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Comparative Analysis of Gluten Proteins in Three Durum Wheat Cultivars by a Proteomic Approach

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Supporting Information

ABSTRACT: The gluten protein composition and expression level influence dough properties and are cultivar and environment dependent. To broaden the knowledge of the durum wheat gluten proteome, three cultivars were compared in two different growing seasons by a proteomic approach. Cultivar-specific and differentially expressed spots in the two years were identified by mass spectrometry. Significant differences were observed among the cultivars: Ofanto showed the lowest protein spot volumes in the high molecular weight (HMW) and low molecular weight (LMW) <35 000 regions and the highest in the LMW 48 000−35 000 region, Latino the lowest in the LMW 48 000−35 000 region, and Simeto an intermediate expression level in both LMW regions. In the warmer year the up-regulation of HMW glutenins, α-gliadins, and a globulin 3 protein and the down-expression of LMW glutenins and γ-gliadins were observed. Among the cultivars, Simeto showed the highest stability across the environments.

KEYWORDS: *durum wheat, gluten protein composition, genotype, proteome analysis*

INTRODUCTION

Durum wheat (*Triticum turgidum* L. ssp. *durum*), one of the most widespread crops in Mediterranean areas, is used for the production of several end products, such as pasta, couscous, and various types of bread. Gluten proteins, accounting for 80% of wheat grain proteins, are recognized as the most important components affecting wheat quality. They are conventionally divided into two groups, the monomeric gliadins (α/β-, γ-, and ω-subunits) and the polymeric glutenins (high and low molecular weight subunits, HMW-GS and LMW-GS, respectively), which together determine pasta-making quality, being responsible for dough viscosity and elasticity properties, respectively.[1] ω- and γ-gliadins are encoded at the *Gli-1* locus, while α/β-gliadins are encoded at the *Gli-2* locus on the short arms of groups 1 and 6 chromosomes, respectively. HMW-GS are subdivided into x- and y-types, both encoded at the *Glu-A1* and *Glu-B1* loci, on the long arm of chromosomes 1A and 1B, respectively. Finally, LMW-GS are encoded by genes at the *Glu-A3* and *Glu-B3* loci, on the short arms of chromosomes 1 (1A and 1B), and also by loci tightly linked to the *Gli-1* and *Gli-2* loci on chromosomes 6 depending on the LMW-GS subgroup.[2] In fact, LMW-GS are classified in different groups (B-, C-, and D-subunits), according to their structural and functional properties. The B-subunits are typical LMW-GS with a peculiar structure encoded by genes on chromosomes 1; instead the C- and D- subunits are gliadin-like LMW-GS encoded by genes on chromosomes 6, structurally similar to gliadins but functionally acting as glutenins, due to their ability to form intermolecular disulfide bonds by means of unpaired cysteine residues.[2] A direct relationship between gluten protein composition and dough properties is well documented.[2,3] In several wheat cultivars the presence of some HMW-GS has been associated with favorable dough properties; in particular, the *Glu-B1 b* allele coding for protein subunits 7 and 8 seems to be responsible for high alveograph *W* values in durum wheat.[4]

On the contrary, other subunits, such as HMW-GS Bx20 also encoded at the *Glu-B1* locus, have a negative effect on dough characteristics and on pasta-making quality, determining a weak gluten.[5] Since HMW-GS alleles are differently correlated with grain technological quality, HMW-GS have become useful protein markers for wheat improvement and cultivar identification and are extensively used as markers in wheat breeding programs for selecting preferable lines.[6]

As well as the presence of some HMW-GS, the favorable dough properties of several durum wheat cultivars have also been associated with LMW-GS. Some LMW-GS encoded at the *Glu-B3* locus affect pasta-making quality; in particular, lines possessing the specific group LMW-2 have superior quality characteristics compared to lines possessing the allelic group LMW-1.[7] It has been suggested that the stronger gluten associated with the LMW-2 type is due to a greater expression of LMW-GS than for the LMW-1 type.[8] Moreover, it is well-known that two γ-gliadins, γ-42 and γ-45, are also markers for weak and strong durum wheat gluten, respectively, due to the genetic linkage with LMW-1 and LMW-2.

There is a considerable interest in determining the structure−function relationships of wheat gluten proteins to understand and predict end-use quality of different cultivars.[9] For this purpose the proteome approach using two-dimensional electrophoresis (2DE) and nano-HPLC−ESI-IT-MS/MS analysis has opened up new possibilities of protein characterization resulting in an accurate and reliable method able to supply

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much information concerning differences in protein due to environmental[10] and genotype[9,11] effects.

Environmental variables are known to influence durum wheat metabolic pathways;[12−14] as a consequence, changes in grain quality may also occur.[10] Concerning the effect of high temperatures on gluten proteins, Dupont et al.[15,16] reported increases in HMW-GS and α-gliadins and a decrease in LMW-GS in response to thermal stress. Moreover, Majoul et al.[17] identified three α-gliadins that increased in response to high temperature, and Yang et al.[18] reported changes in the relative amounts of some α-gliadins, an increase in γ-gliadins and in some LMW-GS, and a decrease in some ω-gliadins when high temperatures occurred in postanthesis. The same authors observed a decrease in α- and γ-gliadins and changes in the relative amounts of some LMW-GS as a consequence of drought. Furthermore, an increase in the HMW-GS/LMW-GS ratio was observed in durum wheat under water stress when also an increase in protein content occurred.[19]

Also the effect of genotype was widely studied in bread wheat; in particular, Skylas et al.[11] in a study conducted on some Australian wheat genotypes, observed that the gliadin protein family could be used to distinguish among wheat cultivars, and that differences in HMW-GS composition can be used to predict dough-handling properties. However, they found that gluten proteome analysis gave only a small contribution to cultivar characterization, differences in metabolic proteins, such as the heat shock proteins, being more effective in cultivar discrimination.

On the contrary, in a study on four Japanese wheat genotypes, Yahata et al.[9] found that 42% of analyzed proteins giving cultivar-dependent qualitative changes were storage proteins such as gliadins and LMW-GS. Moreover, in accordance with Skylas et al.,[11] the authors underlined the difficulty in providing cultivar discrimination for genotypes with similar pedigrees. A comparison of three bread wheat cultivars with different technological characteristics by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), RP-HPLC, and 2DE was also conducted recently by Liu et al.[20] The authors investigated glutenin accumulation patterns in developing grains and their relationships with wheat quality. They observed higher accumulation levels of HMW-GS and LMW-GS as well as 1Bx13 + 1By16 and 1Dx4 + 1Dy12 subunits in superior gluten quality cultivars than in poor ones. Kamal et al.[21] analyzed the diversity of glutenin genes from six bread wheat cultivars by 2DE and MALDI-TOF-MS analysis. The authors identified the glutenin subunits and compared the gluten isoforms among different wheat cultivars in relation to quality for bread and noodles. Despite a lot of proteomic research performed on wheat grain proteins, less information is available on storage proteins of durum wheat and in particular on Italian cultivars. De Angelis et al.[22] compared 10 Italian durum wheat cultivars in terms of protein composition, by 2D electrophoresis analysis, and starch characteristics to define their cooking quality. A gliadin/glutenin ratio of ≤1 was observed for Ciccio, Duilio, Ocotillo, Simeto, Svevo, and Wollaroi, which give a good semolina suitable for cooking. Also Muccilli et al.[4] compared different durum wheat cultivars (Simeto, Svevo, Duilio, Bronte, and Sant'Agata) against the wheat landrace Timilia (used to produce a traditional Sicilian bread) to identify some diagnostic peptides to be used as markers of wheat cultivars in blended flour.

To the best of our knowledge, studies on the comparison of gluten proteome by 2DE and nano-HPLC−IT-MS/MS among different durum wheat varieties are missing. Furthermore, the interaction cultivar × environment of gluten proteome merits investigation to detect cultivar-specific spots regardless of the cultivation environment and to evaluate the stability of protein composition and grain quality across environments. Therefore, to gain deep insight into the durum wheat gluten proteome and to broaden the knowledge about Italian genotypes, in this study three durum wheat Italian cultivars (Ofanto, Latino, and Simeto), characterized by a different quality performance, were compared by a proteomic approach in two different growing seasons. Genotype stability in the two experimental years, in relation to the gluten proteome, was also evaluated. The use of an ion trap mass spectrometer and an optimized chymotryptic protocol has allowed us not only to identify the proteins but also to obtain the amino acid sequence of the enzymatically digested peptides.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Three durum wheat cultivars, Latino, Ofanto, and Simeto, were chosen on the basis of their different technological performances: Simeto, with the highest pasta-making quality,[4,19,22−24] Ofanto, with intermediate quality properties,[24] and Latino, characterized by poor qualitative performance.[25] Moreover, on the batch used for sowing and derived from a previous field trial (2003−2004), the gluten index was evaluated, giving values of 9, 43, and 70 for Latino, Ofanto, and Simeto, respectively. The cultivars were grown in Foggia (Southern Italy; 41°46′ N, 15°30′ E) during two growing seasons (2005 and 2006) in a naturally lit polycarbonate greenhouse. Plants were grown in pots filled with sandy clay loam soil (59% sand, 14% silt, and 28% clay). Sowing was performed in 0.28 m^2 pots at a seed rate of 150 seeds m^{-2} on Jan 1 and 20 in 2005 and 2006, respectively. A randomized complete block experimental design with three replicates was adopted.

On the sowing date, 92 kg ha^{-1} of phosphorus fertilizer was applied, as superphosphate, while nitrogen fertilizer (90 kg ha^{-1}) was applied in two portions: 1/3 as urea at sowing and 2/3 as ammonium nitrate during stem elongation. In both years the irrigation treatment consisted of bringing soil moisture up to field capacity, whenever the threshold of 50% of available soil water content was reached. In the first and second years 189 and 213 mm of water were added, respectively. The average mean temperatures during the crop cycle ranged from 9.9 and 9.5 °C in January to 15.9 and 14.4 °C in June in the first and second years, respectively.

The grain-filling period in the first year was characterized by higher average and maximum temperatures (23.2 °C vs 20.2 °C and 32.5 °C vs 27.6 °C, respectively). Also the number of days with a maximum temperature between 30 and 35 °C and above 35 °C was higher in the first year than in the second one (21 vs 8 and 13 vs 8, respectively).

Grain Quality Parameters. Grain samples were harvested at physiological maturity on June 13, 2005, and June 12, 2006; then the kernel number per spike, 1000 kernel weight (g), kernel weight per spike (g), protein content, and sodium dodecyl sulfate (SDS) sedimentation value were evaluated.

Protein content, determined by the standard Kjeldhal method, was calculated by multiplying the nitrogen concentration by 5.7 and was expressed on a dry weight basis. The SDS sedimentation value, expressed in millimeters, was evaluated according to Dick and Quick.[26] After testing the variance homogeneity in the studied characteristics by Bartlett's test, data of the two years were analyzed together using the analysis of variance (ANOVA) procedure of the M-stat C statistical package (Crop Soil Sciences Department, Michigan State University). The significant differences among the mean values were calculated following Tukey's test.

Gluten Protein 2DE Analysis. Gluten proteins (gliadins and glutenins) were extracted according to Hurkman and Tanaka[27] and then separated by isoelectric focusing (IEF) × SDS−PAGE. Samples containing 200 μg of protein were loaded on each IEF gel. Immobiline DryStrips (pH 3−10, 13 cm length) (GE Healthcare Bio-Sciences AB,

Uppsala, Sweden) were used to perform first-dimension electrophoresis after rehydration using the Ettan IPGphor 3 isoelectric focusing system (GE Healthcare Bio-Sciences AB) according to Ferrante et al.[28] The separation in the second dimension was carried out with an SE 600 apparatus (Hoefer, Inc., Holliston, MA). The strips were equilibrated and then loaded onto 18 × 16 cm vertical polyacrylamide SDS−PAGE gels (T, 12%; C, 1.28%), 1 mm thick. Separation of both proteins and molecular weight markers (10 000−200 000) (GE Healthcare Bio-Sciences AB) was performed at 30 mA per gel at 10 °C. Staining was performed according to Neuhoff et al.[29] with a destaining in tap water. Three replicates were performed on two biological grain samples, giving a total of 18 gels in each year. Destained gels were analyzed using ImageMaster 2D Platinum 6.0 software (GE Healthcare Bio-Sciences AB). Comparison of mean spot volume values between the two crop seasons and the three cultivars was performed using a *t*-test by means of Jump statistical software (version 3.2.5., SAS Institute Inc., 1999). To better discriminate cultivars and environmental differences, the 2DE gels were subdivided into four regions on the basis of protein molecular weight: the high molecular weight (H) region (>60 000), the intermediate (I) region (60 000−48 000), the 48 000−35 000 low molecular weight region (L48−35), and the <35 000 low molecular weight region (L<35). The value 35 000 was chosen as the down threshold of LMW-GS and the upper threshold of most of the gliadins identified in our 2DE gels.

In-Gel Digestion of Protein Spots and Mass Spectrometry Analyses. *In-Gel Chymotrypsin Digestion.* The protein spots excised from 2DE gels were digested by an optimized in-gel chymotrypsin procedure. Gel pieces (3 mm), thoroughly washed with 10 μL of deionized water and dried, were decolorized with a 50 mM ammonium bicarbonate solution and acetonitrile (ACN) (50:50, v/v) (four times for 5 min). After liquid removal the gel pieces were dehydrated for 1/2 h by 30 μL of ACN. The dehydrated gel pieces were incubated with 10 mM dithiothreitol (DTT) at 56 °C for 45 min and then with 55 mM iodoacetamide (IoAA) at room temperature in the dark for 30 min, finally washed with a solution of 50 mM ammonium bicarbonate/ACN (50:50, v/v) with gentle agitation for 10 min, and briefly dehydrated with ACN. The wash step was repeated twice before the gel pieces were dried. Proteins in the gel pieces were digested with 5 μL of chymotrypsin (5 ng/μL) in 25 mM ammonium bicarbonate at 37 °C for 16 h. A 5 μL volume of urea (1 M, pH 8) as the denaturing agent and calcium chloride (1 mM) were added to the solution to promote the enzyme action. After the digestion, peptides were extracted with 5 μL of a 50 mM ammonium bicarbonate solution for 15 min and 5 μL of an ACN/formic acid (FA; 1% in water) solution (50:50, v/v). After 15 min the solution containing the extracted peptides was collected.

Chromatographic Separations of Peptides. Digested proteins were separated by a nano-HPLC−UV system (UltiMate/Famos/Switchos, Dionex LC Packings, Amsterdam, The Netherlands) and analyzed by using an Esquire 3000plus ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an online nanoelectrospray source. The UV detector was set at 214 nm. A C18 PepMap RP trap column (5 mm × 300 μm i.d., 3 μm particle size, 100 Å pore diameter; Dionex LC Packings) was used for concentrating and desalting the injected sample. Chromatographic separations of the peptides were carried out by a C18 PepMap analytical column (15 cm length × 75 μm i.d., 3 μm particle size, 100 Å pore diameter; Dionex LC Packings). The injected sample was loaded for 3 min into the trap column by an isocratic step with ACN/0.1% FA in water (2:98, v/v) at 30 μL/min. Sample elution through the analytical column was obtained at a flow rate of 0.175 μL/min by the following gradient: 30 min linear gradient from 0% A to 55% A; 5 min isocratic step at 10% A and 90% B; step with 100% solvent A for 15 min. Solvent A was ACN/0.1% FA in water (2:98, v/v), and solvent B was ACN/0.1% FA in water (80:20, v/v).

MS/MS Spectrometry Analysis. The Esquire 3000plus was operated in the positive ion mode with a maximum accumulation time of 200 ms and an ion charge control of 100 000 with an average of five spectra in an *m*/*z* range of 400−1400. The peptides were fragmented using the auto-MS/MS system.

MS Data Analysis. Identification of protein spots was performed by submitting the MS and MS/MS spectra, extracted and deconvoluted from chromatograms (using Data Analysis 2.0, Bruker Daltonics), to non-redundant protein sequence databases by using the Mascot search engine algorithm (Matrix Science, London, U.K., www.matrixscience.com) and NCBInr, Swiss-Prot, and MSDB database. Carbamidomethylation of cysteine residues was selected as a fixed modification, whereas oxidation of a methionine residue as a variable modification was included in the searches. The data analysis files were used to search entries under the Viridiplantae category. Peptide mass tolerance of 1.5 Da for precursor peptide ion and MS/MS tolerance of 0.4 Da were set for the window of error for matching the peptide mass values. Protein identification was accepted when Mascot search results delivered scores of ≥50 ($p < 0.05$) for each sample injection, and with the same protein identification as the top hit for the multiple injection (at least twice) of the same sample.

RESULTS AND DISCUSSION

Yield and Quality Parameters. The ANOVA relative to yield components and quality variables showed a significant effect of year, cultivar, and interaction year × cultivar only for protein content and SDS sedimentation value.

In Table 1 the interaction year × cultivar interaction is reported. In both years, Simeto showed the highest values in protein content and thousand grain mass. Also the SDS sedimentation value, a character accounting for both protein content and quality,[19] was the highest for cultivar Simeto in both years. These findings are in agreement with previous works, where Simeto, tested in different locations, showed a high quality performance.[19,23,24] As for Latino, in both the growing seasons, it showed a poor quality performance due to the low SDS value, in agreement with Boggini et al.[25] In the first crop season, characterized by higher temperatures during the grain-filling period, all the cultivars showed higher values for the quality parameters with respect to the second year; this result is probably due to a lower thousand grain mass observed in the first warmer year with respect to the second one, leading to a positive effect on qualitative properties as a concentration effect. This qualitative improvement was more evident for Latino (about 26% and 79% for protein content and SDS, respectively) and less evident for Ofanto (6% and 21% for protein content and SDS, respectively). The variability of the SDS sedimentation value between the two years was higher than that of the protein content, highlighting that a part of this variability may be ascribable to the protein quality.

Table 1. Effect of the Year × Cultivar Interaction on Yield and Quality Parameters[a]

	2005			2006		
	Latino	Ofanto	Simeto	Latino	Ofanto	Simeto
kernel number per spike (no.)	25.4	28.3	23.9	21.8	25.4	23.6
kernel weight per spike (g)	1.2	1.3	1.2	1.2	1.4	1.4
1000 kernel weight (g)	46.7	45.3	50.8	57.1	56.3	59.7
protein content (% d.w.)	17.1 ab	15.7 bc	18.4 a	13.6 d	14.8 cd	16.0 bc
SDS sedimentation value (mm)	76.3 B	74.3 B	95.0 A	42.7 D	61.3 C	62.0 C

[a]Different letters indicate significant differences at 0.05 (small letters) and 0.01 (capital letters) *p* levels according to the Tukey's test.

Table 2. Protein Spots of the Three Cultivars Detected by Image Analysis

		% spot volume[a]				
gel region	spot number	Latino	Ofanto	Simeto	fold variation[b]	p level[c]
H	118	2.96		3.15	0.94	ns
H	123	0.96		1.21	0.79	ns
H	130	1.14		1.16	0.98	ns
H	132	0.25		0.28	0.89	ns
H	143	0.36		0.58	0.62	ns
H	105		3.22			
L48−35	255			0.32		
L48−35	342		1.17	0.95	1.23	ns
L48−35	333		0.27	0.25	1.08	ns
L48−35	277		18.72	16.75	1.12	ns
L48−35	277a	8.82				
L<35	403		1.13	1.03	1.1	ns
L<35	385		0.75	0.67	1.12	ns
L<35	422		2.80	2.67	1.05	ns
L<35	433		0.71	0.48	1.48	ns
L48−35	338	5.01	9.09	7.62	0.55 (L vs O); 0.66 (L vs S); 1.19 (O vs S)	*d*
L48−35	327	0.28	0.15	0.13	1.87 (L vs O); 2.15 (L vs S); 1.15 (O vs S)	*e*
L48−35	328	0.48	0.17	0.22	2.82 (L vs O); 2.18 (L vs S); 0.77 (O vs S)	*e*
L48−35	325	4.89	0.67	0.65	7.3 (L vs O); 7.52 (L vs S); 1.03 (O vs S)	*d*
L<35	347	1.63	1.22	1.29	1.34 (L vs O); 1.26 (L vs S); 0.94 (O vs S)	*d*
L<35	358	4.88	3.66	3.52	1.33 (L vs O); 1.39 (L vs S); 1.04 (O vs S)	*d*
L<35	395	1.90	0.42	0.35	4.52 (L vs O); 5.43 (L vs S); 1.2 (O vs S)	*d*
L<35	420	2.20	1.04	1.23	2.11 (L vs O); 1.79 (L vs S); 0.84 (O vs S)	*d*
L<35	383	1.47	0.65	0.76	2.26 (L vs O); 1.93 (L vs S); 0.85 (O vs S)	*e*
L<35	399	2.82	0.84	1.06	3.36 (L vs O); 2.66 (L vs S); 0.79 (O vs S)	*d*
L<35	402	1.10	1.56	1.24	0.70 (L vs O); 0.89 (L vs S); 1.26 (O vs S)	*e*
L<35	404	2.52	0.66	0.75	3.82 (L vs O); 3.36 (L vs S); 0.88 (O vs S)	*d*
L<35	437	0.46	2.04	2.52	0.22 (L vs O); 0.18 (L vs S); 0.81 (O vs S)	*d*

[a]Mean of two years. [b]L = Latino, O = Ofanto, and S = Simeto. [c]ns = not significant. [d]$p \leq 0.01$. [e]$p \leq 0.05$.

Table 3. Protein Spots of the Cultivar × Environment Interaction Detected by Image Analysis

		% spot volume (2005)			% spot volume (2006)			
gel region	spot number	Latino	Ofanto	Simeto	Latino	Ofanto	Simeto	p level
H	123	1.79		2.18	0.18		0.23	*a*
H	132	0.32		0.33	0.18		0.23	*a*
H	105		3.59			2.85		*a*
I	119	0.42	0.38	0.12	0.10			*a*
I	124	1.80	0.55	0.29	0.73			*a*
I	128	0.22	0.32	0.17	0.11			*a*
I	214	0.61	0.24	0.10	0.24			*a*
I	215	0.75	0.94	1.37	0.29	0.50	0.76	*b*
I	225	0.26	0.44	0.24	0.11			*a*
L48−35	277		15.8	15.30		21.64	18.19	*a*
L48−35	277a	2.22			15.42			*a*
L48−35	338	4.32	6.76	6.60	5.70	11.42	8.63	*b*
L48−35	294	1.04	1.04	1.06	0.76	0.87	0.75	*a*
L<35	358	5.09	3.96	3.61	4.67	3.36	3.43	*b*
L<35	422		2.93	2.94		2.66	2.37	*b*
L<35	420	2.45	1.61	1.62	1.94	0.46	0.83	*b*
L<35	347	2.00	1.39	1.40	1.25	1.05	1.18	*b*
L<35	341	5.46	5.00	4.19	3.94	4.11	3.90	*a*
L<35	385		0.57	0.63		0.93	0.71	*b*
L<35	383	1.22	0.60	0.61	1.72	0.70	0.91	*b*
L<35	402	0.95	1.36	1.07	1.24	1.75	1.41	*b*

[a]$p \leq 0.01$. [b]$p \leq 0.05$.

The positive effect of moderately high temperatures during grain filling on the SDS sedimentation value, gluten index, and P/L ratio, leading to an increase in dough strength, has already been reported in durum wheat.[19,30]

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Table 4. Protein Spot Identification by Nano-HPLC−ESI-IT-MS/MS Analysis and Searching the NCBInr Database Using Mascot (www.matrixscience.com)

spot number	accession number (NCBInr general index)	protein	species	Mascot score	sequence coverage (%)	theor. M_w (kDa); pI	no. of peptide matches	peptide sequence[a]
118	71084277	HMW1Bx7	*Triticum aestivum*	124	19	82.5; 8.7	5	YPTSPQQSGQGQQPGHEQQPGQW; WQPGQGQQPGQGQQGYASPY; YYPISPQQPGQGQQSGQGQPGY; FYPSETTPSQQLQQMIF; YPSVTSSQQGSY
123	71084277	HMW1Bx7	*T. aestivum*	106	16	82.5; 8.7	4	
130	29150726	HMW1By8	*Triticum turgidum* ssp. *durum*	383	21	78.2; 8.63	6	EQTVVPPKGGSF; GTSSQTVQGYYPSVSSPQQGP; HQSGQGQQGYYPSSL; YPTSPQHPGQRQQPGQGQQIGQGQQL; PASLQQPGQGQQGHYTASL; YPTSPQQPGQGKQL
132	29150726	HMW1By8	*T. turgidum* ssp. *durum*	212	16	78.2; 8.63	5	
143	29150726	HMW1By8	*T. turgidum* ssp. *durum*	160	13	78.2; 8.63	4	
105	19171607	HMW1Bx20	*T. turgidum* ssp. *durum*	106	16	86.1; 9.22	5	EQQPVVPSKAGSF; GIPALLRRYYPSVTSSQQGSY; YPTSPQQSGQGQQPGQRQSG; YYPTSPQQPGQL; YPISPQQSGQGQQSGQGQQGY
119	215398470	globulin 3	*T. aestivum*	276	13	66.6; 7.78	8	LRPFDEVSRLLR; FDEVSRLLR; FVVPGLTDADGVGYV; YVAQGEGVLTVIENGEKRSYT; YTVRQGDVIVAPAGSIMHLA; LTAALKTSDERLGSLLG; FVVPPGHPVVEIASSRGSSNLQ; FEINAERNERVWLA; WLAGRNNVIAKLDDPAQELT; LAGRNNVIAKLDDPAQELT
124	215398470	globulin 3	*T. aestivum*	206	6	66.7; 7.78	6	
128	215398470	globulin 3	*T. aestivum*	256	21	66.7; 7.78	10	
214	215398470	globulin 3	*T. aestivum*	241	9	66.7; 7.78	7	
215	215398470	globulin 3	*T. aestivum*	328	2	66.7; 7.78	4	
225	215398470	globulin 3	*T. aestivum*	754	19	66.7; 7.78	9	
342	21930	LMW-GS	*T. turgidum* ssp. *durum*	76	10	32.2; 8.17	2	WQQQPLPPQQTFPQQPPF; WQQSSCHVMQQQCCQQLSQIPEQSRY
403	9931207	LMW-GS (*lmw-gs3* gene)	*T. turgidum* ssp. *durum*	71	10	34.3; 7.56	2	SIILQEQQQGF, QQPQQQLGQQPQEQQVQQGTF
277	4584082	LMW-GS type 2	*T. turgidum* ssp. *durum*	194	19	42.7; 8.73	4	SHIPGLERPSQQQPLPPQQTL; SQQQQPVLPQQPSF; SQQQQPVLPQQPPF; GQQPQQQQLAHGTFLQPHQIAQL
338	2808522	LMW-GS Met type	*T. turgidum* ssp. *durum*	56	6	40.2; 8.14	2	QQQPLPLQQILW; SQQQQQPILL
255	90101517	α-gliadin Gli2-LM2-12	*T. aestivum*	145	22	36.3; 7.66	3	LQQILQQQLIPCRDVVL; LQQQRQQPSSQVSFQQPQQQYPSSQVSF; IPPHCSTTIAPF
385	82880023	α-gliadin Gli2 Du2	*T. turgidum* ssp. *durum*	56	9	33.1; 8.24	2	LQQYQLGQGSFRPSQQNPQAQGSVQPQQ; PPYCTIAP
422	218140824	α-gliadin Gli-turg-1	*T. turgidum* ssp. *durum*	103	11	26.7; 7.73	3	LVQQLQY; LPYPQPQP; QQPQQQYPSGQGSF
333	7209253	α-gliadin	*T. aestivum*	70	22	32.5; 6.99	5	LQLQPFPQPQLSY; RPQQPYPQPQPQY; QQPLQQYPLGQGSF; FQQPLQQYPLGQGSF; YSQPQPF
327	7209253	α-gliadin	*T. aestivum*	68	14	32.2; 6.98	3	
433	56709482	α-gliadin precursor	*T. turgidum* ssp. *durum*	81	18	33.4; 8.58	3	YSQPQPFRPQQPYPQPQPRY; FSFQQPLQQYPLGQGSFR; YIPPYCTIAPFGIF
328	56709482	α-gliadin precursor	*T. turgidum* ssp. *durum*	57	10	33.4; 8.58	2	
341	56709482	α-gliadin precursor	*T. turgidum* ssp. *durum*	61	20	33.4; 8.58	3	
325	72537616	α-gliadin (*Gliw-5* gene)	*T. turgidum* ssp. *dicoccoides*	58	14	31.8; 6.99	3	VRVPVPQLQPQNPSQQQPQEQVPL; YLQLQ PFPQPQLPY; RPQQPYPQPQPQY

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Table 4. continued

spot number	accession number (NCBInr general index)	protein	species	Mascot score	sequence coverage (%)	theor. M_w (kDa); pI	no. of peptide matches	peptide sequence[a]
347	72537616	α-gliadin (*Gliw-5* gene)	*T. turgidum* ssp. *dicoccoides*	53	18	31.8; 6.99	4	
294	72537616	α-gliadin (*Gliw-5* gene)	*T. turgidum* ssp. *dicoccoides*	58	14	31.1; 6.99	3	
358	82880021	α-gliadin Gli2 Du1	*T. turgidum* ssp. *durum*	121	16	36.4; 7.68	4	QQQLIPCRDVVL; HQQRQQPSSQV; SFQQPQQQYPSSQVSF; IPPHCSTTIAPF
395	67464993	α/β-gliadin precursor	*T. aestivum*	54	4	33.2; 7.66	2	YSQPQPFR; YQLLQEL
420	72537614	α-gliadin (*GliTd-4* gene)	*T. turgidum* ssp. *dicoccoides*	54	8	31.4; 7.14	2	YLQLQPFPQPQLPY; YQLLQELCCQHLW
383	30141195	γ-gliadin	*T. turgidum* ssp. *durum*	63	16	32.7; 6.34	6	CEQPQRTIPQPHQT; LQQQMNPCKNF; FQLAQG LGIIQPQQPA; QLEGIRSLV; KTLPTMCNVY
399	30141195	γ-gliadin	*T. turgidum* ssp. *durum*	56	13	32.7; 6.34	5	
402	30141195	γ-gliadin	*T. turgidum* ssp. *durum*	63	16	32.7; 6.34	6	
404	30141195	γ-gliadin	*T. turgidum* ssp. *durum*	63	16	32.7; 6.34	6	
437	34329295	γ-gliadin group III	*T. turgidum* ssp. *durum*	59	10	27.4; 8.15	2	VQGQGIIQPQQPAQL; VLQTLPTMCNVYVP PYCSTIRAPF

[a]Peptide sequences identified in spots with the highest sequence coverage.

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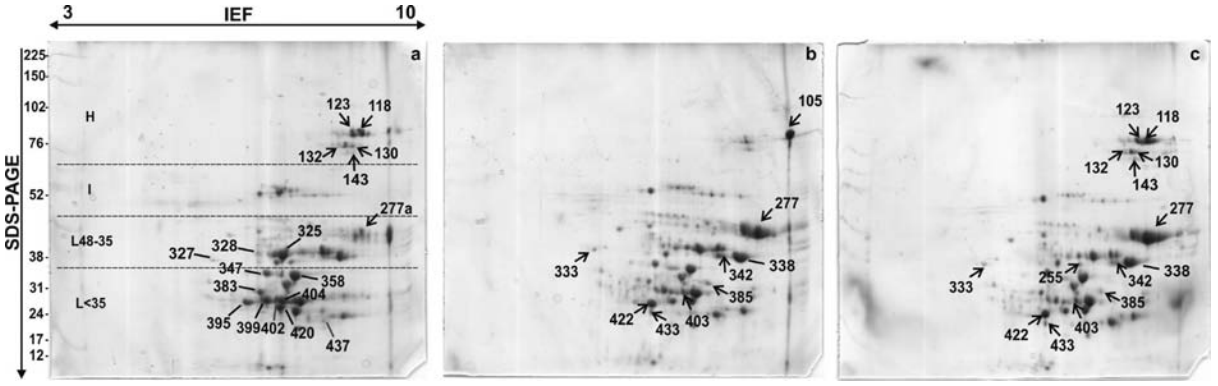

Figure 1. Gluten protein 2DE gels (IEF × SDS−PAGE) of the three cultivars (a) Latino, (b) Ofanto, and (c) Simeto. Arrows label specific spots. Lines indicate differentially expressed spots (black, up-regulated; gray, down-regulated).

Proteome Analysis. *Image Analysis.* The 2DE gels were highly reproducible and showed well-resolved spots without streaking with an average of 133, 127, and 144 spots for Latino, Ofanto, and Simeto cultivars, respectively, and a mean value of 145 and 124 spots in the 2005 and 2006 growing seasons. As for the cultivars, Latino showed the highest number of specific, differentially expressed, and absent spots (13, 25, and 17, respectively) compared to Ofanto (9, 10, and 12, respectively) and Simeto (9, 8, and 2, respectively). Furthermore, with respect to the cultivar × environment interaction, in 2005 a generalized increase of specific spots, with respect to 2006, was observed, more drastic in Ofanto (41 vs 4). Moreover, a marked increase in the up-regulated spots was observed in 2005 only for Latino (17 vs 3).

Only the spots that showed the same cultivar response in the two years (Table 2) and the spots that showed significant differences between the two years in the cultivar × environment interaction (Table 3) were considered for the identification by means of MS/MS analysis (Table 4).

Cultivar Effect. The comparison of the storage protein compositions of the three cultivars under study is shown in Figure 1 and Table 2.

High Molecular Weight Gel Region. Differences among the three cultivars were found within this gel region. The lowest protein expression, evaluated as a percentage of the region spot volume over the total, was found for Ofanto (6.2%) with respect to Latino (9.3%) and Simeto (9.3%). In Latino and Simeto the gel matching and image analysis showed the presence of two spots, 118 and 123, that were always absent in Ofanto (Figure 1 and Table 2), identified by means of nano-HPLC-ESI-IT-MS/MS as HMW1Bx7 (Table 4); furthermore, three spots, 130, 132, and 143, found in both Latino and Simeto cultivars but absent in Ofanto, were identified as HMW1By8 (Figure 1 and Tables 2 and 4) as already reported in the literature.[4,23,25,31] Moreover, Yan et al.[31] reported that Simeto has the 1Bx7 and 1By8 subunits encoded at the *Glu-B1* locus and that the amount of 1By8 subunit was much lower than that of 1Bx7, as evaluated by different separation methods and confirmed in our study (Table 2). In both bread and durum wheat, these alleles have been shown to be associated with high elastic recovery, gluten firmness,[31] and good-quality semolina.[32] All of these spots were overexpressed in Simeto with respect to Latino. The presence of multiple spots for both 1Bx7 and 1By8 subunits was also observed by Dupont et al.,[33] who reported that highly reproducible charge trains are commonly observed in 2DE of flour proteins and hypothesized that they could be a result of naturally occurring in vivo modifications. Moreover, Zhang et al.[34] suggested and confirmed the phosphorylation of the HMW subunits with a specific stain and a simplified protocol of detecting phosphoproteins in 1D gel. On the contrary, Herbert et al.[35] ascribed such a presence to artifactual modifications of the sample prior to and during electrophoresis. However, it has been pointed out that, with a normal sample handling, these modifications, such as deamidation, do not occur.[36] As for 1By8, a minor spot was resolved at slightly different apparent molecular weight from the charge train and could result from proteolysis or post-translational modification.[31,33] In Ofanto we observed the lack of 7 and 8 subunits, which is a constitutive feature of this cultivar, and the presence of a specific spot (105) identified as HMW 1Bx20 (Table 4), also reported in another study.[23] The substitution of two cysteine residues in the N-terminal domain by tyrosines at positions 22 and 37, reported by Shewry et al.,[5] could be responsible for the detrimental effect of 1Bx20 on dough strength by decreasing the number and affecting the pattern of disulfide cross-links in the glutenin polymers, when compared to 1Bx7.

Intermediate Molecular Weight Gel Region. In the intermediate molecular weight gel region, only a few differences among cultivars were found in the total spot protein volumes; in particular, Latino showed the highest protein expression (3.9%) with respect to Ofanto (2.6%) and Simeto (2.5%). On the contrary, more important differences were observed in relation to the environmental effect (see the section "Interaction Cultivar × Environment" for details).

Low Molecular Weight Gel Region. The low molecular weight gel region includes both low molecular weight glutenins and α-, β-, and γ-gliadins. In the 48 000−35 000 molecular weight region, Ofanto showed the highest protein expression value (49.8%) and Latino the lowest (38.6%), while the opposite behavior was observed in the <35 000 molecular weight region: Latino 47.6% and Ofanto 40.9%. Simeto showed in both regions intermediate values (45% and 43.1%, respectively). As far as the specific spot expression is concerned, Ofanto and Simeto showed spots 342 and 403, which were absent in Latino (Figure 1 and Table 2). Spot 342 was identified as an LMW-GS for *Triticum durum* (NCBI code gil 21930) encoded at an unknown locus (Table 4). In fact, despite their important effect on the qualitative properties, the number of studies on LMW-GS is more limited compared to those on HMW-GS, due to their great number and heterogeneity.[37] Spot 403 was identified as an LMW-GS encoded at locus *GluA3*

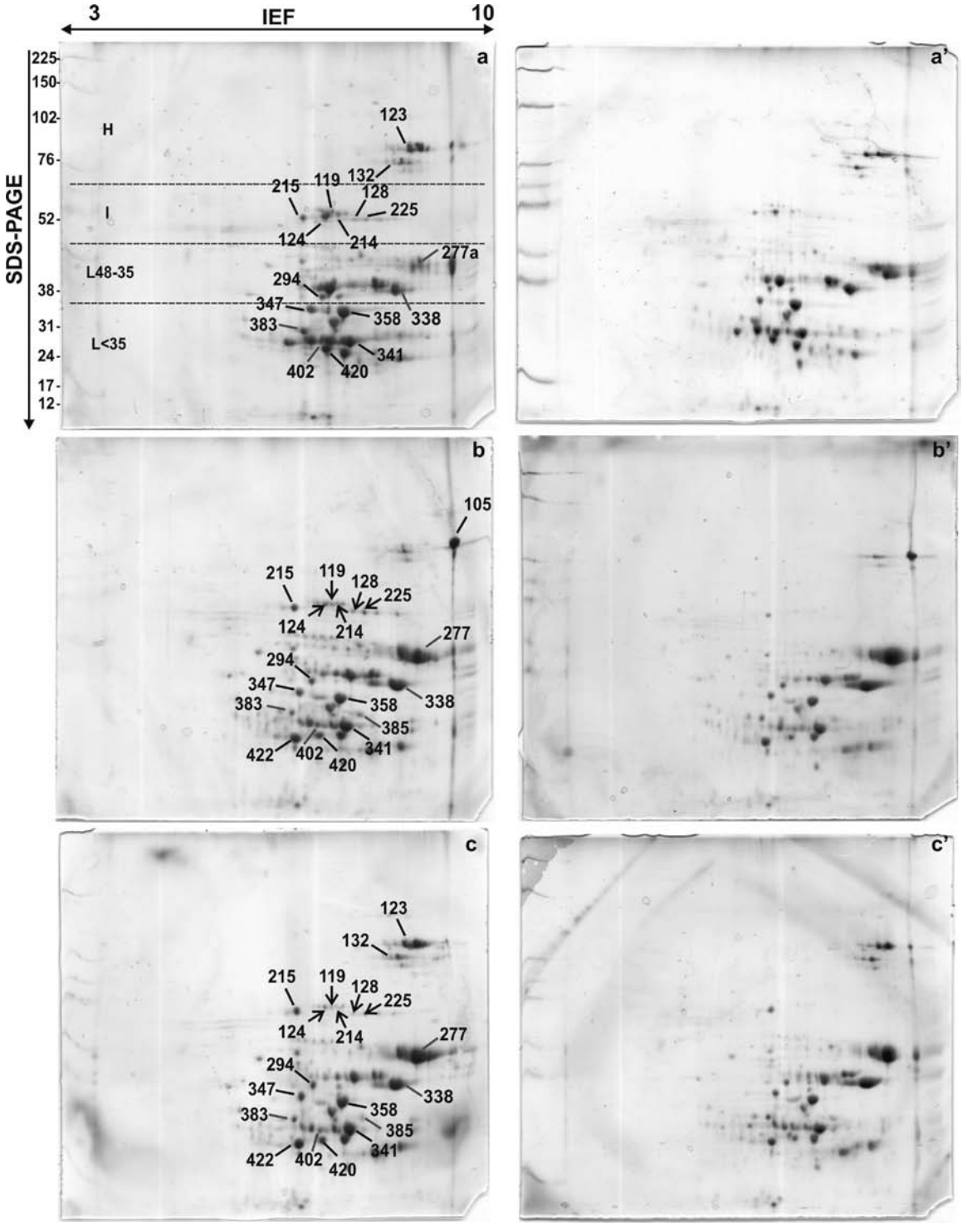

Figure 2. Gluten protein 2DE gels (IEF × SDS−PAGE) in the cultivar × environment interaction (a and a′, b and b′, and c and c′ are Latino, Ofanto, and Simeto in 2005 and 2006, respectively). Arrows label specific spots. Lines indicate differentially expressed spots (black, up-regulated; gray, down-regulated).

from the gene *lmw-gs3* (Table 4). Although LMW-GS encoded by the *GluB3* and *GluD3* loci are the most abundant, those encoded by the *GluA3* locus also influence quality characteristics.[38] Moreover, this protein, reported in the NCBI data bank (gil9931207) and encoded by the *LDNLMW1A3* gene (NCBI code AJ293099), is an LMW-m type with a peculiar N-terminal sequence that has never been previously identified at the protein level. Recently, Masci et al.[38] have shown clear evidence of its expression in the endosperm tissue and its participation in the gluten polymer. Spot 277 was found up-regulated in Ofanto and Simeto and has been identified as an LMW-2. In the literature it is reported that lines possessing the specific group of LMW-2 have superior quality characteristics compared to lines possessing the allelic group LMW-1[8] and that Simeto and Ofanto show LMW-2[23] and Latino shows LMW-1.[25] The identified peptides by tandem mass spectrometry in Latino

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(277a) do not allow discrimination of the small differences between the two proteins (LMW-1 and LMW-2), an insertion of 13 amino acids within the repetitive domain. Therefore, the same identification obtained for the three cultivars was considered correct only for Ofanto and Simeto. Also spot 338 was up-regulated in Ofanto and Simeto with respect to Latino (Figure 1 and Table 2). For this spot the MS/MS analysis allowed the identification of an LMW Met-type[39] encoded at the *GluB3* locus for *T. durum* (Table 4). The proteins encoded at the *GluB3* locus are of particular interest in durum wheat because they play an important role in determining the viscoelastic characteristics of durum wheat flour.[39]

Consistently, with the variation in the LMW-GS expression levels, the three cultivars exhibited changes also in α- and γ-gliadin composition. In particular, one spot (255) was specific for Simeto, four spots (333, 385, 422, 433) were common to Ofanto and Simeto and absent in Latino, and seven spots (325, 327, 328, 347, 358, 395, 420) were up-regulated in Latino with respect to Ofanto and Simeto (Figure 1 and Table 2). All these spots were identified as α-gliadins (Table 4), and in particular, the spot specific for Simeto (255) was identified as the α-gliadin Gli2-LM2-12 reported in the database (NCBI) for *T. aestivum*. Concerning the α-gliadins common to Ofanto and Simeto, spot 385, identified as a Gli2_Du2, is characterized by six cysteine residues involved in three intermolecular disulfide bonds that may influence gluten structure and quality.[40] The α-gliadin Gli-turg1 (spot 422) was characterized by two cysteine residues and defined as a potential chain extender.[41] Spot 333 absent in Latino was characterized by the same NCBI accession number (gi|7209253) obtained for spot 327, which was overexpressed in Latino. The same results were obtained for spots 328 and 433, which were overexpressed and absent in Latino (NCBI accession number gi|56709482), respectively. In particular, both spots 333 and 327 were identified as the same α-gliadin reported in the database for *T. aestivum*,[42] while spots 328 and 433 were the α-gliadin reported in the NCBI database for *T. durum*, cultivar Ofanto. In Latino these proteins showed a higher molecular weight than in Ofanto and Simeto. Also, both spots 325 and 347, overexpressed in Latino but with different molecular weights (Figure 1 and Table 2), have been identified as the same α-gliadin encoded by the gene *Gliw-5* (Table 4). In both cases, a molecular weight different from the one predicted from the sequence of these α-gliadins was observed, as already reported by Dupont et al.[33] Another spot overexpressed in Latino was 358, the α-gliadin Gli2_Du1 (Table 4), similar to gliadin Gli2_Du2 (spot 385).[40] Finally, spot 420 has been identified as the α-gliadin (Table 4) encoded by the *GliTd-4* gene that is potentially functional.[43] Also for this protein, six cysteine residues not randomly distributed, but conserved, in the two unique domains, forming three intramolecular disulfide bonds, which can result in a compact structure, have been reported.[43]

As for γ-gliadins, three spots were up-regulated in Latino with respect to Ofanto and Simeto (spots 383, 399, and 404), while two spots (402 and 437) were down-expressed in Latino. By MS/MS analysis these spots, except 437, led to the identification of the same γ-gliadin subtype (Table 4), similar to, but clearly different from, the γ-45 gliadin, which is a true monomeric form.[44] This γ-gliadin with an odd number of cysteine residues, in particular a ninth cysteine located at position 26 from the N-terminus, is able to form an intermolecular disulfide bond, thus incorporating this protein into the glutenin polymer fraction. Therefore, this γ-gliadin, also called γ-type glutenin, is likely to act as a terminator of growing polymer chains and cause the molecular weight distribution of the glutenin polymer system to be shifted downward.[44] Finally, spot 437 was identified as a γ-gliadin group III (Table 4) having a sequence with a signal peptide followed by five regions, the last three of which contain eight cysteine residues that form four intrachain disulfide bonds.[45]

Interaction Cultivar × Environment. The effect of the cultivar × environment interaction on storage protein expression, evaluated comparing the proteome of each cultivar between the two cropping seasons, is shown in Figure 2 and Table 3.

High Molecular Weight Gel Region. Concerning the variation of the H region between the two growing seasons, three spots were up-regulated in the first warmer season (2005): HMW 1Bx7 (spot 123) and HMW 1By8 (spot 132), both in Latino (fold variations of 9.9 and 1.78 for spots 123 and 132, respectively) and Simeto (with fold variations of 9.52 and 1.43 for spots 123 and 132, respectively), and HMW 1Bx20 (spot 105) in Ofanto (with a fold variation of 1.26). The up-regulation of these HMW glutenin subunits might be specifically related to environmental conditions occurring in the first cropping season that was characterized by higher temperatures. This is in agreement with Dupont et al.,[15,16] who reported an increase of HMW-GS when grains are exposed to high temperature during grain filling.

Intermediate Molecular Weight Gel Region. This gel region seems to be organized as a train spot beginning with the master spot 215, followed by several spots having the same molecular weight but different pI values (shifted toward basic pH) (Figure 2), all identified as the globulin 3 protein codified by the *Glo3A* gene (Table 4). Globulin-like proteins are nonprolamines similar to 7S vicilin-like proteins, and functioning as storage proteins. Globulins are incorporated unspecifically into the gluten network, suggesting that their role in the gluten agglomeration is rather aspecific.[46] At least two copies each of globulin genes *Glo3A*, *Glo3B*, and *Glo3D* were reported for hexaploid wheat and mapped to chromosome 4; at least one of the protein products is reported to be associated with the pathogenesis of type I diabetes (TID) in some susceptible individuals.[47] This region was environmentally dependent in all cultivars investigated. In fact, in the first warmer year, spot 215 was up-regulated in all cultivars with respect to the second year with fold variations of 2.59, 1.88, and 1.80 for Latino, Ofanto, and Simeto, respectively (Table 3). Moreover, the train spot was detected in both years in Latino, showing an up-regulation in the first warmer year with fold variations between the two years of 4.2 for spot 119, 2.46 for spot 124, 2 for spot 128, 2.54 for spot 214, and 2.36 for spot 225. The same train spot was missed in Ofanto and Simeto in the second year (Figure 2 and Table 3). As a consequence, Latino appears to exhibit a constitutive expression of this train spot. It might be hypothesized that high temperatures characterizing the 2005 crop season increased the globulin train expression, in agreement with Altenbach et al.[46] and Laino et al.,[48] who reported an increase in globulin amount during grain development under high temperatures in both *T. aestivum* and *T. durum*. On the contrary, Yang et al.[18] found a decrease in several globulins in response to heat stress and an increase in one globulin in response to water deficit. In a recent paper, Chao-Ying et al.[49] observed isoforms of a globulin (gi|215398470) very similar to ours (gi|215398472), and they

suggest that these isoforms might represent post-translational modifications (PTMs). However, the reason why globulins vary in response to heat and/or water stress is still unknown, and the data suggest that the roles of the globulins should be studied further.[10,48]

Low Molecular Weight Gel Region. Two of the more expressed LMW-GS, LMW type 2 (spot 277) and LMW Met-type (spot 338) encoded at the locus *GluB3*, were down-regulated in the first warmer year with respect to the second one in all three cultivars (Figure 2 and Table 3) with fold variations of 0.76, 0.59, and 0.76 for Latino, Ofanto, and Simeto, respectively. This result is in agreement with Dupont et al.,[16] who reported a decrease in relative spot volume for a major LMW-GS in relation to the high temperature. Furthermore, in the <35 000 molecular weight region, six α-gliadins were up-regulated in the first warmer year with respect to the second one: Gli2_Du1 (spot 358 with fold variations of 1.09, 1.18, and 1.05 for Latino, Ofanto, and Simeto, respectively), Gli-turg 1 (spot 422 with fold variations of 1.10 and 1.24 for Ofanto and Simeto, respectively), α-gliadin encoded by *GliTd-4* (spot 420 with fold variations of 1.26, 3.5, and 1.95 for Latino, Ofanto, and Simeto, respectively), α-gliadin encoded by the *Gliw-5* gene (spot 347 with fold variations of 1.6, 1.32, and 1.19 for Latino, Ofanto, and Simeto, respectively; spot 294 with fold variations of 1.37, 1.19, and 1.41 for Latino, Ofanto, and Simeto, respectively), and the α-gliadin precursor (spot 341 with fold variations of 1.38, 1.22, and 1.07 for Latino, Ofanto, and Simeto, respectively). It is important to note that the last four spots belong to the region that includes both seed storage protein and the α-amylase inhibitor subfamily, which play important roles in the natural defense of plants; recently, an increase of these proteins in response to heat shock has also been reported.[48] Also the protein Gli2_Du2 (spot 385), which was down-regulated in the first warmer year with respect to the second one, belongs to the same region (Figure 2 and Tables 3 and 4). Moreover, these data suggest that environmental conditions characterizing the 2005 season were favorable for the increase in the expression of α-gliadins as reported by Daniel and Triboi,[50] who suggested that high temperatures promote gliadin synthesis. Also Majoul et al.[17] found an increase in α-gliadins in the presence of high temperatures, demonstrating that the presence of heat shock elements in the gliadin gene promotes gliadin synthesis in hot seasons.

Furthermore, the γ-gliadins already mentioned (spots 383 and 402) were down-expressed in the 2005 season (Figure 2 and Table 3). In particular, for spot 383 the fold variations between the first and the second years were 0.71 for Latino, 0.86 for Ofanto, and 0.67 for Simeto, while for spot 402 the fold variations were 0.77 for Latino, 0.78 for Ofanto, and 0.76 for Simeto. As reported above for LMW-GS, a negative effect of higher temperatures was also observed for γ-gliadins. Similar to our results, Daniel and Triboi[50] and Dupont et al.[15] reported a concurrent decrease of both LMW-GS and γ-gliadins when grains were exposed to high temperatures during grain filling.

In conclusion, the three durum wheat cultivars showed significant differences in gluten proteome. In particular, Latino showed the lowest protein spot volume in the LMW 48 000–35 000 region with the absence of the LMW-2 and the down-expression of the LMW Met-type, both of them positively related to dough properties.[39] Moreover, the absence of the other two LMW-GS, one encoded at locus *GluA3* from the gene *lmw-gs3* and one encoded at an unknown locus (NCBI code gil21930), may also negatively affect the qualitative performance of this cultivar. Whereas Latino seemed to be distinguished from the other two cultivars in LMW-GS composition, Ofanto showed major differences in the high molecular weight gel region. In fact, despite a high expression in the LMW 48 000–35 000 region, the lowest protein spot volumes observed in the HMW region for Latino, together with the specific presence of 1Bx20 HMW-GS might explain the lower quality characteristics of Ofanto with respect to Simeto. Finally, in Simeto the up-regulation of 1Bx7 + 1By8 HMW-GS and of LMW-GS, as the LMW Met-type, and the presence of two LMW-GS (one encoded at locus *GluA3* from the gene *lmw-gs3* and one encoded at an unknown locus, NCBI code gil 21930) might be responsible for its known good qualitative performance.

Concerning the year effect, a marked up-regulation of the HMW glutenin subunits and a slight down-expression of LMW glutenins, together with minor changes in α- and γ-gliadins, were observed in the first warmer year. Furthermore, in the first year an up-regulation of a globulin 3 protein, probably involved in the high-temperature stress response also found to be associated with autoimmune disease type I diabetes, was observed. These changes in protein composition were associated with an improvement in technological quality shown by the increase in SDS sedimentation values. These findings are in accordance with evidence from the literature on the improvement of gluten strength under moderately high temperature.[19] In particular, under our experimental conditions thermal stress provided an increase in HMW-GS, which may have allowed the formation of intermolecular disulfide bonds, thus contributing to the creation of large polymers responsible for good dough viscoelastic properties.[20] Among the cultivars investigated, Ofanto and Latino showed a marked variability in the two years concerning both specific and differentially expressed spots, while Simeto showed a more constant spot pattern across the years. Currently, there are large markets for durum wheat grown in traditional areas, both for domestic consumption and for export to developing countries. Therefore, the investigation on cultivar stability, concerning the quality performance across different environments, is an important requirement both for industrial use and for consumers.

However, despite it being well-known that environmental conditions occurring under Mediterranean environments, such as heat and drought stress, may markedly influence the quality of wheat flours, due to changes in gluten protein composition, further studies have to be carried out to obtain deep insight into cultivar and environmental influence on the durum wheat gluten proteome in relation to technological and healthy aspects.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table showing all accession numbers of the identified spots. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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