

Diagonal two-dimensional electrophoresis (D-2DE): a new approach to study the effect of osmotic stress induced by polyethylene glycol in durum wheat (*Triticum durum* Desf.)

N. S. Kacem^{1,2} · S. Mauro¹ · Y. Muhovski¹ · F. Delporte¹ · J. Renaut³ · A. Djekoun² · B. Watillon¹

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Abstract Acclimatization to stress is associated with profound changes in proteome composition. The use of plant cell and tissue culture offers a means to investigate the physiological and biochemical processes involved in the adaptation to osmotic stress. We employed a new proteomic approach to further understand the response of calli to dehydration induced by polyethylene glycol (PEG6000). Calli of three durum wheat genotypes Djenah Khetifa, Oued Zenati and Waha were treated with two concentrations of polyethylene glycol to mimic osmotic stress. Changes in protein relative abundance were analyzed using a new electrophoretic approach named diagonal two-dimensional electrophoresis (D-2DE), combined with mass spectrometry. Total proteins were extracted from 30-day-old calli from three durum wheat genotypes that showed contrasting levels of drought stress tolerance in the field. The combination of one-dimensional electrophoresis and D-2DE gave a specific imprint of the protein extracts under osmotic stress, as well as characterizing and identifying individual target proteins. Of the variously expressed proteins, three were selected (globulin, GAPDH and

peroxidase) and further analyzed using qRT-PCR at the transcriptome level in order to compare the results with the proteomic data. Western blot analysis was used to further validate the differences in relative abundance pattern. The proteins identified through this technique provide new insights as to how calli respond to osmotic stress. Our method of study provides an original and relevant approach of analyzing the osmotic-responsive mechanisms at the cellular level of durum wheat with agronomic perspectives.

Keywords *Triticum durum* · Osmotic stress · PEG · Diagonal two-dimensional electrophoresis · Mature embryo

Introduction

Durum wheat (*Triticum durum* Desf.) is an important food crop in the Mediterranean area, not only because of the large acreage it covers, but also because of its importance in the human diet. In the semi-arid high plains of Algeria, drought is a serious wheat production problem. The general effects of drought on plant growth are well known, but the effects of water deficit at biochemical and molecular levels are not well understood. Plant species possess distinctive indicators of stress tolerance at whole plant, tissue, or cellular level, reviewed by Ahmad et al. [1]. Understanding the drought tolerance mechanisms of wheat involves isolating and characterizing drought stress-related genes and proteins [2]. Many genes induced by osmotic stress have been identified in plants using various functional genomics strategies [3]. However, the loose correlation between mRNA and protein expression has greatly stimulated the application of proteomics to characterize the response to drought stress.

✉ N. S. Kacem
kacem.nadia@yahoo.fr

¹ Department of Life Sciences, Walloon Agricultural Research Centre, Chaussée de Charleroi, 234, 5030 Gembloux, Belgium

² Laboratory of Genetic Biochemistry and Plant Biotechnology, Faculty of Nature and Life Sciences, University Frères Mentouri Constantine, 25017 Constantine, Algeria

³ Environmental Research and Innovation Department (ERIN), Integrative Biology Facility, Luxembourg Institute of Science and Technology, 41, Rue Du Brill, 4422 Belvaux, Luxembourg

In our study, we started looking at the potential use of proteomic tools for identifying osmotic stress-related proteins in durum wheat calli. Although two-dimensional electrophoresis (2DE) allows thousands of individual proteins to be separated into discrete spots in one gel, it has lately come under critic because of its known limitations such as poor representation of low abundant proteins [4], highly acidic/basic proteins, or proteins with extreme size or hydrophobicity [5], and the co-migration of multiple proteins to a single spot which makes comparative quantification rather inaccurate [6]. Several studies have shown that the best way to use a 2D gel-based approach is either to focus on one or a few particular proteins or to reduce the complexity of the sample [6]. In this paper, we describe a relatively simple and reproducible method that addresses the co-migration drawbacks. It is based on a diagonal electrophoretic technique [7] and combines the differential electrophoretic resolution capabilities of two gel buffers systems used in series. So far as we know, no study has used this method to analyze the differential changes in relative protein abundance under osmotic stress in wheat calli. The method provides new insights into the osmotic—responsive mechanisms at the cellular level of durum wheat.

Materials and methods

Plant material

The experiments were carried out on three durum wheat (*Triticum durum* Desf.) genotypes. Based on field trials, one genotype was classified as drought sensitive (Waha) and two as drought tolerant (Oued Zenati and Djenah Khetifa). The wheat germplasm was obtained from the Technical Institute of Field Crops (ITGC) Institut Technique des Grandes Cultures (Station El-khroub Constantine, Algeria).

Callus induction and in vitro selection procedures

Callus cultures were initiated using mature embryos. The seeds were surface sterilized with 70 % ethanol for 15 min and 12 % sodium hypochlorite (NaClO) for 20 min, and then rinsed five times with sterile H₂O. Mature embryos about 2–4 mm long were aseptically excised and then incubated with the scutellum side down on MS induction medium [8] supplemented with 2 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 3 % sucrose and 2.5 g l⁻¹ phytigel (SigmaAldrich). The pH value of the medium was adjusted to 5.7 prior to autoclaving at 110 °C for 30 min. Cultures were maintained at 25 ± 1 °C under 30 µm⁻² S⁻¹ light provided by cool fluorescent light for a

16 h light period and an 8 h dark period. Each treatment was performed in triplicate. Calli were maintained by subculturing every 20 days on the same MS medium with different concentrations of PEG6000 concentrations (0, 10 and 20 %). For proteomic and transcriptomic analysis, 20 % of PEG6000 was used. The osmoticum was added to the media before autoclaving.

Callus growth

Callus growth was assessed by means of the image analysis technique. Images were captured using a Canon EOS 1100D reflex numerical camera equipped with a 60 mm f/2.8 macro USM objective, and they were analyzed using Image Pro Plus 6.2 software (Media Cybernetics) [9]. The resolution of the numerical image was a 25.5 × 25.5 µm square (equal to the size of 1 pixel). The sample consists of embryogenic callus collected 30 days after tissue culture induction. Surface area, mean diameter and ratio between the maximum and minimum diameters were determined for each callus. Analyses were performed in five repetitions for each variety and each treatment.

Protein extraction and one-dimensional gel electrophoresis (1D SDS PAGE)

The total proteins were extracted from the three durum wheat genotypes after grinding and precipitation in solution of 10 % trichloroacetic acid (TCA) in cold acetone [10]. After centrifugation and rinsing, the vacuum-dried pellets were dissolved in 400 µl of rehydration solution (Urea 7 M, Thiourea 2 M, CHAPS 4 %, Tris 30 mM). The supernatant was harvested after 10 min of centrifugation at 20,000g. The protein concentration was quantified using the Bradford method, with BSA as standard [11].

Bands were revealed with Coomassie Blue R-250 and analyzed using ImagQuant 5 and Photocapt 8 software in order to identify differentially expressed polypeptides among control and stressed calli. The proteins were initially screened by detecting those whose relative abundance had been changed by osmotic stress at the callus stage. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10 % polyacrylamide using the Bio-Rad Protean II xi Cell gel size 16 × 16 cm system using the Laemmli buffer system [12].

Diagonal two-dimensional electrophoresis (D-2DE)

Initially, the protein extracts were electrophoresed on 15 % PAGE in Tris-tricine gel buffer, following the Hoefer system [13]. The zone of interest, between 100 and 25 kDa, was excised from the one-dimensional electrophoresis gel, placed orthogonally on top of a second gel and

electrophoresed using the Laemmli buffer [12]. The signals are visualized by silver staining and *Coomassie Blue R-250*.

Spot excision, digestion and identification of proteins

Manually excised gel fragments were washed and desalted with a 50 mM ammonium bicarbonate solution containing 50 % (v/v) methanol followed by 75 % (v/v) acetonitrile solution and dried at 37 °C. Proteins within each gel spot were digested for 6 h at 37 °C in 8 µl of trypsin Gold (5 ng/µl trypsin in 20 mM ammonium bicarbonate) (Promega, Madison, WI, USA). The resulting peptides were dissolved in 50 % (v/v) acetonitrile containing 0.1 % (v/v) trifluoroacetic acid (TFA), dried at 53 °C and spotted on matrix-assisted laser desorption/ionization (MALDI) plates, 0.7 µl of α -cyano-4-hydroxycinnamic acid [7 mg ml⁻¹ acetonitrile 50 % (v/v) containing TFA 0.1 % (v/v)] was added to each extracted peptide mixture. Digestion and spotting were done using the Tecan freedom EVO200 (Tecan, Männedorf, CH). For mass spectroscopy (MS) analysis, MALDI peptide mass spectra were acquired using the AB Sciex 5800 time-of-flight (TOF)/TOF (AB Sciex, Redwood City, CA, USA). For subsequent MS/MS analysis, 10 most intense peaks, excluding known contaminants, were selected and fragmented.

All spectra, MS and MS/MS, were submitted for database-dependent identification using the NCBI nonredundant protein sequence database (<http://www.ncbi.nlm.nih.gov>) downloaded on June 6th, 2014 (40,910,947 sequences) reduced to the green plants Taxonomy (1717, 798 sequences) using an in-house MASCOT server (Matrix Science, <http://www.matrixscience.com>, London, UK). The parameters used for these searches were mass tolerance MS 100 ppm, mass tolerance MS/MS 0.5 Da, fixed modifications cysteine carbamidomethylation, and variable modifications methionine oxidation, simple and double oxidation of tryptophan, tryptophan to kynurenine. Proteins were considered as identified when at least two peptides passed the MASCOT-calculated 0.05 threshold score of 30. However, when a high-scoring peptide (>2 times the 0.05 threshold) was matched and a protein p-value of $<1 \times 10^{-5}$ was obtained, the protein was also accepted. Subsequently, the MASCOT-based results were manually validated.

Immunoblot analysis

The D-2DE results were validated using Western blot analysis with specific anti-globulin and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies. The samples were analyzed by 1D SDS PAGE using (Bio-Rad mini protean III system) in 10 % polyacrylamide (Laemmli buffer) gel and transferred to the nitrocellulose blotting

membrane (GE Healthcare Life Science) using a Bio-Rad Mini Trans-Blot[®] Cell at a constant current of 350 V for 1 h 15 min. After overnight incubation in blocking solution (phosphate buffered saline, PBS, pH 7.4; 0.5 % Tween-20; 2 % non-fat dried milk), the membranes were incubated with either a primary rabbit anti-globulin polyclonal antibody (Beijing Protein Innovation Corporation) or a goat anti-GAPDH antibody, combined subsequently with horseradish peroxidase (GenScript) diluted 1:1000 in a 5 ml blocking solution for 1 h 30 min and then washed three times in PBS supplemented with 0.5 % Tween-20. Immunodetection was conducted using an enhanced chemiluminescence (ECL) and Western blotting detection system (Amersham Biosciences, UK).

RNA extraction, first strand cDNA synthesis and qRT-PCR analysis

Total RNA was isolated from the control and stressed calli using TRI Reagent (MPBio) as specified in the user manual. The quality and purity of the extracted RNA was determined by measuring the absorption at 260 and 280 nm, and the integrity of the preparations was verified with 1.2 % agarose gel electrophoresis. In order to remove possible genomic DNA contamination, 5 µg of total RNA was treated with RNase-free DNase I (Promega) and then subjected to first strand cDNA using random hexamers and the RevertAid First Strand cDNA Synthesis Kit (Life Technologies), following the manufacturer's instructions. The homology search for sequences of the selected clones was performed using the basic local alignment sequence tool (BLAST) (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>; <http://www.plantgdb.org/cgi-bin/blast/PlantGDBblast>). Primer pairs were designed for three selected durum wheat genes (globulin3A, GAPDH and peroxidase) using Primer 3.0 web resource software (<http://bioinfo.ut.ee/primer3-0.4.0/>). Table 1 gives the selected genes and the corresponding designed primers. The transcript accumulation was analyzed using qRT-PCR with a Bio-Rad CFX96 thermocycler. The PCR reactions were performed in triplicate using 0.3 µM of each gene specific primer, 5 µl GoTaq qPCR master mix (Promega) and 100 ng of corresponding cDNA in the final volume of 10 µl.

The cycling conditions were: 2 min at 95 °C, followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s. Melting curve analysis followed each reaction in order to verify its specificity. The actin gene was used as an internal control to normalize all the data and the $\Delta\Delta C_t$ method was used to calculate relative expression levels.

Statistical analysis

The data were analyzed using SPSS20 and MINITAB 17 software. The analysis was carried out using analysis of

Table 1 Primer sequences used in the quantitative real-time PCR (qRT-PCR) analysis to determine the transcript abundance of genes putatively involved in water stress tolerance in wheat

Gene	Forward primers (5'–3')	Reverse primers (5'–3')
Glo3	GCCTCTCCTCGCTAGTTTGA	CTTCTCCTCCTCCTCCTTGC
Actin	ACCTTCAGTTGCCAGCAAT	CAGAGTCGAGCACAATACCAGTTG
GAPDH	CCAGTGCTGCTTGGAATGATG	TGTCCATGCCATGACTGCAA
Peroxidase	ACTGGTTCTGGCCTTCGTTA	ACAGCACGATGCTCTCCAG

Actin transcripts were used as endogenous unregulated references

variance (ANOVA). For each parameter three experimental replicates were performed, followed by a Fisher comparison of means test at 5 % levels. The homogeneous groups were separated using the Newman-Keuls test at 5 % levels.

Results and discussion

Effect of osmotic stress on callus surface growth

Osmotic stress is a common consequence of several abiotic stresses such as drought, high salinity, and low temperature that causes severe cell dehydration and adversely affects plant growth and development. PEG has long been used to investigate the osmoadaptive responses after an osmotic shift or during growth at different osmotic pressures [14].

PEG of high molecular weight is a non-penetrating inert osmoticum. This agent stimulates water stress by acting as osmotic agent which reduces the potential of the medium in where the cells are growing [15]. The response of calli growth to two PEG concentrations was assessed by means of the image analysis technique [9]. Pictures were taken at 30 days after the initiation of the embryogenic callus, digital images were analyzed by using the “Image Pro Plus” software. This non-invasive evaluation of growth allowed us to monitor the evolution of the calli until regeneration without removing them from their in vitro environment, thus without interfering on their development.

The results given in Table 2 showed that there were highly significant differences among the wheat genotypes with distinct responses to two PEG levels (SI and SII, 10 and 20 % PEG, respectively).

Callus growth slowed down with the introduction of 10 % PEG in the culture medium (from 28 to almost 50 %,

compared to control), and slowed further when PEG concentration was increased to 20 % (from 42 to almost 80 %, compared to control) (Table 2). The decrease in growth rate varied from one genotype to another. The most severe growth slowdown was observed in the W variety, where the reduction rate of 48.70 % at the SI stress level (10 % PEG) increased significantly ($p < 0.001$) with increasing stress (SII, 20 % PEG), reaching 78.95 %. The Oued Zenati variety seemed less affected by stress than Waha, with a reduction rate of 39.78 % at the SI level and 59.78 % at the SII level. The Djenah Khetifa variety seemed to be the most tolerant, recording reduction rates of 27.74 and 41.94 % at the SI and SII levels, respectively, and also being the variety that produced the biggest embryogenic calli compared to Oued Zenati and Waha (Table 2). Based on the ability to growth on PEG enriched media, it appears that Djenah Khetifa is the most tolerant genotype under both moderate and high PEG levels (10 and 20 % PEG), followed by Oued Zenati, whereas Waha shows the greatest sensitivity, especially at high levels of PEG (20 %).

These observations suggest that metabolic responses to stress are not general but specific to the genotype. Similar results were obtained in durum wheat callus [16].

Our physiological study was combined with molecular approaches to identify biological factors associated with osmotic stress response in wheat calli.

One-dimensional electrophoresis

1D SDS PAGE can be a useful tool for revealing changes in proteome induced by exposure to osmotic stress induced by polyethylene glycol. Therefore, a comparative 1D SDS-PAGE analysis of total protein extracts was performed on

Table 2 Effects of PEG concentrations on the callus surface (mm^2) in three durum wheat varieties

Genotypes	C	PEG concentrations		Value F	Value $p < 0.05$
		SI	SII		
DK	73.86 $\pm$ 7.66a	53.37 $\pm$ 6.81c	42.88 $\pm$ 7.76d	67.53	0.000
OZ	60.85 $\pm$ 7.08b	36.64 $\pm$ 8.32e	24.47 $\pm$ 10.44f	67.63	0.000
W	43.77 $\pm$ 6.82d	22.45 $\pm$ 4.56f	9.21 $\pm$ 3.79 g	168.08	0.000

Means followed by the same letter are not statistically different according to the Fisher test ($p < 0.05$)

C control, SI 10 % of PEG6000, SII 20 % of PEG6000

callus cultures collected 30 days after culture induction, either without or under 20 % PEG. Obvious differences were observed when comparing Oued Zenati and Waha with Djenah Khetifa (Fig. 1).

1D SDS-PAGE of the water-soluble protein fraction revealed outstanding quantitative and qualitative differences between control and stressed calli in Oued Zenati, more variation in Waha, no detectable change in Djenah Khetifa. The results showed that exposing wheat callus to under 20 % PEG caused the disappearance of some bands, such as the 78, 34, 30 and 29 kDa polypeptides in Waha and the 72 and 29 kDa polypeptides in Oued Zenati. Stress also led to the observation of newly synthesized or more abundant polypeptides that were clearly detected in Waha (37 and 84 kDa) and Oued Zenati (35 and 24 kDa).

No change in the protein relative abundance profile was observed in Djenah Khetifa, the most tolerant variety to PEG6000. In contrast, Oued Zenati and especially Waha (the most susceptible to PEG, Table 2) showed the greatest changes. Together, these results indicate a positive

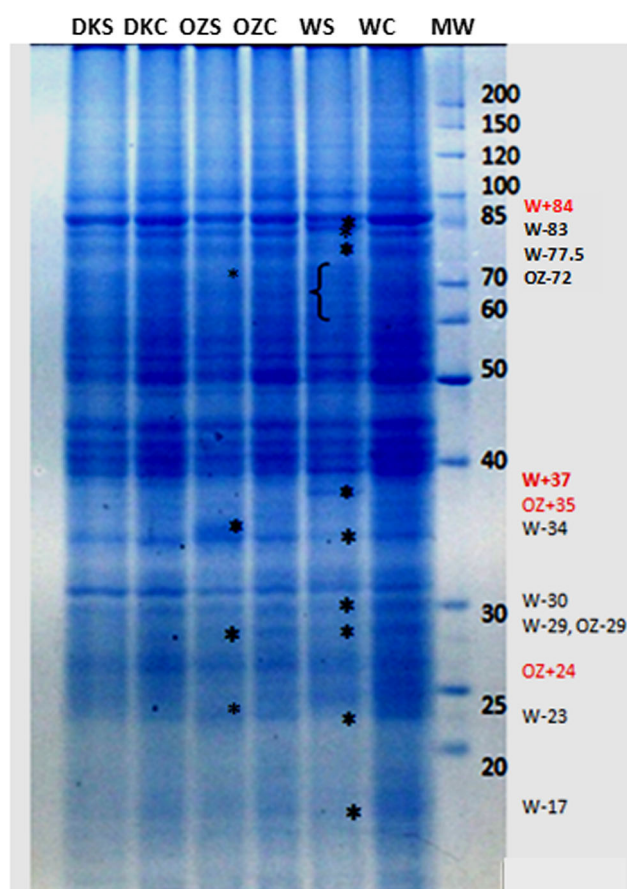


Fig. 1 Electrophoretic pattern of soluble proteins from callus cultures collected 30 days after culture induction, either without or under osmotic stress (20 %PEG). *S* stressed, *C* control, *MW* molecular weight markers in Kilo Daltons, Oued Zenati-OZ, Djenah khetifa-DK and Waha-W

correlation between the changes in the overall protein profile and water stress response at the cellular/tissue levels. These results are in agreement with those reported by Ashrafi et al. [17], where the SDS-PAGE analysis showed that resistant genotypes in experimental fields had lower variation in the seed protein bands than sensitive genotypes that had the highest variation. Some experimental evidence suggests that drought-sensitive species and varieties have higher proteolytic activity than resistant ones [18]. The presence of 20 % PEG affects callus proteins by inducing change (increase or decrease) in the protein bands pattern. Changes in protein expression, accumulation and synthesis have been observed in other species, as rice, after exposure to osmotic stress with PEG6000 [19]. Abdel-Hady and El Neggar [16] found that new proteins induced in response to varying PEG concentrations could be identified and could represent potential biomarkers for drought tolerance in wheat cultivars. Plomion et al. [20] suggested that water stress caused profound changes in cellular metabolism, as well as protein functions. They also reported that many genomic regions were involved in a complex regulation of these changes at the molecular level. It is possible that the synthesis of new proteins under stress conditions in susceptible varieties plays a role in the plant's response to water stress and could constitute a form of adaptation to stress. Stress in plants could be defined as any change in growth conditions that alters or disrupts metabolic homeostasis. This requires an adjustment of metabolic pathways, aimed at achieving a new state of homeostasis, in a process that is usually referred to as acclimation [21]. Some newly synthesized proteins under stress conditions in susceptible varieties might be interpreted as potential candidates in the plant's response to water stress; i.e. the stress signaling networks and cascades that are induced in the attempt to re-establish or maintain cellular homeostasis under water deficiency. A relationship has been reported to exist between the accumulation of osmotic-induced proteins and physiological adaptations during water-deficit stress. From a practical point of view, as stated by Abdel-Hady and El-Naggar [16], the newly induced protein bands could be used as markers for indirect selection for osmotic tolerance induced by PEG and this could support the development of drought-tolerant wheat cultivars.

Two-dimensional electrophoresis

The individual proteins, which were separated by 1D SDS-PAGE, are often associated, *in vivo*, with each other, forming temporary, or stable larger protein complexes. Thus it is difficult to ascertain which proteins form a complex through IEF/SDS-PAGE alone [22]. In the present study, we describe a new electrophoretic procedure that

improves the separation, identification and relative quantification of interesting proteins. We combined two successive electrophoreses within a single experiment, resulting in a method we named ‘diagonal two-dimensional electrophoresis’ (D-2DE), which was subsequently combined with mass spectrometry.

Like any 2D electrophoresis approach (e.g., NE, 2D-DIGE) that aims to map the proteome of a biological system given in the form of spots, this method compares the proteomes of the same biological system (Oued Zenati variety) under different conditions. We chose to analyze the gel area between 25 and 100 kDa because this was the area where most changes in protein accumulation due to water stress were observed.

1D SDS-PAGE is used to separate denatured proteins on the basis of their molecular weight. The first migration was conducted in Tris tricine SDS-PAGE. Tricine, used as the trailing ion, allows a resolution of small proteins at lower acrylamide concentrations than in the Tris glycine-SDS-PAGE system.

1D SDS-PAGE Tris–glycine and Tris-tricine methods are widely used for separating high- and low-molecular mass proteins, respectively. The different separation characteristics of the two techniques relate directly to the strongly differing pK values of the glycine and tricine functional groups that define the electrophoretic mobilities of the trailing ions (glycine and tricine) in relation to the electrophoretic mobilities of proteins [23].

The results obtained showed very good reproducibility. Spots revealed with silver nitrate showed better resolution than with Coomassie Blue. We identified 16 conspicuous new spots in the cut area between 25 and 100 kDa in Oued Zenati stressed tissues, and this was compared with the control. Only the colored spots revealed with Coomassie Blue were identified by mass spectrometry (Fig. 2).

The system resolves most polypeptides and allows the rapid characterization of proteins with a wide range of molecular weights. This analysis method, that addresses the co-migration drawbacks, improved separation and gave highly reproducible gels with good spot resolution.

Protein identification

Only those proteins with significant and reproducible changes were considered to be differentially accumulated. The one large spot colored with Coomassie Blue and the eight spots colored with silver nitrate (Fig. 2) showed significant differences between the stressed and control calli. They were manually excised and analyzed using MALDI-TOF.

Although staining with silver nitrate gives a better resolution than Coomassie Blue staining, the identified proteins were obtained only from gels stained with Coomassie

Blue. The amount of protein was probably too low or the staining protocol incompatible with MALDI. These identified proteins included: glyceraldehyde-3-phosphate dehydrogenase (*Brassica juncea*) (spots 1 and 2); *Triticum urartu* globulin 1S allele (spots 1, 2 and 3), globulin 3-A (*Triticum aestivum*) (spots 3 and 4). Also, in spot 3 and 4 were identified predicted protein (*Hordeum vulgare*), we searched them against Genbank and it revealed a 95 % match with peroxidase (*Triticum urartu*), *Triticum aestivum* cDNA clone H01_1125_plate_3, mRNA sequence (spots 1 and 3) was identified as globulin-1S in *Setaria italica* or globulin 2 in *Zea mays*, with an 81 and 80 % homology, respectively, in spot 4 were identified as the hypothetical protein.

Functionally, the identified proteins were related to protein synthesis and carbohydrate metabolism; the hypothetical protein could be involved in drought stress but what functions they actually have requires further investigation [24]. Details of these identified proteins are given in Table 3. The difference in the mobility of some spots could be attributed to post-translational modifications or allelic variations resulting from in vitro culture maintenance.

Globulins

Two forms of globulins—globulin 1S (gi474323981) and globulin 3A (gi390979705)—were identified under the effect of osmotic stress (Fig. 3). This production could play a role in the acquisition of tolerance to dehydration. Analysis of the two sequences showed a 40.5 and 30.6 % amino acid similarity and identity, respectively (Fig. 3a).

The function of GLB1 is unknown; it is believed to serve as a storage protein [25]. The best characterized monocot globulins are the maize embryo globulins (GLB) 1 and 2, each encoded by a single gene (*Glb1* and *Glb2*) homologous to each other and to globulins of dicots [26]. The three most commonly occurring *Glb1* alleles are designated L, I and S (for Large, Intermediate and Small proteins, respectively). By convention, the protein product of each allele is indicated in the upper case (e.g., *Glb1-S* encodes GLB1-S) [27].

Immunofluorescence studies have found that Glo-3 gene products are located in the developing wheat seed embryo and aleurone layer [28]. A recent study identified Globulin 3A as the first candidate wheat protein to be associated with the development of Type 1 diabetes (T1D) [29].

It has been reported that the exposure of Type I callus (embryogenic callus) to abscisic acid or water stress induced the production of globulin 1 [30]. Few studies have shown an over-expression of globulins under stress, but several have reported this protein being less expressed under stress. Zhang et al. [31] analyzed the accumulation of various storage protein classes from mature grains in wheat

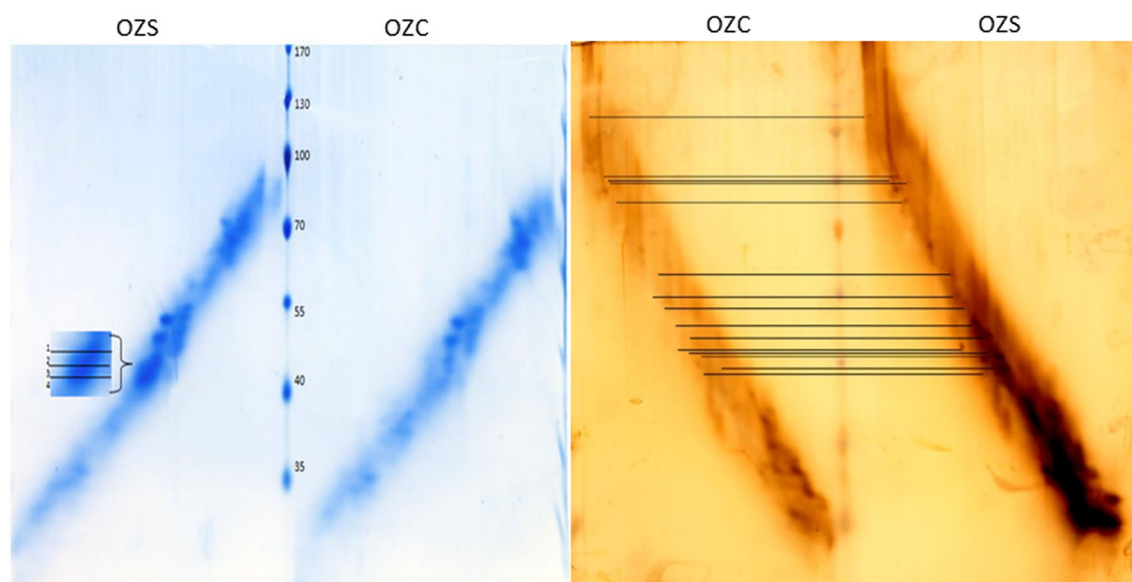


Fig. 2 Diagonal two-dimensional electrophoresis (D-2DE) of total proteins of callus expressing new proteins (*S* stressed) compared with the control (*C*). Proteins were visualized using *Coomassie Blue* (gels on the *left*) and silver nitrate staining (gels on the *right*). Circles and a

zoomed-in view show the proteins of interest identified by MALDI-time-of-flight (TOF)/TOF. The numbering corresponds to differentially expressed proteins. Gels were scanned using Typhoon 9400 (excitation wavelength of 532 nm)

Table 3 Proteins identified by MALDI-TOF/TOF from D-2DE gel electrophoresis of *Triticum durum* calli

Spot ID	Protein name	gilnumber	Species	Mass	Scor	Peptid
B1	Glyceraldehyde-3-phosphate dehydrogenase	gil51102306	<i>Brassica juncea</i>	6719	107	K.LVSWYDNEWGYSTR.V
	Globulin 1S allele	gil474323981	<i>Triticum urartu</i>	55,586	70	R.AFLQPSHHDAEIAFVR.E
		gil414873301				K.VLERFDHELLR.G
		gi/215398468				K.EGEGVIVLLR.G
B2	cDNA clone H01_1125_plate_3, mRNA sequence	AL818841	<i>Triticum aestivum</i>	14,447	113	K.ALPRPFDEVSR.L
						R.AFLQPSHYDAEIAFVR.E
	Globulin 1S	gil474323981	<i>Triticum urartu</i>	55,586	93	R.AFLQPSHHDAEIAFVR.E
B3	Glyceraldehyde-3 phosphate dehydrogenase	gil51102306	<i>Brassica juncea</i>	6719	72	K.EGEGVIVLLR.G
						K.LVSWYDNEWGYSTR.V
	Globulin 3-A	gil390979705	<i>Triticum aestivum</i>	66,627	213	K.ALPRPFDEVSR.L
B4	Globulin 1S	gil474323981	<i>Triticum urartu</i>	55,586	60	R.SFHALAQHDVR.V
						R.DTFNLLEQRPK.I
						K.VLERFDHELLR.G
	Predicted protein	gil326521510	<i>Hordeum vulgare</i>	37,339	156	K.DAFFGQFAR.S
	cDNA clone H01_1125_plate_3, mRNA sequence	AL818841	<i>Triticum aestivum</i>	14,447	94	R.AFLQPSHYDAEIAFVR.E
						R.AFLQPSHYDAEIAFVR.E
	Globulin 3	215398470	<i>Triticum aestivum</i>	66,652	154	K.ALPRPFDEVSR.L
	Predicted protein	326521510	<i>Hordeum vulgare</i>	37,339	91	R.SFHALAQHDVR.V
						R.DTFNLLEQRPK.I
						K.VLERFDHELLR.G
	Hypothetical protein	307105297	<i>Chlorella variabilis</i>	88,357	61	R.GLGDVADLVALSGAHTIGR.S

and found that albumin and gliadin concentrations increased significantly under drought stress, whereas globulin and glutenin did not markedly change in rice and oat, the high content of globulin storage proteins in the grains might contribute to the high nutritional value compared with other cereals, such as barley and wheat [32]. Mushtaq et al. [33] reported that globulin showed a 58 % decrease under osmotic drought stress conditions in rice. The globulin-2 proteins were identified in the proteomic study of a dough liquor fraction prepared from wheat flour [34]. Interestingly, the levels of these proteins in the dough liquor increased when salt or salt plus ascorbate was included in the dough mixture. Salt is frequently included

Fig. 4 Alignments of the amino acid sequences of TaGlob3A and TuGlob1S (a), TaGlob3A, TuGlob1S and rice REG960 (b), and TaGlob3A, TuGlob1S and REG100 proteins (c). Identical amino acids are shown with a *black* background; positive amino acids are shown with *gray* background

in commercial processes to improve breadmaking properties and has been shown to influence water absorption, dough development time, dough stability and loaf volume [35]. Another investigation established that Globulin-2, a member of the cupin superfamily of proteins with similarity to known food allergens, was up-regulated from high temperature, reinforcing the hypothesis that there is a

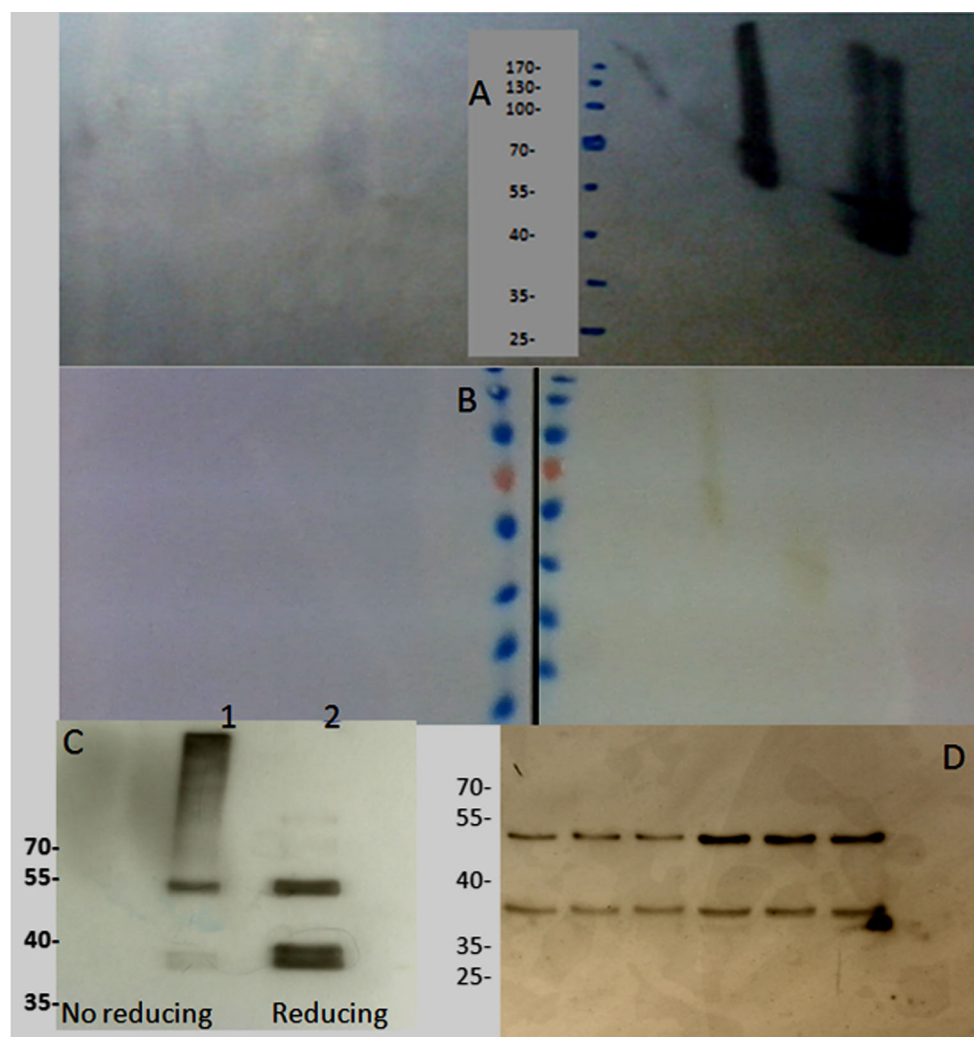


Fig. 3 **a** Western blot analysis with REG 960 antibody on D-2DE gel electrophoresis; control (*left*), stressed (*right*). **b** Nitrocellulose membrane of the Western blot analysis in A; appearance of luminescence on stressed sample (*left*), but not on the control (*right*). The amount of luminescence detected was directly proportional to the amount of protein produced by the gene, being white under drought stress. **c** The extracts were fractionated on non reducing *I* or reducing

2 gel and immunoblotted with REG960 antibodies to show that globulins induced under drought stress were not connected by disulfide bonds. **d** Western blot analysis of GAPDH, with the upper band for stressed samples (4, 5, 6) showing more accumulation than the low band, compared with the control (1, 2, 3). Total proteins extracted from the OZ callus were fractionated using SDS-PAGE (10 %), in triplicate

A

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TuGlob1S : --MKSAVRSWVLAIVLSLCLSLSFASMDADVGRGSR-----RWQSG-----GRS----- : 50
TaGlob3A : MATARARVILFLGLTSLIAFAAAVSASHDEEDRRGSRSLQQCVQRCCQDRPRYSHARCVCQECRDDQQQHGHEGEGEGRGHGRHGEGEREEQGRGRGRGRG : 119
          4 V P L L L AS D E R G R
          *      *      *      *      *      *      *      *      *      *
          20    40    60    80    100   120
          *      *      *      *      *      *      *      *      *      *
TuGlob1S : -----SGR-PYHFGQESYREWAKSRIGHEKVLERDEEL-LRGSIGDYRVAMLDAAFRALFQSHHDAEIAFVREGGVILLNNGRKSRFIREGQDVIVLPAGSVIVYANTHR : 158
TaGlob3A : EGERDEEHGSGRRPYVFCGRFFRRIISDGHFVLRERFEDVSRLLRGLRGVYRVAMLVNRAFVFLSLTDADVGVYVQEGGVILLNNGRKSRFIREGQDVIVLPAGSVIVYANTHR : 238
          GR PY FG S5R 4S HG K L FD L I 1YRVA 6 P PRAF6 P DAD 6 5V 2GEGVL 66 NG 4 S5 6R2GDVIV PAGS16 ANT
          *      *      *      *      *      *      *      *      *      *
          140  160  180  200  220  240
          *      *      *      *      *      *      *      *      *      *
TuGlob1S : SKALRVVMLINVSHPGRFQSEELIGSGDERPQSELSVFSDEVIQAALNRRERDVRVEESKSKGEGE-----IYEAEEQIRELSRSCSRSGGGG--SGSEKEDIQSRSLFGKPKR : 270
TaGlob3A : RRLLVIAKIIHITISVPGKFOFSSAK-----ELASLSKRVIAAALTSRDLGSLGSRQGRGSEEEKSISIVRAEEQIRELSRSCSRSGGGG--SGSEKEDIQSRSLFGKPKR : 349
          4 L 6 66 6S PG4FQ F L S V6 AAL T E 6 6 S4 E E I ASEEQ6REL R S G G
          *      *      *      *      *      *      *      *      *      *
          260  280  300  320  340  360
          *      *      *      *      *      *      *      *      *      *
TuGlob1S : YSNKHGHEHITGDOCHLRKLDMLVITNITRGSMTALKYTRSRIRIVVVEGRDGYFEMACPHISSSGSRERRRHEQERERSHGQGRSSEERSEEGRGRSRSEERBOQHGRFOQK : 389
TaGlob3A : TANRHGRDYADARSFHLAQEDVYVAVNITIGSMTAPYLNTQSKLNVLEG-EGEVDIVCPHLGRSS-ERRRERRRHEQERERSHGQGRSSEERSEEGRGRSRSEERBOQHGRFOQK : 456
          N4HGR 2 H L D6 V 6 NIT GSMTA T S 46 VV6EG G EG CPH6 ERR E EHGR R E2 RR 2 R E22
          *      *      *      *      *      *      *      *      *      *
          360  380  400  420  440  460
          *      *      *      *      *      *      *      *      *      *
TuGlob1S : SRERQVRAQIVSVSIVLPSGHPATFAGNDG-NLRILSGHANNDEEFVFGSNVVKOLDEAANAALPQARELADRVIRAOPE-SVIVAGPQOO-----FVAGD----- : 493
TaGlob3A : QQCHETVRAVSEGSFAFVFPCHPVVLESSRSNLLVVEEENRNERVWLGRRNNVIAKLLDDPAQELTFRRPAREVQEVFRKDCDEGVFVAGPEQOQHEHRSDDRGRGRGDE : 575
          Y VRA 6 GS V6P GHP 6A G NL 66 F 6 A 1E V56 G N V6 LD A L F ARE6 2 FVAGP2QQ R R D
          *      *      *      *      *      *      *      *      *      *
          480  500  520  540  560  580
          *      *      *      *      *      *      *      *      *      *
TuGlob1S : ----- : -
TaGlob3A : AVEAFRLMATAAL : 588
          600

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B

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TuGlob1S : --MKSAVRSWVLAIVLSLCLSLSFASMDADVGRGSR-----RWQSG-----GRS----- : 47
REG960 : --LAKKISSSMARSQAAALILCLSLASNAVWSR-----ERELEDEDEER----- : 49
TaGlob3A : MATARARVILFLGLTSLIAFAAAVSASHDEEDRRGSRSLQQCVQRCCQDRPRYSHARCVCQECRDDQQQHGHEGEGEGRGHGRHGEGEREEQGRGRGRGRG : 117
          m vtp 6 l L sl fa s d e vrg R g E ed2g r h
          *      *      *      *      *      *      *      *      *      *
          20    40    60    80    100   120
          *      *      *      *      *      *      *      *      *      *
TuGlob1S : -----SGSRPYVFCGRFFRRIISDGHFVLRERFEDVSRLLRGLRGVYRVAMLVNRAFVFLSLTDADVGVYVQEGGVILLNNGRKSRFIREGQDVIVLPAGSVIVYANTHR : 154
REG960 : -----GSEGRPYVFCGRFFRRIISDGHFVLRERFEDVSRLLRGLRGVYRVAMLVNRAFVFLSLTDADVGVYVQEGGVILLNNGRKSRFIREGQDVIVLPAGSVIVYANTHR : 157
TaGlob3A : RGEGERDEEHGSGRRPYVFCGRFFRRIISDGHFVLRERFEDVSRLLRGLRGVYRVAMLVNRAFVFLSLTDADVGVYVQEGGVILLNNGRKSRFIREGQDVIVLPAGSVIVYANTHR : 234
          G ggrPFhFG eSR w 43rHG fkvLerF d 6 6g YRVA 6eaaPRAF6qPsh DADe6 5V 2GEGV6v66rnG 4eSSc6R2GDv6ViPAGS16ysA
          *      *      *      *      *      *      *      *      *      *
          120  140  160  180  200  220
          *      *      *      *      *      *      *      *      *      *
TuGlob1S : NTIRKRVVMLINVSHPGRFQSEELIGSGDERPQSELSVFSDEVIQAALNRRERDVRVEESKSKGEGE-----IYEAEEQIRELSRSCSRSGGGGSGSEK--EDIQSRSLFGKPKR : 264
REG960 : NTIRKRVVMLINVSHPGRFQSEELIGSGDERPQSELSVFSDEVIQAALNRRERDVRVEESKSKGEGE-----IYEAEEQIRELSRSCSRSGGGGSGSEK--EDIQSRSLFGKPKR : 261
TaGlob3A : NTIDGRKIVIAKHGRIYADARSFHLAQEDVYVAVNITIGSMTAPYLNTQSKLNVLEG-EGEVDIVCPHLGRSS-ERRRERRRHEQERERSHGQGRSSEERSEEGRGRSRSEERBOQHGRFOQK : 343
          Nth s4wlr6vm66np6StPG F2esf g rp sfls fsd V6gAaINTrR 6 efes ge gel AsEEQ6RELS4scSRg gggsgse i p sL
          *      *      *      *      *      *      *      *      *      *
          240  260  280  300  320  340
          *      *      *      *      *      *      *      *      *      *
TuGlob1S : NTIRKRVVMLINVSHPGRFQSEELIGSGDERPQSELSVFSDEVIQAALNRRERDVRVEESKSKGEGE-----IYEAEEQIRELSRSCSRSGGGGSGSEK--EDIQSRSLFGKPKR : 264
REG960 : NTIRKRVVMLINVSHPGRFQSEELIGSGDERPQSELSVFSDEVIQAALNRRERDVRVEESKSKGEGE-----IYEAEEQIRELSRSCSRSGGGGSGSEK--EDIQSRSLFGKPKR : 261
TaGlob3A : NTIDGRKIVIAKHGRIYADARSFHLAQEDVYVAVNITIGSMTAPYLNTQSKLNVLEG-EGEVDIVCPHLGRSS-ERRRERRRHEQERERSHGQGRSSEERSEEGRGRSRSEERBOQHGRFOQK : 343
          Nth s4wlr6vm66np6StPG F2esf g rp sfls fsd V6gAaINTrR 6 efes ge gel AsEEQ6RELS4scSRg gggsgse i p sL
          *      *      *      *      *      *      *      *      *      *
          360  380  400  420  440  460
          *      *      *      *      *      *      *      *      *      *
TuGlob1S : RGEKPRVSNKHGHEHITGDOCHLRKLDMLVITNITRGSMTALKYTRSRIRIVVVEGRDGYFEMACPHISSSGSRERRRHEQERERSHGQGRSSEERSEEGRGRSRSEERBOQHGRFOQK : 381
REG960 : RGEKPRVSNKHGHEHITGDOCHLRKLDMLVITNITRGSMTALKYTRSRIRIVVVEGRDGYFEMACPHISSSGSRERRRHEQERERSHGQGRSSEERSEEGRGRSRSEERBOQHGRFOQK : 358
TaGlob3A : LEQNPFIANRHGRDYADARSFHLAQEDVYVAVNITIGSMTAPYLNTQSKLNVLEG-EGEVDIVCPHLGRSS-ERRRERRRHEQERERSHGQGRSSEERSEEGRGRSRSEERBOQHGRFOQK : 445
          tg P sN HG4L 2 tgd chhL kLD6 6 6aNITrGSMTAp ynTrst46aV62G Gyf66aCPH6s g SERR erErEHG gRr erEeeggr 2
          *      *      *      *      *      *      *      *      *      *
          480  500  520  540  560  580
          *      *      *      *      *      *      *      *      *      *
TuGlob1S : SRERQVRAQIVSVSIVLPSGHPATFAGNDG-NLRILSGHANNDEEFVFGSNVVKOLDEAANAALPQARELADRVIRAOPE-SVIVAGPQOO-----FVAGD----- : 487
REG960 : SRERQVRAQIVSVSIVLPSGHPATFAGNDG-NLRILSGHANNDEEFVFGSNVVKOLDEAANAALPQARELADRVIRAOPE-SVIVAGPQOO-----FVAGD----- : 464
TaGlob3A : SSSSGGEEQDQOQCHETVRAVSEGSFAFVFPCHPVVLESSRSNLLVVEEENRNERVWLGRRNNVIAKLLDDPAQELTFRRPAREVQEVFRKDCDEGVFVAGPEQOQHEHRSDDRGRGRGDE : 562
          ghG e Ek rry VRAQ6 gsviV6PagHPat 6As nLa66cF 6gAn 1E V56aGrNsv6 qLDdpAk L Fg Are adrv aagp2 f6aGP qq
          *      *      *      *      *      *      *      *      *      *
          600
          *      *
TuGlob1S : --RWQSG-----GRS----- : 493
REG960 : --GSVSDM----- : 470
TaGlob3A : GDRRCGRGRGDEAVEAFRLMATAAL : 588
          rrv Dm

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C

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Taglobulin : MATGRATIPFLGLTSLFAAAVSASHDEEDRRGSRSLQQCVQRCCQDRPRYSHARCVCQECRDDQQQHGHEGEGEGRGHGRHGEGEREEQGRGRGRGRG : 115
TuGlobulin : MATARARVILFLGLTSLIAFAAAVSASHDEEDRRGSRSLQQCVQRCCQDRPRYSHARCVCQECRDDQQQHGHEGEGEGRGHGRHGEGEREEQGRGRGRGRG : 49
osl00ABF98 : MATARARVILFLGLTSLIAFAAAVSASHDEEDRRGSRSLQQCVQRCCQDRPRYSHARCVCQECRDDQQQHGHEGEGEGRGHGRHGEGEREEQGRGRGRGRG : 99
          matr rati ll lfaaa as edrr sl rc q4c qdrP y Arc6qEC d qqq e r hggh rR e rG g eed2GR r
          *      *      *      *      *      *      *      *      *      *
          20    40    60    80    100   120
          *      *      *      *      *      *      *      *      *      *
Taglobulin : EGERDEEHGSGRRPYVFCGRFFRRIISDGHFVLRERFEDVSRLLRGLRGVYRVAMLVNRAFVFLSLTDADVGVYVQEGGVILLNNGRKSRFIREGQDVIVLPAGSVIVYANTHR : 230
TuGlobulin : EGERDEEHGSGRRPYVFCGRFFRRIISDGHFVLRERFEDVSRLLRGLRGVYRVAMLVNRAFVFLSLTDADVGVYVQEGGVILLNNGRKSRFIREGQDVIVLPAGSVIVYANTHR : 150
osl00ABF98 : EGERDEEHGSGRRPYVFCGRFFRRIISDGHFVLRERFEDVSRLLRGLRGVYRVAMLVNRAFVFLSLTDADVGVYVQEGGVILLNNGRKSRFIREGQDVIVLPAGSVIVYANTHR : 202
          Gsr rRPYVFG rS5R 4SdhG v4 L pFd s llrgI 1YRVA 6eanPRAF6 P htDAd 6 5Va2GEGV6 66eNGe4 S5 6R2GDVIVaPAG3I
          *      *      *      *      *      *      *      *      *      *
          120  140  160  180  200  220
          *      *      *      *      *      *      *      *      *      *
Taglobulin : YHANTGRKRVVMLINVSHPGRFQSEELIGSGDERPQSELSVFSDEVIQAALNRRERDVRVEESKSKGEGE-----IYEAEEQIRELSRSCSRSGGGGSGSEKEDIQ : 337
TuGlobulin : YHANTGRKRVVMLINVSHPGRFQSEELIGSGDERPQSELSVFSDEVIQAALNRRERDVRVEESKSKGEGE-----IYEAEEQIRELSRSCSRSGGGGSGSEKEDIQ : 260
osl00ABF98 : YHANTGRKRVVMLINVSHPGRFQSEELIGSGDERPQSELSVFSDEVIQAALNRRERDVRVEESKSKGEGE-----IYEAEEQIRELSRSCSRSGGGGSGSEKEDIQ : 308
          yIaNdgr4kL 6 k6ht6svPG fQ Pfa g p sflsfsK V Aalkts E 6 6lg kg I rASEEQ6RELr asEGG G hwp1ppf s
          *      *      *      *      *      *      *      *      *      *
          240  260  280  300  320  340
          *      *      *      *      *      *      *      *      *      *
Taglobulin : DTNIRKRVVMLINVSHPGRFQSEELIGSGDERPQSELSVFSDEVIQAALNRRERDVRVEESKSKGEGE-----IYEAEEQIRELSRSCSRSGGGGSGSEKEDIQ : 443
TuGlobulin : DTNIRKRVVMLINVSHPGRFQSEELIGSGDERPQSELSVFSDEVIQAALNRRERDVRVEESKSKGEGE-----IYEAEEQIRELSRSCSRSGGGGSGSEKEDIQ : 373
osl00ABF98 : DTNIRKRVVMLINVSHPGRFQSEELIGSGDERPQSELSVFSDEVIQAALNRRERDVRVEESKSKGEGE-----IYEAEEQIRELSRSCSRSGGGGSGSEKEDIQ : 417
          r pfn61e24P4 aN4HGRLy2adarsfH La hD6rVa6vNIT GSMtAp ynTrS 46avV6eG ege E6vCPH6sr gr s2eRerhGkG4wreEeEe rq r
          *      *      *      *      *      *      *      *      *      *
          360  380  400  420  440  460
          *      *      *      *      *      *      *      *      *      *
Taglobulin : --RSGSGSSEEGDQCHETVRAVSEGSFAFVFPCHPVVLESSRSNLLVVEEENRNERVWLGRRNNVIAKLLDDPAQELTFRRPAREVQEVFRKDCDEGVFVAGPE : 555
TuGlobulin : SEEGEGQGHGRBOQKSRYPYQVRAQIVK3SVLPAGHPATFVAGN--DGALLSGVGGANDEEFVFGSNVVKOLDEAANAALPQARELADRVIRAOPE-SVIVAGPQOO : 485
osl00ABF98 : --RSGSGSSEEGDQCHETVRAVSEGSFAFVFPCHPVVLESSRSNLLVVEEENRNERVWLGRRNNVIAKLLDDPAQELTFRRPAREVQEVFRKDCDEGVFVAGPE : 523
          r g e eE22 qgY6t6RAr6srG3vF6P GHP v as dsNlG66cF 6 Ann1Er656aG NsV6kkLD AkeLAF ARE6 e a qes F6AGP2
          *      *      *      *      *      *      *      *      *      *
          580  600
          *      *
Taglobulin : ----- : 588
TuGlobulin : ----- : 493
osl00ABF98 : KSGRRGSGSDDRRRSHRGRGDEAVEAFRLMATAAL : 562
          e e RRr drgrgdeave lma aa

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connection between the heat stress and the accumulation of some classes of wheat allergens [35].

In order to confirm these results we tested three antibodies (REG 960, REG 100 and REG 410), initially directed against the rice globulin [36]. We chose to work with REG 960 because it gave the best results, showing 68.4 % positivity and 20.8 % identity with Glob S1 and Glob3A (Fig. 4b). The alignment between GlobS1 and Glob3A with REG100 gave 76 and 22.5 % positivity and identity (Fig. 4c). With REG 410, however, the recognition rates were very low. Western blot analysis of the Oued Zenati callus revealed a significant increase in globulin under dehydration induced by osmotic stress. The results also indicated that there is a diverse group of globulins in wheat, some of which could be associated with osmotic stress tolerance.

Glyceraldehyde-3-phosphate dehydrogenase

This enzyme was identified in spots 1 and 2 (Table 2) in the Oued Zenati callus in response to water stress, as well as in unstressed calli. Western blot analysis revealed the presence of two isoforms of GAPDH in the protein extracts. The enzyme accumulation was influenced by stress and, interestingly, the two isoforms that were present were regulated differently. Three isoforms of GAPDH have been identified to date: GAPA, GAPB and GAPC. In contrast to the findings in our study, these three isoforms decreased in wheat seedlings after 3 days of salt stress [37]. For many decades, GAPDH has been regarded merely as a housekeeping glycolytic enzyme found mainly in the cytoplasm. Several recent studies, however, have suggested that it is a multifunctional protein [38, 39]. Our results are in agreement with those reported by Laino et al. [40] who studied the effect of high temperature on durum wheat total leaf proteins and showed that there was a GAPDH increase. Using a proteomic approach, it was established that GAPDH activity in *Arabidopsis thaliana* was inhibited by

H₂O₂, suggesting that GAPDH is a direct target of H₂O₂ and might have a role in mediating reactive oxygen species (ROS) signaling in plants [41]. A secondary function identified for GAPDH is as one of the allergens implicated in Baker's asthma [42].

Peroxidase

Peroxidases catalyze the hydrogen peroxide-dependent oxidation of substrates (RH₂), in line with the general equation $RH_2 + H_2O_2 \rightarrow 2H_2O + R$. Most studies have reported an increase in the activity of this enzyme under drought stress [43, 44]. Plants increase the activity of some enzymes in removing ROS, such peroxidase, by stimulating gene expression in order to alleviate the adverse effects of oxidative stress caused by drought stress. Oxidative damage in plants caused by biotic and abiotic stresses is usually reduced by enzymatic antioxidants (superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase) and by non-enzymatic antioxidants [45]. The ability of antioxidant enzymes to scavenge ROS and reduce their damaging effects might confer osmotic resistance on plants.

These results showed the effect of stress on the expression of this protein, despite the involvement of these proteins in other processes; Budak et al. [46] performed proteomics analysis (IEF/SDS-PAGE) using two wild emmer wheat varieties TR39477 and TTD22, and one durum wheat cv. (Kızıltan). After 9 days of drought exposure, 75 differentially expressed proteins were detected with many being common to all three wheat genotypes, e.g., manganese superoxide dismutase (MnSOD), a glutathione transferase (GST) showed increased expression in the durum wheat.

In order to better understand how plants respond and adapt to abiotic stresses, it is therefore important to pay greater attention to the cellular level.

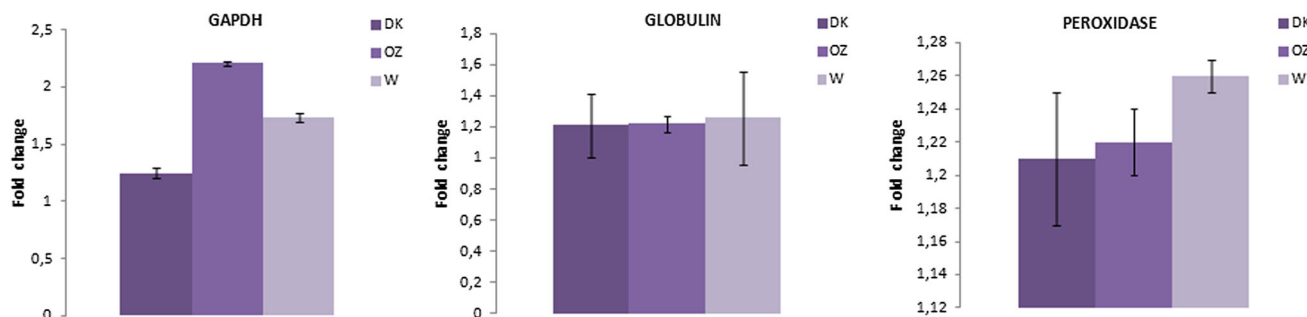


Fig. 5 Fold changes in the accumulation of GAPDH, globulin and peroxidase gene transcripts in the calli of DK, OZ and W under drought stress, obtained using qRT-PCR. The relative quantity of the target gene transcripts was calculated using the comparative $\Delta\Delta C_t$ method. The

stressed calli were quantified relative to the controls after 30 days. Actin was used as an endogenous control to normalize the data for input RNA differences among the samples

qRT-PCR analysis

Three proteins (GAPDH, globulin and peroxidase) identified by MALDI-TOF were examined at the mRNA expression level using qRT-PCR. Target gene expression was quantified using the comparative Ct (threshold cycle value) method of relative quantification ($\Delta\Delta C_t$), as described by Livak and Schmittgen [47]. Primer specificity was tested using conventional PCR, followed by electrophoretic migration through 1.2 % agarose gel. Transcript accumulation on the three selected genes was quantified by qRT-PCR. The results showed that under osmotic stress, GAPDH, globulin and peroxidase were slightly up-regulated (no more than 1.74-fold) over the control, apart from Oued Zenati with GAPDH (Fig. 5). The bars in the histograms indicated no differences between treatments which confirm the reproducibility of the technique.

Generally, the expressions of the GAPDH, globulin and peroxidase protein genes at the mRNA level were not in line with their expression at the protein level.

Our results are in accord with the works of Budak et al. [46] for example for RuBisCO; RT-qPCR revealed a marked decrease in transcript levels in all three genotypes upon drought stress. However, at the proteome level, all genotypes exhibited elevated levels in response to drought. Similarly for Glutathione transferase; the transcript and protein levels of genotype TR39477 showed a contrasting pattern of regulation upon drought stress. Thus, GST appears to be downregulated at the transcriptome level but upregulated at the proteome level in response to drought.

Comparative transcriptomic approaches aimed at highlighting differentially expressed genes have been successfully used against both biotic and abiotic stresses [48, 49]. The identification of differentially expressed genes solely, however, is generally not enough to unravel the underlying molecular mechanisms of water-deficit stress because several transcripts are known to undergo transcriptional, translational and post-translational modifications. Thus, the comparative proteomics approach has emerged as a powerful and promising tool for investigating stress response in plants [50–53].

Conclusion

This study described a simple procedure based on the combination of two successive electrophoreses. It is SDS compatible and provides a rapid characterization of proteins with a wide range of molecular weights, even using a very small amount of proteins. Some of the proteins that changed under dehydration induced by osmotic stress are considered to be allergens, such as globulin and GAPDH,

suggesting that people sensitive to these proteins might be adversely affected by wheat products exposed to stress factors; the over expression of globulins under osmotic stress could improve grain nutritional quality. The correlation between mRNA and protein expressions is low because of various factors, such as different half-lives and post-transcription machinery. Sometimes, proteomic analysis might be more appropriate in the study of water stress in wheat than transcriptomic analysis. The analysis of organ-specific protein abundance provided rich information on the response mechanisms of plants to abiotic stress. Greater focus on these proteins at the cellular level (callus) could contribute to a better understanding of how plants respond and adapt to abiotic stresses and could help identify candidate genes for molecular breeding studies.

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