Egypt. J. Bot. **Vol. 62**, No. 3, pp. 671-685 (2022)

Egyptian Journal of Botany
http://ejbo.journals.ekb.eg/

Biological Aspects and Proline Metabolism Genes Influenced by Polyethylene Glycol and Salicylic Acid in Two Wheat Cultivars

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DROUGHT has become an important challenge in crop cultivation, so detection of drought-tolerant cultivars by screening existing genotypes is a valuable tool in the effort to attain food security worldwide. The drought stress tolerance of two wheat cultivars was determined through treatments with PEG (−0.4MPa) and SA (0.5mM). The resulting data on seed germination and seedling growth indicated that Misr-2 cultivar is more drought-tolerant than Gemaza-12. Under non-stress conditions, Misr-2 had higher pigment values, higher levels of soluble sugars (Glu, Fru, and Suc) and antioxidant enzyme activity (CAT, APX, POX and SOD), but lower proline values than those in Gemaza-12. Gemmaza-12 had higher expression of proline synthesizing gene (P5CS) but lower expression of proline-degrading genes (ProDH and P5CDH) than Misr-2. Under drought conditions, Gemaza-12 needed more osmoregulators and antioxidants than Misr-2 to minimize the negative effects of drought and associated oxidative stresses. Increase percentages of soluble sugars, proline content and antioxidation enzymes were higher in Gemaza-12 than in Misr-2. Under drought stress, stimulation of P5CS and retardation of ProDH and P5CDH were higher in Gemaza-12 than in Misr-2. Although treatment of stressed plants with SA stimulated P5CS gene in both cultivars, retardation of ProDH and P5CDH was more pronounced in Gemaza-12 than in Misr-2. Consequently, we conclude that seed germination and seedling growth could be used to compare drought tolerance of wheat cultivars and seed soaking in SA improves drought tolerance. Our results confirmed that an increase in proline production is one of the symptoms of drought stress in plants.

Keywords: Antioxidants, Drought, Proline metabolism genes, Salicylic acid, Soluble sugars, Wheat.

Introduction

Wheat (*Triticum aestivum* L.), a widely cultivated crop in Egypt and worldwide, is considered one of the most important crops domestically and internationally, especially as the source of staple foods, such as bread (Mohammed & Faisal, 2021; Abd El-Megeed & Mohiy, 2022). Drought is one of the most challenging threats to crop cultivation due to the numerous adverse effects on the biochemical and physiological mechanisms in plants (Loutfy et al., 2011; Hasanuzzaman et al., 2013), consequently, drought causes huge crop losses annually, including loss of grain harvests. Climate change compounds these challenges, with further losses in the annual yield of crops, including wheat. Therefore, selection of more drought-tolerant lines by screening the existing genotypes under artificial drought stress is an essential tool in the task of attaining food security worldwide.

In response to harsh conditions, plants have evolved various protective mechanisms, including the accumulation of organic solutes (osmoprotectants) and compatible solutes (such as proline and glycine betaine) and the modulation of gene expression related to the response to

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Received 27/02/2022; Accepted 09/05/2022
DOI: 10.21608/ejbo.2022.124280.1921
Edited by: Prof. Dr. Reda M. Gaafar, Botany Department, Faculty of Science, Tanta University, 31527 Tanta, Egypt.

oxidative stress (Hassanein et al., 2010; Anjum et al., 2017; La et al., 2019; Abbaspour & Ehsanpour, 2020). Proline accumulation in plants plays several protective roles, including stability of cellular structures and enzymes, scavenging of reactive oxygen species (ROS), and hormonal balance (Anjum et al., 2017; La et al., 2019).

Compared with ideal growth conditions, drought significantly increases the activities of ascorbate peroxidase (APX), peroxidase (POX), phenylalanine ammonia lyase, and superoxide dismutase (SOD), as shown in water-stressed wheat plants treated with salicylic acid (SA) (Aldesuquy & Ghanem, 2015). Under drought conditions, exogenously applied SA was found to increase growth, photosynthetic pigments, total soluble sugars, proline levels, and yield (Abd El-Mageed et al., 2016; Loutfy et al., 2020). Regulation of proline metabolism by SA is an important pathway in the promotion of plant resistance to osmotic stress (Khan et al., 2013; Abbaspour & Ehsanpour, 2020).

The accumulation of ROS (mainly H_2O_2) is associated directly or indirectly with regulation of proline biosynthesis (Rejeb et al., 2015; La et al., 2019). In plants, proline is synthesized through ornithine and glutamate pathways. The last pathway is the main source of proline accumulation in abiotic stressed plants. Hayat et al. (2012) reported that glutamate is converted to glutamic semialdehyde (GSA) by pyrroline-5-carboxylate synthetase (P5CS1 and 2) and spontaneously to pyrroline-5-carboxylate (P5C), which is converted to proline by pyrroline-5-carboxylate reductase (P5CR). In most plant species, P5CS is encoded by two genes and P5CR is encoded by one gene (Armengaud et al., 2004). Conversely, proline catabolism is catalyzed by proline dehydrogenase (ProDH), which converts proline to P5C, which, in turn, is catalyzed by P5C dehydrogenase (P5CDH) to glutamate (Hayat et al., 2012). ProDH is encoded by two genes, whereas P5CDH is encoded by one gene (Ribarits et al., 2007). Although ProDH transcription is activated during rehydration periods, it is repressed by dehydration, leading to proline accumulation during periods of stress (Verbruggen et al., 1996).

In the present study, we chose polyethylene glycol (PEG) to induce drought stress. Because PEG does not penetrate the plant tissues but simply reduces water availability, the response of plants to PEG treatment mimics the response to drought in nature (Rai et al., 2011). The decrease in water potential (Ψw) due to PEG treatment was associated with the accumulation of ROS and enhanced proline synthesis due to the enhanced expression of the proline synthesis genes P5CS and P5CR (La et al., 2019).

Seed germination and seedling growth parameters are influenced by stress intensity and genetic background (Hassanein et al., 2012; Hassanein et al., 2020). Expressed drought tolerance during *in vitro* seed germination is strongly correlated to the response under field conditions (Hellal et al., 2018). In the present study, we aimed to estimate the response of two wheat cultivars to PEG-induced drought stress at an early stage of growth. The effects of PEG stress were evaluated based on the crosslink between some related physiological aspects. We found that the expression of proline metabolism genes as well as the growth of the two wheat cultivars differed under artificial drought conditions (treatment with SA).

Materials and Methods

Plant material and growth conditions

Seeds of two wheat (*Triticum aestivum* L) cultivars (Misr-2 and Gemaza-12) were obtained from the Agricultural Research Centre, Ministry of Agriculture, Giza, Egypt. Misr-2 was selected from the materials introduced from CIMMYT (International Maize and Wheat Improvement Center in Mexico) under the name of "2nd EBWYT 2006/2007" to the Wheat Research Department, Field Crops Research Institute, Agricultural Research Center (ARC), Egypt. The pedigree and selection history of the new cultivar are SKAUZ/BAV92 (CMSS96M03611S-1M-0105Y-010M-010SY-8M-OY-OS). The new bread wheat cultivar Gemaza-12 had been selected from CIMMYT materials grown at El-Gemaza Agricultural Research Station, ARC, in the 2005/2006 season. The cross name and pedigree of the new cultivar is OTUS/3/SARA/THB//VEE (CMSS97YOO227 S-5Y-010M-010Y-010M-2Y-1M-0Y-OGM). Seeds were sterilized with sodium hypochlorite solution (5%) for 5min and washed thoroughly with sterilized distilled water. To determine the recommended concentration of PEG, nutrient solutions [0.5 strength Hoagland's solution (Hoagland & Arnon, 1950); pH 5.5] supplemented with different concentrations

of polyethylene glycol 6000 (0.0, −0.1, −0.2, −0.3, −0.4, or −0.5MPa) were used. Seeds were germinated on Whatman filter paper in Petri dishes containing nutrient solutions under lab conditions for 7 days. To determine the effect of PEG and SA, sterilized seeds were divided into two groups. The first group was soaked in distilled water (control) and the second group in 0.5mM SA for 12h. Nutrient solutions were renewed every 2 days. The osmotic potentials of PEG 6000 were calculated as described by Michael & Kaufman (1973). The seeds were left to germinate and the seedlings to continue growing in nutrient solutions with or without PEG or SA for 2 weeks. Triplicate samples were used for each treatment (15 seeds each). The seedlings were then harvested and washed three times with double distilled water. Root and shoot growth parameters were determined, and the seedlings were quickly frozen at -20°C for further biochemical investigation.

Determination of photosynthetic pigments

The procedure for the extraction of the measurement of chlorophyll a and b and carotenoids from the fresh leaves follows the protocol of Metzner et al. (1965). In addition, total photosynthetic pigments were calculated. The obtained results were presented as the average of four observations over three replications for each treatment.

Determination of soluble sugars using high-performance liquid chromatography (HPLC)

For the determination of soluble sugars, frozen plant tissues of the two wheat cultivars were extracted using 80% ethanol (1mL·g FM^{-1}) at 80 °C for 30 min. Extracts were incubated at 0 °C for 30 min and centrifuged at 10,000*g* for 5min. The supernatants were dried in a vacuum at 50 °C and dissolved in 50% acetonitrile. The obtained impurities were removed by filtration (Millipore Millex-GV; pore size, 0.22μm). Samples (20μL) were injected into a carbohydrate analysis column (Shodex NH2P-504E, 250mm × 4.6mm i.d., Showa Denko, Tokyo, Japan) of an HPLC pump (L-7000, Hitachi, Tokyo). Columns were eluted with 75% acetonitrile (v/v) at a flow rate of 0.5mL·min^{-1}. Monosaccharides and oligosaccharides were determined using a refractive index detector (L7490, Hitachi) equipped with a chromatographic data processor (D-2500, Hitachi).

Assays of enzyme activities

Catalase (EC 1.11.1.6) activity was estimated spectrophotometrically (Aebi, 1984). CAT activity was estimated by the decrease in absorbance at 240nm for 1min due to the consumption of H_2O_2 (E= 0.036mM·cm^{-1}). The prepared reaction mixture consisted of 50mM phosphate buffer containing 0.1μM EDTA, 20mM H_2O_2 (pH 7.0), and 0.05mL of enzyme extract. One CAT unit is the amount of enzyme necessary to decompose 1μmol·min^{-1} H_2O_2.

Peroxidase (EC 1.11.1.7) activity was estimated as described by Zhang (1992). Assay mixture was prepared to contain 50mM potassium phosphate buffer [(pH 6.8), 0.1mM EDTA, 5 mM guaiacol, and 0.3mM H_2O_2 (30%)]. Absorbance increase due to the oxidation of guaiacol (E= 26.2mM·cm^{-1}) was measured spectrophotometrically at 470nm. POX activity was then calculated in terms of μM of guaiacol oxidized·min^{-1}·g^{-1} fresh weight of plant tissue at 25°C ± 2°C.

Ascorbate peroxidase (EC 1.11.1.11) activity was estimated according to Nakano & Asada (1981). The reaction mixture contained 50mM phosphate buffer containing 0.5mM EDTA, 0.5mM ascorbate, 0.1mM H_2O_2, and 0.05mL of enzyme extract (pH 7.0). The decrease in ascorbate concentration was associated with the decline in absorbance at 290nm for 1min. Enzyme activity was calculated using the extinction coefficient (E= 2.8mM·cm^{-1}) for ascorbate.

Superoxide dismutase (EC 1.15.1.1) activity was estimated as described by Giannopolitis & Ries (1977). SOD activity was assayed because of its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture consisted of 50mM sodium phosphate buffer containing 13mM methionine, 2mM riboflavin, 75mM NBT, 100mM EDTA (pH 7.8), and 0.05mL of enzyme extract. The increase in absorbance at 560 nm followed the production of blue formazan. The unit of SOD activity was the amount of enzyme that inhibits the nitroblue tetrazolium photoreduction (extinction factor (E)= 10.3mM·cm^{-1}).

Proline determination

Free proline contents were determined according to Bates et al. (1973). Briefly, 100mg of powdered tissue was homogenized in 10mL of 3% sulfosalicylic acid (SSA) for 10min, followed by filtration. A 2mL volume of the filtrate was

mixed with 2mL of glacial acetic acid and 2mL of acid ninhydrin for 1h at 90°C. The product was extracted in 4mL toluene and measured colorimetrically at 520nm against toluene. A standard curve with proline was used for the final calculations. The results were presented as the average of three replicate estimations for each treatment.

Semiquantitative RT-PCR

RNA was isolated from plants grown under different conditions using RNAiso (TaKaRa, Shiga Japan), following the manufacturer's instructions. The quality of the RNA was investigated through electrophoresis using 1% agarose gel containing ethidium bromide, and the concentration was determined spectrophotometrically. The isolated RNA was heated for 1min at 65°C and stored in ice. Reverse transcription was performed using high-capacity cDNA reverse transcription (Applied biosynthesis, Japan) in a 20µL reaction with 1µg of total RNA, 25× dNTPs, 10× reverse transcription buffer, 10× random primers, and 50 U/µL reverse transcriptase (Maryland, USA). The reaction mixtures were incubated for 10 min at 25°C, 120 min at 37°C, 5 s at 85°C, and then at 4°C. TE buffer (1M Tris pH 8, 0.5M EDTA, and MilliQ H_2O) was used to dilute the cDNA solution (100µL). The PCR reaction was performed with 30 cycles, and each cycle contained three steps: denaturation at 98°C for 10s, annealing at 55°C for 30s, and extension at 72°C for 1min in a reaction mixture containing 2.5mM dNTPs, 10× EX Taq Buffer, and TaKaRa Ex Taq HS 0.25 units and 0.5pmol primers. The specific primers used for P5CS, P5CDH, and ProDH detection (Table 1) were synthesized by Fasmac, Japan (https://fasmac.co.jp/en/). As a positive control, actin RT-PCR was used for all RT-PCR reactions. Specific primers were also used, where the forward and reverse primers were wheat actin F and wheat actin R, respectively, (Table 1). The products of the PCR reaction were visualized using electrophoresis on a TBE (0.89 M Tris, 0.89 M boric acid, and 0.02M EDTA) and 1% agarose gel containing 0.5 mg/mL ethidium bromide. The bands of gene transcripts (mRNA) on the gel were photographed, and the density of each band was analyzed using ImageJ (https://imagej.nih.gov/ij/download.html).

Statistical analysis

The experiments were based on a completely random design, and the data were described using standard deviation, following the process of Snedecor & Cochran (1980). All data were subjected to analysis of variance (ANOVA, SPSS 16) to test for the significant difference between the means (n= 3) of measured variables. Tukey's test was run at $P< 0.05$ to determine the level of significance.

Results

Under unstressed conditions, the germination rate was 100% for the two wheat cultivars by the fourth day (Table 2). Artificially decreasing the water potential through the application of PEG (less than −1 MPa) resulted in a difference in the ability of the two cultivars to germinate. A further reduction of the water potential to −0.4 MPa resulted in a marked difference in germination rates of the two cultivars: 80% germination in the case of Misr-2, compared to only 30% for Gemaza-12. Under a more pronounced drought condition (water potential of −0.5MPa), 55% of Misr-2 seeds germinated by the seventh day whereas Gemaza-12 did not germinate at all.

TABLE 1. Characteristics of primers (Fasmac, Japan) used to detect the proline metabolism genes

Primer name	Primer sequence	Annealing temperature (°C)	Gene
TaP5CS 1468-1487F	5′-GCTCTAGCAATCCGAAGTGG	55	P5CS
TaP5CS 1569-1550R	5′-TGGAATCACGCTGGTTATGA	55	
TaP5CDH 713-732F	5′-TGATCAGGTTGCTTCACGAG	55	P5CDH
TaP5CDH 935-916R	5′-GTGAGCTCCCAGTGAAGAGG	55	
TaProDH 1284-1303F	5′-GAGCAAGTACCTGCCCTACG	55	PDH
TaProDH 1427-1408R	5′-GCGTTCTTGAACCTCCTGAC	55	
Wheat actin F	5′-GCCACACTGTTCCAATCTATGA	55	A positive control for all RT-PCR reactions
Wheat actin R	5′-GCGGTTGTTGTGAGGGAGTA	55	

TABLE 2. Effect of drought stress (MPa) on seed germination for 7 days

Day	% percentage of germination					
	Control	-0.1 Mpa	-0.2 Mpa	-0.3 Mpa	-0.4 Mpa	-0.5 Mpa
			Misr-2			
1	64	59	55	50	42	28
2	90	80	76	70	51	38
3	100	93	80	78	60	44
4	100	100	86	85	73	50
5	100	100	90	90	80	55
6	100	100	98	90	80	55
7	100	100	98	90	80	55
			Gemaza-12			
1	70	52	42	35	13	0
2	90	69	52	43	20	0
3	95	85	60	50	24	0
4	100	96	68	63	30	0
5	100	100	78	68	30	0
6	100	100	88	70	30	0
7	100	100	90	70	30	0

When the seed germination of the two cultivars took place in the nutrient solution containing PEG (-0.4MPa), the seedling fresh and dry weights of the two cultivars decreased (Table 3). The decrease in seedling fresh weight was less pronounced in Misr-2 (32.0%) than in Gemaza-12 (41.4%). This decrease in seedling fresh weight was improved when plants were treated with SA, resulting in the reductions in fresh weights of 13.0% and 20.8% for Misr-2 and Gemaza-12, respectively. In addition, shoot length decreased under PEG, with decreases ranging from 24.6% (Gemaza-12) to 27.4% (Misr-2). Compared to plants treated only with PEG, the decrease in shoot length was improved using SA, with a reduction of 11% in both cultivars. By contrast, root length increased when plants were exposed to PEG, regardless of the presence or absence of SA. When a combination of PEG and SA was applied, the increase in root length of Misr-2 (104.1%) was higher than that of Gemaza-12 (89.7%).

TABLE 3. Effects of salicylic acid (0.5mM) and drought stress [PEG (−0.4MPa)] on the fresh mass and dry mass of 15-day-old seedlings of two wheat cultivars

Treatment	Shoot			Root		
	FM (g)	DM (g)	Length (cm)	FM (g)	DM (g)	Length (cm)
			Misr-2			
Control	0.753 ± 0.006^{b}	0.089 ± 0.003^{b}	25 ± 0.5^{b}	0.250 ± 0.005^{b}	0.026 ± 0.004^{b}	7.35 ± 0.30^{d}
PEG	0.550 ± 0.004^{d}	0.076 ± 0.001^{c}	18.16 ± 0.57^{d}	0.132 ± 0.002^{d}	0.016 ± 0.004^{c}	12 ± 0.005^{b}
SA	0.887 ± 0.006^{a}	0.095 ± 0.002^{a}	30.66 ± 0.53^{a}	0.295 ± 0.005^{a}	0.032 ± 0.005^{a}	8.83 ± 0.28^{c}
SA+PEG	0.679 ± 0.002^{c}	0.069 ± 0.001^{c}	22.33 ± 0.56^{c}	0.194 ± 0.004^{c}	0.025 ± 0.005^{b}	15 ± 0.005^{a}
			Gemaza-12			
Control	0.492 ± 0.007^{b}	0.058 ± 0.004^{b}	19.66 ± 0.76^{b}	0.139 ± 0.001^{b}	0.019 ± 0.002^{b}	6.5 ± 0.5^{d}
PEG	0.293 ± 0.005^{d}	0.042 ± 0.001^{d}	14.83 ± 0.76^{d}	0.077 ± 0.003^{d}	0.013 ± 0.001^{c}	10.33 ± 0.57^{b}
SA	0.586 ± 0.004^{a}	0.071 ± 0.002^{a}	22.83 ± 0.28^{a}	0.174 ± 0.005^{a}	0.027 ± 0.004^{a}	7.5 ± 0.5^{c}
SA+PEG	0.386 ± 0.006^{c}	0.049 ± 0.001^{c}	17.5 ± 0.5^{c}	0.114 ± 0.003^{c}	0.017 ± 0.005^{b}	12.33 ± 0.57^{a}

FM: Fresh mass and DM: Dry mass.

Values not followed by the same letter are significantly different at $P < 0.05$

Pigment content (chl. a, chl. b, and carotenoids), expressed in mg·g^{-1} F.w., was higher in Misr-2 than in Gemaza-12 in the case of both the control groups and PEG stress treatment (Table 4). Compared with the control, the concentrations of chlorophyll a, chlorophyll b, and carotenoids (total pigments) decreased significantly when the plants of the two cultivars were subjected to PEG stress, and the reduction in total pigment content of Gemaza-12 (26.2%) was higher than that of Misr-2 (20.8%). The recorded decrease in the concentration of these pigments due to PEG was improved when plants were treated with SA [Gemaza-12 (10.7%) and Misr-2 (9.1%)]. The negative effect of PEG stress on carotenoid concentration was completely avoided using SA.

The Misr-2 cultivar accumulated total soluble sugars (glucose, fructose, and sucrose) under control (66.53mg·g^{-1} F.w.) or PEG stress (127.08mg·g^{-1} F.w.) more than those of Gemaza-12 under control condition (35.79mg·g^{-1} F.w.) and PEG stress (70.76mg·g^{-1} F.w.) (Table 5). The highest increase in the concentration of each sugar was recorded when plants were subjected to drought stress and treated with SA. In that case, the increases in glucose, fructose, and sucrose contents (compared to control) were 115.8%, 244.0%, and 179.0%, respectively, in the Gemaza-12 plants compared to 133.7%, 226.1%, and 114.4% in the Misr-2 plants. These results also indicated that among the sugars, fructose showed the highest increase (%). In the drought-stressed Gemaza-12 plants, the mean percentage of increase in the three sugars compared with the control was 97.7%, higher than what was found in the Misr-2 plants (91.0%).

The effects of PEG and SA on the activities of the antioxidant enzymes (CAT, APX, POX, and SOD) of the two wheat cultivars are shown in Table 6. The activities of CAT, APX, POX, and SOD under control or PEG stress conditions in Misr-2 were higher than those in Gemaza-12. Compared with the control, the increases in the activities of the enzymes CAT (68.2%), APX (138.3%), POX (72.6%), and SOD (116.4%) of Gemaza-12 plants under the influence of PEG were higher than those of the Misr-2 cultivar: 27.7%, 116.3%, 60.9%, and 77.3%, respectively. A further increase in the activity of the three enzymes (APX, POX, and SOD) was detected when stressed plants were treated with SA, and the estimated values for Gemaza-12 were higher than those for Misr-2. Catalase activity decreased in Gemaza-12 under the influence of PEG with or without SA but increased significantly in Misr-2 under the same conditions.

When seedlings of either cultivar were exposed to a nutrient medium containing PEG as a stress factor, there was a significant increase in proline concentration. The proline content (mg·g^{-1} F.w.) of Misr-2 under control or PEG stress was lower than that of Gemaza-12 (Table 7). The increase in proline content of the plant shoots (150.7%) and roots (306.3%) of Gemaza-12 was greater than the corresponding values in Misr-2: shoots (89.6%) and roots (132.9%). Compared to the control, SA resulted in a decrease in proline concentration in the shoots and roots of both wheat cultivars. When the nutrient medium was supplemented with PEG and SA, the increases in proline concentrations of the shoots (53.1%) and roots (203.9%) of Gemaza-12 as well as the roots of Misr-2 (44.7%) were greater than the corresponding values of the control group but lower than the values measured in plants exposed to PEG only. Proline concentration was not influenced in Misr-2 shoots when plants were exposed to PEG and SA together.

TABLE 4. Effects of salicylic acid (0.5mM) and drought stress (−0.4MPa) on the leaf chlorophyll and carotenoid contents of 15-day-old seedlings of two wheat cultivars

Treatment	Chl. a	Chl. b	Carotenoid	Total pigment
		Misr-2		
Control	1.745 ± 0.011^{b}	0.630 ± 0.004^{b}	0.490 ± 0.005^{b}	2.865^{b}
PEG	1.385 ± 0.007^{d}	0.490 ± 0.006^{d}	0.395 ± 0.003^{d}	2.27^{d}
SA	1.887 ± 0.007^{a}	0.681 ± 0.005^{a}	0.534 ± 0.002^{a}	3.102^{a}
SA+PEG	1.570 ± 0.006^{c}	0.545 ± 0.003^{c}	0.489 ± 0.002^{c}	2.604^{c}
		Gemaza-12		
Control	1.504 ± 0.004^{b}	0.543 ± 0.005^{b}	0.431 ± 0.002^{b}	2.478^{b}
PEG	1.112 ± 0.007^{d}	0.358 ± 0.001^{d}	0.358 ± 0.003^{c}	1.828^{d}
SA	1.795 ± 0.011^{a}	0.560 ± 0.006^{a}	0.512 ± 0.006^{a}	2.867^{a}
SA+PEG	1.337 ± 0.005^{c}	0.455 ± 0.001^{c}	0.422 ± 0.003^{b}	2.214^{c}

The values of photosynthetic pigment contents are expressed as mg g^{-1} fresh weight.
Values not followed by the same letter are significantly different at $P< 0.05$.

TABLE 5. Effects of salicylic acid (0.5mM) and drought stress (−0.4MPa) on the soluble sugar contents of two wheat cultivars

Treatment	Glu	Fru	Suc	Total sugars
		Misr-2		
Control	34.77 ± 0.33^{d}	13.05 ± 0.67^{d}	18.71 ± 0.16^{d}	66.53^{d}
PEG	62.78 ± 0.96^{b}	33.22 ± 0.11^{b}	31.08 ± 0.12^{b}	127.08^{b}
SA	49.92 ± 0.22^{c}	19.40 ± 0.34^{c}	24.17 ± 0.15^{c}	93.49^{c}
SA+PEG	81.25 ± 0.45^{a}	42.55 ± 0.28^{a}	40.12 ± 0.15^{a}	163.92^{a}
		Gemaza-12		
Control	18.12 ± 0.11^{d}	7.55± 0.12^{d}	10.12 ± 0.06^{d}	35.79^{d}
PEG	31.44 ± 0.27^{b}	19.11 ± 0.11^{b}	20.21 ± 0.11^{b}	70.76^{b}
SA	23.22 ± 0.08^{c}	11.21 ± 0.14^{c}	14.15 ± 0.07^{c}	48.58^{c}
SA+PEG	39.11 ± 0.07^{a}	25.97 ± 0.21^{a}	28.24 ± 0.08^{a}	93.32^{a}

The values are expressed as mg g^{-1} fresh weight.

Values not followed by the same letter are significantly different at $P< 0.05$

TABLE 6. Effects of salicylic acid (0.5mM) and drought stress (−0.4MPa) on the activities of four enzymes involved in the antioxidation systems of two wheat cultivars

Treatment	CAT	APX	POX	SOD
		Misr-2		
Control	63.00 ± 0.27^{b}	41.92 ± 0.22^{d}	55.71 ± 0.55^{d}	46.42 ± 0.22^{d}
PEG	80.48 ± 0.55^{a}	90.68 ± 0.39^{b}	89.61 ± 0.39^{b}	82.31 ± 0.36^{b}
SA	72.84 ± 0.29^{d}	73.06 ± 0.36^{c}	76.00 ± 0.11^{c}	54.62 ± 0.45^{c}
PEG + SA	95.42 ± 0.49^{c}	119.67 ± 0.33^{a}	110.26 ± 0.29^{a}	112.78 ± 0.87^{a}
		Gemaza-12		
Control	45.19 ± 0.22^{b}	25.67 ± 0.45^{d}	42.78 ± 0.33^{d}	33.42 ± 0.65^{d}
PEG	76.01 ± 0.51^{a}	61.18 ± 0.30^{b}	73.85 ± 0.68^{b}	72.31 ± 0.29^{b}
SA	36.57 ± 0.69^{c}	42.21 ± 0.34^{c}	63.96 ± 0.83^{c}	44.62 ± 0.34^{c}
PEG + SA	42.10 ± 0.55^{b}	83.14 ± 0.72^{a}	95.41 ± 0.29^{a}	90.78 ± 0.72^{a}

The values of CAT, APX, POX and SODare expressed as µg $H_2O_2 min^{-1} µg^{-1}$ protein, µg ascorbate $min^{-1} µg^{-1}$ protein, µg guaiacol $min^{-1} µg^{-1}$ protein and U g FW respectively.

Values not followed by the same letter are significantly different at $P< 0.05$.

TABLE 7. Effects of salicylic acid (0.5mM) and drought stress(−0.4MPa) on the proline content of two wheat cultivars

Treatment	Shoot	Root
	Misr-2	
Control	2.98±0.18^{c}	0.85 ±0.02^{c}
PEG	5.65±0.28^{a}	1.98±0.17^{a}
SA	2.45±0.13^{d}	0.76±0.02^{c}
PEG + SA	2.98±0.23^{b}	1.23±0.16^{b}
	Gemaza-12	
Control	4.56±0.12^{c}	1.27±0.03^{c}
PEG	11.43±0.10^{a}	5.16±0.08^{a}
SA	2.89±0.08^{d}	1.12±0.03^{d}
PEG + SA	6.98 ±0.11^{b}	3.86±0.10^{b}

The values of proline are represented as mg g^{-1} fresh weight.

Values not followed by the same letter are significantly different at $P< 0.05$.

In Figure 1, we present the expression patterns of the genes P5CS, ProDH, and P5CDH in the Misr-2 (Fig. 1A) and Gemaza-12 (Fig. 1B) cultivars under the influence of PEG and SA. In the plant shoots and roots of Misr-2 (Fig. 1A), the expression of the P5CS gene was hardly detected when plants were grown under nonstress (control) conditions, even with SA. Under stress (PEG) conditions, the expression of P5CS was upregulated, and further upregulation of P5CS was detected in the roots and shoots of stressed plants treated with SA. By contrast, P5CS expression in Gemaza-12 was clearly upregulated in the roots and shoots of treated plants, with the exception of the shoots of plants treated with SA. In general, P5CS expression in Misr-2 was lower than that in Gemaza-12. In addition, P5CS expression in roots was higher than that in the shoots of either cultivar.

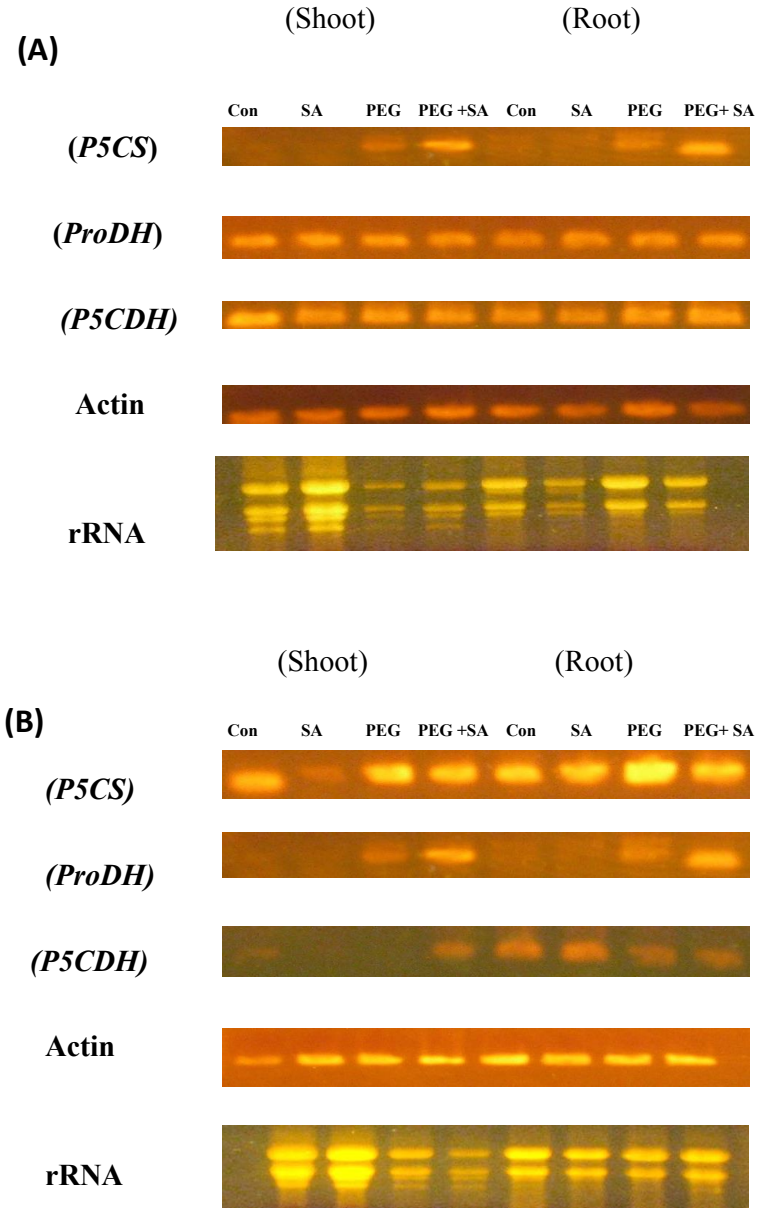

Fig. 1. RT-PCR profile of the induction of proline responsible genes (P5CS, ProDH, and P5CDH) in the shoots and roots of a tolerant wheat cultivar (Misr-2; A) and sensitive wheat cultivar (Gemaza-12; B) under water deficit (drought) stress (−0.4MPa) and treatment with salicylic acid (0.5mM) [Total RNA was isolated from the shoots and roots of 2-week-old seedlings of both wheat cultivars. Each lane was loaded with 5µg of total RNA, which was fractionated on 1% agarose gel]

In Misr-2 plants, the expression of the genes ProDH and P5CDH was clearly upregulated in roots and shoots, regardless of the presence or absence of either PEG or SA. In the Gemaza-12 cultivar, ProDH gene expression was not detected in the shoots of unstressed plants, either with or without SA. Although the expression of ProDH was scarcely observed in the shoots and roots of plants subjected to PEG stress, an increase in ProDH expression was detected in plants subjected to a combination of PEG and SA. Under the unstressed conditions, P5CDH was expressed at low levels in plant shoots and roots and was higher in the roots than in the shoots. P5CDH expression was not detectable in Gemaza-12 shoots under the influence of SA alone or in combination with PEG. By contrast, Gemaza-12 roots showed slight expression of ProDH under nonstress conditions with or without SA, but the expression level decreased in plants subjected to PEG alone or in combination with SA.

Discussion

Seed germination potential was substantially influenced by stress intensity and genotype. The germination rate of Misr-2 under PEG drought stress was higher than that of Gemaza-12. Traits associated with seed germination and seedling growth were used to detect genetic variation among genotypes as screening criteria for drought tolerance (Avramova1 et al., 2016; Cai et al., 2020; Othmani et al., 2021). In fava beans, growth parameters were more effective indicators of salinity tolerance when measured at the vegetative phase rather than the seed germination stage (Hassanein et al., 2020).

The superior drought tolerance of Misr-2 compared to Gemaza-12 was confirmed by studying seedling growth under PEG stress condition (Idrissi et al., 2015). In the present study, we found a lesser decrease in seedling fresh weight in Misr-2 compared to Gemaza-12, and this loss was ameliorated by the application of SA, where the decrease was higher in drought-sensitive than drought-tolerant cultivar. Proline is synthesized in leaves (Bojorquez-Quintal et al., 2014) and transported to the roots, where it is involved in osmotic regulation (Ferreira Júnior et al., 2018). Under drought stress, the level of proline in root tips was significantly higher than that in the root base. It has been reported that osmotic potentials differ over the length of the root and can regulate root length and mass (Yamaguchi & Sharp, 2010). In our work, the increase in root length of the drought-tolerant wheat cultivar was higher than that of the drought-sensitive cultivar under drought stress. The accumulation of proline—due to the enhancement of the P5CS gene expression and the resultant inhibition of ProDH and P5CDH—supplies energy for plant growth and survival and enhances the ability of plants to tolerate the applied stress (Ghobadi et al., 2013). The data on seed germination and growth parameters that we have collected in the present study indicate that the Misr-2 cultivar is more drought-tolerant than Gemaza-12. In studies of drought indices, Gemaza cultivars, including Gemaza-12, were found to be sensitive to both drought and salinity stresses (Gadallah et al., 2017; Ebaid et al., 2019; Mansour et al., 2020). The Gemaza-12 cultivar was recommended for cultivation under nonstressful environments (Farag et al., 2019).

Under both control and PEG conditions, pigment contents in Misr-2 plants were higher than those in Gemaza-12. Pigments provide plants with sugars and energy, which are necessary for plant metabolism and growth. It is not surprising, therefore, that the Misr-2 plants, which maintained the concentration of pigments not much lower than the levels in the control plants, were more tolerant to the applied stress condition (−0.4MPa) than the Gemaza-12 plants and had higher growth values. Conversely, an increase in pigment content and photosynthetic activity leads to increased levels of ROS, proline, and enzymatic and nonenzymatic antioxidants (Abid et al., 2018). In the present study, carotenoid, which has a nonenzymatic antioxidant effect, was found to have a positive correlation with proline content, as was reported previously by Ghobadi et al. (2013).

In both wheat cultivars in our study, the application of PEG and/or SA resulted in an increase in the concentration of glucose, fructose, and sucrose, and the highest increase was recorded when PEG and SA were applied together. The mean increase in the three sugars of Gemaza-12 (179.6%) was higher than that of Misr-2 (151.4%). This result suggests that the role of soluble sugars in controlling osmotic potential, photosynthetic activity, and ROS balance is more pronounced in Gemaza-12 than in Misr-2, as was explained by Couée et al. (2006). In addition, the accumulation of sucrose activates P5CS expression and proline accumulation (Wang et al., 2019).

The activity of each of the tested enzymes increased when the wheat cultivars were grown under PEG stress with or without SA treatment. Stomatal closure during drought stress decreased CO_2 uptake and overexcitation of the reaction centers of photosystem II (Ahmed et al., 2009), leading to the formation of ROS (Fu & Huang, 2001). Therefore, the activity of antioxidant enzymes, such as CAT, APX, POX, and SOD, enhanced and coincided with the increase in proline content (Uchida et al., 2002; Agarwal & Pandey, 2004) and was correlated with drought tolerance (Wang et al., 2014). Compared with the control group, the increase in the activity of APX, POX, and SOD in Gemaza-12 plants under the influence of PEG was higher than the corresponding measures in the Misr-2 plants, even when the plants were treated with PEG and SA. The mitigation of the adverse effects of drought by SA may be due to the increased levels of nonenzymatic antioxidants, such as carotenoids, in addition to the enzymatic antioxidants CAT, APX, SOD, POD, and glutathione reductase (Durner & Klessig, 1995; Abbaspour & Ehsanpour, 2016a, b; La et al., 2019). We found that the application of SA decreased catalase activity in Gemaza-12 even with PEG whereas it significantly increased this activity in Misr-2. The inhibition of CAT and APX by SA has been previously reported (Durner & Klessig, 1995).

To date, the effect of accumulated proline on drought-stressed plants is controversial (Liu et al., 2019). There are two opinions: either proline accumulates to enhance stress tolerance, or it is a symptom of stress injury (Liu et al., 2019). In the present study, in which the shoots and roots of the wheat cultivars were exposed to PEG, the significant increase in proline content may be due to the inhibition of protein synthesis, a decrease proline utilization or degradation, or an increase in proline synthesis and enhanced protein hydrolysis (Jiang et al., 2020; Tarabih & EL-Eryan, 2020). Under the applied concentration of PEG, the proline content in the roots and shoots of Gemaza-12 was higher than that of Misr-2. In barley, no relation was detected between proline accumulation and salinity tolerance (Chen et al., 2007), and proline content was higher in a drought-sensitive cultivars of *Artemisia aucheri* Boiss (Liu et al., 2019) and potato (Abbaspour & Ehsanpour, 2020) compared with a drought-resistant cultivar . Our data support the theory that proline accumulation under drought conditions is a sign of stress-induced plant injuries.

When plants were subjected to PEG and SA together, the concentration of proline increased—especially in the plant roots—and was higher than the concentration of proline in the control group but lower than that of plants exposed to PEG only. Proline accumulation has been attributed to the increase in P5CS activity due to SA application (Abbaspour & Ehsanpour, 2020).

In plants, biosynthesis of proline from glutamic acid via P5C is the main route for proline accumulation under stressed conditions. This pathway is catalyzed by P5CS and P5CR. In Misr-2 shoots and roots under nonstress conditions, we found that the P5CS gene was hardly expressed, even with the application of SA, whereas it was clearly detected in the Gemaza-12 plants. Moreover, the proline content of Gemaza-12 shoots and roots was higher than that of Misr-2. There was a positive correlation between the measured proline content and P5CS expression, as was reported by Liu et al. (2019). Our assessment of the germination and growth of the two cultivars indicates that Misr-2 is more resistant to drought and has both a lower concentration of proline and lower expression of P5CS; the opposite is true for Gemaza-12 (the drought-sensitive cultivar). In other plant species (*A. thaliana*), P5CS and P5CR transcripts are correlated with proline content in plant organs, except in the roots, where the proline content is lower than that in the shoots. The low proline content associated with P5CS expression in the roots of both wheat cultivars may be due to the export of proline via xylem from the roots to the shoots (Yoshiba et al., 1995). In addition, under nonstress conditions, proline content is controlled by the expression of proline-degrading genes (ProDH and P5CDH), and thus, it was lower in Gemaza-12 than in Misr-2.

Under PEG stress, the expression of P5CS in Misr-2 was lower than that in Gemaza-12. Conversely, the expression of proline catabolic genes, namely, ProDH and P5CDH, was higher in Misr-2 than in Gemaza-12. Soluble sugars were accumulated to a greater degree in Misr-2 compared with Gemaza-12. Accumulation of soluble sugars has been shown to activate P5CS expression, resulting in the accumulation of proline (Wang et al., 2019), and accumulation of soluble sugars occurs upon exogenous application of proline (Gadallah, 1999). The results of our study complicate the proposed role of proline accumulation in drought tolerance (La et al.,

2019; Abbaspour & Ehsanpour, 2020), given that proline synthesizing genes were expressed to a greater degree (resulting in proline accumulation) in the drought-sensitive cultivar compared with the drought-resistant cultivar. In addition, the drought tolerance of the two wheat cultivars was proportional to the expression of proline-catabolizing genes, which was higher in the drought-tolerant cultivar than in the sensitive cultivar. It seems that when proline accumulation is maximized under drought conditions, the genes responsible for the proline degradation pathway (ProDH and P5CDH) function to convert proline to glutamate (Adamipour et al., 2020). In a drought-tolerant potato genotype, the expressions of the genes ProDH and P5CDH were found to be higher than those in the drought-sensitive genotype under the influence of severe drought stress (Liu et al., 2019). The proline content of the drought-tolerant potato genotype was lower than that of the drought-sensitive genotype under PEG stress. Consequently, the downregulation of P5CS and P5CR and upregulation of ProDH and P5CDH may be necessary in drought-tolerant genotypes (Rajaeian et al., 2017; Liu et al., 2019). The data obtained in our study support this hypothesis and confirm the conclusion made by Liu et al. (2019) that proline accumulation results as a symptom of stress injury and cannot be viewed as an indicator of stress tolerance in plants.

In both cultivars, the expression of P5CS in plants treated with PEG and/or SA was higher than that in the control group. In Misr-2, the expression of the P5CS gene was enhanced in stressed plants and further enhanced when plants were treated with SA. The opposite effect was found in Gemaza-12. Abbaspour & Ehsanpour (2020) reported that P5CS activity increased in parallel with proline accumulation levels under drought stress. P5CS activity increased when drought-stressed plants were pretreated with SA. In a previous study of the Misr-2 cultivar, treatment of drought-stressed plants with SA enhanced the expression of proline synthesis-related genes (P5CS and P5CR) and no change in the expression of proline degradation-related genes (ProDH and P5CDH) was detected (La et al. 2019). Consequently, the proline content of Misr-2 plants treated with PEG and SA was the same as of that of the control. Stimulation of ProDH by SA without a change in proline content has been reported in the case of other plant species (Abbaspour & Ehsanpour, 2020).

Conclusion

The measurements of the rates of seed germination and seedling growth can be used to compare the drought tolerance of wheat cultivars. The integration of analytical methods (physiological assays, biochemistry, and molecular biology) in this study confirms that the increase in proline production is one of the symptoms of drought stress in plants. The use of SA to improve drought tolerance of different wheat cultivars is recommended.

Acknowledgement: We would like to thank the staff members of the Crop Plants Department at the Cairo Agricultural Research Center for providing the wheat used in this study.

Data availability statement: The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest: The authors whose names are listed in this manuscript certify that they have no affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

Authors' contributions: NL, AMH, MI, and JMS proposed the idea of this study. NL and MI designed the experimental work and made the measurements. AMH analyzed and interpreted the data and wrote the manuscript. JMS performed the calculations and statistical analysis, participated with AMH in the analysis and interpretation of the data, revised the manuscript, checked the manuscript for plagiarism, and acted as the corresponding author. All authors participated in the drafting of the manuscript and have read and approved the final draft.

Ethics approval: Not applicable.

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تأثُر الجوانب البيولوجية وجينات أيض البرولين بالبولي إيثيلين جليكول وحمض الساليسليك في صنفين من القمح

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يمثل الجفاف تحديًا مهمًا يؤخر نمو وانتاجية النباتات المنزرعة، ولذا فإن تحديد الصنف الذي يتحمل الجفاف عن طريق فحص الأنماط الجينية الموجودة يعد أداة أساسية لتحقيق الأمن الغذائي في جميع أنحاء العالم. في هذه الدراسة تم تحديد قدرة صنفين من القمح على تحمل إجهاد الجفاف باستخدام تركيز0.4 Mpa- من البولي إيثيلين جليكول وتركيز 0.5 ملي مولار من حمض الساليسليك. أشارت بيانات إنبات البذور ونمو البادرات إلى أن صنف مصر-2 كان أكثر تحملًا للجفاف من صنف جميزة-12. تحت ظروف عدم الاجهاد (الكنترول أو العينة الضابطة)، كان لدى الصنف مصر-2 قيم أعلى من الأصباغ النباتية والسكريات الذائبة (جلوكوز وفركتوز وسكروز) وكذلك الانزيمات المضادة للأكسدة (الكاتاليز والأسكوربيت بيروأكسيديز والبيروكسيديز والسوبرأكسيد ديسميوتيز) ولكن قيم أقل من البرولين، من تلك الموجودة في الصنف جميزة -12.وقد وجد أن صنف جميزة-12 كان لديه تعبيرًا جينيًا أعلى لجين تخليق البرولين (إنزيم تخليق برولين 5 كربوكسيلات) ولكن تعبيرًا أقل لجينات تكسير البرولين (برولين ديهيدروجينيز و برولين 5 كربوكسيلات ديهيدروجينيز) مقارنة بالصنف مصر-2. تحت ظروف الجفاف، احتاج الصنف جميزة-12 إلى مزيد من منظمات الاسموزية ومضادات الأكسدة أكثر من الصنف مصر-2 المقاوم للجفاف وذلك لتقليل الآثار السلبية لنقص الماء وما يرتبط بها من ضغوط الأكسدة. لذلك، فإن نسبة الزيادة في السكريات الذائبة ومحتوى البرولين والانزيمات المضادة للأكسدة كانت أعلى في الصنف جميزة-12 منها في الصنف مصر-2. تحت ظروف الجفاف، كان تحفيز التعبير الجيني لإنزيم تخليق برولين 5 كربوكسيلات P5CS و تثبيط التعبير الجيني لإنزيم البرولين ديهيدروجينيز و إنزيم البرولين كربوكسيلات ديهيدروجنيز ProDH و P5CDH في صنف جميزة-12 أعلى منه في صنف مصر-2. على الرغم من أن المعالجة بحمض الساليسليك للنباتات المجهدة بالجفاف أدت إلى تحفيز التعبير الجيني لإنزيم تخليق برولين 5 كربوكسيلات P5CS في كلا الصنفين، إلا أن تثبيط التعبير الجيني للجينات المسؤلة عن تخليق إنزيمي البرولين ديهيدروجينيز ProDH والبرولين كربوكسيلات ديهيدروجينيز P5CDH في صنف جميزة-12 كان أكثر وضوحًا من مصر-2. وعليه، فقد خلصت هذه الدراسة إلى أنه يمكن استخدام إنبات البذور ونمو البادرات لمقارنة تحمل صنف قمح للجفاف بصنف بآخر، كما أدى نقع البذور في حمض الساليسليك إلى تحسين تحمل الجفاف. وقد أكدت هذه الدراسة أن زيادة إنتاج البرولين تكون من أعراض الجفاف في النباتات.