2212, 2328, 2348, 2482, 2255, 2237 and 2232). Spots 2323, 1812, 1707 and 1703 were identified as Avenins-3 and the spot 2331 corresponded to Heat shock protein in 81-3. In some spots, unique peptides allowed the identification of more than one protein. PCA was represented in Supplementary Fig.

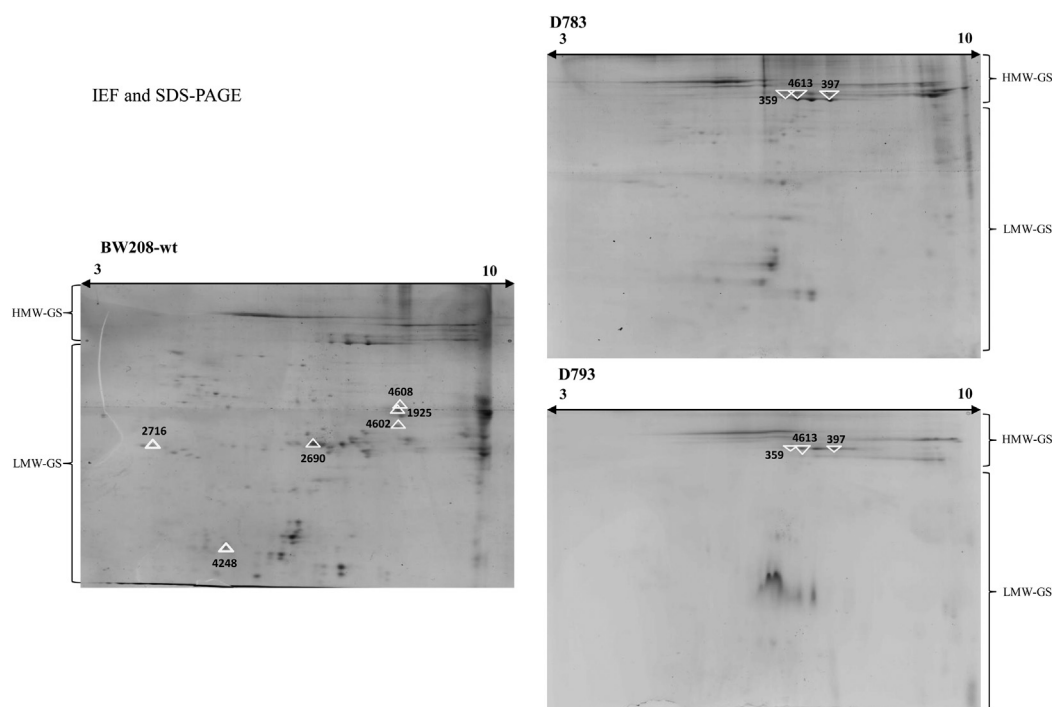


Fig. 2. 2-DE map of the comparative glutenins subunits analysis from wild-type BW208 and transgenic lines D783 and D793 with a range of pH 3–10. Differentially accumulated spots are marked with up (for over-accumulated) and down (for down-accumulated) triangles using the control line as reference.

Table 5

List of differentially expressed metabolic protein spots comparing the three lines used in this study.

Spot	ANOVA	Fold change	Protein	Accession	Species	Score/%Cov	Average normalized volumes		
							BW208	D783	D793
2646	4.37E−06	(+) 7.5	Serpin-Z2A	Q9ST57*	<i>T. aestivum</i>	530/23	2.64E+07a	9.17E+06b	3.51E+06b
			Serpin-Z1B	P93693*	<i>T. aestivum</i>	80/9			
6223	4.74E−07	(+) 4.6	Beta-amylase	P16098*	<i>Hordeum vulgare</i>	135/9	1.46E+07a	3.86E+06b	3.16E+06b
			Enolase	Q42971*	<i>Oryza sativa</i>	212/15			
4305	2.91E−06	(−) 3.6	Alpha-amylase inhibitor 0.53	P01084*	<i>T. aestivum</i>	125/20	3.88E+06	1.41E+07	1.08E+07
			Serpin-Z1B	P93693*	<i>T. aestivum</i>	81/7			
2172	0.002	(+) 3.3	Serpin 1	COLF30#	<i>T. aestivum</i>	88/10	4.46E+06a	1.59E+06b	1.35E+06b
2096	7.57E−07	(+) 3.1	Beta-amylase	P93594*	<i>T. aestivum</i>	347/28	1.04E+07a	3.87E+06b	3.40E+06b
6219	8.20E−08	(−) 2.6	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	503/19	5.36E+06a	1.18E+07b	1.40E+07b
			Serpin-Z2A	Q9ST57*	<i>T. aestivum</i>	82/9			
5506	0.033	(−) 2.5	Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	674/68	2.26E+07	3.43E+07	5.77E+07
			Alpha-amylase inhibitor CM3	P17314*	<i>T. aestivum</i>	255/53			
2879	2.58E−05	(−) 2.4	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	2385/59	5.05E+06a	1.06E+07b	1.21E+07b
2931	4.58E−07	(−) 2	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	137/9	4.91E+06a	6.11E+06ab	9.84E+06b
2882	9.16E−06	(−) 1.9	Serpin 1	COLF30#	<i>T. aestivum</i>	273/28	5.17E+06a	8.72E+06b	9.64E+06b
6228	3.67E−05	(−) 1.8	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	1263/59	9.41E+06a	1.36E+07ab	1.69E+07b
2936	1.31E−04	(−) 1.8	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	142/15	2.98E+06a	3.28E+06a	5.86E+06b
6218	1.68E−04	(−) 1.8	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	301/12	1.28E+07	2.05E+07	2.30E+07
			Serpin-Z2B	P93692*	<i>T. aestivum</i>	64/8			
2754	3.61E−04	(−) 1.7	Serpin-Z2B	P93692*	<i>T. aestivum</i>	169/20	8.21E+06	1.04E+07	1.36E+07
2565	0.016	(+) 1.7	Serpin 1	COLF30#	<i>T. aestivum</i>	132/9	6.13E+07a	3.63E+07b	3.81E+07b

The criteria used was Mascot score >20 and Expect <0.05; the columns correspond to: Spot, number assigned to each identified protein; ANOVA, the ANOVA value for each spot; Fold Change of the normalized volumes among the spots in the control and in both transgenic lines; Protein, protein names corresponding to each identification; Accession, protein identify as referred to in UniProt database (asterisks indicate that sequences have been reviewed with Swiss Prot; pads indicate unreviewed sequences TrEMBL); Species, species to which the identified protein belongs; Score, the Mascot total ion score; %Cov, the per cent sequence coverage. (−) and (+) symbols in the fold change indicate whether the protein expression is up (+) or down (−) regulated in the control with respect to the transgenic(s). The letters indicate the significant differences between the samples for each spot according to the data obtaining with the pairwise comparisons. The spots without letters mean that no significant differences were found in the comparison by pairs.

S4E where we can observe separated cluster on the basis of line. The spots resulting from the comparisons between pairs are represented in the Supplementary Fig. S6 and the identification of these spots are exhibited in the Supplementary Table S8.

4. Discussion

The proteomic results reported in this work, are complementary to previous characterizations [24–26,39] carried out on these transgenic lines.

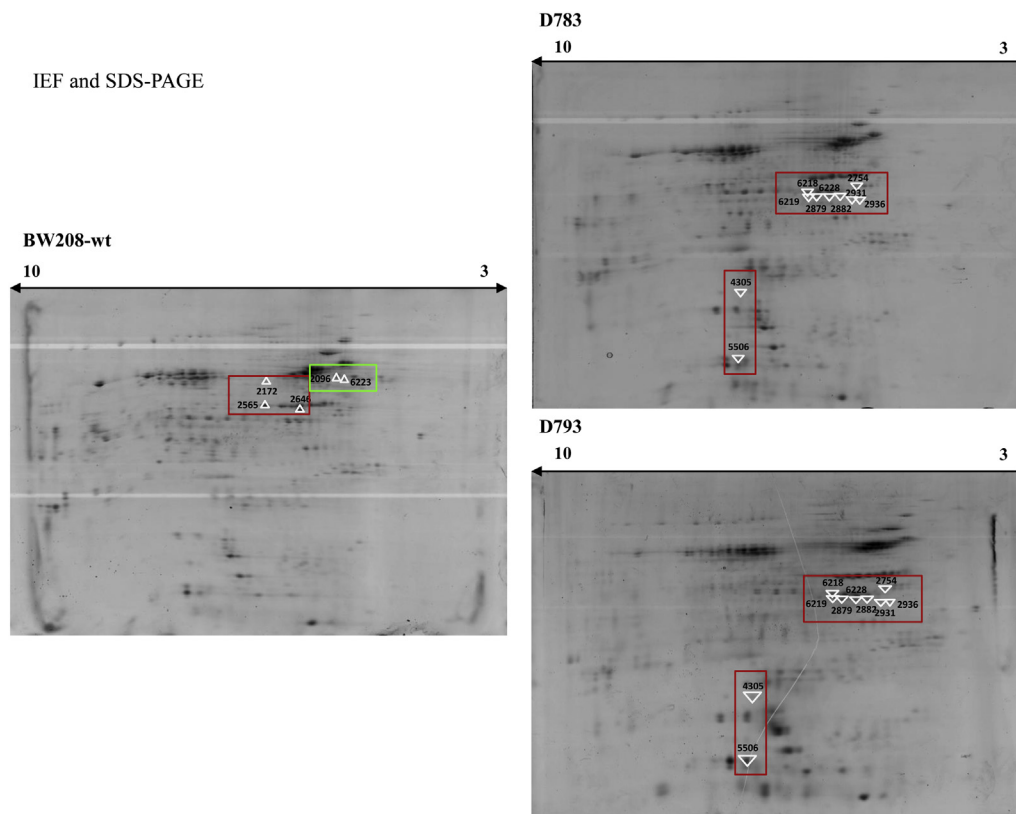


Fig. 3. 2-DE map of the comparative metabolic proteins analysis from wild-type BW208 and transgenic line D793 with a range of pH 3–10. Differentially accumulated spots were marked with up (for over-accumulated) and down (for down-accumulated) triangles using the control line as reference. The spots within the red rectangles belong to serpins, while the green rectangle encompasses the β-amylases.

Table 6
List of differentially expressed CM-like spots comparing the three lines used in this study.

Spot	ANOVA	Fold change	Protein	Accession	Species	Score/%Cov	Average normalized volumes		
							BW208	D783	D793
2323	2.24E–13	(–) 4.5	Avenin-3	M8ANS4#	<i>T. urartu</i>	756/19	1.81E+06a	4.47E+06b	8.12E+06b
1812	3.33E–16	(–) 4.1	Avenin-3	M8ANS4#	<i>T. urartu</i>	786/19	1.30E+07a	2.91E+07b	5.33E+07b
824	4.24E–12	(+) 4	Gamma-gliadin	Q94G97#	<i>T. aestivum</i>	54/5	3.98E+06a	1.86E+06b	1.00E+06c
1707	6.34E–10	(–) 3.3	Avenin-3	M8ANS4#	<i>T. urartu</i>	756/19	3.13E+06a	7.35E+06b	1.05E+07b
1722	1.38E–11	(–) 3.2	Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	108/20	9.95E+06a	2.21E+07b	3.18E+07b
			Alpha-amylase inhibitor 0.53	P01084*	<i>T. aestivum</i>	106/33			
2325	1.05E–13	(–) 3	Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	171/43	1.21E+07a	2.39E+07b	3.34E+07b
1703	5.13E–07	(–) 2.9	Avenin-3	M8ANS4#	<i>T. urartu</i>	756/19	2.80E+06a	5.32E+06b	8.15E+06b
2331	2.34E–08	(–) 2.8	Heat shock protein 81-3	Q07078*	<i>Oryza sativa</i>	264/7	8.66E+06a	2.02E+07b	2.39E+07b
2255	1.21E–09	(–) 2.5	Alpha-amylase/trypsin inhibitor CM1	P16850*	<i>T. aestivum</i>	558/36	7.37E+07a	1.22E+08ab	1.87E+08b
			Alpha-amylase inhibitor 0.53	P01084*	<i>T. aestivum</i>	210/31			
			Alpha-amylase/trypsin inhibitor CM2	P16851*	<i>T. aestivum</i>	172/25			
2328	4.48E–11	(–) 2.5	Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	1117/67	3.25E+07a	5.24E+07ab	8.27E+07b
			Alpha-amylase/trypsin inhibitor CM2	P16851*	<i>T. aestivum</i>	524/30			
			Alpha-amylase/trypsin inhibitor CM1	P16850*	<i>T. aestivum</i>	72/18			
2482	1.08E–10	(–) 2.4	Alpha-amylase inhibitor 0.53	P01084*	<i>T. aestivum</i>	2483/89	3.43E+07a	5.75E+07ab	8.34E+07b
			Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	236/19			
2191	3.61E–11	(–) 2.3	Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	16,724	8.58E+06	1.50E+07	1.99E+07
2212	4.05E–11	(–) 2.3	Alpha-amylase/trypsin inhibitor CM4	P17314*	<i>T. aestivum</i>	9483/69	4.93E+07a	7.67E+07ab	1.11E+08b
			Alpha-amylase/trypsin inhibitor CMd	P11643*	<i>Hordeum vulgare</i>	2248/28			
			Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	173/46			
2348	9.63E–11	(–) 2.3	Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	19,046/94	7.86E+07a	1.28E+08ab	1.81E+08b
			Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	832/51			
2257	7.41E–12	(–) 2.2	Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	7663/74	5.75E+07a	8.01E+07ab	1.27E+08b
			Alpha-amylase/trypsin inhibitor CM1	P16850*	<i>T. aestivum</i>	908/25			
2226	2.59E–14	(–) 2.1	Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	16,499/69	5.48E+07a	8.60E+07ab	1.17E+08b
			Alpha-amylase/trypsin inhibitor CMd	P11643*	<i>Hordeum vulgare</i>	3813/28			
			Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	1483/88			
2237	2.62E–09	(–) 2	Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	1706/29	3.95E+07a	5.36E+07ab	7.84E+07b
			Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	696/52			
2232	7.54E–08	(–) 2	Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	12,746/94	7.30E+07a	1.03E+08ab	1.45E+08b
			Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	1538/51			
			Alpha-amylase/trypsin inhibitor CMd	P11643*	<i>Hordeum vulgare</i>	160/28			
813	4.26E–09	(+) 1.9	Granule-bound starch synthase 1, chloroplastic/amyloplastic	P04713*	<i>Zea mays</i>	363/11	2.21E+06	1.64E+06	1.14E+06

The criteria used was Mascot score >20 and Expect <0.05; the columns correspond to: Spot, number assigned to each identified protein; ANOVA, the ANOVA value for each spot; Fold Change of the normalized volumes among the spots in the control and in both transgenic lines; Protein, protein names corresponding to each identification; Accession, protein identify as referred to in UniProt database (asterisks indicate that sequences have been reviewed with Swiss Prot; pads indicate unreviewed sequences TrEMBL); Species, species to which the identified protein belongs; Score, the Mascot total ion score; %Cov, the per cent sequence coverage. (–) and (+) symbols in the fold change indicate whether the protein expression is up (+) or down (–) regulated in the control with respect to the transgenic(s). The letters indicate the significant differences between the samples for each spot according to the data obtaining with the pairwise comparisons. The spots without letters mean that no significant differences were found in the comparison by pairs.

4.1. Prolamins

The enzyme used for peptide digestion was trypsin. This enzyme cleaves the chains of peptides in the amino acids lysine or arginine, except when they are followed by proline. Therefore, the proteomic analysis of gliadins and glutenins is noteworthy difficult due to these prolamins contain a low content of arginine and lysine which gives rise to tryptic peptides of high molecular mass which are not suitable for MS/MS fragmentation; moreover, gliadins show a high content of proline leading to very low number of the resulting tryptic peptides suitable for MS analysis [33]. However, trypsin has still to be considered the method of choice in proteomic analysis, wherever possible, due to its high specificity. In this specific case, in spite of this complexity of protein mixture, the peptides obtained allowed to identify the different protein spots (Tables 3 and 4). Finally, less than expected α -gliadins were detected in the control, and we attribute this lack to the typical poor 2D resolution of this area (Fig. 1) (for comparison, match with Fig. 6 present in Scossa et al., [31]).

The two transgenic lines here used were transformed for silencing the expression of most of the gliadin genes. As a confirmation of what reported in Gil-Humanes et al. [24,25,27], the proteomic analysis here performed shows that both transgenic lines present a lower expression of gliadin genes (Table 3 and Fig. 1) and a higher abundance of HMW-GS (Table 4 and Fig. 2) with respect to the reference genotype [33]. Most of

the proteins that are differentially accumulated belong to γ -gliadins, HMW-GS and LMW-GS (Tables 3 and 4). A down-accumulation of gliadins (in this case, most γ -gliadins and some α/β -gliadins) in the transgenic genotypes with respect to the control is expected, because the corresponding genes were the targets for silencing. In Table 3, most of the proteins resulting up-accumulated in the transgenic lines belong to LMW-GS, and the explanation might reside in the fact that, because the transgenic genotypes do not have most of the gliadins normally present, the detection of minor proteins, hidden by the most abundant gliadins, would be facilitated. LMW-GS actually should not be present because unreduced extracts were loaded. However, it is known that glutenin subunits can be co-extracted with gliadins because they can form oligomers that are soluble without reducing agents [49], but still they should be present as monomers only after reduction, that has not been used in this procedure. Thus, it is reliable that such proteins identified as LMW-GS could correspond to minor γ -gliadins, since both present a high degree of homology [44]. For the same reason, the γ -gliadins recognized by proteomic analysis as up-accumulated in the control genotype in the glutenins fractions (Table 4), are realistically gliadin-like LMW-GS [44], because the monomeric gliadin fraction was removed prior to glutenin extraction. Moreover, these γ -gliadins identified are located in the LMW region in the 2D gel (Fig. 2). Thus, as expected, they are silenced in transgenic lines, similarly to typical γ -gliadin genes, because of the high sequence similarity.

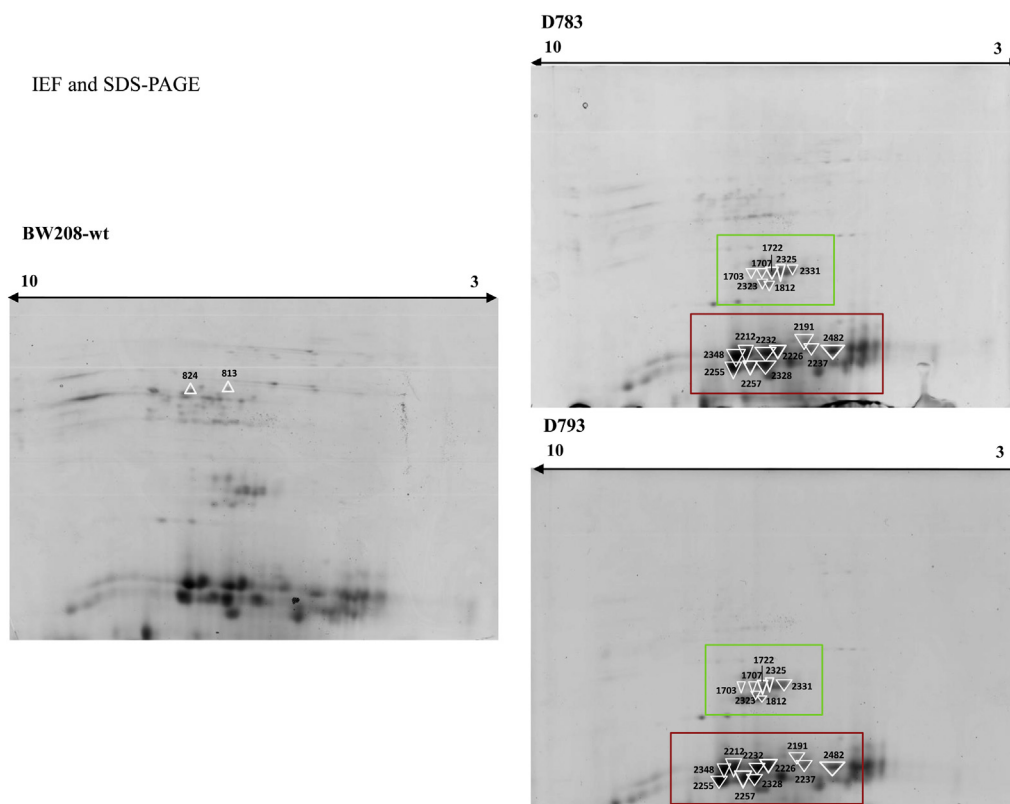


Fig. 4. 2-DE map of the comparative CM-like proteins analysis from wild-type BW208 and transgenic line D793 with a range of pH 3–10. Differentially accumulated spots are marked with up (for over-accumulated) and down (for down-accumulated) triangles using the control line as reference. The spots within the red rectangles belong mainly to avenins, while the green rectangle encompasses the α -amylase/trypsin inhibitors.

The increment in the glutenin fractions in the transgenic lines, is in agreement with other authors [24,25,27] who used both SDS-PAGE and HPLC analyses, and this increment might be due to a compensation effect due to the absence of most gliadin proteins. Such compensation effects have been previously observed in a transgenic wheat genotype (derived from the control genotype Bobwhite, the same used in this paper) over-accumulating a specific LMW-GS [31], and was explained by the recruitment of transcription factors by the multiple transgenes there present. Similar compensation effects were also reported in maize and rice, where the silencing of a group of storage proteins leads to a rearrangement of other storage proteins [50,51].

The presence of gliadin-like proteins in the glutenin fraction accounts for the scarcity of spots corresponding to LMW-GS in 2D gel obtained from the line D793, which showed higher silencing of gliadins than D783 (Fig. 2). Thus, we can observe a slight reduction of these LMW-GS spots in the line D783 respect to the control and a lack of LMW spots in the line D793 (Fig. 2). This lack of LMW-GS in the line D793 is in agreement to Gil-Humanes et al. [27], who reported that the silencing of gliadins in the line D793 was associated with a significant decrease in the LMW-GS and a significant increase in the HMW-GS, compared to the control. Moreover, the results obtained in our study are in agreement with Gil-Humanes et al. [25] that classified line D783 as “high-LMW”, with a similar LMW-GS content in comparison to the wild-type, and line D793 as “low-LMW”, with a reduced LMW-GS content relative to the control.

Furthermore, the HMW-GS Dy10 resulted up-accumulated in the transgenic genotypes (Table 4 and Fig. 2). The HMW-GS Dy10 is actually present in a single copy, since it is coded by a single gene, thus the presence of three different polypeptides might be likely attributed to the presence of procedure artifacts, due to glutamine deamidation, highly abundant in HMW-GS [52] and LMW-GS [46]. This increment in the HMW-GS could improve the bread-making quality since they promote

the formation of larger glutenin polymers contributing to the elasticity of the dough [25].

Regarding the comparison between the two transgenic lines, D783 showed a higher expression of gliadins than line D793 (Supplementary Table S1C and Supplementary Fig. S1C), in agreement to Gil-Humanes et al. [24], who reported that the reduction of gliadins with the ω/α hpRNA constructs was 69.8% and 87.9% in lines D783 and D793, respectively, in comparison to that of the wild type.

4.2. Metabolic proteins

Structural and metabolic proteins, present in wheat grain composition, comprise a wide range of proteins with several functions such as components of cell walls, proteins involved in defense, transport, growth, gene transcription, protein synthesis and enzymes.

Regarding the transgenic lines, we obtained mainly spots corresponding to serpins (serine protease inhibitors), identified as a putative allergen for Bakers' asthma in the albumin/globulin fraction [53,54]. These peptidase inhibitors are involved in defense activities related to biotic and abiotic stresses, regulation and/or programming cell death [55]. Besides, the main differences with regard to serpins accumulation were found between the wild- type and line D793 (Fig. 3 and Table 5), which has a greater silencing level respect to D783 [24]. Thus, given that lines with reduced levels of all gliadins groups (ω/α -hpRNA construct) showed an increase in serpins, it could indicate that such modifications may activate the process of response to stresses.

4.3. CM-like proteins

This protein fraction includes mostly α -amylase or α -amylase/trypsin inhibitors, along with some α - and γ -gliadin type proteins [3]. Both α -amylase or α -amylase/trypsin inhibitors and gluten proteins belong to

the prolamin superfamily. Most of the differentially accumulated polypeptides resulted more abundant in the transgenic genotypes, especially in the line D793, with respect to the control (Fig. 4). This might be the consequence of a compensation effect due to the silencing of gliadins, thus it is possible to speculate that amino acid reserve is mostly used for the synthesis of protease inhibitors (and glutenins), instead of gliadins [31]. These changes are greater in line D793, probably as a consequence of the greater silencing of gliadins with respect to the line D783 [24,25, 27]. These proteins of the α -amylase/trypsin inhibitor family were described as allergens, especially for the Baker's asthma [56,57].

In this analysis, Avenin-like proteins were identified in four spots as over-accumulated in both lines D793 and D783 (Table 6). These proteins are considered as storage proteins and they are also prolamins, thus its increased expression level might be related to a compensation effect as for the other prolamins. The two spots up-accumulated in BW208 corresponded to a γ -gliadin and a granule-bound starch synthase (Table 6), that was previously identified as a new putative allergen [15].

In summary, in this work two transgenic lines with strong down-regulation of gliadins by RNAi were studied. As a result of the strong gliadins down-regulation, the HMW-GS, metabolic and chloroform/methanol soluble proteins were increased in the transgenic lines. The line more affected respect to this proteomic variation was D793 which presents the γ -gliadin promoter, with respect to the line D783 with the D-hordein promoter. We have confirmed that the γ -gliadin promoter has a greater effect on the silencing of gliadins and consequently, the accumulation of the remaining proteins is increased.

5. Conclusion

Basing on these results, further *in vivo* analysis will be needed to determine if these transgenic lines could be used for food preparation for celiac or other gluten intolerant groups. Regarding celiac patients, the amount of food products made using the low-gliadin flour should be quantified, since such lines still present other gluten proteins in their composition which trigger celiac disease. Gil-Humanes et al. [39], in fact, determined that a safe daily intake for celiac patients, with products made with the transgenic line D793, would be about 67 g of flour.

Finally, given to the wheat respiratory allergy or Bakers' asthma are associated to the albumin and globulin proteins and these transgenic lines contain greater amount of these protein than the wild-type, the use of this low-gliadin wheat by allergic people would not be advised. For this collective, assessments such as immunoblotting using sera from patients with wheat allergy must be performed in relation to the different wheat related pathologies.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2017.06.010>.

Conflict of interest

The authors declare no conflict of interest.

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Table S1. List of differentially expressed gliadins spots resulting from the three pairwise comparisons.

Spot	ANOVA	Fold change	Protein	Accession	Species	Score/%Cov	Average Normalised Volumes	
<i>A. Data corresponding to comparison between BW208 and D783</i>							BW208	D783
217	1.99E-07	(-) 2.4	Gamma-gliadin	R9XV62#	<i>T. aestivum</i>	96/12	3.00E+07	1.26E+07
			LMW-GS	P10385;D2DII3#	<i>T. aestivum</i>	192/9		
			LMW-GS	R4JAQ1#	<i>T. aestivum</i>	72/10		
226	5.11E-08	(+) 3.3	LMW-GS B3-2	D3UAL7#	<i>T. aestivum</i>	155/28	1.03E+06	3.40E+06
245	7.77E-08	(+) 3	LMW-GS	B8XU58#	<i>T. monococcum</i>	123/12	1.01E+06	3.02E+06
279	2.10E-02	(-) 1.7	Gamma-gliadin	P08453*	<i>T. aestivum</i>	164/4	3.84E+06	2.24E+06
			LMW-GS	P10385*	<i>T. aestivum</i>	54/4		
<i>B. Data corresponding to comparison between BW208 and D793</i>							BW208	D793
217	9.8E-12	(-) 4.6	Gamma-gliadin	R9XV62#	<i>T. aestivum</i>	96/12	3.00E+07	6.5E+06
			LMW-GS	P10385;D2DII3#	<i>T. aestivum</i>	192/9		
			LMW-GS	R4JAQ1#	<i>T. aestivum</i>	72/10		
243	6.64E-06	(-) 3.1	Alpha/beta-gliadin A-I	P04721*	<i>T. aestivum</i>	93/10	3.43E+07	1.10E+07
273	3.23E-04	(-) 2	Gamma-gliadin	B8XU48#	<i>T. monococcum</i>	73/12	1.18E+07	6.06E+06
279	7.07E-04	(-)2.3	Gamma-gliadin	P08453*	<i>T. aestivum</i>	164/4	3.84E+06	1.67E+06
			LMW-GS	P10385*	<i>T. aestivum</i>	54/4		
<i>C. Data corresponding to comparison between D783 and D793</i>							D783	D793
217	6.03E-06	(+) 1.9	Gamma-gliadin	R9XV62#	<i>T. aestivum</i>	96/12	1.26E+07	6.51E+06
			LMW-GS	P10385;D2DII3#	<i>T. aestivum</i>	192/9		
			LMW-GS	R4JAQ1#	<i>T. aestivum</i>	72/10		
243	1.46E-04	(+) 2.4	Alpha/beta-gliadin A-I	P04721*	<i>T. aestivum</i>	93/10	2.62E+07	1.10E+07
273	2.00E-03	(+) 1.6	Gamma-gliadin	B8XU48#	<i>T. monococcum</i>	73/12	9.70E+06	6.06E+06

The criteria used was Mascot score > 20 and Expect < 0.05; the columns correspond to: Spot, number assigned to each identified protein; ANOVA, the ANOVA value for each spot; Fold Change of the normalized volumes among the spots in the control and in both transgenic lines; Protein, protein names corresponding to each identification; Accession, protein identify as referred to in UniProt database (asterisks indicate that sequences have been reviewed with Swiss Prot; pads indicate unreviewed sequences TrEMBL); Species, species to which the identified protein belongs; Score, the Mascot total ion score; %Cov, the per cent sequence coverage. (-) and (+) symbols in the fold change indicate whether the protein expression is up (+) or down (-) regulated in the transgenic line (A, B) and D783 (C) respect to the control.

Table S3. List of differentially expressed glutenins spots resulting from the comparison between BW208 and the two transgenic lines.

Spot	ANOVA	Fold change	Protein	Accession	Species	Score/%Cov	Average Normalised Volumes	
A. Data corresponding to comparison between BW208 and D783							BW208	D783
359	4.55E-06	(+) 4.5	HMW-GS DY10	P10387*	<i>T. aestivum</i>	455/15	1.03E+06	4.64E+06
1925	6.12E-06	(-) 3.3	Gamma-gliadin	P08453*	<i>T. aestivum</i>	83/4	1.09E+06	3.28E+05
4613	1.53E-05	(+) 4.4	HMW-GS DY10	P10387*	<i>T. aestivum</i>	515/16	2.25E+06	9.97E+06
397	1.27E-04	(+) 3.7	HMW-GS DY10	P10387*	<i>T. aestivum</i>	969/26	3.92E+06	1.44E+07
4608	6.42E-04	(-) 2.4	Gamma-gliadin	P08453*	<i>T. aestivum</i>	233/4	5.21E+05	2.14E+05
4602	0.025	(-) 2	Gamma-gliadin	Q94G97C	<i>T. aestivum</i>	83/5	1.48E+06	7.56E+05
2716	3.30E-02	(-) 1.8	Uncharacterized protein	W5E3J3#	<i>T. aestivum</i>	836/60	2.71E+05	1.47E+05
4248	1.90E-02	(-) 1.8	16.9kDa class I heat shock protein	P12810*	<i>T.aestivum</i>	153/36	5.54E+05	3.08E+05
B. Data corresponding to comparison between BW208 and D793							BW208	D793
359	3.62E-08	(+) 4.9	HMW-GS DY10	P10387*	<i>T. aestivum</i>	455/15	1.03E+06	5.08E+06
1925	3.52E-06	(-) 3.7	Gamma-gliadin	P08453*	<i>T. aestivum</i>	83/4	1.09E+06	2.92E+05
4613	1.76E-06	(+) 4.3	HMW-GS DY10	P10387*	<i>T. aestivum</i>	515/16	2.25E+06	9.68E+06
397	1.26E-05	(+) 3.8	HMW-GS DY10	P10387*	<i>T. aestivum</i>	969/26	3.92E+06	1.49E+07
4608	3.41E-05	(-) 3	Gamma-gliadin	P08453*	<i>T. aestivum</i>	233/4	5.21E+05	1.76E+05
4602	1.36E-04	(-) 3.2	Gamma-gliadin	Q94G97C	<i>T. aestivum</i>	83/5	1.48E+06	4.60E+05
					<i>T. aestivum</i> x			
2690	7.00E-03	(-) 2	Gamma-gliadin	B6DQD9V	<i>Lophopyrum elongatum</i>	86/5	3.05E+06	1.53E+06
2716	7.00E-03	(-) 2.3	Uncharacterized protein	W5E3J3#	<i>T. aestivum</i>	836/60	2.71E+05	1.20E+05
4248	2.00E-03	(-) 2.1	16.9kDa class I heat shock protein	P12810*	<i>T.aestivum</i>	153/36	5.54E+05	2.67E+05

The criteria used was Mascot score > 20 and Expect < 0.05; the columns correspond to: Spot, number assigned to each identified protein; ANOVA, the ANOVA value for each spot; Fold Change of the normalized volumes among the spots in the control and in both transgenic lines; Protein, protein names corresponding to each identification; Accession, protein identify as referred to in UniProt database (asterisks indicate that sequences have been reviewed with Swiss Prot; pads indicate unreviewed sequences TrEMBL); Species, species to which the identified protein belongs; Score, the Mascot total ion score; %Cov, the per cent sequence coverage. (-) and (+) symbols in the fold change indicate whether the protein expression is up (+) or down (-) regulated in the transgenic(s) with respect to the control.

Table S6. List of differentially expressed metabolic protein spots resulting from the three pairwise comparisons.

Spot	ANOVA	Fold Change	Protein	Accession	Species	Score/%Cov	Average Normalised Volumes	
A. Data corresponding to comparison between BW208 and D783							BW208	D783
6219	3.66E-06	(+) 2.2	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	503/19	5.36E+06	1.18E+07
			Serpin-Z1C	Q9ST58*	<i>T. aestivum</i>	303/19		
			Serpin-Z2A	Q9ST57*	<i>T. aestivum</i>	82/9		
6223	6.60E-05	(-) 3.8	Beta-amylase	P16098*	<i>Hordeum vulgare</i>	135/9	1.46E+07	3.86E+06
			Enolase	Q42971*	<i>Oryza sativa</i>	212/15		
2096	6.12E-05	(-) 2.7	Beta-amylase	P93594*	<i>T. aestivum</i>	347/28	1.04E+07	3.87E+06
2646	2.00E-03	(-) 2.9	Serpin-Z2A	Q9ST57*	<i>T. aestivum</i>	530/23	2.64E+07	9.17E+06
			Serpin-Z1B	P93693*	<i>T. aestivum</i>	80/9		
2882	9.05E-06	(+) 1.7	Serpin 1	C0LF30#	<i>T. aestivum</i>	273/28	5.17E+06	8.72E+06
2879	1.09E-05	(+) 2.1	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	2385/59	5.05E+06	1.06E+07
2172	7.00E-03	(-) 2.8	Serpin 1	C0LF30#	<i>T. aestivum</i>	88/10	4.46E+06	1.59E+06
2565	2.00E-03	(-) 1.7	Serpin 1	C0LF30#	<i>T. aestivum</i>	132/9	6.13E+07	3.63E+07

Table 4 (continued)

<i>B. Data corresponding to comparison between BW208 and D793</i>							BW208	D793
6219	1.52E-06	(+) 2.6	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	503/19	5.36E+06	1.40E+07
			Serpin-Z1C	Q9ST58*	<i>T. aestivum</i>	303/19		
			Serpin-Z2A	Q9ST57*	<i>T. aestivum</i>	82/9		
2931	1.11E-06	(+) 2	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	137/9	4.91E+06	9.84E+06
6223	1.31E-07	(-) 4.6	Beta-amylase	P16098*	<i>Hordeum vulgare</i>	135/9	1.46E+07	3.16E+06
			Enolase	Q42971*	<i>Oryza sativa</i>	212/15		
2096	6.35E-06	(-) 3.1	Beta-amylase	P93594*	<i>T. aestivum</i>	347/28	1.04E+07	3.40E+06
2646	2.21E-07	(-) 7.5	Serpin-Z2A	Q9ST57*	<i>T. aestivum</i>	530/23	2.64E+07	3.51E+06
			Serpin-Z1B	P93693*	<i>T. aestivum</i>	80/9		
2882	6.65E-05	(+) 1.9	Serpin 1	C0LF30#	<i>T. aestivum</i>	273/28	5.17E+06	9.64E+06
2879	1.86E-04	(+) 2.4	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	2385/59	5.05E+06	1.21E+07
6228	1.82E-04	(+) 1.8	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	1263/59	9.41E+06	1.69E+07
2936	1.00E-03	(+) 2	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	142/15	2.98E+06	5.86E+06
2172	3.00E-03	(-) 3.3	Serpin 1	C0LF30#	<i>T. aestivum</i>	88/10	4.46E+06	1.35E+06
2565	2.90E-02	(-) 1.6	Serpin 1	C0LF30#	<i>T. aestivum</i>	132/9	6.13E+07	3.81E+07
<i>C. Data corresponding to comparison between D783 and D793</i>							D783	D793
2936	7.63E-06	(-) 1.8	Serpin-Z1A	Q41593*	<i>T. aestivum</i>	142/15	3.28E+06	5.86E+06

The criteria used was Mascot score > 20 and Expect < 0.05; the columns correspond to: Spot, number assigned to each identified protein; ANOVA, the ANOVA value for each spot; Fold Change of the normalized volumes among the spots in the control and in both transgenic lines; Protein, protein names corresponding to each identification; Accession, protein identify as referred to in UniProt database (asterisks indicate that sequences have been reviewed with Swiss Prot; pads indicate unreviewed sequences TrEMBL); Species, species to which the identified protein belongs; Score, the Mascot total ion score; %Cov, the per cent sequence coverage. (-) and (+) symbols in the fold change indicate whether the protein expression is up (+) or down (-) regulated in the transgenic(s) (A, B) and D783 (C) with respect to the control.

Table S8. List of differentially expressed cm-like spots resulting from the three pairwise comparisons.

Spot	ANOVA	Fold Change	Protein	Accession	Species	Score/%Cov	Average Normalised Volumes	
A. Data corresponding to comparison between BW208 and D783							BW208	D783
1812	2.93E-10	(+) 2.2	Avenin-3	M8ANS4#	<i>T. urartu</i>	786/19	1.30E+07	2.91E+07
2325	1.09E-09	(+) 2.1	Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	171/43	1.21E+07	2.39E+07
2323	2.38E-07	(+) 2.5	Avenin-3	M8ANS4#	<i>T. urartu</i>	756/19	1.81E+06	4.47E+06
824	5.22E-09	(-) 2.1	Gamma gliadin	Q94G97#	<i>T. aestivum</i>	54/5	3.98E+06	1.86E+06
1722	2.46E-07	(+) 2.2	Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	108/20	9.95E+06	2.21E+07
			Alpha-amylase inhibitor 0.53	P01084*	<i>T. aestivum</i>	106/33		
1707	9.59E-07	(+) 2.3	Avenin-3	M8ANS4#	<i>T. urartu</i>	756/19	3.13E+06	7.35E+06
2331	5.47E-07	(+) 2.3	Heat shock protein 81-3	Q07078*	<i>Oryza sativa</i>	264/7	8.66E+06	2.02E+07
1703	3.95E-05	(+) 1.9	Avenin-3	M8ANS4#	<i>T. urartu</i>	756/19	2.80E+06	5.32E+06
B. Data corresponding to comparison between BW208 and D793							BW208	D793
1812	7.37E-12	(+) 4.1	Avenin-3	M8ANS4#	<i>T. urartu</i>	786/19	1.30E+07	5.33E+07
2226	9.35E-11	(+) 2.1	Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	16499/69	5.48E+07	1.17E+08
			Alpha-amylase/trypsin inhibitor CMd	P11643*	<i>Hordeum vulgare</i>	3813/28		
			Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	1483/88		
2325	1.53E-10	(+) 3	Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	171/43	1.21E+07	3.34E+07
2323	3.56E-10	(+) 4.5	Avenin-3	M8ANS4#	<i>T. urartu</i>	756/19	1.81E+06	8.12E+06
824	4.84E-10	(-) 4	Gamma gliadin	Q94G97#	<i>T. aestivum</i>	54/5	3.98E+06	1.00E+06
2257	4.03E-09	(+) 2.2	Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	7663/74	5.75E+07	1.27E+08
			Alpha-amylase/trypsin inhibitor CM1	P16850*	<i>T. aestivum</i>	908/25		
1722	1.42E-08	(+) 3.2	Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	108/20	9.95E+06	3.18E+07
			Alpha-amylase inhibitor 0.53	P01084*	<i>T. aestivum</i>	106/33		

Table 5 (continued)

2212	6.83E-09	(+) 2.3	Alpha-amylase/trypsin inhibitor CM4	P17314*	<i>T. aestivum</i>	9483/69	4.93E+07	1.11E+08
			Alpha-amylase/trypsin inhibitor CMd	P11643*	<i>Hordeum vulgare</i>	2248/28		
			Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	173/46		
2328	2.75E-09	(+) 2.5	Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	113/67	3.25E+07	8.27E+07
			Alpha-amylase/trypsin inhibitor CM2	P16851*	<i>T. aestivum</i>	92/30		
			Alpha-amylase/trypsin inhibitor CM1	P16850*	<i>T. aestivum</i>	72/18		
2348	2.59E-08	(+) 2.3	Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	19046/94	7.86E+07	1.81E+08
			Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	832/51		
2482	2.72E-11	(+) 2.4	Alpha-amylase inhibitor 0.53	P01084*	<i>T. aestivum</i>	2483/89	3.43E+07	8.34E+07
			Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	236/19		
1707	1.59E-07	(+) 3.3	Avenin-3	M8ANS4#	<i>T. urartu</i>	756/19	3.13E+06	1.05E+07
2255	7.88E-08	(+) 2.5	Alpha-amylase/trypsin inhibitor CM1	P16850*	<i>T. aestivum</i>	558/36	7.37E+07	1.87E+08
			Alpha-amylase inhibitor 0.53	P01084*	<i>T. aestivum</i>	210/31		
			Alpha-amylase/trypsin inhibitor CM2	P16851*	<i>T. aestivum</i>	172/25		
2237	1.47E-08	(+) 2	Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	1706/29	3.95E+07	7.84E+07
			Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	696/52		
			Granule-bound starch synthase 1,					
813	1.68E-08	(-) 1.9	chloroplastic/amyloplastic	P04713*	<i>Zea mays</i>	363/11	2.21E+06	1.14E+06
2331	1.25E-06	(+) 2.8	Heat shock protein 81-3	Q07078*	<i>Oryza sativa</i>	264/7	8.66E+06	2.39E+07
2232	2.98E-06	(+) 2	Alpha-amylase inhibitor 0.19	P01085*	<i>T. aestivum</i>	12746/94	7.30E+07	1.45E+08
			Alpha-amylase/trypsin inhibitor CM3	P17314*	<i>T. aestivum</i>	1538/51		
			Alpha-amylase/trypsin inhibitor CMd	P11643*	<i>Hordeum vulgare</i>	160/28		
1703	8.96E-06	(+) 2.9	Avenin-3	M8ANS4#	<i>T. urartu</i>	756/19	2.80E+06	8.15E+06

Table 5 (continued)

<i>C. Data corresponding to comparison between D783 and D793</i>							D783	D793
824	1.05E-04	(+) 1.9	Gamma gliadin	Q94G97#	<i>T. aestivum</i>	54/5	1.86E+06	1.00E+06

The criteria used was Mascot score > 20 and Expect < 0.05; the columns correspond to: Spot, number assigned to each identified protein; ANOVA, the ANOVA value for each spot; Fold Change of the normalized volumes among the spots in the control and in both transgenic lines; Protein, protein names corresponding to each identification; Accession, protein identify as referred to in UniProt database (asterisks indicate that sequences have been reviewed with Swiss Prot; pads indicate unreviewed sequences TrEMBL); Species, species to which the identified protein belongs; Score, the Mascot total ion score; %Cov, the per cent sequence coverage. (-) and (+) simbols in the fold change indicate whether the protein expression is up (+) or down (-) regulated in the transgenic line (A, B) and D783 (C) with respect to the transgenic(s).