Ascorbate–glutathione cycle confers salt tolerance in Egyptian lentil cultivars

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Abstract In order to investigate the salt tolerance mechanism in Egyptian lentils, five cultivars were grown under different salinity levels. The results indicated that Giza 9 is salt-tolerant, while Giza 4 is a salt-sensitive lentil cultivar, as Giza 9 showed the highest germination percentage and relative water content, the lowest decline in root and shoot length, and the highest proline content. Relative gene expression of *ADH*, *CYS3*, *APX*, *SOD*, and *GR* was significantly upregulated in Giza 9 roots after 24 h of high salt treatment (250 mM), while *CAT* transcripts level was non-significantly reduced (0.5-fold) relative to control. However, in Giza 4, only *CAT* gene was significantly upregulated in both root (2.3-fold) and shoot (8.5-fold) tissues. Investigation of ascorbate–glutathione enzymatic activities revealed significantly higher ascorbate peroxidase and glutathione reductase activities in Giza 9 roots compared to its shoots. Moreover, endogenous reduced glutathione (GSH) content as well as reduced ascorbate content were significantly high in the roots of Giza 9 compared to Giza 4. These results strongly indicate the involvement of ascorbate–glutathione cycle in the tolerance mechanism of Giza 9. Furthermore, exogenous application of 350 μ M of GSH assisted Giza 4 seedlings to tolerate salinity stress. As far as we know, this is the first work reporting the involvement of ascorbate–glutathione cycle in salt tolerance of lentil.

Keywords *Lens culinaris* · ROS · Salt tolerance · qRT-PCR · Antioxidant defense

Introduction

Lentil (*Lens culinaris* Medikus) is a self-pollinating diploid ($2n = 14$) plant. It has a haploid genome size of approximately 4064 Mbp (Arumuganathan and Earle 1991). It is not only a pivotal economic winter-legume crop cultivated in the Mediterranean region but is also grown worldwide as food for human (Sarker and Erskine 2006). It has the ability to survive on relatively poor soils and under adverse environmental conditions, especially high temperature and drought (Singh et al. 2017). However, like many other legume species, lentil is classified as a salt sensitive plant (Bhatnagar-Mathur et al. 2013).

It has been shown that soil and water salinity limits plant growth and productivity through production of osmotic stress, ion toxicity, and stimulation of oxidative stress (Bandeoglu et al. 2004; Ouda et al. 2008). Salinity stress causes enormous alterations in the signaling pathways as well as the regulation of gene expression that have effects on the growth and development of plants (Büyük and Aras 2015). Two essential groups of genes induced by salt-stress have been identified in Arabidopsis. The first category involves regulatory proteins, for example protein kinases, transcription factors, and enzymes required to phospholipids metabolism. The second category includes functional genes, which encode defending proteins versus abiotic stress such as detoxification enzymes, mRNA-binding proteins, and water channel proteins (Cramer et al. 2011).

The effects of salt (NaCl) stress on response of antioxidants have been examined in various plant species including wheat, rice, potato, mulberry, maize, tomato, citrus, pea, and lupins. Such studies uncovered that the antioxidant response is nicely associated with sensitivity and tolerance of the investigated plant cultivars. Depending on the studied plant species, various antioxidant enzymes

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are involved in the response to NaCl stress (Bandeoglu et al. 2004; Aghaei et al. 2009; Sofo et al. 2015).

High salinity induces overproduction of reactive oxygen species (ROS), which causes cellular and oxidative damage to lipid membranes, proteins and DNA, thereby disrupting cellular metabolism in several plant species such as *Pisum sativum* L. (Hernández et al. 2000), citrus plants (Tanou et al. 2009), and *Carthamus tinctorius* L. (Karray-Bouraoui et al. 2011).

Enzymatic antioxidants include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), enzymes of ascorbate–glutathione (ASA-GSH) cycle such as ascorbate peroxidase (APX, EC 1.11.1.11), ascorbate free radical reductase (AFRR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and glutathione reductase (GR, EC 1.6.4.2). On the other hand, ascorbate (ASA), glutathione (GSH), carotenoids, phenolics, and tocopherols are corresponding to nonenzymic antioxidants inside the cell. Several researchers have described increased activities of many antioxidant defense enzymes in plants to fight oxidative stress induced by various environmental stresses such as salt-stress (Chen et al. 2007).

It has been previously indicated that high levels of ASA, GSH and harmonized superior activities of SOD, CAT, GPX, APX, and GR enzymes can function as the crucial determining component for identifying salt tolerance in seedlings of rice (Mishra et al. 2012). In a similar way, the salt-tolerant *Plantago maritima* exhibited an improved defense mechanism averse to oxidative damage resulted from salt stress by increasing enzymatic activities of SOD, CAT, GR, and APX than did the salt-sensitive *Plantago media* (Hediye Sekmen et al. 2007).

In this study, we aimed to determine the most salt-tolerant and sensitive lentil cultivar(s) among the five mostly cultivated Egyptian cultivars. In addition, the effects of high salinity (salinity shock) on gene expression profiles of antioxidant enzymes were compared in the most salt-tolerant (Giza 9) and most salt-sensitive (Giza 4) lentil cultivars. Furthermore, we attempted to unravel the possible biochemical and molecular mechanism(s) leading to salt-tolerance in Egyptian lentil at seedling stage.

Materials and methods

Seeds of five Egyptian lentil cultivars (Giza 4, Giza 9, Giza 51, Giza 370, and Sinai 1) were obtained from Agriculture Research Center, Giza, Egypt.

Growing conditions and salinity experiments

The seeds of the five cultivars were germinated in the dark on filter paper moistened with 10 ml distilled water for

3 days in sterile Petri dishes. Out of each cultivar, 10 seedlings were then transferred into Petri-dishes containing distilled water (control) or different concentrations of NaCl (34, 68, 137, 274, and 548 mM) and kept for 6 days at 25 ± 1 °C under 8 h day length. In addition, for determining the most salt tolerant and salt sensitive lentil cultivars, 25 seeds of each cultivar were directly germinated in five different concentrations of NaCl (34, 68, 137, 274, and 548 mM) and the germination percentage of each cultivar was calculated. The emergence of the radicals from seed coats was used as an indicator of complete seed germination.

For exogenous GSH treatment experiment, Giza 4 (most salt-sensitive lentil cultivar) was used. Three treatments were conducted: control (3 day-old Giza 4 seedlings in dist. H₂O), 3 day-old Giza 4 seedlings treated with 250 mM NaCl solution, and 3 day-old Giza 4 seedlings treated with a solution containing 250 mM NaCl plus 350 µM exogenous GSH (350 µM GSH final concentration was prepared using 500 µM GSH stock solution). GSH was maintained at 350 µM in the solution during the treatment period (24 h) and three replicates were done for each treatment.

For quantitative real-time PCR (qRT-PCR) and glutathione-ascorbate pathway analyses, 250 mM salt shock experiments were performed on the Giza 4 (salt-sensitive) and Giza 9 (salt-tolerant) lentil cultivars. The growing conditions were the same as described above. Three day-old germinated seedlings of both cultivars were treated with 250 mM NaCl (salt shock) for 24 h. The salt shock experiments, qRT-PCR analyses, and quantification of glutathione-ascorbate enzymatic activities were conducted at the Department of Plant science, University of Manitoba, Canada.

Measurement of plant growth (root and shoot length)

Root and shoot lengths of 6 days old control and treated lentil seedlings were measured using a measuring ruler and then mean values of three replicates were calculated.

Relative water content (RWC%)

RWC% was quantified as described by Turner (1981). It was determined using the following equation: $RWC = (FW - DW)/(TW) \times 100$, where FW is the fresh weight of shoots, TW is the full turgor shoot weights measured after left floating in distilled water at room temperature (24 °C) for 24 h in the light, and DW is the dry weight estimated after drying the seedlings (shoots) at 70 °C until a constant weight was attained.

Determination of potassium (K⁺) and sodium (Na⁺) concentrations

Concentrations of potassium and sodium ions were estimated according to Sadeghi (2011). First, seedlings were dried overnight at 70 °C, then ground into a fine powder. Afterward, 0.1 g was transferred to a test tube containing 10 ml of 0.1 N acetic acid and heated in a water bath (80 °C) for 2 h. Subsequently, samples were cooled down to room temperature (RT) and left for overnight. The concentration of sodium and potassium was then determined using Inductively Coupled Plasma Spectrometer (Perkin Elmer Model Optima 7000 DV, Germany).

Determination of proline content

Proline concentration was measured calorimetrically following Bates et al. (1973) method. 0.5 g of fresh seedlings of both Giza 4 and Giza 9 were hand-homogenized in 10 ml of 3% aqueous sulfosalicylic acid. The homogenates were centrifuged at 5000 rpm for 5 min. Then, 2 ml of the supernatants were allowed to react with 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid. Afterwards, the mixture was boiled for 30 min and 5 ml of toluene was added directly after the red color has appeared. The absorbance of the produced red color upper layer was measured at 520 nm using UV–Vis Dual Beam Spectrophotometer (Labomed UV Spectro, USA).

Total RNA isolation and reverse transcription

Total RNA was extracted with TRI reagent[®] (Sigma-Aldrich, USA), treated with DNase I (RNase-free, Roche, Germany), and used for cDNA synthesis with the High Capacity cDNA Reverse Transcription Kit[™] (Applied Biosystems, USA) following the manufacture's protocol. The concentration of total RNA was quantified with NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcription was performed in 20 µl reaction volume at 37 °C for 120 min and the enzyme was inactivated at 80 °C for 5 min.

Quantitative real-time PCR (qRT-PCR) analysis

Gene quantification was performed in a CFX96 Touch[®] real time thermo-cycler (Bio-Rad Laboratories Inc., Singapore) in a 10 µl reaction volume containing 5 µl of SsoFast[™] EvaGreen[®] Supermix (Bio-Rad, USA), 10 pmol of each primer and 4 µl of 1:20 diluted cDNA. The conditions used for amplification were as follow: 98 °C for 2 min, 40 cycles of 98 °C for 5 s, 60 °C for 5 s followed by a melting curve from 65 °C to 95 °C in 0.5 °C steps.

The sequences of the primers used in the qRT-PCR analysis are presented in Table 1.

The *LcActin* gene was used as an internal control and qRT-PCR amplifications were performed in triplicate. PCRs were repeated when the standard deviation of PCR replicates was higher than 0.2. Expression level of the selected genes was first normalized with the *Actin* (*LcActin*) gene and then represented relative to the controls by calculating fold changes in expression levels as described by Livak and Schmittgen (2001).

Assessment of enzymatic activities of the ascorbate–glutathione cycle

In order to quantify the activities of the enzymes that are involved in the ascorbate–glutathione cycle, extracts were performed separately on root and shoot tissues from control and treated Giza 4 and Giza 9 seedlings using 50 mM Tris-HCl buffer pH 7.2 (Stasolla and Yeung 2001). Both root and shoot homogenates were centrifuged at 10,000 g for 20 min at 4 °C and then the supernatants were used for measuring the enzymatic activities.

Determination of the endogenous glutathione and ascorbate contents, as well as enzymatic reactions of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and ascorbate free radical reductase (AFRR) were performed as described by Stasolla and Yeung (2001). The reduced and oxidized glutathione (GSH and GSSG) were determined by the 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB)/GSSG reductase recycling assay as described by Stasolla et al. (2004). All values for enzyme activities are the means of determinations on three separate extracts for each sample. All enzymatic activities were measured using 800TS Absorbance Reader (BioTek[®] Instruments, USA).

Statistical analysis

Significance of difference between mean values obtained from 3 independent experiments was determined by one-way analysis of variance (ANOVA) at 95% confidence interval. The standard errors among means were calculated by descriptive statistics test in GraphPad Prism ver.7.

Results

Salt tolerance screening of Egyptian lentil cultivars

After germination of the five lentil cultivars in different NaCl concentrations, Giza 9 showed the highest germination percentages of 93.33%, 73.33%, and 3.33% in the range from 68 to 274 mM, while Giza 4 exhibited the

Table 1 Oligonucleotides used for qRT-PCR expression analysis of six salinity-related and antioxidant genes (*LcAPX*, *LcADH*, *LcCAT*, *LcCSY3*, *LcCuZn-SOD* and *LcGR*) in the two most salt-tolerant and salt-sensitive lentil cultivars Giza 9 and Giza 4, respectively

Gene name	Primer ID	Primer sequence (5'–3')	Tm	Primer length	%GC	References
Aldehyde dehydrogenase (<i>ADH</i>)	<i>LcADHf</i>	TTCAACCAGGGGCAATGTTG	62.56	20	50	Singh et al. (2017)
	<i>LcADHr</i>	ATGCGCGTGCCCTTGATTTC	64.08	20	50	
Citrate synthase 3 (<i>CSY3</i>)	<i>LcCSY3f</i>	GCGCTTTCATCTTTCATCCTG	63.86	22	50	Singh et al. (2017)
	<i>LcCSY3r</i>	AGCTGCTGCAATTGTTGGTG	60.82	20	50	
Superoxide dismutase (chloroplast Cu ²⁺ and Zn ²⁺ containing <i>CuZn-SOD</i>)	<i>LcSODf</i>	CTTCCATATCCATGCCTTG	54.93	19	47	Aksoy (2008)
	<i>LcSODr</i>	AGTACTCTGCCACCAGCAT	58.51	20	55	
Catalase (<i>CAT</i>)	<i>LcCATf</i>	CCTGTCATTGTGCGTTTCTC	57.71	20	50	Aksoy (2008)
	<i>LcCATr</i>	CTCCACCTTAATGGCCTCT	58.99	20	55	
Glutathione reductase (chloroplast/mitochondrial <i>GR</i>)	<i>LcGRf</i>	GAAATTGCTAGTCTATGCGTCA	55.82	22	41	Aksoy (2008)
	<i>LcGRr</i>	AGCAAACCTCCAAGGCAATGT	58.24	20	45	
Ascorbate peroxidase (chloroplast/stromal <i>APX</i>)	<i>LcAPXf</i>	CTTCTCCAGCCGATCAAAGA	58.94	20	50	Aksoy (2008)
	<i>LcAPXr</i>	AGGACATTGGTCAGGTCCAG	58.37	20	55	
<i>Actin</i>	<i>LcActf</i>	GTTCCACAATGTTCCTGGT	58.13	20	50	Vaghefi et al. (2013)
	<i>LcActr</i>	ATTCTGCCTTTGCAATCCAC	58.58	20	45	

Lentil *Actin* (*LcAct*) was used as the housekeeping gene for normalization of expression levels

lowest germination percentages (63.32%, 32.63%, and 0%). By contrast, control seeds of all five lentil cultivars attained 100% germination (Fig. 1a). On the other hand, root and shoot measurements showed that NaCl treatments significantly ($p > 0.01$) reduced root and shoot lengths of the five tested lentil cultivars in a dose-dependent manner. The highest reduction in root and shoot lengths was found in cultivar Giza 4 followed by Giza 370, Giza 51, Sinai 1, and Giza 9 (Fig. 1b, c).

Similarly, K⁺/Na⁺ ratios were also reduced by NaCl treatments compared to controls. A significant decrease ($p > 0.01$) in K⁺/Na⁺ ratios was noticed in the five examined cultivars at different NaCl concentrations compared to the controls. Under 548 mM NaCl treatment, lowest K⁺/Na⁺ ratio was found in cultivars Giza 4, Giza 370, and Giza 51, while the highest K⁺/Na⁺ ratio was observed in cultivar Giza 9 followed by Sinai 1 (Fig. 1d).

Increasing NaCl concentration (from 34 to 548 mM) caused a stepwise reduction in RWC% in all five lentil cultivars (Fig. 1e). Over this range of NaCl concentrations, the highest RWC% was determined in cultivar Giza 9 followed by cultivar Sinai 1, whereas the lowest RWC% was observed in Giza 4, Giza 51, and Giza 370, respectively. Under 548 mM NaCl, the observed decrease in RWC% was found to be significant ($p > 0.01$) for the five examined cultivars, even though, it was insignificant in case of cultivar Giza 9.

Based on the above-mentioned results, Giza 9 is considered to be the most salt-tolerant Egyptian lentil cultivar among the five chosen cultivars, while Giza 4 is the most

salt-sensitive one. Therefore, Giza 9 and Giza 4 cultivars were selected for further investigations.

Under salt stress, with increasing NaCl concentration, a significant increase in proline content was found in both cultivars (Giza 9 and Giza 4). The lowest proline content was observed in controls of both Giza 4 (0.005 mg/g dry weight) and Giza 9 (0.01 mg/g dry weight). At 68 mM NaCl, a decrease in proline content in both Giza 4 and Giza 9 was observed, after which a gradual increase was observed. In Giza 9, the highest proline content (0.035 mg/g dry weight) was observed at 548 mM NaCl, while in Giza 4, the highest content (0.025 mg/g dry weight) was shown at 274 mM NaCl (Fig. 1f).

Expression analysis of antioxidant and salt related genes

In order to compare the expression levels of aldehyde dehydrogenase (*ADH*), citrate synthase 3 (*CSY3*), ascorbate peroxidase (chloroplast/stromal *APX*), superoxide dismutase (chloroplast copper and zinc-containing superoxide dismutase *CuZn-SOD*), catalase (*CAT*), and glutathione reductase (chloroplast/mitochondrial *GR*) of the salt-tolerant (Giza 9) and salt-sensitive (Giza 4) lentil cultivars, seedlings of the two Egyptian cultivars were exposed to 250 mM of NaCl treatment (salt shock) for 24 h. Both root and shoot tissues were utilized to isolate total RNA samples. The transcripts of these previously mentioned genes were analyzed using the qRT-PCR technique.

Fig. 1 Effect of different NaCl treatments (34, 68, 137, 274, and 548 mM) on germination percentage (**a**), K^+/Na^+ ratio (**b**), percentage of relative water content (RWC%, **c**), root and shoot length (**d** and

e) of five Egyptian lentil cultivars and proline content (**f**, mg/g dry weight) in the two most salt-tolerant and salt-sensitive cultivars Giza 9 and Giza 4, respectively

With respect to controls, the obtained results showed that the relative gene expression (fold change) of five (*ADH*, *CYS3*, *APX*, *CuZn-SOD*, and *GR*) of the studied genes was significantly upregulated in Giza 9 root tissues after 24 h of salt treatment, while level of *CAT* transcripts was non-significantly reduced (by 0.5-fold) (Fig. 2a). Expression levels of *ADH*, *CYS3*, *APX*, *CuZn-SOD*, and *GR* were increased by 3.0, 3.9, 7.3, 8.7, and 15.5-fold, respectively. However, only *APX* and *CSY3* expression levels were significantly increased (by 1.3- and 0.3-fold, respectively) in Giza 9 shoot tissues compared to controls (Fig. 2b). In Giza 4, only *CAT* gene was significantly increased (by 2.3-fold) in root tissues (Fig. 2a). In addition, about 0.5-fold reduction in *APX* expression level was observed, while *GR* transcript level was non-significantly elevated (by 2.1-fold). Similar to root tissues, *CAT* gene was significantly upregulated (by 8.5-fold) in shoot tissues

(Fig. 2b), while *CuZn-SOD* gene expression was repressed (by 0.49-fold) and that of *GR* was markedly up-regulated (by 5.9-fold), but statistically it was a non-significant increase.

Enzyme activities of ascorbate–glutathione cycle

In order to investigate the response of enzymes involved in the ascorbate–glutathione cycle to salt stress (250 mM NaCl), the activities of these enzymes were assessed (Fig. 3). Enzymatic activities of AFRR and APX were significantly high in root tissues of Giza 9 after 24 h of salt treatment, with 113.7 and 106.4 nmol of metabolized substrate mg^{-1} protein min^{-1} for AFRR and APX, respectively. Interestingly, APX activity was highly significant in root tissues of Giza 9 control (26.2 nmol of metabolized substrate mg^{-1} protein min^{-1}) compared with

Fig. 2 Effect of NaCl treatment (250 mM) on the relative expressions (fold change) of six salinity-related and antioxidant genes (*LcAPX*, *LcADH*, *LcCAT*, *LcCSY3*, *LcCuZn-SOD* and *LcGR*) in the root (a) and shoot (b) of the two most salt-tolerant and salt-sensitive lentil cultivars Giza 9 and Giza 4, respectively measured by quantitative real time-polymerase chain reaction (qRT-PCR). mRNA levels were normalized with respect to lentil *Actin* (*LcAct*) gene and are expressed relative to those of controls. Data are mean $\pm$ SE of three biological replicates and two technical replicates. Asterisk refers to a significance difference between gene expression in the two most salt-tolerant and salt-sensitive cultivars Giza 9 and Giza 4, respectively

root tissues of Giza 4 (9.6 nmol of metabolized substrate mg^{-1} protein min^{-1}). However, APX was significantly low in Giza 9 shoot tissues (15.7 nmol of metabolized substrate mg^{-1} protein min^{-1}) under salt treatment compared with Giza 4 (29.2 nmol of metabolized substrate mg^{-1} protein min^{-1}). No significant difference between the activities of DHAR was observed in root and shoot tissues of Giza 9 and Giza 4 under salt treatment and in controls (Fig. 3).

Similar to APX, GR activity was significantly high in root tissues of Giza 9 under salt treatment (80.0 nmol of metabolized substrate mg^{-1} protein min^{-1}) and Giza 9 control (47.2 nmol of metabolized substrate mg^{-1} protein min^{-1}) compared with Giza 4 (Fig. 4a). The reduced ascorbate content (ASC) was significantly high in the root tissues of Giza 9 (95.2 nmol g^{-1} fresh weight) after 24 h under salt treatment compared to Giza 4 (53.9 nmol g^{-1} fresh weight) (Fig. 4a).

Moreover, contents of endogenous GSH and GSSG were measured in root tissues. The obtained results showed that

under high salt stress (250 mM NaCl), endogenous content of GSH was significantly increased in Giza 9 (606.3 nmol g^{-1} fresh weight) compared to Giza 4 (313.4 nmol g^{-1} fresh weight) (Fig. 4b). However, endogenous content of GSH in non-stressed Giza 9 and Giza 4 cultivars were 279.5 and 259.1 nmol g^{-1} fresh weight, respectively. On the other hand, the endogenous GSSG contents were nearly the same in root tissues of both cultivars under non-stressed (controls) and stressed (250 mM NaCl treatment) conditions.

Under high salt stress condition, the ratio of endogenous reduced to total glutathione (GSH/GSH + GSSG) was significantly high in the roots of Giza 9 cultivar (0.93) compared to Giza 4 (0.87) (Fig. 4c). On the contrary, GSH/GSH + GSSG ratios were identical in both cultivars under non-stressed condition. From the ascorbate–glutathione analyses, it seems that cultivar Giza 9 switched the glutathione and ascorbate pools towards the reduced forms.

In order to examine the effect of exogenous GSH on the salt tolerance of Giza 4 cultivar, the salt shock experiment was repeated for Giza 4. Two treatments were used, 250 mM NaCl alone and a combination of 250 mM NaCl and 350 μM of GSH. RWC% of the seedlings of each treatment and the control was measured. The results showed a highly significant ($p < 0.05$) increase in the RWC% (74%) of the seedlings treated with 250 mM NaCl and 350 μM of GSH compared to 58% in case of 250 mM NaCl alone (Fig. 4d). In contrast, the difference between RWC% of the combined treated seedlings and that of control seedlings was insignificant. Such findings indicate that GSH assisted Giza 4 seedlings to tolerate salinity shock and retain high RWC% similar to that of control seedlings.

Discussion

In the present study, we endeavored to decipher the mechanism of salt-tolerance in the Egyptian lentils. First, out of the five investigated cultivars, the most salinity-tolerant and salinity-sensitive cultivars were determined based on seed germination percentage under different NaCl concentrations and seedlings growth parameters (root and shoot length, RWC%, as well as K^+/Na^+ ratio). The results indicated that, in presence of NaCl in the range of 34–548 mM, Giza 9 was the most salt-tolerant cultivar, while Giza 4 was the most salt-sensitive one. It has been reported that seed germination under saline conditions was considered as simple, quick, and reliable way to evaluate salt-tolerance of crop plants like lentil (Sidari et al. 2008).

In our study, there was a substantial reduction in growth parameters of the five lentil cultivars under different salt concentrations in a dose-dependent manner. The five

Fig. 3 Activities of the enzymes included in metabolism of ascorbate and glutathione metabolism in the root and shoot of two lentil cultivars Giza 4 and Giza 9. GR, glutathione reductase; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; AFRR, ascorbate free radical reductase. Three days old seedlings of Giza 4 and Giza 9 were treated with 250 mM NaCl and after 24 h of salinity

shock, enzymatic activities were assayed. GR, 1 unit = 1 nmol GSH oxidized mg^{-1} protein min^{-1} ; APX, 1 unit = 1 nmol ASC oxidized mg^{-1} protein min^{-1} ; AFRR, 1 unit = 1 nmol NADH oxidized mg^{-1} protein min^{-1} ; DHAR, 1 unit = 1 nmol ASC reduced mg^{-1} protein min^{-1} . Values are means $\pm$ SE of three biological experiments

investigated lentil cultivars responded diversely to increase of salt levels. Such findings are in agreement with previously reported studies on lentil cultivars (Ashraf and Waheed 1993; Bandoğlu et al. 2004; Kökten et al. 2010). In addition, the impact of salinity on germination percentage and seedling features has been extensively reported in legume plants (Kumawat et al. 2018). Reduction of seed germination under salinity stress was suggested to be due to decreased water content and synthesis of hydrolytic enzymes, which restrict the hydrolysis of stored food in seeds (Kumar et al. 2009; Ologundudu and Ajayi 2016).

In the present investigation, the highest RWC% was found in cultivar Giza 9, while the lowest RWC% was observed in Giza 4. This finding is in agreement with the result of Sharaf (2008), who showed that the highest RWC% reduction was observed in Giza 4 cultivar. The K^+/Na^+ ratio is considered an appropriate criterion for evaluating tolerance response to salinity stress in plants (Ashraf et al. 2007). In our study, Sinai 1 showed the highest K^+/Na^+ ratio under the highest NaCl levels (274 and 548 mM)

followed by Giza 9, which was in accordance with the results of Sharaf (2008) and Singh et al. (2017).

It is well known that proline is one of the most crucial osmo-protectants in plant, where under salinity stress, nearly all plants display an extraordinary rise in their proline levels (Bandoğlu et al. 2004; Wu et al. 2013). A similar trend has been observed in our study, where proline contents of Giza 4 and Giza 9 increased with increasing salinity. However, Giza 9 displayed higher proline content than cultivar Giza 4. The results of proline content clearly confirmed that Giza 9 is the most salt-tolerant lentil cultivar as salt-tolerant plants compile high proline content during salt stress conditions (Bavei et al. 2011).

To unravel the mechanism of salt tolerance of Giza 9 and understand its molecular basis, relative expression of six antioxidant and salinity related genes was determined in Giza 9 and Giza 4 lentil cultivars using quantitative real-time PCR. The results showed that five genes (*ADH*, *CYS3*, *APX*, *CuZn-SOD*, and *GR*) were significantly upregulated in roots of Giza 9 cultivar, thus indicating that the root system seems to be more affected than the shoot during

Fig. 4 Endogenous levels of reduced ascorbate (ASC) and its oxidized forms: ascorbate free radicals (AFR) and dehydroascorbate (DHA) (a) and of reduced (GSH) and oxidized (GSSG) glutathione (b) in the roots of 3 days old seedlings of Giza 4 and Giza 9 treated with 250 mM NaCl for 24 h. The redox state of glutathione is presented as the ratio between GSH and total (GSH + GSSG)

glutathione (c). Relative water content (RWC%) of 3 days old seedlings of Giza 4 after treatment with 250 mM NaCl and with 250 mM NaCl + 350 μM for 24 h. Values are means ± SE of three biological experiments. Asterisk refers to a significance difference between treatments

salinity shock (250 mM NaCl) treatment for 24 h in both cultivars. However, *APX*, *CuZn-SOD*, and *GR* transcripts were highly abundant (7.3, 8.7 and, 15.6-fold change, respectively) in Giza 9 compared to Giza 4 roots. Bandoğlu et al. (2004) demonstrated that *CuZn-SOD* was highly induced under salinity stress in lentil roots, which is in accordance with the results of the present study.

Interestingly, the observed significant upregulation of *APX* and *GR* genes (8.7 and 15.6-fold change, respectively) evidenced that ascorbate–glutathione cycle might be involved in the removal of electrons from hydrogen peroxide (H_2O_2), which are generated by salinity shock in Giza 9 roots (Fig. 5). Under stress environments, numerous metabolic reactions yield ROS. Nevertheless, plants have their own effective system for scavenging ROS, thus defending their destructive oxidative reactions (Gossett et al. 1994; Sofo et al. 2015; Yang and Guo 2018). It has been reported that *APX* and *GR* genes are up-regulated in plants indicating less peroxisomal abundance of CAT

Fig. 5 Schematic representation of ascorbate–glutathione cycle. ASC, reduced ascorbate; AFR, ascorbate free radicals; DHA, dehydroascorbate; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O , water; H_2O_2 , hydrogen peroxide; $NADP^+$, nicotinamide adenine dinucleotide phosphate oxidized form; $NADPH$, nicotinamide adenine dinucleotide phosphate reduced form; AFRR, ascorbate free radical reductase; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase

activity (Apel and Hirt 2004). Similar observation was shown in this study, where *CAT* transcripts were significantly down-regulated in Giza 9 roots compared to Giza 4.

An important role of antioxidant defense mechanism in plants has been attributed to the ascorbate–glutathione pathway, which is catalyzed by a group of four enzymes as shown in Fig. 5. To confirm the results obtained with gene expression analyses, the activities of enzymes involved in ascorbate–glutathione cycle were investigated under the same salinity stress (250 mM NaCl) condition. Results of these metabolic investigations showed that salinity stress has fundamental impacts on glutathione and ascorbate metabolism through alteration of the endogenous contents of GSH, GSSG, and ASC in the Giza 9 roots (significant increase in the endogenous contents of GSH, GSSG, and ASC were observed). The results of this study clearly suggested that Giza 9 plants changed the glutathione and ascorbate pools toward reduced forms (reduced glutathione GSH and reduced ascorbate ASC). These metabolic alterations were attributed to the capability of Giza 9 to escalate the activities of GR, APX, and AFRR enzymes under salt stress in the root system. In rice, it has been recently shown that APX, CAT, GR and SOD antioxidant activities boosted the protection against oxidative stress (Kumar et al. 2018; Rossatto et al. 2017). In contrast, a remarkable increase in the GR activity in the leaves of lentil seedlings under salt stress has been reported by Bandoğlu et al. (2004).

In stressed plants, it has been demonstrated that activities of the antioxidant enzymes are generally considered as a measure of stress tolerance of the plant genotypes. Therefore, in our study the activities of antioxidant enzymes in salt-tolerant (Giza 9) and salt-sensitive (Giza 4) cultivars were assessed to uncover the probable involvement of ascorbate–glutathione cycle in the mechanism of salt-tolerance observed in Giza 9 cultivar. The obtained results revealed that the ascorbate–glutathione cycle might be involved in the mechanism of salt tolerance in Giza 9, which showed the most tolerance against salinity. Similar results were reported by Gossett et al. (1994) in cotton cultivars and Sairam and Srivastava (2002) in wheat genotypes.

In this study, exogenous GSH treatment of the salt-sensitive Giza 4 lentil cultivar resulted in a significant ($p < 0.05$) rise in RWC% under salt stress (250 mM NaCl). Consequently, it is possible to suggest that GSH application is effective to render Giza 4 seedlings more robust to thrive under salt stress, which corresponds to the results obtained by Sofo et al. (2015). This indicated that exogenous application of GSH increases the endogenous counterpart, therefore lentils could be grown in high salt containing soils by priming the seeds with GSH.

Conclusion

Salinity tolerance screening showed that Giza 9 and Giza 4 are the most salt-tolerant and most salt-sensitive Egyptian lentil cultivars, respectively. The molecular characterization of these two lentil cultivars evidenced that Giza 9 is the most salt-tolerant cultivar. The qRT-PCR analyses demonstrated that *APX*, *CuZn-SOD* and *GR* transcripts were highly up-regulated in the roots of the Giza 9 cultivar. Assessment of the enzymatic activities of the ascorbate–glutathione cycle pointed to their plausible involvement in the mechanism of salt tolerance in Giza 9. The exogenous application of GSH elevated the endogenous counterpart in cultivar Giza 4. These results suggested that priming of salt-sensitive lentil seeds with GSH can alleviate salinity stress. Moreover, lentils could be genetically modified to express high glutathione content through manipulation of one or more of the enzymes involved in the ascorbate–glutathione cycle. To our knowledge, this is the first report on the potential mechanism of salt-tolerance in Egyptian lentil cultivar Giza 9.

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Author contributions RG conceived and planned the experiments. RG and MS carried out the experiments. RG performed the analysis and interpretation of the results and took the lead in writing the manuscript.

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