DOI:10.1021/jf100215t

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Differences in the Triticale (X Triticosecale Wittmack) Flag Leaf 2-DE Protein Profile between Varieties and Nitrogen Fertilization Levels

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Nitrogen nutrition is one of the major factors limiting the growth and production of crop plants. Limited information on proteome changes occurring in response to nitrogen amount have been available up to now. We used 2-DE to investigate proteome differences between two triticale varieties and the changes caused by nitrogen nutrition deficit in the flag leaf tissue. Some physiological features, such as the number of tillers per plant, SPAD index, dry weight, and protein content were measured previous to the proteomic analysis. Statistical analysis identified 29 differential protein spots in the selected pairwise comparisons of experimental conditions and correlated with the expression cluster revealed by the principal component analysis. The 29 protein spots were subjected to matrix-assisted laser desorption ionization time of flight (MALDI-TOF) to deduce their possible functions. Many of these changes referred to enzymes involved in photosynthesis, metabolic pathways implicated in the balance of the energy, and redox status of the cell. This work provides a first characterization of the proteome changes that occur in response to nitrogen deficit in flag leaves of triticale plants.

KEYWORDS: Triticale proteomics; flag leaf proteome; nitrogen fertilization

INTRODUCTION

Triticale (X Triticosecale Wittmack) is a cereal crop which was developed by man rather than through natural evolution. A wheat (*Triticum*)−rye (*Secale*) cross, triticale was initially developed to combine the positive traits of both parent types: vigor and winter hardiness as well as the higher protein content of rye combined with higher quality gluten and the baking properties of wheat. The higher yield potential and plumper kernels of modern triticale cultivars have resulted in lower kernel protein levels, which are similar to those of common bread wheats (*1*, *2*).

Nitrogen is one of the main inputs on cereals in high-input agricultural systems. Differences in nitrogen uptake and efficiency favor spring and winter triticales when compared to those of other small grains. Genetic variability for these traits among triticales could be exploited in future breeding efforts. Limited pollution risks could be achieved either with low fertilizer rates or cultivars that absorb nitrogen better. Field experiments have shown that genetic variability for nitrogen uptake exists in small grains. Therefore, selecting new crop varieties exhibiting improved nitrogen use efficiency (NUE) and adapting agricultural practices to reduce the use of nitrogen fertilizers represents a challenge for both breeders and farmers (*3*).

A recent comparison between triticale and wheat indicates that triticale accumulates more nitrogen during heading and physiologic maturity than does wheat (*4*). The difference in nitrogen accumulation is maximum under lower levels of nitrogen application, indicating that triticales are better crops for soils with low nitrogen fertility. In relation to protein quality, it appears that triticale is either richer than both parental species in many essential amino acids, particularly in the most limiting ones, or intermediate between them dependent on the protein content of the grain (*5*).

Metabolic processes, based on protein, leading to increases in vegetative and in reproductive growth and yield are totally dependent upon the adequate supply of nitrogen (*6*). Both field and laboratory investigations in several plants such as tomato, maize, or sunflower have demonstrated that increasing the supply of nitrogen fertilizer increases growth and photosynthesis. Nitrogen deprivation reduces leaf production and individual and total leaf area for photosynthesis (*7*−*9*). An increase in nitrogen availability results in higher leaf nitrogen content. This results in a strong positive correlation between photosynthesis and leaf nitrogen content for many species, as has been reported in wheat, maize, rice, and sunflower. (*10*−*13*).

It is generally difficult to identify good candidate genes among species that have large genomes, such as wheat. Nitrogen fertilization level can be studied at the transcriptomics and proteomics levels. Wang and co-workers (*14*) identified several genes in *Arabidopsis* differentially expressed in response to different concentrations of nitrate, using 5524 genes/clones microarray. For this purpose, large RNA collections and technical developments are necessary prior to carrying out such an approach on

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 Published on Web 03/24/2010

nonmodel species. At another level, two-dimensional gel electrophoresis (2-DE) has been used to study differentially abundant proteins. This approach removes some of the limitations encountered at the DNA/RNA level mainly due to post-translational modifications and large differences in mRNA and protein turnover (*15*). Dhugga and co-workers (*16*) have reported a specifically nitrate-induced protein in the maize root membrane, but they worked on only one genotype, obtaining no information to explain genetic variability within a species. Bahrman and co-workers (*17*) have reported that the nitrogen level effect is highly significant for the number of tillers per plant, aerial dry weight, and nitrogen content in wheat. Although the improvement in technologies for protein study and the widening of gene sequences have made it possible to study plant proteomes, only limited information on proteome changes occurring in response to nitrogen amount has been available up to now. Thus, Bahrman and co-workers used the 2-DE to compare the proteome profiles of leaves (*17*) and roots (*18*) of two wheat varieties exposed to different levels of nitrogen. These works pointed out some significant differences, correlated with nitrogen availability during plant growth in the protein profiles of both organs. More recently, protein pattern changes in wheat (*19*) and maize (*20*) have been evaluated in response to different nitrogen nutrition levels. These authors found that changes in proteins were involved in photosynthesis as well as being directly implicated in nitrogen assimilation and some stress responses.

We aim to characterize the soluble protein profile of the flag leaf tissue of two triticale varieties selected on the basis of previous studies carried out in the field and in growth chambers (*21*), as well as changes caused by nitrogen nutrition deficits. We selected flag leaf tissue because of its great importance since it makes up about 75% of the leaf area that effectively contributes to grain filling.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Two winter triticale varieties (AD7291 and Rakita) were selected on the basis of physiological differences observed (grain protein content, resistance to low temperatures, average yield) in previous works carried out in the field and in growth chambers (*21*, *22*). Both displayed the most extreme differential responses to winter and to nitrogen deficit, with Rakita being the most resistant variety and AD7291 the less resistant, used as the standard.

Seeds were germinated at room temperature on filter paper for 3 days and after germination were transplanted individually into 500 cm^3 pots containing 35 g of dry perlite. Plants were grown in a growth chamber at 25/18 °C day/night temperature and 80% air relative humidity using a 16 h day period with 350 μmol m^{-2} s^{-1} photosynthetically active radiations. Four pots per variety and treatment were employed. Each pot received Hoagland nutritional solution (*23*) containing either 1 or 5 mM $CaNO_3$ so that the plants received 6 or 32 mg N/day (hereafter, these concentrations are noted as N1 and N2, respectively). These nitrogen concentrations were selected on the basis of previous studies carried out by Bahrman and co-workers (*17*) as low and high concentration, respectively. Pots were arranged in a split-plot pattern with nitrogen treatment assigned to the main plots. The number of tillers per plant was recorded every fifth day after the beginning of tillering. Leaf greenness was measured from the beginning of tillering until the end of stems elongation phases, on sampled fresh leaves using a hand-held leaf greenness meter (SPAD-502, Minolta, Japan), hereafter referred to as SPAD. The average of three readings (top, center, and base of the leaf blade) per leaf was used as a greenness score. This parameter was taken as indicative of the visible appearance of the leaves. The dry weight per single plantlet also was measured.

At the end of the stem elongation stage (56 days after sowing), when the flag leaves appeared, these were collected, frozen in liquid nitrogen, and stored at −80 °C until protein extraction.

Protein Extraction and Two-Dimensional Gel Electrophoresis (2-DE). A soluble fraction of proteins was extracted according to the TCA−acetone precipitation protocol (*24*) with some minor modifications. Leaf samples (ca. 2 g fresh weight) from three independent replicates per treatment and variety were ground with liquid nitrogen in the presence of glass powder by using a precooled mortar and pestle. The powder was suspended in −20 °C cold acetone containing 10% v/v TCA and 0.07% w/v DTT (4 mL per g of fresh tissue) and sonicated for 10 min on ice at 50 MHz by using an Ultrasonic Homogeneizer 4710 Series (Cole-Parmer). After standing for 1 h at −20 °C, the samples were centrifuged at 48400*g* for 30 min at 4 °C. The pellet was washed twice by resuspension in cold (−20 °C) acetone containing 0.07% w/v DTT, placed at −20 °C for 30 min, and centrifuged at 27200*g* for 15 min at 4 °C. The resulting pellet was lyophilized for 10 min and resuspended in sample buffer, containing 8 M urea, 2% w/v CHAPS, 0.5% v/v Bio-Lyte 3−10 carrier ampholytes, 20 mM DTT, and Bromophenol blue traces. Samples were sonicated for 5 min in an Ultrasonic bath and incubated for 1 h at 35 °C. Samples were centrifuged at 27200*g* for 15 min at room temperature, and soluble proteins were determined by RCDC Protein Assay Kit (BioRad), according to the manufacturer's instructions.

The protein profile of triticale flag leaf tissue was preliminarily analyzed by 2-DE on 7 cm length. For this, IPG strips (BioRad) 7 cm wide pH gradients were used, and 17 cm 5−8 pH gradient was used for preparative purposes. Strips were passively rehydrated for at least 12 h with 125 μL (7 cm) or 300 μL (17 cm) of sample buffer, containing 150 and 200 μg of protein, respectively. Strips were loaded onto a PROTEAN IEF System (BioRad, Hercules, CA, USA) and focused at 20 °C with increasing linear voltage according to the manufacturer's instructions: 250 V−4000 V until reaching 10000 V h for 7 cm IPG strips and 250 V−10000 V until reaching 50000 V h for 17 cm IPG strips. After IEF, strips were equilibrated by soaking first for 10 min in 375 mM Tris-HCl buffer at pH 8.8, 6 M urea, 2% SDS, and 20% glycerol solution containing 2% DTT, and then for 10 min in the same solution containing 135 mM iodoacetamide.

Second dimension SDS−PAGE was performed using the Mini-Protean 3 System (7 cm strips) or the PROTEAN IIxi Cell System (BioRad) for the 17 cm strips. Electrophoresis was carried out at 20 °C in 13% house-made polyacrylamide gels until bromophenol blue reached the end of the gel, by using a constant voltage of 200 V (60 min) for small gels and 30 mA (60 min) plus 50 mA (240−300 min) for preparative gels. Broad molecular range markers (BioRad) containing myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovoalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa) were loaded beside the strip.

Small size gels were Coomassie stained according to Neuhoff and co-workers (*25*). Preparative gels were stained with CBB G-250 according to the procedure reported by Mathesius and co-workers (*26*). Gel images were captured with a GS800 imaging densitometer (BioRad) and initially analyzed with the PDQuest Advanced version 8.0.1 software (BioRad) using 10-fold over background as a minimum criterion for presence/absence. This software assigns unique standard spot numbers to each protein spot, termed SSP. Spot detection and matching errors were corrected manually on the basis of the respective group consensus data. Normalized spot volumes (individual spot intensity/normalization factor) calculated for each gel on the basis of total quantity in valid spots were determined and these values used to designate the differential protein spots.

Identification of Proteins by Mass Spectrometry. Spots were excised manually from gels and digested with modified porcine trypsin (sequencing grade; Promega) by using a ProGest (Genomics Solution) digestion station. The digestion protocol used was that of Schevchenko and co-workers (*27*), with minor variations. Gel plugs were destained by incubation (twice for 30 min) with a solution containing 200 mM ammonium bicarbonate in 40% acetonitrile at 37 °C, then being subjected to three consecutive dehydratation/rehydration cycles with pure acetonitrile and 25 mM ammonium bicarbonate in 40% acetonitrile, respectively, and finally dried at room temperature for 10 min. Then, 20 μL of trypsin, at a concentration of 12.5 ng/μL in 25 mM ammonium bicarbonate was added to the dry gel pieces, and the digestion proceeded at 37 °C for 12 h. Peptides were extracted from gel plugs by adding 10 μL of 1% (v/v) trifluoracetic acid (TFA) and incubating for 15 min. Samples (3 μL) were deposited onto MPep Chips prespotted with CHCA (Sunyx, Germany) using the thin layer affinity method (*28*) following the manufacturer's

Table 1. Evaluation of Physiological Features on the Two Triticale Varieties in Response to N-Treatment[a]

	AD7291		Rakita	
	N1	N2	N1	N2
tillers/plant	1.86 a	5.14 b	5.43 c	6.85 d
SPAD index	37.60 a	53.20 c	42.60 b	55.10 d
plant dry weight (g)	4.18 a	7.95 c	6.54 b	9.27 d
protein contents (mg/g)	1.82 a	3.34 c	2.45 b	3.85 d

[a] Values with letters in common in each row are not significantly different ($p<0.05$, LSD test). N1 corresponds to the deficit of nitrogen (6 mg N/day), and N2 corresponds to the normal level (32 mg N/day).

instructions. The MS analysis was performed in a MALDI-TOF (4700 Proteomics Analyzer, Applied Biosystems) mass spectrometer in the m/z range 800 to 4000, with an accelerating voltage of 20 kV. Spectra were internally calibrated with peptides from trypsin autolysis (M^+H^+ = 842.509, M^+H^+ = 2211.104).

Searching was performed in the protein database MSDB using the MASCOT search engine (Matrix Science Ltd., London; http://www.matrixscience.com). A detailed analysis of peptide mass mapping data was performed using GPS ExplorerTM software v 3.5 (Applied Biosystems), allowing the following parameters: species *Viridiplantae*, one missed cleavage, 100 ppm mass tolerance, and cysteine carbamidomethylation and methionine oxidation as possible modifications. The confidence in the peptide mass fingerprinting matches was based on the score level and confirmed by the accurate overlapping of the matched peptides with the major peaks of the mass spectrum.

Statistical Analysis of Protein Expression Data. For statistical treatment and cluster analysis of protein abundance values, the web-based software NIA array analysis tool was utilized ((*29*) available at http://lgsun.grc.nia.nih.gov/anova/index.html). This software tool selects statistically valid protein spots based on analysis of variance (ANOVA). After uploading the data table (Supporting Information) and indication of biological replications, the data were statistically analyzed using the following settings: error model max (average, actual), 0.01 proportion of highest variance values to be removed before variance averaging, 10 degrees of freedom for the Bayesian error model, 0.05 FDR threshold, and zero permutations. First, hierarchical clustering was performed to check the entire data set, and the results were represented in dendrograms using the cluster function of the software. Second, the entire data set was analyzed by PCA using the following settings: covariance matrix type, three principal components, 1-fold change threshold for clusters, and 0.6 correlation threshold for clusters. PCA results were represented as a biplot, with proteins more abundant in those experimental situations located in the same area of the graph. Protein spot data for this analysis were recorded in Supporting Information. Third, pairwise comparisons of protein spot mean expression values were performed with the software tool using the following settings: 0.05 FDR and 1-fold change threshold. Fourth, histograms representing log average protein spot values were downloaded using the software.

RESULTS

Triticale Varieties' Properties. Two triticale varieties differing in the mean content of crude protein in grain (11.27% to AD7291 and 12.29% to Rakita variety without applying nitrogen fertilizers) (*30*) were subjected to different nitrogen fertilization levels. The following parameters were evaluated, and data were analyzed by one-way ANOVA test (**Table 1**): number of tillers per plant, with Rakita forming more tillers per plant (6.85) than AD7291 (5.14) under normal nitrogen level, but both forming less tillers/plant at deficient nitrogen level (N1) being significantly lower in AD7291; SPAD index, with a clear tendency toward increasing at the higher level of nitrogen nutrition in both varieties; the plant dry weight and the protein content in the flag leaf tissue were also significantly lower at N1 than N2 nitrogen levels.

Two-Dimensional Gel Electrophoresis and Mass Spectrometry Analysis. According to the results of small sized gels (**Figure 1**), a deeper analysis was performed by using the narrowest pH gradient, 5 to 8 and 17 cm in length. For each of the conditions analyzed (genotypes and treatments), three replicates corresponding to independent protein extracts were made. Following CBB staining of the gels, an average of 495 ± 113 spots was resolved (**Figure 2**).

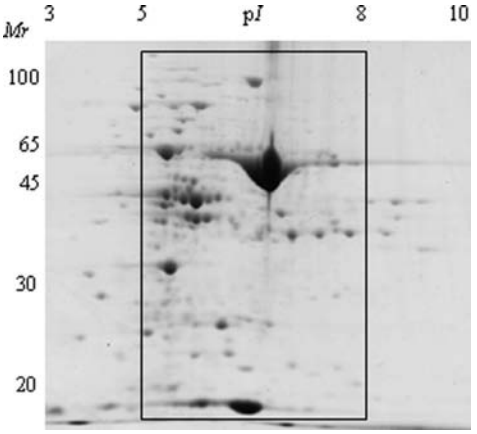

Figure 1. Representative 2-DE gel CBB stained in the 3−10 pH range corresponding to the flag leaf proteome from Rakita plants. Most proteins were concentrated in the 5 to 8 pH range.

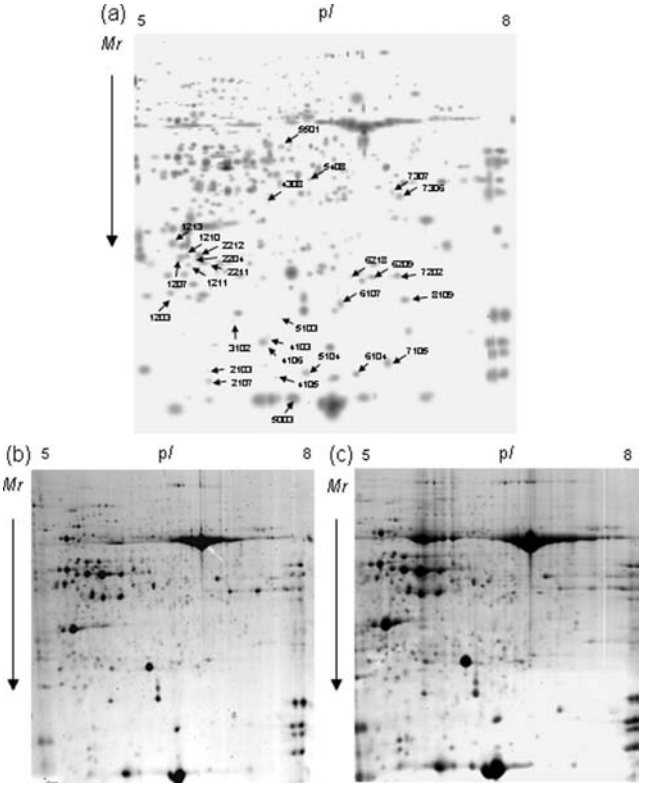

Figure 2. Location of 29 variable protein spots on a virtual two-dimensional (2-D) gel (**a**). Representative Coomassie-stained 2-D protein gels: (**b**) AD7291 variety; (**c**) Rakita variety.

After normalization of protein spot images using PDQuest software and manual verification, 29 potentially differential (qualitative and quantitative) protein spots were detected. To illustrate this, **Figure 3** shows SSPs 2107, 5408, and 6218 on real gels, and **Figure 2** shows all of the differential protein spots on virtual gels. **Table 2** summarizes the features of the experiment.

Statistical Analysis of Protein Abundance and Expression Cluster Analysis. The 29 differential protein spots were processed to

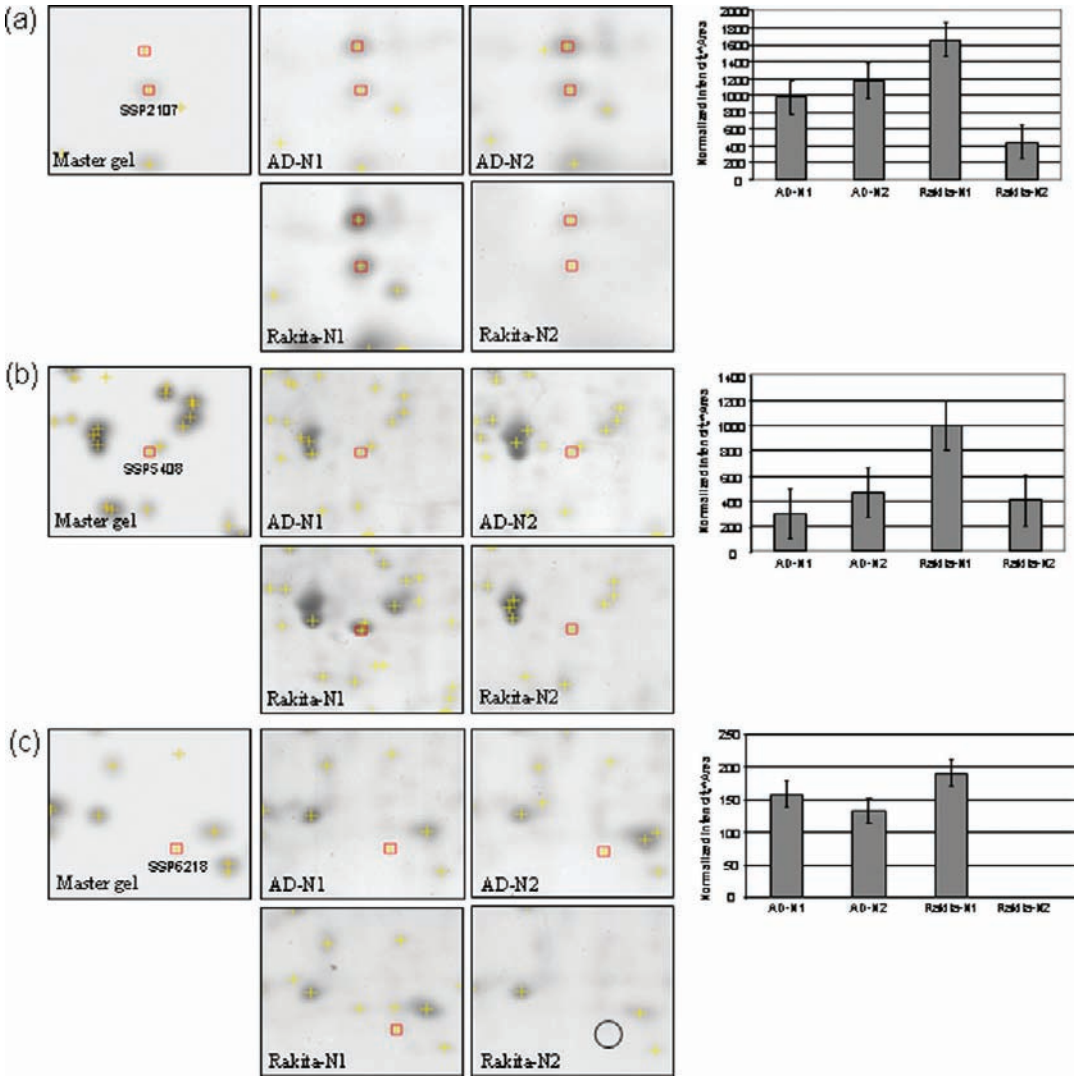

Figure 3. Protein spot images and expression profiles. Three protein spots were selected as examples to illustrate differential expression profiles: (**a**) SSP 2107; (**b**) SSP 5408; (**c**) SSP 6218. On the left, close-up regions of the Coomassie-stained 2-D gels are shown (from the left to right and top to bottom: AD-N1, AD-N2, Rakita-N1, Rakita-N2). The positions of the protein spots on the gels are marked by cross hairs. The respective differential protein spots, matched throughout all presented gels, are marked by boxed cross hairs if they were detectable and by a circle if they were not detectable. On the right, graphs of mean expression values for the respective protein spot are shown (standard deviations were calculated from the means of the three repetitions).

select protein spots with significant and reproducible patterns across the biological repetitions using the web-based NIA array analysis software tool developed by Sharov and co-workers (*29*). This software uses analysis of variance (ANOVA) for statistical analysis of a large data set with multiple variables and subjects. Expression cluster analysis can be performed by a variety of methods including principal component analysis (PCA) (*29*).

First, we used the NIA array analysis tool for hierarchical clustering of biological experiments and their repetitions. We found that the experimental conditions could be divided into two large clusters in a dendrogram, namely, cluster 6 (RakitaN2 and RakitaN1) and cluster 5 (ADN2 and ADN1) (**Figure 4a**). This clustering indicated that AD7291 plants (clustering closest together) had protein expression profiles similar to those shown by Rakita plants (clustering closest together) grown under the two experimental conditions (N1 and N2 nitrogen levels) across the experiments. The hierarchical clustering of biological repetitions confirmed that the data were reproducible for the experiments (Supporting Information).

Protein Identification and Analysis with Respect to Expression Patterns. All of the 29 differential protein spots were analyzed by MALDI-TOF, and the MS spectra were used to screen a *Viridiplantae* index of the nonredundant MSDB database (**Table 3**). Of the 29 protein spots analyzed, 14 were successfully identified with a high probability score for 12 of them. A number of proteins were represented by more than one spot with slightly different *Mr* and p*I* values, suggesting that these changes in the proteome can be attributed to post-transcriptional modification or different members of the same functional family (small shift in the p*I*). Thus, for example, the protein triose-phosphate isomerase (P46225) matched the spots 1211 and 2211, the ascorbate peroxidase (Q945R5) protein matched the spots 1210, 2204, and 2212, and the superoxide dismutase (Q96123) protein matched the spots 2103 and 2107 (**Table 3**).

The identified proteins fall into 6 functional categories (**Table 3**): photosynthesis, glycolisis/glyconeogenesis, metabolism of cofactors and vitamins, amino acid metabolism, metabolic process, and stress-related proteins, with the latter being the largest. Considering the PCA analysis (**Figure 4b**), redox and stress regulation proteins were found in the categories of proteins correlated with PC1 (negative direction) and PC2 (positive direction). Photosynthetic proteins were found in the categories

Table 2. Summary of the Features of the Two-Dimensional Experiment

feature	number of protein spots
average of total spots resolved by 2-DE gels CBB stained	495 ± 113
average of spots consistently present in all three replicates	320 ± 85
differential protein spots[a]:	
total	29 (6% of spots resolved)
between genotypes under N1 nitrogen level	18
between genotypes under N2 nitrogen level	17
in AD7291 under deficit of nitrogen (N1)	6
in Rakita under deficit of nitrogen (N1)	20
total identified spots	14
unique proteins[b]	10

[a] The 29 differential abundant proteins were selected using the web-based NIA array analysis software. N1 nitrogen level corresponds to the deficit of nitrogen (6 mg N/day), and N2 corresponds to the normal level (32 mg N/day). [b] The number of different proteins identified.

of proteins correlated with PC1 (positive and negative direction). Metabolic proteins were found in the categories of proteins correlated with PC2 (positive and negative direction) and PC3 (positive direction). According to the method described by Sharov and co-workers (*29*), the degree of protein expression change within a specific PC was measured by the slope of regression of log-transformed protein expression versus the corresponding eigenvector multiplied by the range of values within the eigenvector. If the degree of protein expression change exceeded the 1-fold change threshold (as defined in the settings), the protein spot was considered to be associated with the PC (**Figure 4c**). Mean log expression intensities for all 29 protein spots identified by PCA are shown in **Figure 5**. In short, we found associations with protein spot expression patterns in the majority of the identified proteins.

Stress and redox regulation proteins were found in all of the pairwise comparisons of expression (**Figure 6**) except the AD7291 N1 versus N2 nitrogen level, with 5 out of 6 more abundant in

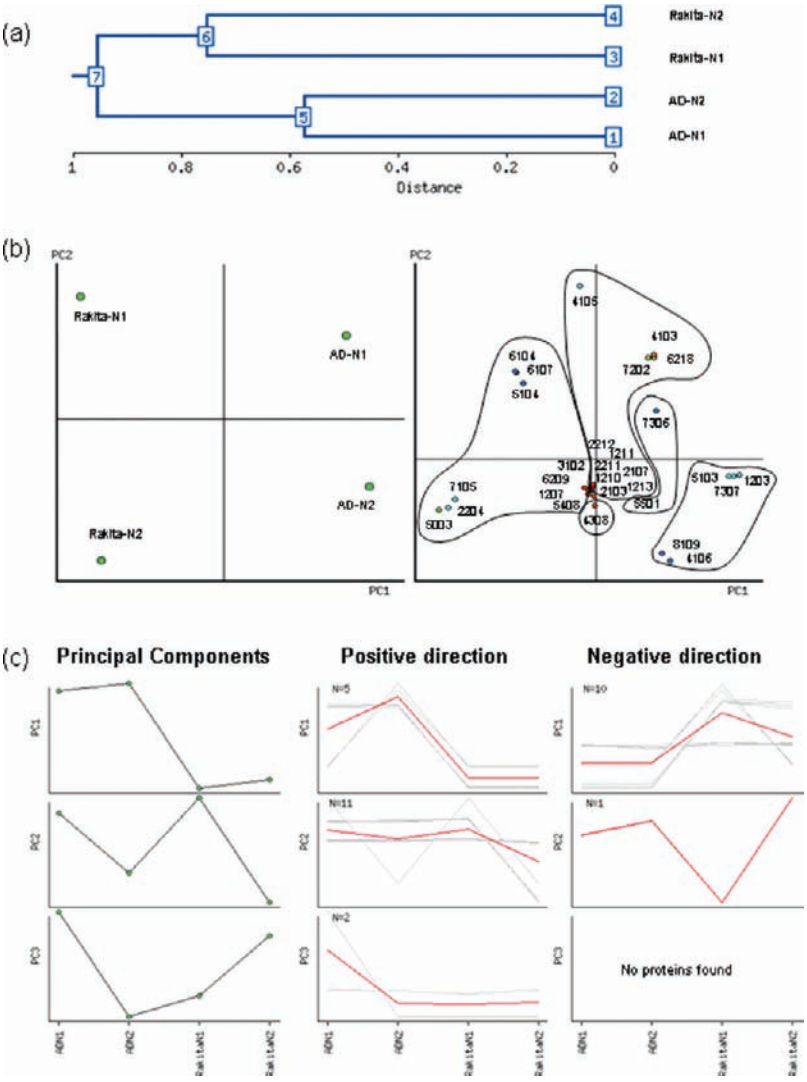

Figure 4. Statistical protein expression cluster analysis of varieties and nitrogen-deficiency responses using the ANOVA-based NIA array analysis tool (*29*). (**a**) Dendrogram showing hierarchical clustering of experimental conditions. The expression clusters are numbered from 1 to 7. (**b**) Two-dimensional biplots showing associations between experimental samples and protein spots generated by principal component analysis (PCA). Samples (left) and protein spots (right) were plotted in the first two component space. A short distance between samples and protein spots in the component space is indicative of similarity in expression profiles. Protein spots correlating together and in the same direction as a PC group are enclosed by a line. (**c**) Protein spot expression clustering based on PCA. For each PC, two clusters of proteins were identified that were positively and negatively correlated with the PC. Protein clustering was performed sequentially starting from the first PC. Proteins that were already clustered with a PC were not included in the clusters associated with subsequent PCs. Protein spots identified in this analysis are recorded in Supporting Information.

Table 3. List of Identified Proteins Classified According to Their Function[a]

SSP	protein function	species (MSDB accession number)	score/% coverage	*Mr*/p*I* experimental (theoretical)	functional category
4106	cytochrome B6−F complex like-protein	*Hordeum vulgare* (O48611)	133/91	22.1/6.1 (10.0/5.8)	photosynthesis
5003	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	*Triticum aestivum* (Q9FRZ5)	231/72	18.4/6.4 (19.7/9.1)	photosynthesis
5103	ribulose 1,5 bisphosphate carboxylase/oxygenase	*Solmsia calophylla* (Q95A34)	197/49	24.0/6.3 (23.6/6.9)	photosynthesis
1211	triose-phosphate isomerize, cytosolic	*Secale cereale* (P46225)	171/45	29.4/5.6 (31.9/6.0)	glycolysis/ glyconeogenesis
2211	triose-phosphate isomerize, cytosolic	*Secale cereale* (P46225)	259/65	29.6/5.7 (31.9/6.0)	glycolysis/ glyconeogenesis
5501	glutamate-1-semialdehyde 2,1-aminomutase	*Hordeum vulgare* (P18492)	62/28	47.6/6.1 (49.7/6.4)	metabolism of cofactors and vitamins
4308	cysteine synthase	*Triticum aestivum* (P38076)	203/60	37.6/6.2 (34.2/5.5)	amino acid metabolism
1213	putative hydrolase	*Oryza sativa* (Q94I53)	96/18	31.8/5.4 (41.4/9.2)	metabolic process
5408	heat shock 70 kDa protein, mitochondrial	*Solanum tuberosum* (Q08276)	65/18	40.5/6.5 (73.3/6.4)	stress related
1210	ascorbate peroxidase	*Hordeum vulgare* (Q945R5)	241/53	30.3/5.6 (28.0/5.1)	redox and stress related
2204	ascorbate peroxidase	*Hordeum vulgare* (Q945R5)	175/43	29.9/5.6 (28.0/5.1)	redox and stress related
2212	ascorbate peroxidase	*Hordeum vulgare* (Q945R5)	97/46	30.4/5.7 (28.0/5.1)	redox and stress related
2103	superoxide dismutase	*Triticum aestivum* (Q96123)	103/46	20.5/5.7 (20.4/5.3)	redox and stress related
2107	superoxide dismutase	*Triticum aestivum* (Q96123)	95/28	19.7/5.7 (20.4/5.3)	redox and stress related

[a] Experimental mass (*Mr*, kDa) and p*I* were calculated with PDQuest software (BioRad) and standard molecular mass markers. The software assigns a standard spot number to each spot (SSP). Theoretical values were retrieved from the protein database.

Rakita N1 versus the N2 nitrogen level. A high proportion of protein spots not identified (14 out of 15) were found to be regulated by variety factor, while a significant number of them (13) were found to be regulated by nitrogen level factor. In general, a clear trend toward greater abundance of proteins could be observed in the Rakita variety compared with that of AD7291, as well as under nitrogen deficit.

DISCUSSION

Physiological data of both varieties growing on different nitrogen levels (see **Table 1**) show that the nitrogen level effect was highly significant for the number of tillers per plant as well as for the SPAD index values. A clear tendency toward increasing of these parameters at the higher level of nitrogen nutrition could be observed. Similarly, more leaf dry matter was accumulated in the N2 level of fertilization during the tillering and stem elongation period.

The relationship between crop growth and the amount of nitrogen applied has been determined in innumerable experiments and has been frequently discussed (*31*, *6*). However, an analysis of the metabolic and physiological aspect of plant response to nitrogen supply is necessary for the development of methods to improve N-application to crops, in breeding and for assessing the potential of genetic manipulation, to improve N-use efficiency and genetic yield potential (*6*).

The soluble protein profile of triticale flag leaf tissue was studied by using 2-DE and mass spectrometry. The results discussed here are limited, according to the methodology used, to just a small soluble fraction of the whole proteome, determined by the extraction protocol and the 2-DE separation technique utilized, with 5−8 p*I* and 10−100 kDa *Mr* as the best range to resolve most of the solubilized proteins, as was revealed in previous experiments, and above the detection limit for Coomassie staining. With these experimental conditions, an average of 495 spots was resolved, this being the distribution pattern observed in leaf tissue from other plant species (*32*, *33*).

For statistical cluster analysis, we employed a software tool designed for the analysis of biological gene chip data (*29*), although it is also well suited to the analysis of protein abundance data. In our work, the three replicates from each experiment condition yielded similar protein expression profiles (**Figure 4c**). Thus, 29 protein spots showed reproducible differential expression behavior in pairwise comparisons of experimental conditions, which were selected for further MS analysis.

With regard to protein identification, about 79% of identified proteins corresponded to cereal species, 43.1% of which belonged to *Secale cereale* and *Triticum aestivum*, and the remaining 35.9% belonged to *Hordeum vulgare*, a cereal grain closely related to rye and wheat.

Out of the 29 analyzed spot proteins, only 14 could be matched against the MSDB database. This low percentage of identified proteins is typical of those species which are absent or underrepresented in public databases. However, a high percentage of identified proteins (79%) matched with specific proteins of wheat, rye, and barley, and in most cases, a close similarity between experimental and theoretical *Mr* and p*I* values was observed.

We discuss each functional group and the behavior pattern observed for the conditions studied (varieties and response to nitrogen nutrition level). Protein differentiating varieties matched photosynthesis (spots 4106, 5003, and 5103), metabolism (spots 1211, 1213, 4308, and 5501), and stress-related proteins (spots 1210, 2103, 2107, 2204, and 5408). A small subunit of RubisCo protein (spot 5003) was identified in larger amounts in Rakita and another form of RubisCo (spot 5103) in smaller amounts when varieties were compared. Up to 75% of leaf nitrogen is found in the chloroplasts (*34*, *35*), most of it invested in ribulose bisphosphate carboxylase alone. Consequently, lower rates of photosynthesis under nitrogen limitation conditions are often attributed to reduction in chlorophyll content and Rubisco activity (*36*−*38*, *8*). This could occur in the AD7291 variety, as revealed by our proteomic results, with the photosynthetic complex cytochrome B6−F like-protein (spot 4106) decreasing in N1 level conditions. However, none of the photosynthetic proteins identified were found to be decreased in the Rakita variety under nitrogen deficit. Rakita shows other physiological features, regardless of the nitrogen nutrition level, as it forms more tillers per plant than AD7291 and higher dry weight and higher content of protein per gram of fresh flag leaves. Nevertheless, we suggest that Rakita showed a better adaptive mechanism to the nitrogen deficit.

Glutamate-1-semialdehyde 2,1-aminomutase (GSAT) (spot 5501) is the second enzyme in the C(5) pathway of tetrapyrrole biosynthesis found in most bacteria, in archaea, and in plants (*39*). These chloroplastic enzymes are involved in chlorophyll synthesis. However, no relationship has been found with photosynthetic

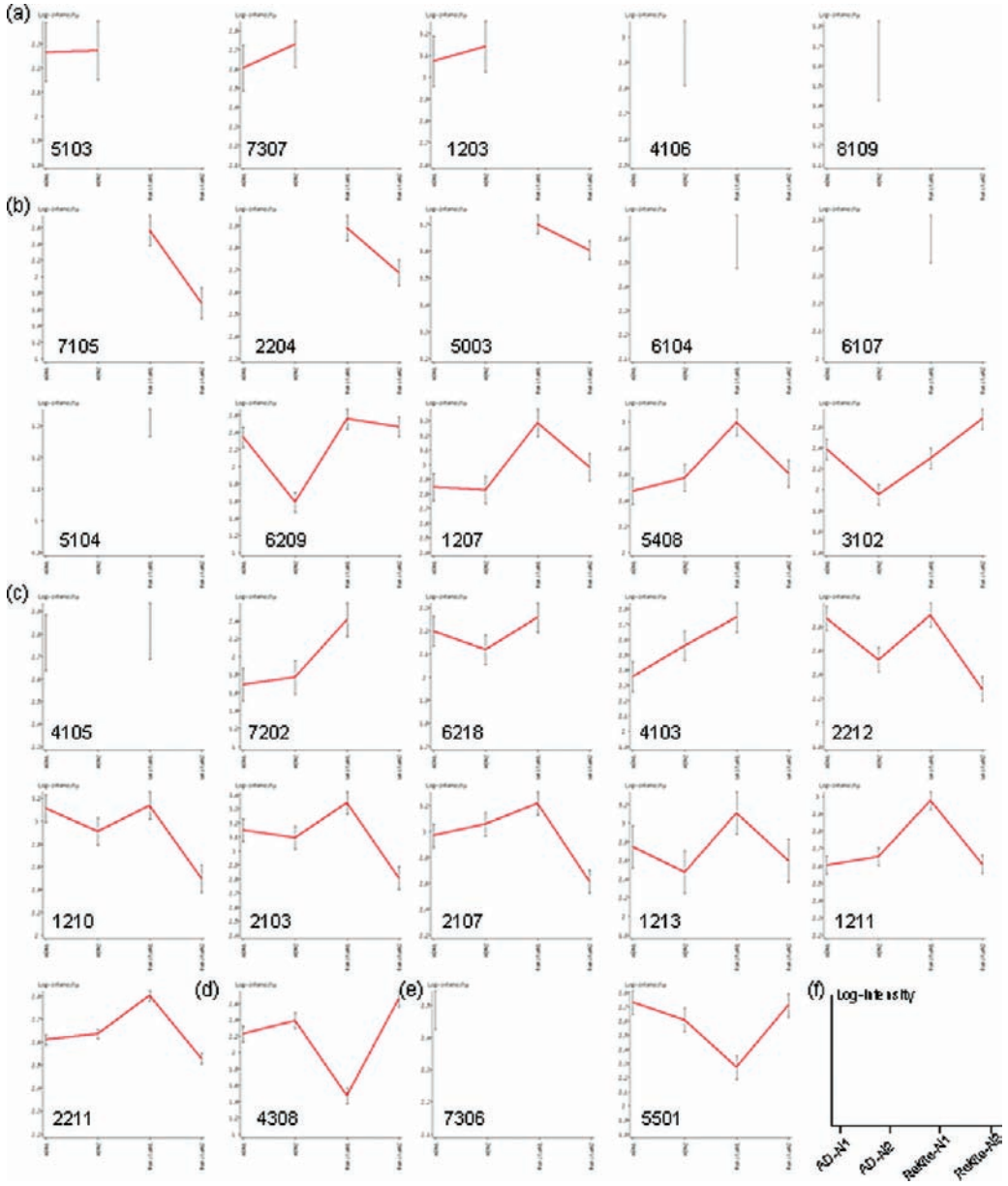

Figure 5. Mean log expression intensities for protein spots identified by PCA and pairwise comparisons. (**a**) Protein spots positively correlated with PC1. (**b**) Protein spots negatively correlated with PC1. (**c**) Protein spots positively correlated with PC2. (**d**) Protein spots negatively correlated with PC2. (**e**) Protein spots positively correlated with PC3. (**f**) Legend for graphs in **a**−**e**. The mean log intensity values were calculated from the sample replications.

efficiency, as reported by Nogaj and co-workers (*40*). They found the rate of chlorophyll synthesis in *Chlamydomonas reinhardtii* not directly controlled by the expression levels of the mRNAs for GSAT or by the cellular abundance of this enzyme protein. In our work, GSAT makes a difference between both triticale varieties studied under deficient nitrogen level (N1), which was found in a higher amount in the AD7291 variety than in Rakita. Also, we found lower levels of this protein in Rakita under deficit nitrogen conditions.

Proteins belonging to different metabolic processes such as triose-phosphate isomerase (spots 1211 and 2211) and putative hydrolase (spot 1213) were higher in the Rakita variety growing at N1 nitrogen level conditions. Cytosolic triose-phosphate isomerases are enzymes involved in biosynthesis and degradation of carbohydrates, fatty acid biosynthesis, and/or the oxidative pentose phosphate pathway. Several enzymes of these ATP-generating pathways, including triose-phosphate isomerase, were seen to be induced upon saline and water stress in cultured cells of rice and in maize (*41*, *42*). This coordinated induction is thought to be essential for the activation of the entire energy-producing pathway to maintain homeostasis in stressed cells. However, the change in accumulation of triose-phosphate isomerase contrasts with a recent proteomic study performed on wheat by Prinsi and co-workers (*20*). These authors found that the induction of this enzyme was positively related to nitrogen availability. This discrepancy could be associated with different genetic traits of the species utilized, as well as possibly being linked to different experimental approaches adopted in the two studies.

Only one identified enzyme, cysteine synthase (spot 4308), was found to be decreased in Rakita under N1 level conditions. Cysteine and methionine are mandatory for the synthesis of proteins in plastids. The synthesis of the C/N-backbone of cysteine is catalyzed by cysteine synthase, a multienzyme complex formed by two enzymes, serine acetyltransferase and *O*-acetylserine, and is provided through serine, thus directly coupling photosynthetic processes and nitrogen metabolism to sulfur

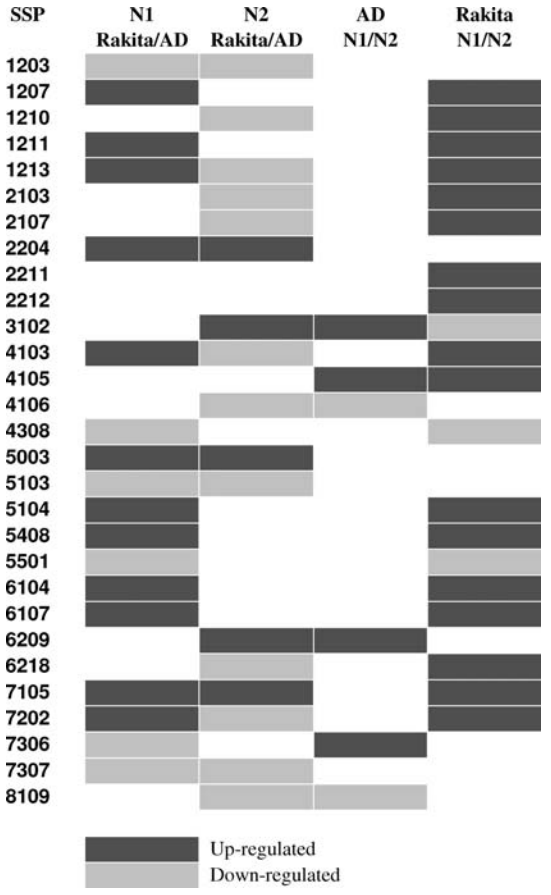

Figure 6. Expression patterns for 29 protein spots based on pairwise comparisons of their protein expression values. Four pairwise comparisons were selected from hierarchical clustering results. The shading illustrates whether protein spots were more abundant (dark gray), less abundant (light gray), or not regulated (white) in the respective pairwise comparison. The first column represents protein expression changes when comparing Rakita with AD varieties under N1 nitrogen level conditions, the second column Rakita versus AD under N2 nitrogen level conditions, the third column AD under N1 versus N2 nitrogen level, and the fourth column Rakita under N1 versus N2 nitrogen level.

assimilation (*43*, *44*). Nakamura and co-workers (*45*) found that transcript accumulation of four rice genes encoding cysteine synthase was coordinately regulated by the availability of sulfur, nitrogen, and light. Bahrman and co-workers (*17*) found that the induction of methionine synthase was positively related to nitrogen availability. This is in agreement with our results, and we found the same behavior in cysteine synthase.

A group of antioxidant enzymes and stress response such as ascorbate peroxidase (APX), superoxide dismutase (SOD), and heat shock protein (Hsp) was increased under N1 level conditions in the Rakita variety. The imposition of abiotic stresses such as drought, cold, and heat, can give rise to excess concentrations of reactive oxygen species (ROS) in plant cells, which are potentially harmful since they initiate the peroxidation and destruction of lipids, nucleic acids, or proteins. In a plant, APX and SOD are very well functioned antioxidant enzymes that protect the cells from a wide range of biotic and abiotic stresses including from ROS. There are many works relating antioxidant enzymes to the resistance to various abiotic stresses in different plants (*46*−*49*). Similarly, the 70 kDa Hsp has been related to biotic and abiotic stresses (*50*, *51*). Specifically, there are some works that observed changes in SOD and Hsp proteins and transcripts in rice and wheat under different levels of nitrogen nutrients (*52*, *53*, *19*). This response is not seen in the AD7291 variety, again suggesting a different adaptive mechanism to nitrogen deficits.

CONCLUSIONS

Triticale is today producing yields equivalent to or better than those for wheat or barley, and differences with respect to other cereals such as wheat in nitrogen accumulation at low levels of nitrogen application have been reported. Our work provides a first step to understand the molecular and physiological control of nitrogen assimilation under varying nitrogen supply conditions in the varieties studied. We found differences in the protein profile of leaf flag tissue and in response to nitrogen fertilization in the two triticale varieties studied related to the physiological features of each. Thus, 29 spot proteins changed when varieties and treatments (different nitrogen nutrition level) were compared, with 24 of them altered under a deficit of nitrogen level. Our analysis showed that nitrogen-dependent protein spots were characterized by different expression patterns as well as by different protein functions. The enzymes of biosynthetic pathways (cysteine synthase) decreased, and enzymes belonging to energy-producing pathways (triose-phosphate isomerases and hydrolase) were seen to be induced in the Rakita variety under N1 conditions to maintain homeostasis in stressed cells, as observed in other abiotic stresses. We found a general increase in the Rakita variety of stress-related proteins when nitrogen availability was low, with no changes to the photosynthetic proteins. However, a decrease in the chloroplastic enzymatic complex was found in AD7291 under nitrogen starvation. Besides the proteomics results, based on the physiological parameters, we suggest that Rakita showed a better efficiency in the use of nitrogen, especially at low fertilizer rates, which can be used by breeders to improve nitrogen use efficiency and to adapt agricultural practices to reduce the use of nitrogen fertilizers, limiting pollution risks. Nevertheless, integration of physiological, agronomic, and biochemical (genomic and proteomic) approaches to study the whole-plant nitrogen response using a large collection of candidates will be essential to elucidate the regulation of nitrogen use efficiency and to provide key target selection criteria for breeding programs.

ABBREVIATIONS USED

APX, ascorbate peroxidase; ANOVA, analysis of variance; CBB, Coomassie Brilliant Blue; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1propane sulfonate; CHCA, α-cyano-4-hydroxycinnamic acid; DTT, dithiothreitol; FDR, false discovery rate; GSAT, glutamate-1-semialdehyde 2,1-aminomutase; Hsp, heat shock protein; IEF, isoelectric focusing; IPG, immobilized pH gradient; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; PCA, principal component analysis; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SOD, superoxide dismutase; SPAD, soil plant analysis development; TCA, trichloroacetic acid; TFA, trifluoracetic acid; 2-DE, two-dimensional electrophoresis.

ACKNOWLEDGMENT

The MS analysis was carried out in the UCO-SCAI proteomics facility, a member of the ProteoRed network.

Supporting Information Available: Data set of protein expression intensity values for protein spots selected due to prospective differential expression behavior and data of protein expression cluster analysis based on PCA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review January 19, 2010. Revised manuscript received March 4, 2010. Accepted March 10, 2010. We thank University of Cordoba and Junta de Andalucía for financial support (AGR-164 Agricultural and Plant Biochemistry and Proteomics). H.K.K. was a Ph.D. student at Agricultural University-Plovdiv, Bulgaria with an Exchange Study Mobility in Frame of ERASMUS grant.