Effects of drought on expression patterns of genes encoding the antioxidant enzymes associated with chloroplasts in wheat

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Abstract

Reactive oxygen species lead to cellular damage and in plants exposed to drought stress, an increasing expressions of genes encoding antioxidant enzymes play important protective roles. The aim of this study was to evaluate response of drought tolerant ('Arg' and 'Roshan') and drought sensitive ('Arta' and 'Navid') wheat cultivars to oxidative stress caused by drought. Relative water content (RWC), water loss rate (WLR), free proline content, malondialdehyde (MDA) accumulation, and peroxidase (POX) activity were measured after 2, 4, 6, and 8 h of dehydration. The tolerant cultivars had a higher RWC and lower MDA, proline content, POX activity and WLR as compared to the sensitive cultivars. Real-time quantitative PCR was used to measure the expressions of genes encoding antioxidant enzymes in chloroplastic thylakoids and stroma. The expressions of chloroplastic Cu/Zn superoxide dismutase, thylakoid-bound ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and chloroplastic glutathione reductase genes were up-regulated in the tolerant cultivars. A direct relationship between physiological traits and increased gene expressions was observed for both sensitive and tolerant cultivars. Overall, increasing gene expressions protect the plants from oxidative damage caused by dehydration stress and improves tolerance to this stress.

Additional key words: ascorbate-glutathione cycle, malondialdehyde, proline, relative water content, ROS scavenging, water-water cycle.

Introduction

Water deficit is considered as the most common environmental stress and a major constraint to plant productivity (Hernández *et al.* 2012). Many factors affect the plant responses to drought stress such as plant genotype, growth stage, severity and duration of stress (Chaves *et al.* 2003), gene expression (Denby and Gehring 2005), respiration activity (Carbo-Ribas *et al.* 2005), photosynthetic activity (Flexas *et al.* 2004), and other environmental factors (McDonald and Davies 1996, Rizhsky *et al.* 2002). Drought stress leads to oxidative stress by increasing the accumulation of reactive oxygen species (ROS). The ROS attack lipids, proteins, saccharides, nucleic acids and, eventually, cause cell death (Smirnoff 1993). Plants exposed to stresses use various approaches including physiological, biochemical, and molecular mechanisms to counteract their harmful effects. One of the most important defense mechanisms against drought is activation of the antioxidant system, which scavenges ROS and keeps an adequate redox balance.

The most important antioxidant enzymes are involved in water-water and ascorbate-glutathione (AsA-GSH) cycles (Asada 1999, Hernández *et al*. 2009). Because of the electron flow from water in photosystem II to water in photosystem I that occurs in this process across thylakoid membranes, water-water cycle takes part in ROS detoxification and scavenging excess photon energy (Peroni *et al*. 2007). The most important issue in this cycle is rapid sweeping O_2^- and H_2O_2 by Cu/Zn superoxide

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbic acid; CAT - catalase; DHAsA - dehydroascorbate; DHAR - dehydroascorbate reductase; GR - glutathione reductase; GSH - glutathione; GSSG - oxidized glutathione; MDA - malondialdehyde; MDAR - monodehydroascorbate reductase; MDAsA - monodehydroascorbate; POX - peroxidase; RWC - relative water content; sAPX - stromatal APX; SOD - superoxide dismutase; tAPX - thylakoid-bound ascorbate peroxidase; WLR - water loss rate.

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dismutase (SOD, as the major isoform of SOD, EC 1.15.1.1, in almost all plant species) or Fe SOD (in several plant species) and thylakoid-bound ascorbate peroxidase (tAPX) at the site of production before they affect the target molecules (Asada 1999, Peroni *et al*. 2007). The SOD can protect chloroplasts from the threat of increasing O_2^- by converting it to H_2O_2 and O_2. Then, tAPX enzyme converts H_2O_2 into water using two AsA molecules (Peroni *et al.* 2007). In addition to water-water cycle, AsA-GSH cycle located in the stroma of chloroplast prohibits the accumulation of ROS (Asada 1992). The AsA-GSH cycle provides another position for reducing H_2O_2 to water. The AsA-GSH cycle is active in most subcellular organelles such as mitochondria and cytoplasm, in spite of the fact that it is present in chloroplast. The enzymes APX (EC 1.11.1.11), monodehydroascorbate reductase (MDAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and glutathione reductase (GR, EC 1.8.1.7) participate in AsA-GSH cycle and depending on their activities in certain organelles have various isoforms; for example, stromal APX (sAPX), chlMDHAR, chlDHAR, and chlGR in chloroplasts. During this cycle, APX reduces H_2O_2 through AsA, and the product of this reaction is MDAsA that is reduced with MDAR to AsA (Asada 1999). The MDAsA may also be converted to dehydroascorbate (DHAsA) through non-enzymatic disproportionation and then DHAR reduces DHAsA to AsA, which is connected with GSH oxidation (Sečenji *et al.* 2010) to oxidized glutathione (GSSG). Since GSH is one of the most important antioxidants in plant cells, GSSG is rapidly reduced to GSH by GR using NAD(P)H as a reducing agent (Sečenji *et al.* 2010).

The tolerance of plants to abiotic stresses, such as drought, is related to the activation of antioxidant enzymes, which has been proven by several studies (Kang and Saltveit 2002, Simova-Stoilova *et al.* 2008, Xiaoqin *et al*. 2009, Yang *et al.* 2011, Chakraborty and Pradhan 2012). Increasing the expression of antioxidant enzyme encoding genes in order to protect against oxidative damage caused by drought stress can be considered as one of the molecular changes to increase the tolerance of plants to drought stress (Hernández *et al.* 2012). In this regard, the best way to understand the activity of antioxidant enzymes under stress conditions is to examine the expression of their genes. Several studies have been conducted to investigate the activity of antioxidant enzymes under stress conditions in wheat, but few studies have investigated expression of their genes; especially, the whole cycle in a particular organelle has been rarely studied. Increasing the expression of APX, SOD, and catalase (CAT) genes and their activities in barley genotypes, was observed under severe drought stress, especially in the drought resistant genotype (Harb *et al*. 2015). Peroxidase (POX), cytosolic APX (cytAPX), and CAT genes show higher expressions in a tolerant cultivar of canola under severe drought stress as compared to a susceptible cultivar (Hosseini *et al.* 2015). Sečenji *et al*. (2010) stated that the low expression of genes encoding APX, DHAR, and GR isoforms in a susceptible cultivars of wheat indicated that they do not have sufficient protection against oxidative stress caused by water deficiency. High expressions of the enzymes encoding genes of AsA-GSH cycle play an important role in protecting against cold stress in wheat (Baek and Skinner 2003) and eggplant (Chen *et al.* 2011). It has been proven that high expressions of the genes encoding antioxidant enzymes in plants improves the protection against ROS caused by abiotic stresses (Baek and Skinner 2003).

The aim of this investigation was to study the expressions of genes encoding the antioxidant enzymes in the thylakoid (water-water cycle) and stromal (AsA-GSH cycle) sections in wheat chloroplasts with the exception of the sAPX gene and to evaluate the physiological and biochemical responses of four cultivars to dehydration. Contrary to previous works, the present study is focused on the expression of these genes in one organelle.

Materials and methods

Plants, growth conditions, and dehydration: Seeds of uniform size from 'Arta' and 'Navid' (susceptible) and 'Arg' and 'Roshan' (tolerant) wheat cultivars that were obtained from the Seed and Plant Improvement Institute, Karaj, Iran, were washed with distilled water and sterilized using 0.1 % (m/v) $HgCl_2$ for 5 min and then washed with deionized water. Next, 10 seeds of each cultivar were sown in pots filled with sterilized soil. Seedlings were grown in a greenhouse (Guilan University, Iran) under a 16-h photoperiod, an irradiance of 300 μmol $m^{-2} s^{-1}$, day/night temperatures of 20 - 24/16 °C, and a 65 - 75 % relative humidity. They were regularly irrigated with tap water.

The dehydration treatment was used as the best drought stress simulator (Malatrasi *et al.* 2002, Liu and Baird 2003, Rampino *et al.* 2006). Two-week-old seedlings of the similar leaf size were transferred on dry filter paper and left at room temperature for 0 (control), 2, 4, 6, and 8 h. After completing each stage of stress, samples were frozen in the liquid nitrogen and stored at -80 °C for gene expression and biochemical assessments.

Total RNA extraction and quantitative real-time PCR: Total RNA was extracted from leaves by an RNA isolation kit (*DENAzist*, Mashhad, Iran) according to recommended protocol with a slight modification. After purifying the genomic DNA from RNA, cDNA was synthesized by a synthesis kit of the first strand cDNA (*RevertAid*, *Thermo Fischer Scientific*, San Jose, USA) according to the manufacturer's recommendation using oligo (dt) 15 as a primer. Quantitative real-time PCR was performed on a *BioRad-C1000* (Hercules, USA) real-time detection system using *GreenHot Master Mix* (*BIORON*, Germany). Each reaction containing 6.25 mm^3 of 2× *Green Hot Master Mix* (including active *Syber Green* dye and *Super Hot Taq* DNA polymerase), 1 mm^3 of cDNA template, 4.25 mm^3 of sterile deionized water, and 0.5 mm^3 of each primer in a total volume of 12.5 mm^3 was carried out three times under the following protocol: 95 °C for 3 min, 45 cycles at 95 °C for 30 s, an appropriate primer-specific annealing temperature for 30 s, and at 72 °C for 30 s. In order to verify the uniqueness of real-time PCR products, the following two criteria were used: *1*) a single peak melting curves measured at the end of each PCR reaction, and *2*) a single band on agarose gel electrophoresis. The expression level of each target gene was normalized by comparing with *18srRNA* as a reference gene and the internal control. The relative quantification of the gene expression was carried out using the $2^{-\Delta\Delta CT}$ methodology (Livak and Schmittgen 2001).

Primer design: Sequences of the genes under study were identified by searching in the *NCBI* databases (www.ncbi.nlm.nih.gov). All genes, except GR, were designed based on the wheat gene sequences related to chloroplast organelle using the *Primer 3* program (http://primer3.ut.ee). The chloroplastic GR sequence related to barley (*Hordeum vulgare* L.) was used to design the GR primer. The sequences of MDAR and DHAR genes were aligned from the chloroplastic MDAR and DHAR sequences of different cereal species (such as rice, barley, and maize) by the *tcoffee* program (http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee), and the sections of sequences having a very high overlap were chosen for the primer designing. Primer sequences, amplified fragment length, and accession numbers of genes are listed in Table 1 Suppl.

Assay of physiological and biochemical characteristics: In order to measure relative water content (RWC), fresh mass (FM) of the leaves was immediately recorded after imposing 0, 2, 4, 6, and 8 h of drought stress. The leaves were then immersed in distilled water for 4 h under ambient temperature. After 4 h, samples were slightly dried with a tissue paper and their water-saturated mass (WSM) was determined. Then the samples were dried at 80 ºC for 24 h and their dry mass (DM) was measured. RWC was determined according to Barrs and Weatherley (1962) using the following formula: RWC [%] = [(FM - DM) / (WSM - DM)] × 100.

After imposing 0, 2, 4, 6, and 8 h of dehydration stress, the first fully expanded leaf was cut and its FM was immediately recorded. The leaf was then dried at 80 ºC for 24 h and total DM was determined. Water loss rate (WLR) was computed based on Suprunova *et al.* (2004) with a slight modification: WLR [g h^{-1} g^{-1}(DM)] = [(FM_{Tx} - FM_{Tx+2})] / [DM × (Tx+2 - - Tx)], where Tx = 0, 2, 4, and 8 h.

The enzyme extract for the analysis of POX activity and lipid peroxidation was prepared as follows: 0.5 g of leaf powder was homogenized in 1 cm^3 of 10 mM extraction buffer [consisting of 0.68 % (m/v) KH_2PO_4 and 0.87 % (m/v) $K_2HPO_4 \cdot 12\ H_2O$, pH 7.2]. The solution was then centrifuged at 12 000 *g* and 4 ºC for 15 min. The supernatant was used to measure POX activity and lipid peroxidation. In order to determine lipid peroxidation, 1 g of polyvinylpyrrolidone and 0.0186 g Na_2-EDTA were added to the extraction buffer.

Activity of POX (EC 1.11.1.7) was determined according to Chance and Maehly (1955): 485 mm^3 of 40 mM H_2O_2 and 485 mm^3 of 20 mM guaiacol were mixed together at a low temperature and then 30 mm^3 of the enzyme extract was added. The changes in absorbance at 470 nm for 1 min were observed by a spectrophotometer (*T80+UV-VIS*, *PG Instruments*, Coventry, UK). The enzyme concentration was determined by the Lambert-Beer law and a guiacol peroxidase coefficient of absorbance was 26.6 mM^{-1} cm^{-1}. One unit of POD activity was defined as an absorbance change of 0.01.

Lipid peroxidation was assayed as malondialdehyde (MDA) content produced through a thiobarbituric acid reaction according to Heath and Packer (1968): 600 mm^3 of the enzyme extract was mixed with 600 mm^3 of 20 % (m/v) trichloroacetic acid. The mixture was kept at a temperature of 95 ºC for 30 min in a hot water bath and then immediately cooled on ice and centrifuged at 10 000 *g* and 4 °C for 10 min. The supernatant was used for determining absorbance at 532 nm, from which the absorbance of other non-specific pigments at 600 nm was deducted. Content of MDA was determined using a coefficient of absorbance of 155 mM^{-1} cm^{-1}.

Proline content was measured as described by Bates *et al*. (1973): 0.1 g of powdered leaf sample was homogenized in 1.5 cm^3 of 3 % (m/v) sulphosalicylic acid and after centrifuging the reaction mixture at 9 000 *g* for 12 min, 1 cm^3 of glacial acetic acid and 1 cm^3 of 2.5 % (m/v) ninhydrin (1.25 g of ninhydrin dissolved in a mixture of 30 cm^3 of glacial acetic acid and 20 cm^3 of 6 M phosphoric acid) were added to supernatant. The mixture was heated at 100 ºC for 60 min, and then the reaction was stopped by incubating in an ice bath. Then, 2 cm^3 of toluene was added to the mixture and mixed vigorously for 15 - 20 s. The absorbance of the red upper phase was measured at 520 nm. A standard curve was used for calculating proline content.

Data analysis: Statistical analyses of physiological and biochemical attributes, including the Tukey test for comparing means, were performed by the *SAS v. 9* software (*SAS Institute*, Chicago USA) using three replications. Furthermore, principal component analysis was carried out by the *Minitab* statistical software (*v. 14*), using all 10 attributes under study, for comparing wheat responses to dehydration stress between stress-tolerant and stress-susceptible genotypes. In order to remove the scale effect, correlation matrix, instead of covariance matrix, was utilized to extract the principal components.

Results

The highest expression of the *chlCu/Zn SOD* gene was observed in 'Roshan' (Fig. 1*A*). The expression was 7- and 8-times higher than in the control after 6 and 8 h of dehydration, respectively. After 2 h of dehydration, gene expression was maximum in 'Arg', and then it severely decreased at 4 h of stress. Afterwards, at 6 h, it was about 7-times higher, but after 8 h, only a slight increase in expression was observed in relation to the control. Regarding the susceptible 'Arta' and 'Navid', *chlCu/Zn SOD* gene expression decreased with the onset of stress, and this reduction was higher in 'Arta' as compared to 'Navid' at all time points.

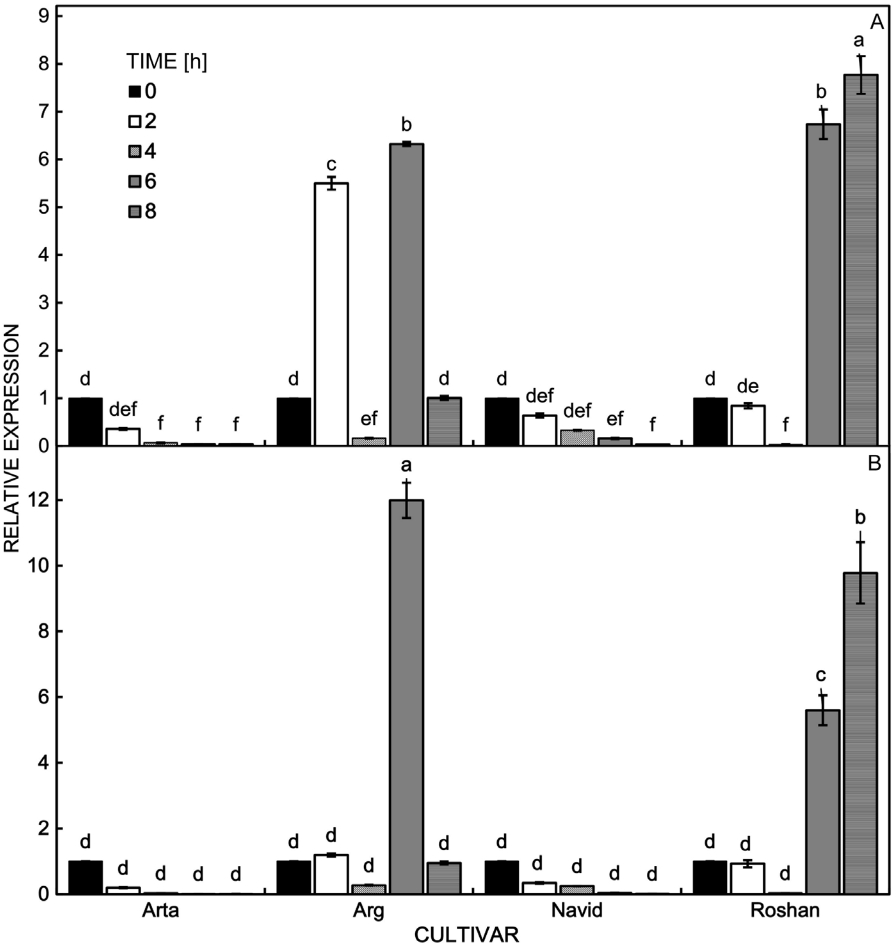

Fig. 1. Expression patterns of genes encoding enzymes of thylakoid water-water cycle in wheat cultivars Arta, Arg, Navid, and Roshan. Means ± SEs, $n = 3$. Means with different letters are significantly different at $P \leq 0.05$ based on the Tukey test. Relative expressions of *chlCu/Zn SOD* (*A*) and *tAPX* (*B*).

Expression of the *tAPX* gene in the non-tolerant 'Arta' and 'Navid' decreased during water stress. The expression of the *tAPX* gene in 'Arg' increased slightly at 2 h of stress and, then increased 12-fold at 6 h as compared to the control. Expression in tolerant 'Roshan' at 6 and 8 h of stress was 6- and 10-times higher than in the control, respectively (Fig. 1*B*).

Increasing the expression of the *MDAR* gene relative to the control for susceptible 'Arta' was observed only at 2 h of the stress and the expression of this gene decreased with increasing the stress period (Fig. 2*A*). In tolerant 'Arg', the expression increased only at 6 h of stress and decreased in other time points. For sensitive 'Navid', the gene expression was higher than in the control during the whole stress except after 4 h when the reduction of gene expression was observed. In tolerant 'Roshan', expression increased about 4-times at 2 and 6 h of dehydration, and after 8 h, it increased 8-times as compared to the control.

The expression of the *DHAR* gene was low in 'Arta' and 'Navid' and decreased as the stress period increased (Fig. 2*B*). In 'Arg', the highest expression of *DHAR* was observed at 6 h of stress, when it was higher than in other cultivars at all time points. At 2 h of stress, the expression of this gene in 'Arg' increased by about 4-times as compared to the control. But at 4 and 8 h of stress, expression decreased. In 'Roshan', early hours of stress were associated with the reduction in *DHAR* expression, but after 6 and 8 h, *DHAR* expression was about 2- and 5-times higher than in the control.

The maximum expression of the *chlGR* gene in 'Arg' was observed at 6 h of the stress, which was approximately 10-times higher than in the control. In 'Arta', the highest expression was obtained after 2 h of stress, and then a decreasing trend was observed. For 'Navid', a slight increase in gene expression was observed after 2 and 6 h of dehydration. In 'Roshan', the expression showed an upward trend with increasing stress duration except for 4 h of stress.

Relative water content of all the cultivars decreased gradually as the stress duration increased. However, tolerant 'Arg' and 'Roshan' had a significantly higher RWC than susceptible 'Arta' and 'Navid' at all time points (Fig. 3*A*).

Water loss rate of 'Arta' and 'Navid' was significantly higher than that of 'Arg' and 'Roshan' (Fig. 3*B*). Water loss rate of 'Arta' and 'Navid' gradually decreased as

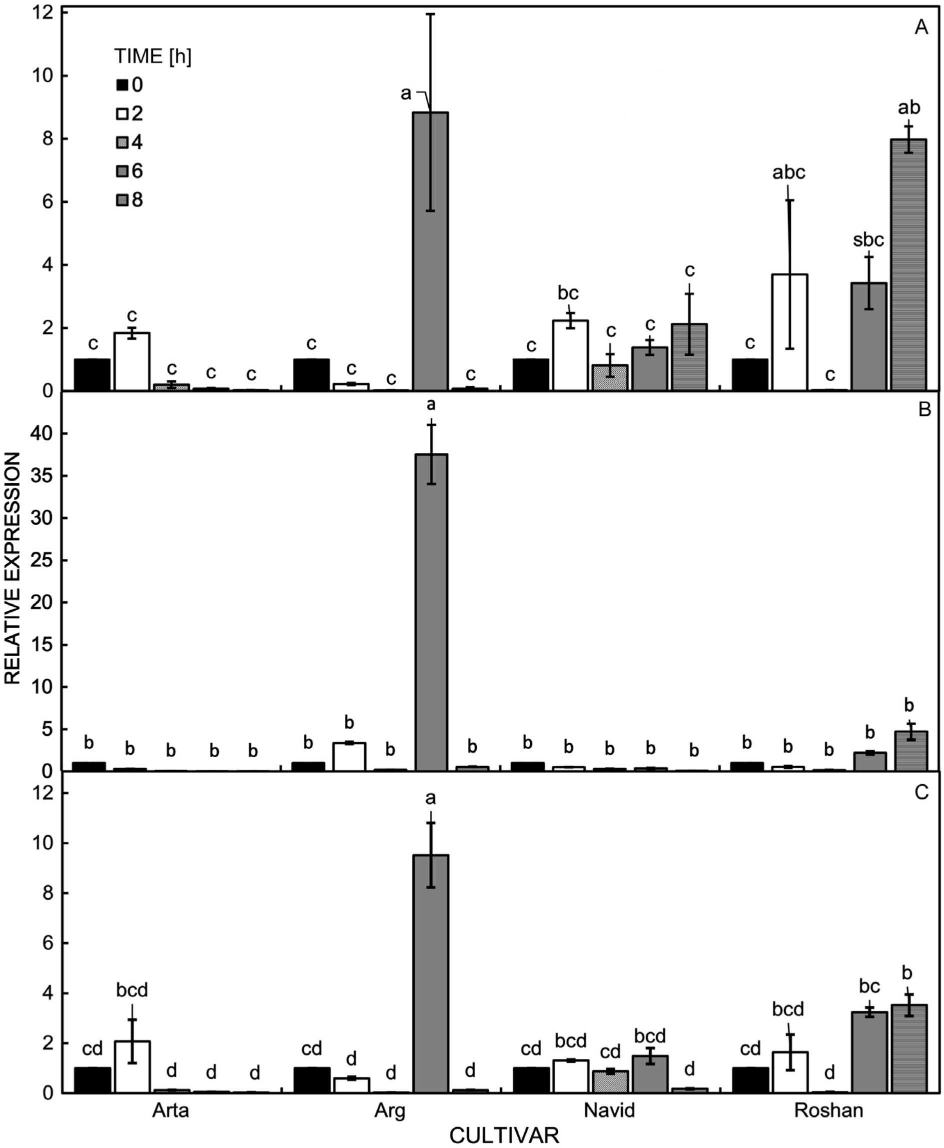

Fig. 2. Expression patterns of genes encoding enzymes of stromal section without the *sAPX* gene in wheat cultivars 'Arta', 'Arg', 'Navid', and 'Roshan'. Means ± SEs, $n = 3$. Means with different letters are significantly different at $P \leq 0.05$ based on the Tukey test. Relative expressions of *MDAR* (*A*), *DHAR* (*B*), and *chlGR* (*C*).

stress duration increased. However, in 'Roshan', WLR at 2, 4, and 8 h of stress did not differ significantly.

Irregular activity of POX was observed in the studied cultivars at various time points (Fig. 4*A*). There was no significant difference between stress and non-stress treatments in 'Roshan', whereas in 'Arg', POX activity was significantly higher at 4 h of stress as compared to the control. Generally, a higher POX activity was observed under both stress and non-stress conditions in susceptible 'Arta' and 'Navid' as compared to tolerant 'Arg' and 'Roshan'.

Generally, susceptible 'Arta' and 'Navid' showed a higher content of MDA, especially at 6 h of stress, compared to tolerant 'Arg' and 'Roshan'. Content of MDA in 'Navid' increased at 2, 4, and 6 h of stress and then decreased at 8 h. Although a slight increase in MDA content in 'Arg' was observed at 2, 4, and 6 h of stress, it was not significantly different from the control. In 'Roshan', MDA increased significantly at 2 h of stress in comparison to the control. Content of MDA decreased significantly at 8 h of stress as compared to 2, 4, and 6 h in both the tolerant cultivars (Fig. 4*B*).

Susceptible 'Arta' and 'Navid' showed a higher proline accumulation, especially after 6 and 8 h of dehydartion, than the tolerant cultivars. However, proline content for tolerant 'Roshan' at 4 h of stress was as high as that in the susceptible cultivars. Yet, during the next hours, its proline accumulation decreased so that the amount of proline at 8 h was not significantly different from the control. It should be mentioned that proline content of tolerant 'Arg' was also significantly lower than in the susceptible cultivars at non-stress conditions and remained almost constant

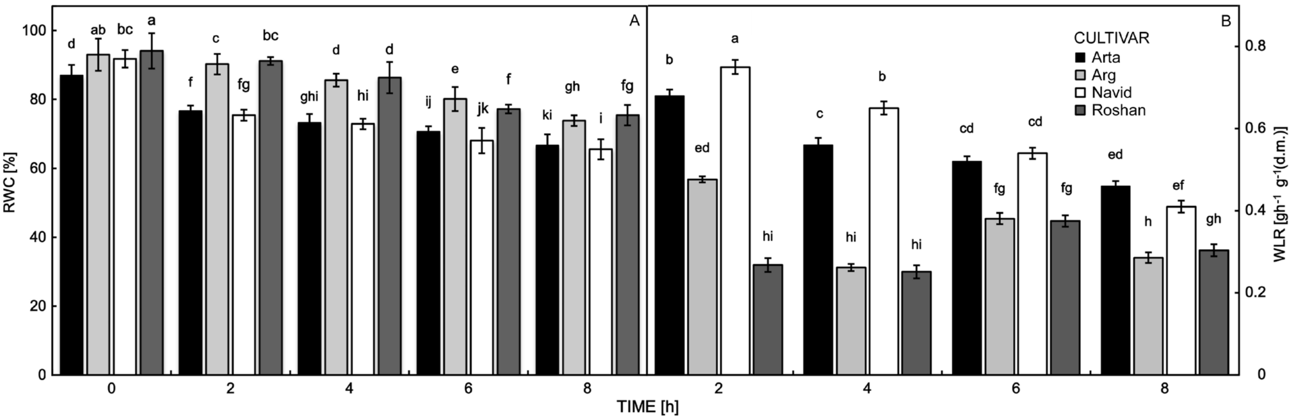

Fig. 3. Relative water content (RWC; *A*) and water loss rate (WLR, *B*) in wheat cultivars Arta, Arg, Navid, and Roshan under dehydration stress. Means ± SEs, *n* = 3. Means with different letters are significantly different at $P \leq 0.05$ based on the Tukey test.

during the whole stress, except at 6 h, when it decreased significantly as compared to the control.

The results of principal component analysis shows that the first two principal components (PCA1 and PCA2) accounted for 69 % of the total variation. Distributing the cultivars under the different time of dehydration based on the first and second components resulted in three groups (Fig. 1 Suppl.). Susceptible 'Arta' and 'Navid' (within the circle) were separated completely from tolerant 'Arg' and 'Roshan'. However, the tolerant genotypes were divided into two groups, mainly by the duration of dehydration stress; at a longer drought stress (6 and 8 h), they were grouped together, except for 'Arg' at 8 h of stress.

The PCA1 is characterized mainly by lower expressions of *tAPX*, *chlGR*, *MDAR*, *chlCu/Zn SOD*, and *DHAR*. The PCA2 is determined mostly by a higher RWC and lower WLR and MDA. Thus, the susceptible cultivars had higher PCA1 and lower PCA2 scores (*i.e.*, lower expressions of *tAPX*, *chlGR*, *MDAR*, *chlCu/Zn SOD*, and *DHAR*, and higher WLR and MDA content). On the other hand, the tolerant cultivars had mainly lower PCA1 scores (*i.e.*, higher expressions of *tAPX*, *chlGR*, *MDAR*, *chlCu/Zn SOD*, and *DHAR*, especially at a longer dehydration), and also higher PCA2 scores (a higher RWC and lower WLR and MDA), except for 'Arg' at 6 h of stress with the lowest amount of the PCA2 score.

Discussion

The increasing expression of genes encoding antioxidant enzymes acts as a protector of plant cells against oxidative stress caused by drought (Sečenji *et al.* 2010). The antioxidant enzymes associated with water-water and AsA-GSH cycles play an important role in stress tolerance (Hernández *et al.* 2009). Generally, the expression of the *chlCu/Zn SOD* gene in the tolerant cultivars was higher than in the susceptible cultivars under water stress (Xu *et al.* 2011). Increasing *Cu/Zn SOD* expression in transgenic tobacco as compared to a wild-type improves the antioxidant defense system of chloroplast and increases tolerance to salinity, water, and osmotic stresses (Badawi *et al.* 2004). A higher expression of *Cu/Zn SOD* gene from *Avicennia marina* in the transgenic rice induces its better tolerance to drought stress than it is in a non-transgenic genotype (Parshanth *et al.* 2008). Furthermore, a higher expression of *Cu/Zn SOD* and an increased activity of the respective enzyme results in an improved tolerance to salinity in rice seedlings (Kaminaka *et al.* 1998).

An increased expression of the *MDAR* gene was observed in the tolerant cultivars as compared to the susceptible ones. Various studies have reported the increase of *MDAR* expression under different stresses such as salinity, heat, drought, and cold in different plants (Ozturk *et al.* 2002, Chew *et al.* 2003, Yoon *et al.* 2004, Leterrier *et al.* 2005, Eltayeb *et al.* 2006, Sečenji *et al.* 2010). However, in Kentucky bluegrass, a decrease in *MDAR* expression has been reported under drought stress by Xu *et al.* (2011). In our study, an increasing expression of this gene in the tolerant cultivars can be a reason for an increased MDAR activity resulting in a higher AsA content and a better tolerance to stress.

The susceptible cultivars showed a less *DHAR* gene expression as compared to the tolerant cultivars. This decrease in gene expression of the sensitive cultivars may be associated with a decreased activity of the related enzyme (Hosseini *et al.* 2015). The DHAR regenerates AsA from DHAsA (Asada 1999). As observed in this study, the decrease in expression of genes responsible for AsA regeneration (*MDHAR* and *DHAR*) in the sensitive cultivars caused a greater sensitivity to oxidative stress imposed by drought stress. Sečenji *et al.* (2010) observed an increased expression of chloroplast and cytosolic *DHAR* genes in tolerant cultivars of wheat under drought stress. Baek and Skinner (2003) reported that under cold stress, a tolerant wheat cultivar has a higher expression of these genes relative to a susceptible cultivar. In transgenic tobacco, an increased expression of *DHAR* results in an increased salinity tolerance (Eltayeb *et al.* 2006). In another study, *DHAR* expression in white clover increases under cold and cadmium stresses but remains constant under drought stress (Zhang *et al.* 2015).

In the present study, the tolerant cultivars generally showed a higher expression of the *GR* gene. Lascano *et al.*

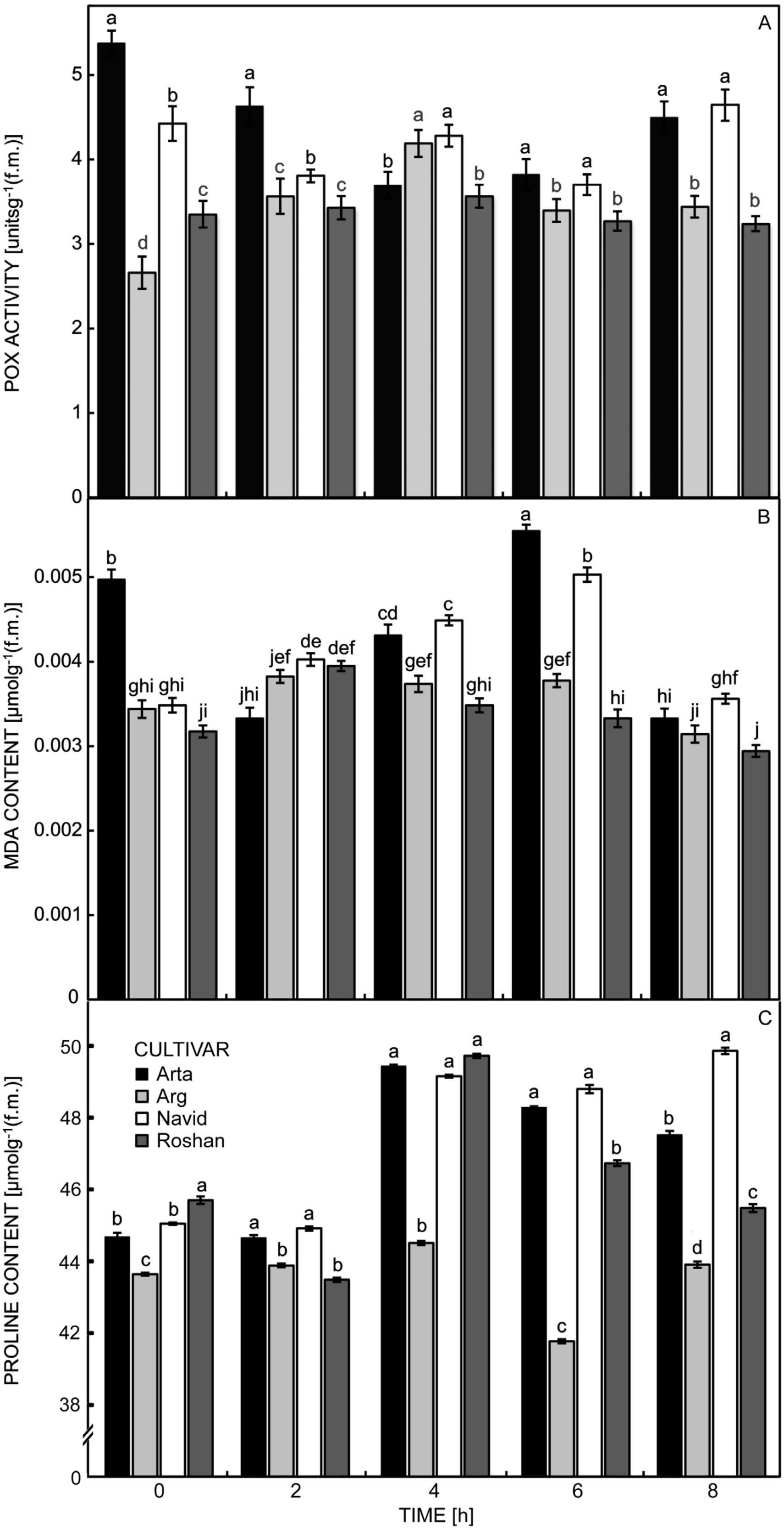

Fig. 4. Peroxidase (POX) activity (*A*), malondialdehyde (MDA) accumulation (*B*), and proline content (*C*) in wheat cultivars Arta, Arg, Navid, and Roshan under dehydration stress. Means ± SEs, $n = 3$. Means with different letters are significantly different at $P \leq 0.05$ based on the Tukey test.

(2003) reported that tolerant wheat genotypes has a higher content of GSH than non-tolerant genotypes. An increase in the expression of *GR* in tolerant cultivars of wheat has also been reported by Sečenji *et al*. (2010).

The pattern of expressions of *chlCu/Zn SOD* and *tAPX* genes was almost similar in the tolerant cultivars. Their expression increased in 'Arg' after 2 and 6 h and in 'Roshan' after 6 and 8 h of dehydration. However, in the sensitive cultivars, the expressions of both genes decreased with stress duration. An increase in the expression of the *chlCu/Zn SOD* gene in the tolerant cultivars was likely accompanied by an increased activity of the respective enzyme and as a result, by a greater amount of O_2^- converted to H_2O_2. Chloroplast uses APX to detoxify H_2O_2. As noted in this study, *APX* gene expression also increased and probably also the activity of APX, which neutralizes H_2O_2 to H_2O and O_2. An increased expression of *Cu/Zn SOD* and *APX* genes under drought stress has also been reported in chickpea (Mittler and Zilinskas 1994), Kentucky bluegrass (Xu *et al*. 2011), and white clover (Zhang *et al*. 2015).

Improvement in stress tolerance has been associated with an increase in the expression of genes encoding antioxidant enzymes (Baek and Skinner 2003, Xu *et al*. 2011). The AsA-GSH cycle is active in most cellular organelles, such as mitochondria, chloroplast, and cytoplasm, and consists of APX, MDAR, DHAR, and GR (Hernández *et al.* 2009). Two crucial non-enzymatic antioxidants are GSH and AsA and their redox state is provided from AsA-GSH cycle. Expressions of the genes encoding antioxidant enzymes related to AsA-GSH cycle show that tolerant 'Arg' and 'Roshan' had higher expressions of them than susceptible 'Arta' and 'Navid', which increased the AsA and GSH redox state and improved tolerance of these cultivars to drought stress. These results are consistent with the findings of Baek and Skinner (2003), Sečenji *et al*. (2010) and Li *et al*. (2013) in wheat, Sultana *et al*. (2012) in rice, Ozturk *et al*. (2002) in barley, and Lee *et al.* (2007) and Eltayeb *et al*. (2007) in tobacco.

The tolerant cultivars had a higher RWC and a lower WLR than the non-tolerant genotypes, which is consistent with the finding of Rampino *et al.* (2006). The RWC is an appropriate indicator for screening drought tolerant cultivars and provides basic information about response to drought (Rampino *et al*. 2006, Wu *et al*. 2014). It has also a positive relationship with cell membrane stability (Mao *et al.* 2010).

The POX is not involved directly in water-water cycle, but it is one of the most important components of enzymatic defense that contributes to H_2O_2 detoxification. The POX activity in susceptible 'Arta' and 'Navid' was higher under both stress and non-stress conditions than in tolerant 'Arg' and 'Roshan'. These results are similar to the findings of Yang *et al*. (2011). In spite of the fact that peroxidases and catalases are present in most ROS-producing organelles, they are not very important for scavenging H_2O_2 caused by drought stress, and APX instead is very important for H_2O_2 sweeping (Hernández *et al*. 2012, Ara *et al*. 2013). As it was observed, the tolerant cultivars exhibited a higher expression of the *APX* gene, but the activity of POX in the tolerant cultivars was lower than in the susceptible genotypes.

The major product of membrane peroxidation is MDA. In our study, MDA content in the tolerant cultivars was lower than in the susceptible cultivars under higher drought stress. 'Arg' and 'Roshan' were more successful than 'Arta' and 'Navid' in protecting the integrity of membrane and had a lower MDA content. Similar results were reported in different plants under stress conditions (Sairam and Srivastana 2001, Bai *et al*. 2006, Moussa and Abdel-Aziz 2008).

Grouping cultivars based on principal component analysis mostly confirmed the results of evaluating the response of susceptible and tolerant cultivars to the dehydration stress for each of the studied attributes separately and the tolerant cultivars resisted better to drought stress as compared with the susceptible cultivars.

As concerns proline content, the response to drought stress was irregular in tolerant 'Arg' and 'Roshan'. A significant decrease was observed at some time points of drought stress and a significant increase at another time points. However, proline content increased in susceptible 'Arta' and 'Navid' after 4 h of dehydration. Increasing proline accumulation in the non-tolerant cultivars compared to the tolerant cultivars has also been reported by other researchers (Aziz *et al.* 1998, Ain-Lhout *et al.* 2001, Peuke *et al.* 2002, Vaidyananthan *et al.* 2003, Theerakulpisut *et al.* 2005, Rampino *et al.* 2006, Poustini *et al.* 2007, Nounjan *et al.* 2012, Tavakoli *et al.* 2016). A lower proline accumulation in the tolerant cultivars at higher stress as compared to the sensitive cultivars may be due to the higher expressions of genes encoding antioxidant enzymes, which lead to better ROS scavenging. Thus, the accumulation of H_2O_2 as a proline synthesis stimulator decreases (Yang *et al*. 2009). As shown in Fig. 4*C*, the maximum proline accumulation in 'Roshan' was after 4 h of dehydration when expressions of antioxidant enzyme encoding genes were lowest (Fig. 1 and 2). Water stress is inevitably associated with increasing oxidative stress due to an increased accumulation of ROS, especially O_2^- and H_2O_2, in chloroplasts, mitochondria, and peroxisomes (Chakraborty and Pradhan 2012). Tolerant cultivars are better capable of scavenging ROS, including H_2O_2. A manifold increase in proline content along with a decreasing leaf potential and RWC is known issue (Rampino *et al*. 2006). Thus, another reason for the lower proline content in the tolerant cultivars can be attributed to the greater ability of these cultivars to maintain RWC due to less WLR.

Proline accumulation is one of the various plant strategies for improving osmotic adjustment and maintaining cellular functions. However, the reports on the importance of proline accumulation in conferring tolerance to drought stress are still contradictory and highly controversial (Hare and Cress 1997). Although proline content is generally higher in stress-tolerant than in stress-sensitive plants, some reports have shown no positive relationship between proline accumulating potential and drought resistance (Hanson *et al.* 1977) and some others, similar to our results, have shown a larger proline content in sensitive cultivars than in tolerant cultivars under drought and salt stresses (Aziz *et al.* 1998, Ain-Lhout *et al.* 2001, Peuke *et al.* 2002, Vaidyananthan *et al.* 2003, Demiral and Türkan 2005, Theerakulpisut *et al.* 2005, Rampino *et al.* 2006, Poustini *et al.* 2007, Nounjan *et al.* 2012, Tavakoli *et al.* 2016). Therefore, it is not decided yet whether or not the increase in proline content can confer increased drought tolerance in plants (De Ronde *et al.* 2000, Nayyar and Walia 2003, Rampino *et al*. 2006).

Conclusions

The increase in the expression of genes encoding antioxidant enzymes in thylakoidal and stromal sections of chloroplasts in the tolerant cultivars improved their protection against oxidative damage caused by water stress as compared to the susceptible cultivars. Therefore, RWC was higher and WLR and MDA were lower in the tolerant cultivars than in the susceptible cultivars, possibly due to decreased cell membrane damage. However, proline accumulation was lower in the tolerant cultivars as compared to the susceptible ones due to better scavenging ROS including H_2O_2 (as a result of higher expressions of *tAPX*, *chlGR*, *MDAR*, *chlCu/Zn SOD*, and *DHAR* genes) and a greater ability to maintain RWC. Although POX activity of the tolerant cultivars was lower than in the susceptible cultivars, these cultivars used other antioxidant defense components.

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