

Role of defense enzymes, proteins, solutes and $\Delta 1$ -pyrroline-5-carboxylate synthase in wheat tolerance to drought

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Abstract Growth parameters of 17-day-old wheat seedlings (Sids4, Sd; Gmiza10, Gm) were similar under normal control conditions; however, water deficit for the following 16 days (up to 33 days after sowing, DAS) led to greater reductions in Gm than in Sd, suggesting that Sd was more tolerant to drought than Gm. At the same time, drought led to significant losses of several proteins in Sd, despite the similarities detected in both cultivars under normal conditions to the extent that 70 % were lost in Gm on the 33rd DAS relative to only 40 % in Sd. In contrast to Sd, drought decreased catalase (CAT) and peroxidase (POX) isozymes and Rubisco as well as the expression of $\Delta 1$ -pyrroline-5-carboxylate synthase (*P5CS*) gene in Gm concomitant with slight effects, if any, on soluble sugars, glycine betaine (GB), free amino acids and proline. Three CAT isozymes and ten POX isozymes were detected in both cultivars; nonetheless, drought down expressed some of these isozymes particularly in Gm. The impacts of drought on Rubisco were greater in Gm than in Sd. The accumulation of soluble sugars, GB, free amino acids and proline by drought was higher in Sd than in Gm and the magnitude increased with drought severity. Also, drought led to a significant rise in *P5CS* in Sd, but caused a decline in Gm. After rewatering, *P5CS* increased in Gm, but decreased in Sd to reach the level of control. The accumulation of proline in Sd synchronous with increases in *P5CS* transcription concludes that drought adaptation is efficient only in the tolerant cultivar. In support, rewatering decreased *P5CS* elevation caused by drought in Sd, concluding that

this mechanism of adaptation is effective only under stress conditions.

Keywords Drought tolerance · Proteins · Osmolytes · Oxidative stress · *P5CS* expression · Wheat

1 Introduction

Drought is a worldwide problem constraining global crop production. It causes reductions in plant growth and inhibitions in cell division (Khedr et al. 2011a), production of reactive oxygen species (ROS) as well as changes in activity and upregulation of antioxidant enzymes (Nemat Alla et al. 2014). ROS typically result from the excitation of O_2 to form 1O_2 or from the transfer of one, two or three electrons to O_2 to form, respectively, O_2^- , H_2O_2 or HO (Mittler, 2002; Nemat Alla et al. 2008). ROS cause cellular damage through oxidation of lipids, proteins and nucleic acids.

To cope with these harsh conditions, plants respond and adapt to stress by complex molecular responses, including changes in isozymal function of antioxidants including catalase (CAT) and peroxidase (POX) and some organic compounds including osmoprotectants or compatible solutes. These substances are small molecules compatible with the cellular metabolism that accumulate in the cytoplasm as osmolytes to balance external osmotic pressure and help organisms survive extreme osmotic stress. CAT and POX participate in the detoxification and elimination of the generated ROS (Mittler 2002; Asada 2006; Nemat Alla et al. 2008, 2014). Among the compatible solutes, glycine betaine (GB) is a particularly effective protectant against abiotic stress and mitigates the damaging effects of oxidative stress by activating or stabilizing ROS-scavenging enzymes and/or

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repressing the production of ROS (Sakamoto and Murata 2002). In addition, carbohydrates that accumulate under various stresses in higher plants (El-Bashiti et al. 2005) confer protective effects by the stabilization of membranes and lipid assemblies at very low hydration and of biological macromolecules (e.g., proteins) in the folded state. Under water stress, a decrease in proteins could reflect either diminished synthesis or increased breakdown, leading to higher levels of free amino acids (Navari-Izzo et al. 1990). Proline functions as a molecular chaperone able to protect protein integrity and enhance the activities of different enzymes. Several studies have attributed an antioxidant feature to proline, suggesting ROS-scavenging activity and proline acting as a singlet oxygen quencher (Matysik, et al. 2002). Proline biosynthesis is controlled by $\Delta 1$ -pyrroline-5-carboxylate synthase (P5CS) and is encoded by the *P5CS* gene (Chen et al. 2009).

Wheat is the first strategic crop in the world; it is the staple food for billions of people. Due to its paramount importance, studying the drought tolerance of wheat is very important for using arid and reclaimed lands in the cultivation of drought-tolerant cultivars to narrow the gap between production and consumption necessities. Therefore, an attempt was performed to check the influence of drought on growth parameters, proteins, defense enzymes activities, compatible solutes as well as *P5CS* expression in two wheat cultivars varying in drought tolerance to find out the basic differences and the regulatory roles of the studied parameters in drought tolerance.

2 Materials and methods

2.1 Plant material and growth conditions

A preliminary experiment was conducted on several wheat cultivars (*Triticum aestivum*) obtained from the Wheat Research Department, Agricultural Research Center, Egypt, using polyethylene glycol (8000) to select the most and the least tolerant cultivar to drought. 30 dry grains of each cultivar exposed to osmotic stress were allowed to germinate in Petri dishes containing about 20 g of dry soil moistened with 10 ml of 15 % PEG solution. The dishes were sealed with parafilm and left at 25 °C for a week for germination. The cultivar which recorded the highest germination percentage was considered as the highest drought-tolerant cultivar, while the one that recorded the lowest germination percentage was considered as the least tolerant one; they were, respectively, Sids4 (Sd) and Gmiza10 (Gm).

Seeds of the two cultivars were sown in a mixture of sand and clay culture (1:1) in 25 cm-diameter plastic pots (10 seeds per pot) under field conditions in a greenhouse. After 10 days, seedlings were thinned to five and watered twice a

week. One week later (on the 17th day after sowing, DAS), the pots were divided into two groups: one was left as control, while the other was restricted to drought by withholding watering for the subsequent 16 days (to 33 DAS). Plants were collected after 0, 3, 6, 9, 13 and 16 days of dehydration (on 17, 20, 23, 26, 30 and 33 DAS, respectively). The design was repeated twice and the treatments were arranged in triplicate such that at least six biological replicates were used for every parameter ($n = 6$). Soil water capacity was measured by drying a known weight of soil sample at 105 °C for 72 h, or until a constant weight was reached. The soil capacity was maintained at 42 % for normal conditions; however, it was maintained under drought conditions at 33, 26, 21, 17 and 13 % on the 20th, 23rd, 26th, 30th and 33rd DAS, respectively.

In a separate experiment, rewatering was resumed 9 days of dehydration (on the 26th DAS) and a recovery sample was collected 2 days later. Sample collection took place 3–5 h after the beginning of the photoperiod. Shoots of some individuals were separated and used for determination of growth parameters (fresh weight, dry weight and relative water content, RWC), while leaves of other individuals were separated, frozen immediately in liquid nitrogen and stored at –80 °C for subsequent analyses.

2.2 Extraction of soluble proteins for SDS-PAGE

Frozen leaves (50 mg) were ground in potassium phosphate buffer (50 mM, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride, 2 mM α -dithiothreitol, 0.1 mM EDTA and 20 % polyvinylpyrrolidone. After centrifugation at $12,000\times g$ for 15 min, the supernatant was used as total soluble protein extract. Protein was resolved as described by Laemmli (1970). Protein content was determined by reaction with Brilliant Blue G and absorbance was measured at 595 nm (Bradford 1976).

2.3 Electrophoretic analysis of catalase, peroxidase and Rubisco

Catalase (CAT) and peroxidase (POX) were analyzed using native polyacrylamide gel electrophoresis (native-PAGE). Total soluble proteins were separated on nondenaturing polyacrylamide gels without mercaptoethanol and SDS using the BioRad Mini Protean 3 equipment (Laemmli 1970). The resolving gel contained 10 % acrylamide and stacking gel contained 4 % acrylamide. Protein was loaded onto the gel and separated at 4 °C.

CAT gels were soaked in 3.27 mM H_2O_2 for 25 min, rinsed twice in distilled water and stained in a freshly prepared solution containing 1 % potassium ferricyanide and 1 % ferric chloride (Anderson et al. 1995). POX gels were washed in distilled water and soaked in a solution

containing 25 mg benzidine dihydrochloride with two drops of glacial acetic acid, 50 ml distilled water and five drops of 1 % freshly prepared H₂O₂ (Larsen and Benson 1970). The proteins of Rubisco were resolved on 12 % acrylamide at 200 V for 30 min. The gels were stained with brilliant blue R-250 (Bio-Rad) and then destained with 20 % methanol. The gels were dried using Bio-Rad gel drier and scanned, and bands were quantified using LabImage V 2.7.2 software.

2.4 Determination of soluble sugars, glycinebetaine, free amino acids and free proline

Soluble sugars were extracted from frozen leaves (50 mg) with 80 % ethanol and centrifuged at 4000×g for 10 min; the supernatant was evaporated to dryness and then resolved in distilled water (Schortemeyer et al. 1997). An aliquot was mixed with anthrone reagent, heated for 10 min, cooled in ice bath for 30 min and the absorbance recorded at 623 nm (Schlüter and Crawford 2001). Glycine betaine (GB) was extracted from frozen leaves with deionized water and centrifuged at 4000×g. The supernatant was diluted to 1:1 with 2 N H₂SO₄, aliquots were cooled in ice water bath for 1 h then cold KI–I₂ reagent was added, stirred and stored at 4 °C for 16 h. After centrifugation at 10,000×g for 15 min, the supernatant was carefully aspirated and the periodide crystals were dissolved in 1,2-dichloroethane and absorbance was measured after 2 h at 365 nm (Grieve and Grattan 1983). Free amino acids were extracted from frozen leaves with 80 % ethanol and centrifuged at 8000×g for 15 min. After cooling to room temperature, the absorbance was read at 570 nm using ninhydrin test (Šircelj et al. 2005). Proline was extracted from frozen leaves with 3 % sulfosalicylic acid and centrifuged at 4000×g. An aliquot was reacted with glacial acetic acid and acidic ninhydrin for 1 h at 100 °C. The reaction was terminated in an ice bath and extracted with 10 ml toluene. The chromophore-containing toluene was warmed to room temperature and absorbance was measured at 520 nm (Bates et al. 1973).

2.5 RNA isolation and cDNA preparation

Total RNAs were extracted from leaves (500 mg) using easy-BLUE™ TRI-reagent (iNtrON Biotechnology) (Chomczynski et al., 1997). DNA was removed from RNA using RNase-free DNaseI (Thermo scientific) following the instruction protocol. The purity of total RNA was assessed at 260/280 nm. The quality of the RNA was assured with ethidium bromide stain analysis by agarose gel electrophoresis. About 0.1–1 µg DNA-free RNA in distilled water (DEPC treated) was added to Maxime RT PreMix tubes with Oligo dT primer to a total volume of 20 µl. The

cDNA synthesis reaction was performed using a Thermal cycler. The mixture was incubated at 45 °C for 60 min followed by 5 min at 95 °C. The reactant was diluted by adding 20–50 µl sterile water then stored at –20 °C for RT-PCR. To check if the cDNAs were properly synthesized, 18S rRNA (amplicon length of about 800 bp) was amplified by PCR conditions. These results were used as positive controls of cDNAs.

2.6 Semi-quantitative PCR for P5CS

The RT-PCR reaction was performed to amplify the *P5CS* gene using the designed primers according to the sequence of *Triticum aestivum P5CS* gene (*wP5CS*) available at the NCBI database <http://www.ncbi.nlm.nih.gov> under the accession number AB193551. The forward and reverse primers sequences were CCAAGTGAACCTTCATCGAA and AACAAAGCAACGTCTCCATTG, respectively. The RT-PCR reactions were performed as follows: initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 45 s, extension at 72 °C for 30 s, and final extension for 5 min at 72 °C. For semi-quantitative analysis, 10 µl of PCR products were collected after 26 and 32 cycles before reaching the plateau phase. For ascertaining equal RNA loading in the RT reaction, 18S rRNA was used as an internal control and the fold expression of the target genes was normalized accordingly. The forward and reverse primer sequences of 18S rRNA were CCACCCATA-GAATCAAGAAAGAG and GCAAATTACCCAATCCT-GAC, respectively. The band volumes of the resolved gel images were measured by LabImage V 2.7.2.

2.7 Statistical analysis

The experiment was repeated twice and the treatments were arranged in triplicates in a complete block randomized design and the mean values ± SE were for $n = 6$. The design consisted of 144 pots [2 cultivars × 2 sets (2 treatments) × 3 pots per set (3 replications per treatment each pot contained 5 plants) × 2 experimental repetitions × 6 intervals]. Analysis of variance (ANOVA) was calculated at $p > 0.05$ for the full data of each interval, starting from the 17th DAS up to the 33rd DAS.

3 Results and discussion

In Fig. 1, there is no great difference in growth parameters of both Sd and Gm wheat cultivars under normal control conditions throughout the whole experiment. The progress in fresh weight, dry weight or RWC of both cultivars was most likely similar with the elapse of time. Nevertheless,

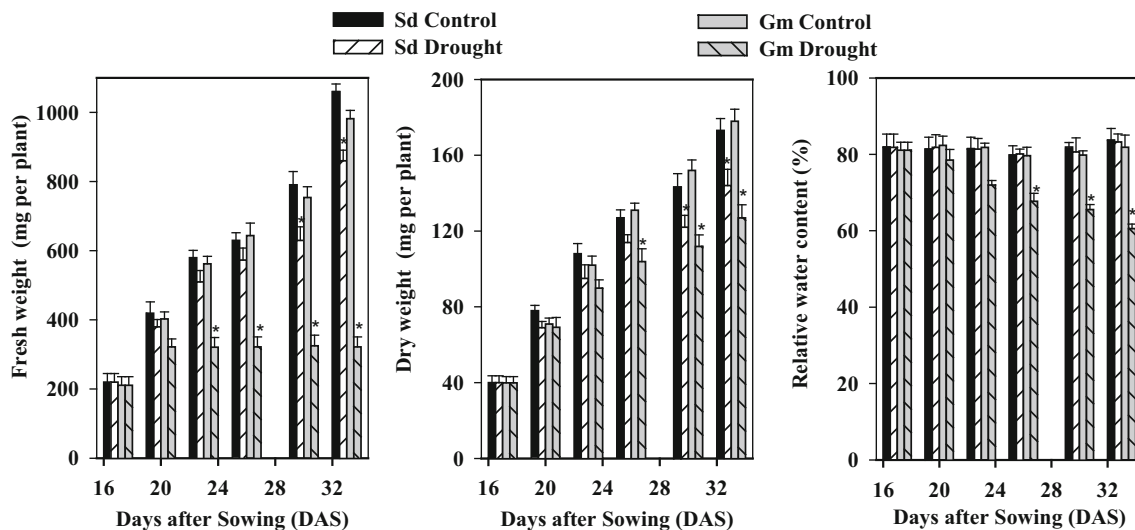


Fig. 1 Changes in fresh weight, dry weight and relative water content of 17-day-old seedlings of two wheat cultivars, Sids (Sd) and Gmiza (Gm), following drought stress for the subsequent 16 days (up to

33 days after sowing, DAS). Values are means ($n = 6$) \pm SE. Asterisk shows significant difference at $P \leq 0.05$ from the untreated control

the progress of these parameters upon imposing water withholding showed differential retractions depending on the cultivar and the duration of drought. Shoot fresh weight of Sd was significantly decreased from the 30th DAS onward, and this reduction was fast in Gm starting from the 23rd DAS. Similarly, drought significantly decreased shoot dry weight of Sd and Gm cultivars from the 30th and the 26th DAS, respectively. In the same pattern, RWC of Sd seemed to be unaffected by drought; however, there was a significant reduction in Gm starting from the 26th DAS. The magnitude of decrease in these growth parameters was usually higher in Gm than in Sd, so that the fresh weight of drought-treated Sd and Gm on the 33rd DAS represented about 81 and 33 % of the respective control value, respectively. Meanwhile, drought reduced the dry weight of Sd and Gm to about 83 and 71 %, respectively, and RWC to about 99 and 74 %, respectively, of the control value.

In general, drought tolerance refers to the degree to which plants adapt to drought conditions. The physiological responses of plants to drought include maintenance of water balance for improving the water-use efficiency, synthesis of compatible solutes and alleviation of ROS as well as activation of genes involved in the perception of drought stress such as that governs the accumulation of proline. The greater reductions in growth of Gm than Sd reveal that Sd could tolerate drought more than Gm. The decrease in fresh weight under drought conditions might suppress cell expansion and cell growth due to the low turgor pressure and reducing meristems (Khedr et al. 2011a). These effects seemed higher in Gm than Sd, probably due to RWC. In confirmation, RWC was significantly reduced in Gm, but mostly unchanged in Sd. RWC is very responsive to drought stress and has been

shown to correlate well with drought tolerance. Therefore, the unchanged RWC in Sd synchronous with the slight decrease in fresh and dry weight relative to Gm reveals that Sd could be considered as more tolerant to drought than Gm. RWC is considered an alternative measure of plant water status, reflecting the metabolic activity in tissues. Protein is the most effective metabolite regulating plant growth and adaptation to harsh conditions. Simova-Stoilova et al. (2010) found that the protein content was significantly decreased in the drought-sensitive cultivar and little protein loss was detected in the drought-resistant cultivar. The loss of protein in the present results might be evidenced as changes in polypeptide composition.

Although both cultivars had similar polypeptide patterns under normal control conditions, drought induced varied modifications in protein profiles of both cultivars (Fig. 2; Table 1). SDS-PAGE showed 20 polypeptides ranging from 15 to 254 kDa, most of which were detected in both cultivars under control conditions. Nonetheless, drought led to losses of some polypeptides, and the loss was augmented in Gm particularly with increase in the severity of drought. The 200 and 95 kDa polypeptides were lost by drought in Gm on the 20th DAS. In addition to these polypeptides, the 72, 43, 40, 34, 27 and 25 kDa polypeptides were downregulated in Gm on the 23rd DAS, while the 43 kDa only was downregulated in Sd. The loss increased gradually up to the end of the experiment, so that on the 33rd DAS 70 % were missed in Gm relative to the disappearance of only 40 % in Sd.

These changes confirm the effects of drought stress on protein in plants. Nevertheless, some results showed decreases, while some others detected increases in total protein (Yang et al. 2009; Nemat Alla and Hassan 2012;

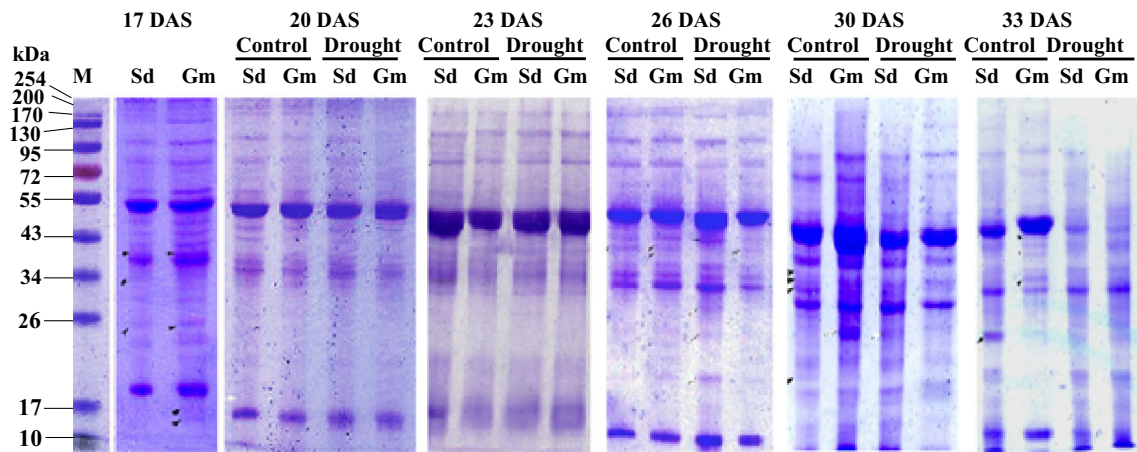


Fig. 2 Changes in SDS-PAGE leaf proteins of 17-day-old seedlings of two wheat cultivars, Sids (Sd) and Gmiza (Gm), following drought stress for the subsequent 16 days (up to 33 days after sowing, DAS)

Table 1 Changes in SDS-PAGE leaf proteins of 17-day-old seedlings of two wheat cultivars, Sids (Sd) and Gmiza (Gm), following drought stress for the subsequent 16 days (up to 33 days after sowing, DAS)

DAS		17		20				23				26				30				33			
Band no.	M wt (kDa)			Control		Drought		Control		Drought		Control		Drought		Control		Drought		Control		Drought	
		Sd	Gm	Sd	Gm	Sd	Gm	Sd	Gm	Sd	Gm	Sd	Gm	Sd	Gm	Sd	Gm	Sd	Gm	Sd	Gm	Sd	Gm
1	254	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	200	+	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+	+	-	-	-	-
3	170	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	113	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	95	+	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-
6	88	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	-	-
7	72	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-
8	61	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	55	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
10	54	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	43	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	-	+	+	+	-
12	40	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	-	-	+	+	-	-
13	37	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	-	-	-
14	34	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	-	-	+	+	-	-
15	27	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-
16	25	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	-	-
17	22	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-
18	19	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-
19	17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-
20	15	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-

This detection is based on data interpolation from Fig. 2

+ Presence; - absence

Nemat Alla et al. 2012). Such decreases could be due to intensified degradation of proteins as well as the overall inhibition in protein synthesis. Some evidences suggested that drought-sensitive species have higher proteolytic activity compared to resistant ones (Hieng et al. 2004).

Despite the similarity of protein profile in both cultivars under normal conditions in the present results, drought resulted in dramatic losses in Gm leading to disappearance of several polypeptides that still function in Sd. These findings reveal that these polypeptides might represent the

basic differences in drought tolerance between both cultivars, as they could have an important role in the metabolic pathways in the drought-adaptive strategy. Hence, the changes in protein profile could result from alterations of some functional enzymes related to stress, such as antioxidant enzymes and Rubisco.

A preliminary check for the functionality of proteins in control lost by drought indicated that most of them were active as enzymic antioxidants (data not shown). In particular, the polypeptides 95, 88, 72, 43, 40, 34, 27, 25 and 22 kDa exerted activities similar to that of both CAT and POX; however, the 37 and 19 kDa polypeptides exerted activities restricted to that of POX. Anyway, a profound study for identification of these particular proteins needs to be performed in a further study. The activities of CAT and POX are crucial for plants as a battery for scavenging of the stress-generated ROS (Mittler 2002; Nemat Alla and Hassan 2006, 2007; Nemat Alla et al. 2008, 2014). CAT activity was significantly inhibited by drought in Gm from the 23rd DAS onward; however, no effects were detected in Sd (Fig. 3). Data of the electrophoretic analysis of CAT isozymes reveals that there was only one CAT isozyme (III) expressed on the 17th DAS while it increased to mostly three isozymes by the lapse of time and the expression was more pronounced in Sd than in Gm (Fig. 3; Table 2). The CAT III isozyme dominates in both cultivars under drought or control conditions throughout the whole experiment. Similarly, CAT II in Sd was not affected by drought; however, it was downregulated in Gm on the 20th DAS and the 33rd DAS. Nonetheless, CAT I was lost by drought from the 26th DAS onward in Gm, but only on the 26th DAS in Sd. This loss of CAT isozymes seemed to be in accordance with the disappearance of some protein with a consequent inhibition in CAT function as defense enzyme. Du et al. (2008) indicated that plants have evolved multiple CAT isozymes. Plant CAT is involved in scavenging of H_2O_2 during drought stress and other abiotic stress conditions (Nemat Alla et al. 2008, 2014). The expression of CAT isozymes in the present work decreased in the sensitive cultivar, Gm, particularly at the last interval (severe drought). These findings declare another strategy in Sd cultivar to tolerate drought through controlling ROS homeostasis, while the sensitive cultivar lacked this strategy.

On the other hand, significant inhibition of POX activity by drought was restricted only to Gm from the 23rd DAS up to the end of the experiment (Fig. 4). Moreover, there were two isozymal POX (I and II) under control and drought conditions up to the 20th DAS in both wheat cultivars (Fig. 4; Table 3). Furthermore, three other isozymes (POX III, IV and V) were upregulated on the 23rd

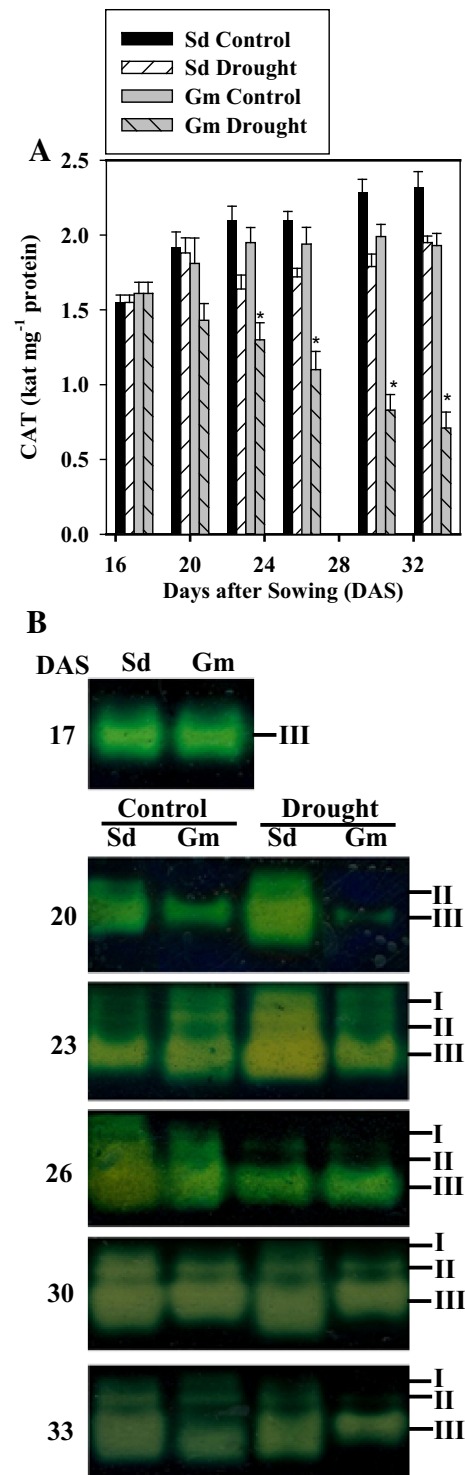


Fig. 3 Changes in catalase (CAT) isozymes of leaves of two wheat cultivars, Sids (Sd) and Gmiza (Gm), following drought stress for the subsequent 16 days (up to 33 days after sowing, DAS). **a** CAT activity; values are means ($n = 4$) \pm SE. Asterisk shows significant difference at $P \leq 0.05$ from the untreated control. **b** Native-PAGE electrophoretic analysis of CAT

Table 2 Changes in catalase isozymes of leaves of two wheat cultivars, Sids (Sd) and Gmiza (Gm), following drought stress for the subsequent 16 days (up to 33 days after sowing, DAS)

DAS	Isozymes	Control		Drought	
		Sd	Gm	Sd	Gm
17	III	+	+	-	-
20	II	+	-	+	-
	III	+	+	+	+
23	I	+	+	+	+
	II	+	+	+	+
	III	+	+	+	+
26	I	+	+	-	-
	II	+	+	+	+
	III	+	+	+	+
30	I	+	+	+	-
	II	+	+	+	+
	III	+	+	+	+
33	I	+	+	+	-
	II	+	+	+	-
	III	+	+	+	+

This detection is based on data interpolation from Fig. 3

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Fig. 4 Changes in peroxidase (POX) isozymes of leaves of two wheat cultivars, Sids (Sd) and Gmiza (Gm), following drought stress for the subsequent 16 days (up to 33 days after sowing, DAS). **a** POX activity; values are mean ($n = 4$) \pm SE. Asterisk shows significant difference at $P \leq 0.05$ from the untreated control. **b** Native-PAGE electrophoretic analysis of POX

DAS in Sd by drought, but only POX V was detected in Gm. More isozymes (up to POX VII) were detected on the 26th DAS restricted to Sd with no further upregulation in Gm. With the elapse of time up to 30 DAS, POX isozymes increased to eight in only Sd that was unaffected by drought; however, neither control nor treated Gm plants exhibited any of POX VII and VIII in addition to the loss of POX III, IV and VI by drought. Moreover, another POX (IX) was upregulated in Sd on the 33rd DAS only under drought conditions. These findings are in conformity with the missed proteins that might have POX activity. The present results reveal that drought did not change the induction of POX isozymes in Sd; moreover, a new isozyme (POX IX) was upregulated. Nonetheless, some isozymes were missed in Gm probably due to loss of some proteins. The disappearance of some isozymes by drought may affect CAT function as an effective quencher of ROS. The CAT/POX system might act cooperatively to remove H₂O₂ at a minimal expense of reducing power and at a maximal rate. Li and Wang (2002) suggested that CAT and POX

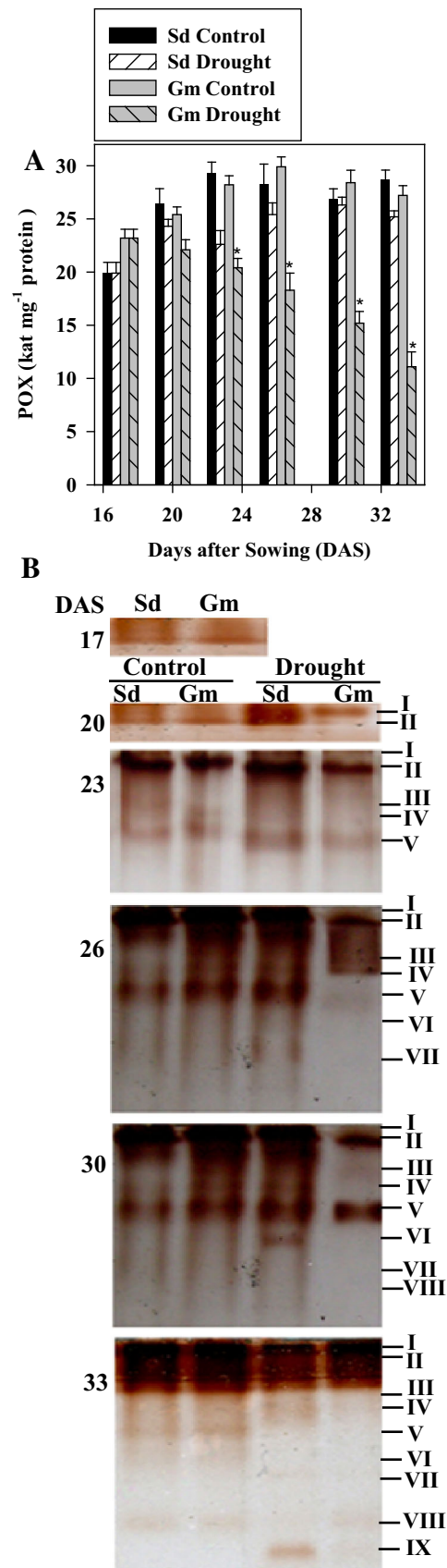


Table 3 Changes in peroxidase isozymes of leaves of two wheat cultivars, Sids (Sd) and Gmiza (Gm), following drought stress for the subsequent 16 days (up to 33 days after sowing, DAS)

DAS	Isozymes	Control		Drought	
		Sd	Gm	Sd	Gm
17	I	+	+	-	-
	II	+	+	-	-
20	I	+	+	+	+
	II	+	+	+	+
23	I	+	+	+	+
	II	+	+	+	+
	III	+	+	+	-
	IV	+	+	+	-
26	V	+	+	+	+
	I	+	+	+	+
	II	+	+	+	+
	III	+	+	+	-
	IV	+	+	+	-
	V	+	+	+	+
	VI	+	+	+	-
30	VII	+	+	+	-
	I	+	+	+	+
	II	+	+	+	+
	III	+	+	+	-
	IV	+	+	+	-
	V	+	+	+	+
	VI	+	+	+	-
33	VII	+	-	+	-
	VIII	+	-	+	-
	I	+	+	+	+
	II	+	+	+	+
	III	+	+	+	-
	IV	+	+	+	-
	V	+	+	+	-
	VI	+	+	+	-
VII	+	+	+	-	
33	VIII	+	+	+	-
	IX	-	-	+	-

This detection is based on data interpolation from Fig. 3

+ Presence; - absence

participate in the regulating mechanism of liquorice cells withstanding a water stress environment. In addition to CAT and POX, Rubisco is also expected to be affected by drought. It is the most abundant plant protein, accounting for 12–30 % of total leaf nitrogen in C3 plants (Makino et al. 2003; Khedr et al. 2011a, b).

Rubisco protein was mostly alike in both cultivars under control conditions (Fig. 5). From the 20th DAS and up to the end of the experiment, the quantity of Rubisco was

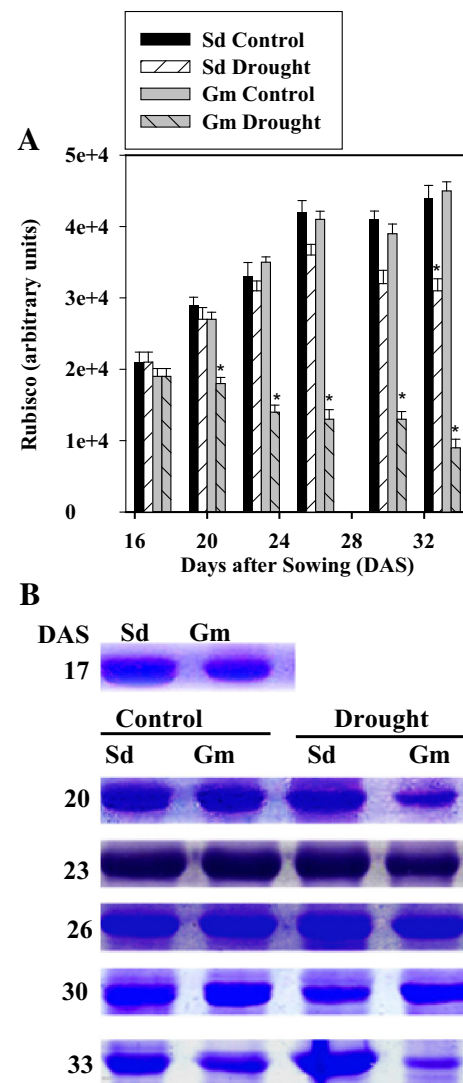


Fig. 5 Changes in Rubisco of leaves of two wheat cultivars, Sids (Sd) and Gmiza (Gm), following drought stress for the subsequent 16 days (up to 33 days after sowing, DAS). **a** Rubisco content; values are mean ($n = 4$) \pm SE. Asterisk shows significant difference at $P \leq 0.05$ from the untreated control. **b** SDS-PAGE of Rubisco

retarded by drought stress in Gm only. However, a significant decrease was detected in Sd on the 33rd DAS only, and the impact of drought stress on the expression of Rubisco was higher in Gm than in Sd. The decreased Rubisco during water stress is a direct consequence of the ionic conditions created inside cells as cell water content decreases (Parry et al. 2002; Khedr et al. 2011a, b). Moreover, plants suffering from drought go through adaptations to survive such periods through regulation of some enzymes, metabolites and genes (Khedr et al. 2011b). Therefore, in the present study, not only the enzymatic proteins were checked, but also some of the protective compatible solutes such as sugars, GB, free amino acids and proline were tested.

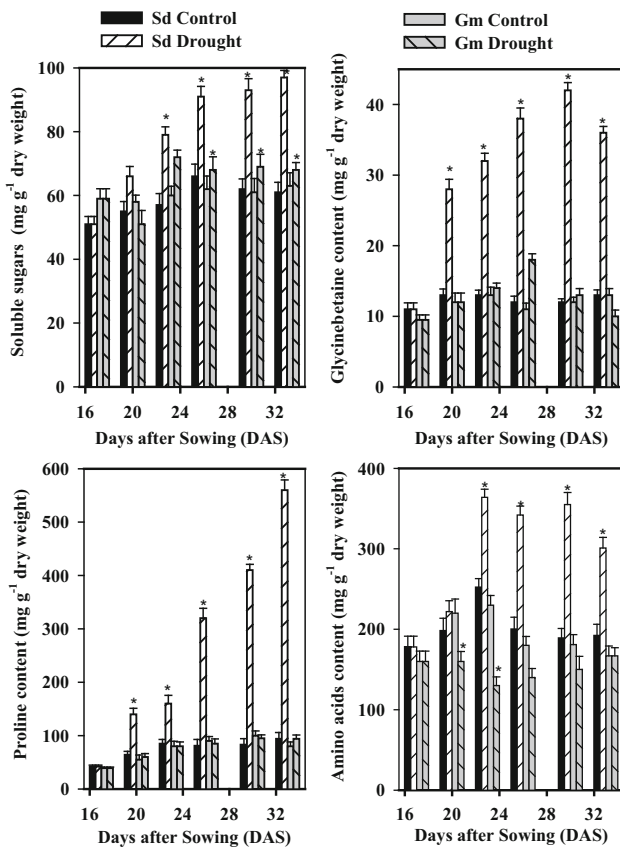


Fig. 6 Changes in soluble sugars, glycinebetaine, free amino acids and proline of leaves of two wheat cultivars, Sids (Sd) and Gmiza (Gm), following drought stress for the subsequent 16 days (up to 33 days after sowing, DAS). Values are mean ($n = 6$) \pm SE. Asterisk shows significant difference at $P \leq 0.05$ from the untreated control

Drought resulted in a significant accumulation in soluble sugars from the 23rd and the 26th DAS onward in Sd and Gm, respectively (Fig. 6). The magnitude of accumulation was higher in Sd than in Gm and it increased with increasing the drought severity. Similarly, drought significantly accumulated GB in Sd after 3 days of applying drought (on the 20th DAS) and continued until the end of the experiment; nonetheless, there was no significant accumulation in Gm during the whole experiment. Free amino acids were unaffected in Sd 3 days after drought treatment, but significantly decreased in Gm during the first 6 days of drought and then became non-significant afterward. However, amino acids in Sd were significantly accumulated by drought from the 23rd DAS up to the end of the experiment. However, non-significant changes were detected in Gm from the 26th DAS onward, before which amino acids were significantly decreased. Synchronous with these increases, proline was significantly accumulated in Sd plants 3 days after withholding water (on the 20th DAS) up to the end of the experiment. Nevertheless, there was

no significant difference in stressed Gm plants relative to control values throughout the experimental period.

So significant increases in soluble sugars, GB, amino acids and proline were induced in Sd by drought; however, there was a little, if any, increase in Gm. These findings support that this accumulation could be related to drought tolerance. In accordance, Chen and Murata (2008) reported that GB accumulation is correlated with the level of tolerance. They concluded on possible roles for GB under such conditions, including the stabilization of complex proteins and membranes, protection of the transcriptional and translational machineries and intervention as a molecular chaperone in the refolding of enzymes. Moreover, the increased accumulation of intracellular compatible solutes, such as proline and GB, results in an increase in cellular osmolarity that can drive influx of water or reduce the efflux, thus providing the turgor necessary for cell expansion under stress conditions (Kavi Kishor et al. 2005). On the other hand, free amino acids have several roles in plants as osmolytes, detoxify heavy metals, regulate ion transport and stomatal opening, affect the synthesis and activity of enzymes and influence gene expression and redox homeostasis (Rai 2002). Pinheiro et al. (2004) concluded that the increase in free amino acids in plants under drought is usually correlated with adaptation to water deficit. This conclusion is in accordance with the increased accumulation of amino acids by drought in Sd, concluding that the accumulation of amino acids is a mechanism in the tolerant cultivar to resist severe stress. Furthermore, proline acts as a molecular chaperone stabilizing the structure of proteins, as well as a regulator of cellular redox potential and an antioxidant controlling free radical levels (Reddy et al. 2004). Vendruscolo et al. (2007) concluded that transgenic wheat plants that accumulated higher proline than wild type exhibited less lipid peroxidation of membranes during drought, indicating a role for proline in reducing ROS damages during drought. In the present study, the increases in proline content were more pronounced in the tolerant cultivar, Sd, than the sensitive one, Gm. In agreement, Ying-hong (2009) found that the drought-resistant mulberry plants showed a marked increase in the proline content compared to the sensitive ones. Proline accumulation due to water stress might result from inhibited degradation, impaired incorporation of proline into proteins or stimulated synthesis that is catalyzed by pyrroline-5-carboxylate synthetase (P5CS) and encoded by the *P5CS* gene (Chen et al. 2009).

The transcript level of *P5CS* gene of Sd and Gm cultivars was unaffected by drought for 3 days; thereafter, a significant increase was detected in Sd up to the end of the experiment on the 26th DAS (Fig. 7). On the other hand, a non-significant effect was observed in Gm during the fol-

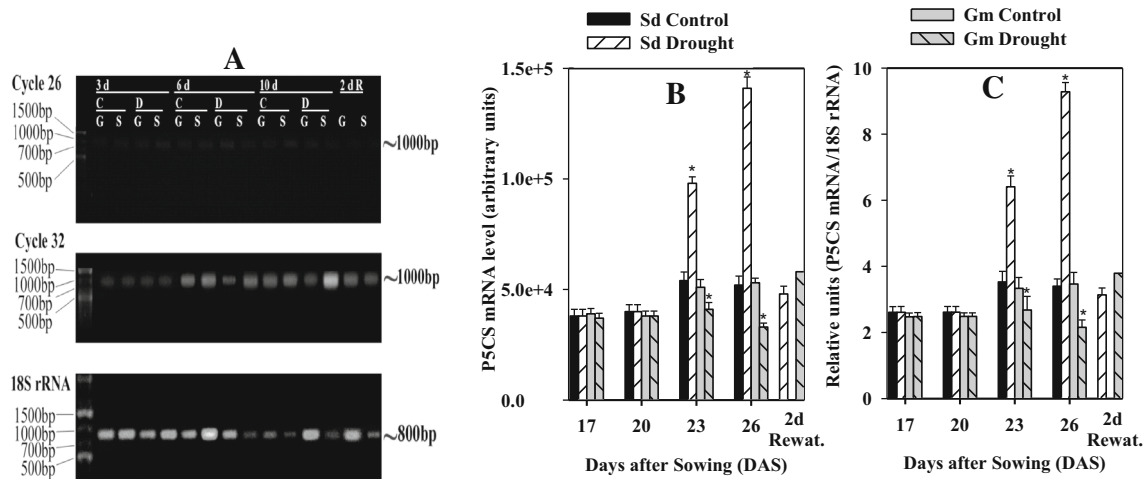


Fig. 7 Semi-quantitative RT-PCR of *P5CS* of two wheat cultivars, Sids (Sd) and Gmiza (Gm), following drought stress for the subsequent 10 days (up to 26 days after sowing, DAS). Rewatering was imposed 2 days later. **a** Ethidium bromide-stained gels. **b** Quantification of expression in terms of band volumes; values are means

($n = 3$) \pm SE. **c** The relative expression in the bands of each treatment after normalization with the internal control *18S rRNA* compared to the control treatment. Asterisk shows significant difference at $P \leq 0.05$ from the untreated control

lowing 3 days; nonetheless, a significant decline set in afterward. By the end of the experiment, the transcript level of *P5CS* gene following drought treatment increased in Sd to 173 %, but decreased in Gm to 38 % relative to the respective control values. After rewatering, the expression of *P5CS* decreased in Sd to reach levels nearly that of the untreated control; however, there was a great increase in Gm. Under these circumstances of water stress removal, gene expression in Gm was higher than levels detected under drought or control conditions, respectively, by 76 or 21 %.

The increases in the transcript level of the *P5CS* gene in Sd by drought synchronous with proline accumulation show that proline biosynthesis and *P5CS* expression depend on tolerance to drought. These findings are in conformity with the observations of Kavi Kishor et al. (2005), who suggested that proline synthesis is regulated not only at the level of enzyme activity, but also influenced by the level of *P5CS* gene expression. In this account, Thippeswamy et al. (2010) correlated the expression of safflower *P5CS* under drought stress with proline accumulation. In addition, Abu-Romman et al. (2011) showed that proline accumulation in barley was accompanied with upregulation of *HvP5CS*. Therefore, a close relation was established between the accumulation of proline by drought in the present results and the expression of *P5CS*, confirming their association. So, one of the adaptations of the tolerant cultivar, Sd, to drought is to synthesize proline via the upregulation of *P5CS*; such adaptation is lacking in the sensitive cultivar, Gm. On the contrary, the elevation of *P5CS* expression in Sd was greatly retracted upon rewatering to reach nearly the control levels, supporting that this

mechanism of adaptation to drought is efficient in the tolerant cultivar during stress only.

In conclusion, although the growth parameters of the two wheat cultivars, Sd and Gm, were mostly similar under normal control conditions, drought resulted in greater reductions in Gm than in Sd suggesting that Sd is more tolerant to drought than Gm. Meanwhile, drought induced a dramatic loss in several polypeptides in Gm despite the similarity detected in both cultivars under normal conditions, revealing that these polypeptides might have important roles in the metabolic pathways in the drought-adaptive strategy. In contrast to Sd, drought decreased isozymes of CAT, POX and Rubisco as well as *P5CS* gene expression in the sensitive cultivar, Gm, accompanied by slight, if any, effects on soluble sugars, GB, free amino acids and proline, showing their importance in drought tolerance. Moreover, the accumulations of proline in Sd is synchronous with increases in the transcript level of *P5CS*, showing that drought tolerance is *P5CS* dependent. In support, rewatering decreased the drought-induced elevation of *P5CS* in Sd, showing that this mechanism of adaptation is efficient in the drought-tolerant cultivar only under stress conditions.

Conflict of interest The authors declare that they have no conflict of interest.

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