J Plant Growth Regul (2011) 30:436–444
DOI 10.1007/s00344-011-9206-z

Effect of Selenium on Ascorbate–Glutathione Metabolism During PEG-induced Water Deficit in *Trifolium repens* L.

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Received: 13 September 2010 / Accepted: 25 February 2011 / Published online: 23 March 2011

Abstract To elucidate the effect of selenium (Se) on the ascorbate–glutathione (ASC–GSH) cycle under drought stress, the activities of antioxidant enzymes and the levels of molecules involved in ASC–GSH metabolism were studied in *Trifolium repens* seedlings subjected to polyethylene glycol (PEG)-induced water deficit alone or combined with 5 μM Na_2SeO_4. Compared to the control, H_2O_2, thiobarbituric acid reactive substances (TBARS), ascorbate (ASC), dehydroascorbate (DHA), and glutathione disulfide (GSSG) contents increased, whereas a constant content of glutathione (GSH) and decreases in ASC/DHA and GSH/GSSG ratios were observed in the presence of PEG. The activities of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) were upregulated, except for monodehydroascorbate reductase (MDHAR) activity during PEG-induced water deficit. Se application decreased the contents of H_2O_2, TBARS, DHA, and GSSG, increased the levels of GSH and ASC, and inhibited the decreases of ASC/DHA and GSH/GSSG ratios. Although it did not affect APX activity significantly, Se addition improved the activities of MDHAR, DHAR, and GR. Furthermore, GR activity showed the highest increase followed by that of DHAR and MDHAR in decreasing order. These data indicated that fluctuations in ASC–GSH metabolism resulting from Se may have a positive effect on drought stress mitigation, and the regulation in the ASC–GSH cycle can be attributed mainly to GR and DHAR in PEG + Se-treated *T. repens* seedlings.

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Keywords Ascorbate peroxidase · Glutathione reductase · Monodehydroascorbate reductase · Selenium · *Trifolium repens* L.

Introduction

Drought is one of the main environmental factors that adversely affect plant growth, productivity, and survival. Drought stress is often associated with increased levels of reactive oxygen species (ROS), represented by the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), and singlet oxygen ($^1O_2$) (Smirnoff 1993; Chaves and others 2003; Bray 2004). If not effectively and rapidly removed from plants, excessive levels of ROS can damage a wide range of cellular macromolecules such as lipids, enzymes, and DNA (Asada 1994). Ascorbate (ASC) and glutathione (GSH) are two crucial nonenzymatic compounds involved in defense against oxidative stress. ASC reacts chemically with $^1O_2$, $O_2^{\bullet -}$, and •OH, and acts as the natural substrate of many plant peroxidases (Mehlhorn and others 1996). Moreover, ASC is involved in other functions such as plant growth, gene regulation, and modulation of some enzymes (Briviba and others 1997; Horemans and others 2000; Smirnoff 2000). GSH scavenges $^1O_2$ and H_2O_2 and is oxidized to glutathione disulfide (GSSG) when it acts as an antioxidant and redox regulator (Briviba and others 1997; Smirnoff 1993, 2000). The ascorbate–glutathione (ASC–GSH) cycle is the recycling pathway of ASC and GSH regeneration (Fig. 1). Thus, the ASC–GSH cycle plays an important role in maintaining the contents of ASC and GSH in plants. In this cycle, ascorbate peroxidase (APX) catalyzes reduction of H_2O_2 to water, with ASC as electron donor. ASC oxidation produces monodehydroascorbate (MDHA), which disproportionately

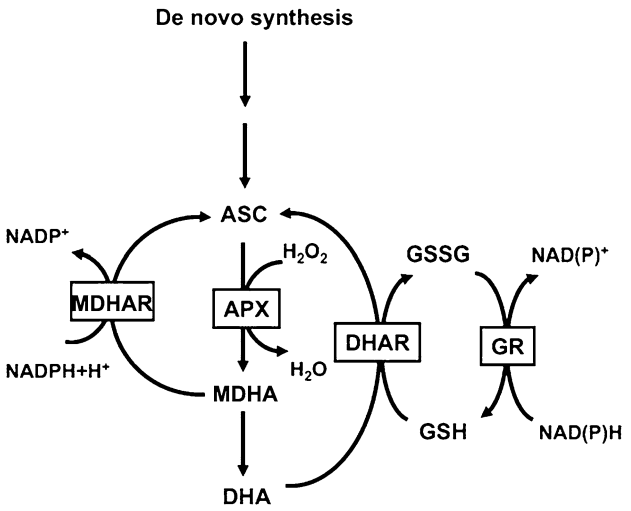

Fig. 1 Schematic representation of the ascorbate–glutathione system

and spontaneously changes to dehydroascorbate (DHA) or is enzymatically reduced to ASC by monodehydroascorbate reductase (MDHAR). DHA is also reduced to ASC by dehydroascorbate reductase (DHAR), using GSH as an electron donor. The GSSG produced in this cycle is then converted back to GSH by glutathione reductase (GR) (Horemans and others 2000; Smirnoff 2000). In this way, ASC and GSH are regenerated and H_2O_2 is scavenged.

Selenium (Se) is an essential micronutrient for animal and human nutrition, but whether it is essential to plants remains controversial. However, several reports have shown a protective role of Se against oxidative stress by increasing the GSH–Px activity and decreasing lipid peroxidation in higher plants (Hartikainen 2005) and in the green algae *Chlamydomonas reinhardtii* (Yokota and others 1988; Takeda and others 1993). In animal cells, Se has been found to influence the level of DNA methylation (Davis and others 2000; Xu and others 2000; Takiguchi and others 2003). There are also indications that Se may enhance the spontaneous disproportion of superoxide radicals and thus reduce the need for their scavenger superoxide dismutase (SOD) (Hartikainen and others 2000). Se may also protect plants from fungal infection and invertebrate phloem-feeders (Hanson and others 2004). Other biological functions of Se, including increased tolerance of plants to UV-induced oxidative stress as well as a promotive growth effect in aging seedlings, have been previously reported (Hartikainen 2005). Furthermore, Se-induced changes in the activities of oxidoreductase enzymes were identified in wheat (Nowak and others 2004), soybean (Djanaguiraman and others 2005), and coffee cell suspension cultures (Gomes-Junior and others 2007). On the basis of the antioxidative role of Se, we hypothesized that selenium application would affect ASC–GSH metabolism under water deficit. Thus, we studied the polyethylene glycol (PEG)-induced change in the ASC–GSH cycle, as well as the impact of Se added to the medium as a protector against water deficit through effects on ASC–GSH metabolism.

The experiment was performed on *Trifolium repens*, which is grown extensively in the temperate and the subtropical regions of the world as one of the most important forage legumes and cover crops. *T. repens* is agronomically highly valuable because it adds enormous amounts of nitrogen to soils by means of the N_2-fixing bacterium *Rhizobium trifoli* in the roots. Previous studies with respect to the responses of *T. repens* to water stress have focused on the activities of antioxidant enzymes (Bermejo and others 2006; Lee and others 2007, 2009; Wang and Li 2008; Wang and others 2008), and only a few reports provide information about Se application in *T. repens* (Smith and Watkinson 1984; Wu and Huang 1992; Mora and others 2008). Moreover, the role of the ASC–GSH cycle and the contribution of Se to ASC–GSH metabolism in water-stressed *T. repens* are still unknown, and none of the reports published so far have analyzed the whole complex reactions of components of the ASC–GSH pathway in *T. repens*. Therefore, in the present study the hypothesis that external Se will mitigate PEG-induced water stress through regulation of ASC–GSH metabolism was tested. To determine the role of Se in regulation of ASC–GSH metabolism in *T. repens* under water stress, we investigated the effect of Se on activities of antioxidant enzymes and the levels of molecules involved in the ASC–GSH cycle in *T. repens* exposed to PEG-induced water deficit.

Materials and Methods

Plant Material and Water Stress Treatments

Seedlings of white clover (*Trifolium repens* L.) cultivar 'Syrian Selection' were established as apical cuttings consisting of two to three nodes in sand–peat potting mix. The experiment was conducted in growth chambers. The 40-day-old seedlings from the beginning of cuttage were transplanted to plastic jugs of half-strength Hoagland nutrient solution. The ambient temperature throughout the day and night was 25 ± 2°C, relative humidity was 60%, photoperiod was 14 h light/10 h dark, and photon flux density was 200 μmol m^{-2} s^{-1}. The 55-day-old seedlings comprising at least three mature stolons were used for the experiment.

On the basis of a previous experiment (data not shown), media containing PEG 6000 to create an osmotic potential of −1.0 MPa and culture time up to 5 days were chosen to investigate the protective role of Na_2SeO_4 at the concentration of 5 μM. The 55-day-old uniform *T. repens*

seedlings were selected and divided into three groups for different treatments: one group was cultured in half-strength Hoagland nutrient solution containing both 5 μM Na_2SeO_4 and PEG 6000 with an osmotic potential of −1.0 MPa; the second group was cultured in the same nutrient solution but contained PEG 6000 (−1.0 MPa) alone; and the last group, cultured in the same basic nutrient solution free of 5 μM Na_2SeO_4 and PEG 6000, was used as the control. Each treatment had 25 replicate jugs and each jug contained five seedlings. Fifteen jugs, five jugs for each of the three treatments, were arranged randomly in one growth chamber. At 9:00 a.m. of 0, 1, 2, 3, 4, and 5 days thereafter, the *T. repens* seedlings were selected randomly in the same chamber, and leaves on the second node of stolons of the selected seedlings were collected for various assays. Each biochemical assay was performed in quintuplicate by using plants in five different chambers.

H_2O_2 and Lipid Peroxidation Assay

Lipid peroxidation was estimated as thiobarbituric acid reactive substances (TBARS). H_2O_2 and TBARS assays were done as we have described previously (Wang and Song 2009). Leaves were powdered with acetone (w/v = 1/10) and filtered through eight layers of gauze cloth. After addition of 0.15 g active carbon, 0.2 ml of 20% $TiCl_4$ in HCl and 0.2 ml of ammonia were added to 1 ml of the supernatant. After centrifugation the supernatant was discarded and the pellet was dissolved in 3 ml of 1 M H_2SO_4 and a spectrum measurement was made at 410 nm. The H_2O_2 content in leaves was calculated from the absorbance at 410 nm compared with the standard curve. For TBARS assay, leaves were homogenized in 0.1% trichloracetic acid (TCA) (w/v = 1/10) and centrifuged at 15,000×*g* for 15 min. To the 1.0-ml aliquot of the supernatant 4.0 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated at 95°C for 30 min in the laboratory electric oven and then cooled in an ice bath. After centrifugation at 10,000×*g* for 10 min, the absorbance of the supernatant was recorded at 532 nm. The TBARS content was calculated according to its extinction coefficient of 155 mM^{-1} cm^{-1}.

Analysis of ASC, GSH, Total Ascorbate, and Total Glutathione

ASC and DHA were measured according to the method of Hodges and others (1996). Total glutathione, GSSG, and GSH were measured according to the method of Griffith (1980). A 0.5-g aliquot of leaves was homogenized in 1.0 ml of ice-cold 2 M $HClO_4$. The homogenate was filtered through three layers of cheesecloth and then centrifuged at 15,000×*g* for 5 min. The supernatant was neutralized with 5 M K_2CO_3 to pH 4.5 for ascorbate determination and to pH 6.5 for glutathione determination.

ASC was measured spectrophotometrically by reading the absorbance at 265 nm due to ascorbate oxidation by ascorbate oxidase. For measurements of total ascorbate (ASC + DHA), 300 μl of neutralized extract were added to 3 ml of a reaction mixture containing 20 mM dithiothreitol (DTT) in 50 mM Hepes-KOH (pH 7.0). After incubation for 10 min at 25°C in a water bath, 100 μl of 0.5 M N-ethylmaleimide was added to remove DTT. The reaction was started with the addition of 5 units of ascorbate oxidase. For ASC determination, 300 μl of neutralized extract was added to 3 ml of a reaction mixture containing 150 mM K_3PO4 (pH 7.4), 5 mM EDTA, and 5 units of ascorbate oxidase. The concentration of DHA was calculated as the difference between total ascorbate and ASC.

The levels of GSH were measured spectrophotometrically by monitoring the reduction of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm. For measurements of total glutathione (GSH + GSSG), 150 μl of neutralized extract was added to 3 ml of 0.3 mM NADPH, 150 μl of 6 mM DTNB, and 1 unit of GR. For GSSG determination, 150 μl of neutralized extract was incubated with 2 μl of 2-vinylpyridine for 1 h at 25°C and then added to 3 ml of 0.3 mM NADPH, 100 μl of 6 mM DTNB, and 1 unit of GR. The concentration of GSH was calculated as the difference between total glutathione and GSSG.

Analysis of APX, MDHAR, DHAR, and GR

Enzymes were extracted according to the method of Grace and Logan (1996). Each frozen leaf sample (1.0 g) was ground into a fine powder in liquid N_2 with a mortar and pestle. Fine powder was homogenized in 10 ml of 50 mM KH_2PO_4 (pH 7.5), containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.3% (v/v) Triton X-100, and 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). For estimations of the activities of APX, MDHAR, and DHAR, 1 mM ASC and 2 mM β-mercaptoethanol were added to the homogenizing buffer to prevent inactivation of the respective enzyme. For determination of GR activity, 1 mM DTT and 5 mM $MgCl_2$ were added to the homogenate. The homogenate was centrifuged at 15,000×*g* for 20 min at 4°C. The obtained supernatant was desalted on a Sephadex™ G-25 M column and then used for the enzyme activity assays.

APX activity was measured spectrophotometrically by recording the decrease in ascorbate content at 290 nm (Nakano and Asada 1981). One hundred microliters of enzyme extract was added to a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM

ASC, 0.1 mM EDTA, and 1 mM H_2O_2. The reaction started with the addition of H_2O_2 and an absorption coefficient of 2.8 mM^{-1} cm^{-1} was used for calculations. One unit of APX activity was defined as the amount of enzyme that oxidizes 1 μmol of ASC per minute at 20°C.

MDHAR activity was tested by following the decrease in absorbance at 340 nm due to NADH oxidation (Hossain and others 1984). One milliliter of enzyme extract was added to a reaction mixture containing 50 mM potassium phosphate (pH 7.6), 0.3 mM NADH, and 2.5 mM ASC. The reaction was started by adding ascorbate oxidase (Sigma) to produce MDHA, and an absorption coefficient of 6.2 mM^{-1} cm^{-1} was used for calculations. One unit of MDHAR activity was defined as the amount of enzyme that oxidizes 1 nmol of NADH per minute at 25°C.

DHAR activity was determined by monitoring the increase in absorbance at 265 nm due to ASC production (Hossain and Asada 1984). One milliliter of enzyme extract was added to a reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5), 0.1 mM EDTA, 0.5 mM DHA, and 2.5 mM GSH. The nonenzymatic reduction of DHA by GSH was subtracted. An absorption coefficient of 14.6 mM^{-1} cm^{-1} was used for calculations. One unit of DHAR activity was defined as the amount of enzyme that produces 1 nmol of ASC per minute at 25°C.

GR activity was measured by following the decrease in absorbance at 340 nm due to NADPH oxidation (Carlberg and Mannervik 1985). Two hundred microliters of enzyme extract was added to a reaction mixture containing 1.5 ml of 0.1 M potassium phosphate buffer (pH 7), 150 μl of 20 mM GSSG, 1 ml of distilled water, and 150 μl of 2 mM NADPH (dissolved in Tris–HCl buffer, pH 7), in a final volume of 3.0 ml. An absorption coefficient of 6.2 mM^{-1} cm^{-1} was used for calculations. One unit of GR activity was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per minute at 25°C.

Protein concentration was measured using bovine serum albumin as standard according to the method of Bradford (1976).

Data Analysis

The experimental design was a split-plot design with time as the main plot and treatment as the subplot. The main plot formed a randomized complete block design (RCBD) with six levels as 0–5 days. The subplot consisted of three treatments within each main plot: treatment without Se and PEG, with only PEG, and with both PEG and Se. Each value was presented as mean ± standard error of the mean (SEM), with a minimum of five replicates. Data were analyzed using the PROC GLM procedure of SAS as a randomized complete block design. $p \leq 0.05$ was considered as significant.

Results

Hydrogen Peroxide and Thiobarbituric Acid Reactive Substances

H_2O_2 and TBARS contents in *T. repens* seedlings were proportionally enhanced with time under the PEG-induced water deficit, but remained constant in the control seedlings across the experiment. Thus, the differences of H_2O_2 and TBARS contents between PEG-treated plants and the control were significant at each observation day (Fig. 2). Se application reduced the contents of H_2O_2 and TBARS, but they were still higher than in control plants from the second day of observation. Therefore, the highest accumulation of H_2O_2 and TBARS was observed in PEG-treated seedlings, followed by PEG + Se-treated seedlings and the control in decreasing order from 2 to 5 days, and the differences of H_2O_2 and TBARS contents among the culture days were also significant in both PEG-treated and PEG + Se-treated seedlings.

The Ascorbate and Glutathione Pools

A pronounced change in the ASC pool was observed in PEG-treated *T. repens* seedlings. The levels of ASC and DHA remained unchanged in the control but increased significantly in a time-dependent manner in PEG-treated seedlings (Fig. 3a, b). The value of the ASC/DHA ratio showed a steady decrease in the PEG-treated seedlings (Fig. 3e) from 0 to 4 days but did not decrease further at 5 days. Addition of Se increased the ASC content but decreased the DHA content notably except for the control day (day 0); thus, it inhibited the decrease of the ASC/DHA ratio markedly in PEG + Se-treated seedlings compared to PEG-treated seedlings (Fig. 3a, b, e). The ASC and DHA contents among the whole six culture days and the ASC/DHA ratio among the first 5 days differed significantly in PEG-treated and PEG + Se-treated seedlings.

The content of GSH remained almost constant in both the PEG-treated plants and the control during the experimental period (Fig. 3c). However, the level of GSSG was continuously elevated with time in PEG-treated seedlings (Fig. 3d). Thus, the value of the GSH/GSSG ratio decreased steadily with time in PEG-treated seedlings in contrast to the control (Fig. 3f) across the experiment. Application of Se affected GSH and GSSG differently. The GSH content was elevated whereas the GSSG content was reduced at each culture day, so the decrease in the GSH/GSSG ratio was lessened significantly and the value of the GSH/GSSG ratio was even higher than in the control at 1 and 2 days in PEG + Se-treated seedlings (Fig. 3c, d, f). The GSH in PEG + Se-treated seedlings and the GSSG and GSH/GSSG ratio in both PEG-treated and PEG +

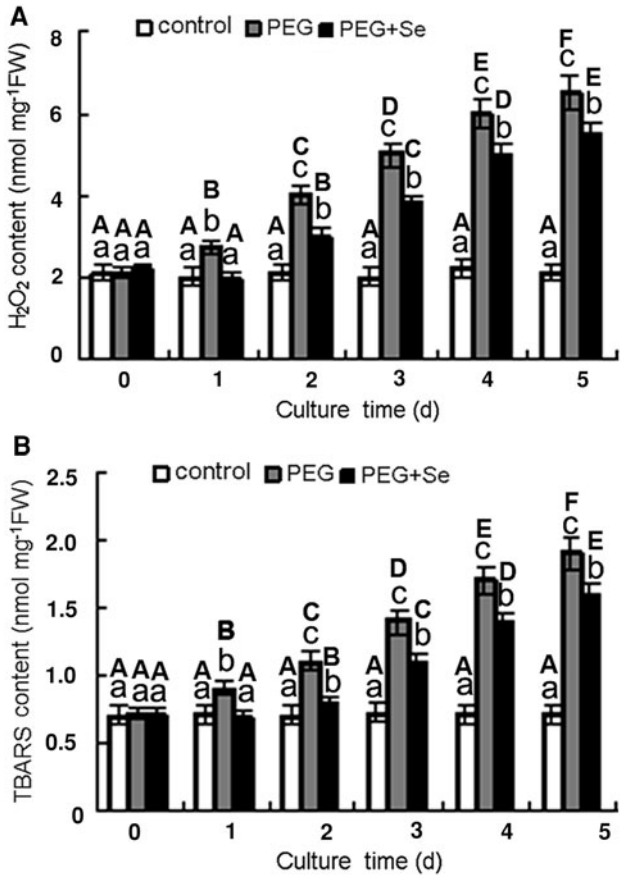

Fig. 2 The contents of H_2O_2 (**a**) and TBARS (**b**) in leaves of *T. repens* during the PEG-induced water-deficit period. The differences among different treatments and culture days were both analyzed; each value is the mean ± SE of five independent measurements. *Bar values* not sharing a common superscript (a–c) at the same day differ significantly at $p \leq 0.05$ among PEG + Se-treated, PEG-treated, and the control plants, whereas bar values not sharing a common superscript (A–F) in the same treatment differ significantly at $p \leq 0.05$ among culture days

Se-treated seedlings were markedly different among the experiment days.

The Activities of the Enzymes Involved in Ascorbate and Glutathione Metabolism

Compared to the control, activities of APX and DHAR in PEG-treated seedlings increased with time (Fig. 4a, c). The enhancement was directly related to the duration of water deficit. For example, the activities of APX and DHAR at 4–5 days were much higher than at 0–3 days. Similar to that of APX and DHAR, GR activity also showed a gradual increase with time in PEG-treated seedlings (Fig. 4d). The increase of GR activity was much lower than that of APX and DHAR activities. MDHAR activity did not change significantly in PEG-treated seedlings compared to the control (Fig. 4b) across the experiment.

Se application influenced the activities of APX, DHAR, MDHAR, and GR differently. APX activity was not affected significantly by Se, whereas the activities of MDHAR, DHAR, and GR were improved in PEG + Se-treated seedlings compared to PEG-treated seedlings (Fig. 4) at each observation day. Furthermore, GR activity showed the highest increase followed by DHAR and MDHAR activities, and the activity increases were reduced with time in PEG + Se-treated seedlings. For example, at 3 days seedlings treated with PEG + Se showed activity increases of 45.7% for GR and 25.3% for DHAR, but only 15.5% for MDHAR in comparison with seedlings treated with only PEG. GR activity increased by 55.6, 48.1, 45.7, 30.4, and 25.3% at 1, 2, 3, 4, and 5 days, respectively, in PEG + Se-treated seedlings when compared with PEG-treated seedlings. Furthermore, the activities of APX, DHAR, and GR differed notably among the culture days in both PEG-treated and PEG + Se-treated seedlings, whereas the MDHAR activity differed significantly only in PEG + Se-treated seedlings.

Discussion

During the period of PEG-induced water deficit, *T. repens* seedlings showed significant increases with time in H_2O_2 content and lipid peroxidation (Fig. 2), which is consistent with our previous observation (Wang and Li 2008; Wang and others 2008). The increases of H_2O_2 and TBARS contents indicated that oxidative stress occurred. Smaller amounts of TBARS and H_2O_2 accumulated in PEG + Se-treated plants than in plants treated with only PEG, suggesting that Se could reduce lipid peroxidation and alleviate oxidative stress induced by water deficit. These results were similar to previous reports (Cartes and others 2005; Djanaguiraman and others 2005) in which Se application reduced the contents of TBARS and H_2O_2.

Although the role of Se as an antioxidant in plants has been previously reported (Xue and others 2001; Seppänen and others 2003; Cartes and others 2005, 2006), the contribution of Se to the ASC–GSH cycle in *T. repens* subjected to water deficit has not been known. In the present study, constant decreases in ASC/DHA and GSH/GSSG ratios were observed in the presence of PEG (Fig. 3e, f). The activities of APX, DHAR, and GR were upregulated by PEG except for MDHAR activity (Fig. 4). Se application decreased the contents of DHA and GSSG, increased the levels of GSH and ASC, and inhibited the decrease of ASC/DHA and GSH/GSSG ratios (Fig. 3). Moreover, addition of Se increased the activities of DHAR, MDHAR, and GR differently, with GR activity showing the greatest increase followed by DHAR and MDHAR activities, and activity increases decreased with time (Fig. 4). These

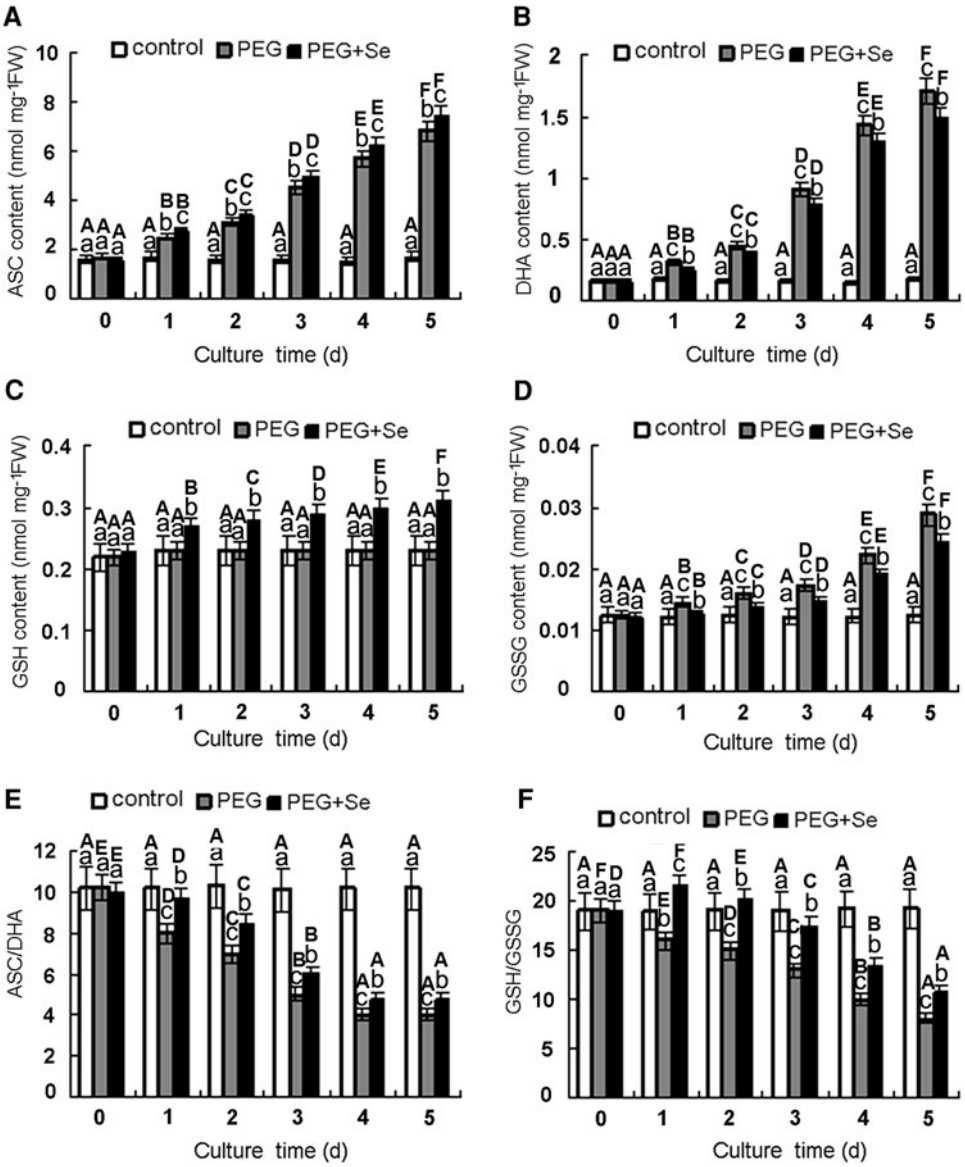

Fig. 3 The contents of ASC (**a**), DHA (**b**), GSH (**c**), GSSH (**d**), and the values of ASC/DHA (**e**) and GSH/GSSG (**f**) ratios in leaves of *T. repens* during the PEG-induced water-deficit period. The differences among different treatments and culture days were both analyzed; each value is the mean ± SE of five independent measurements. *Bar values* not sharing a common superscript (a–c) at the same day differ significantly at $p \leq 0.05$ among PEG + Se-treated, PEG-treated, and the control plants, whereas bar values not sharing a common superscript (A–F) in the same treatment differ significantly at $p \leq 0.05$ among culture days

results further confirmed that external Se alleviated PEG-induced oxidative stress through regulation of enzymes involved in ASC–GSH metabolism.

ASC and GSH are two important compounds of the antioxidant system in plants (Horemans and others 2000; Smirnoff 2000). During the period of PEG-induced water deficit, the levels of ASC and DHA increased differently, resulting in a shift of the ASC/DHA ratio toward an oxidated state (Fig. 3a, e). Except for de novo synthesis, the observed increase of ASC in the presence of PEG may be the result of the increased DHAR activity. In ASC–GSH recycling, MDHA can disproportionately and spontaneously change to DHA or be enzymatically reduced to ASC by MDHAR. DHA is also reduced to ASC by DHAR, using GSH as an electron donor (Arrigoni 1994; Smirnoff 1996; Noctor and Foyer 1998). Enhanced DHAR activity rather than MDHAR activity was observed in PEG-treated plants (Fig. 4b, c), which suggests that DHAR rather than MDHAR contributes mainly to the increase of ASC in the ASC–GSH cycle. Se application increased the activities of DHAR and MDHAR but did not affect APX activity (Fig. 4). Thus, the DHA content decreased and ASC content increased, so the ASC/DHA ratio increased to a reduced state in PEG + Se-treated seedlings compared to PEG-treated seedlings.

As far as GSH is concerned, the content remained almost constant (Fig. 3c), whereas the level of GSSG was continuously elevated during the PEG-induced water-deficit period (Fig. 3d). Consequently, the value of the GSH/GSSG ratio decreased significantly compared to the control (Fig. 3f). Similar results were also found in several plants under stress conditions (Jiménez and others 1998; Kuzniak and others 1999; Belmonte and others 2005). Although GR

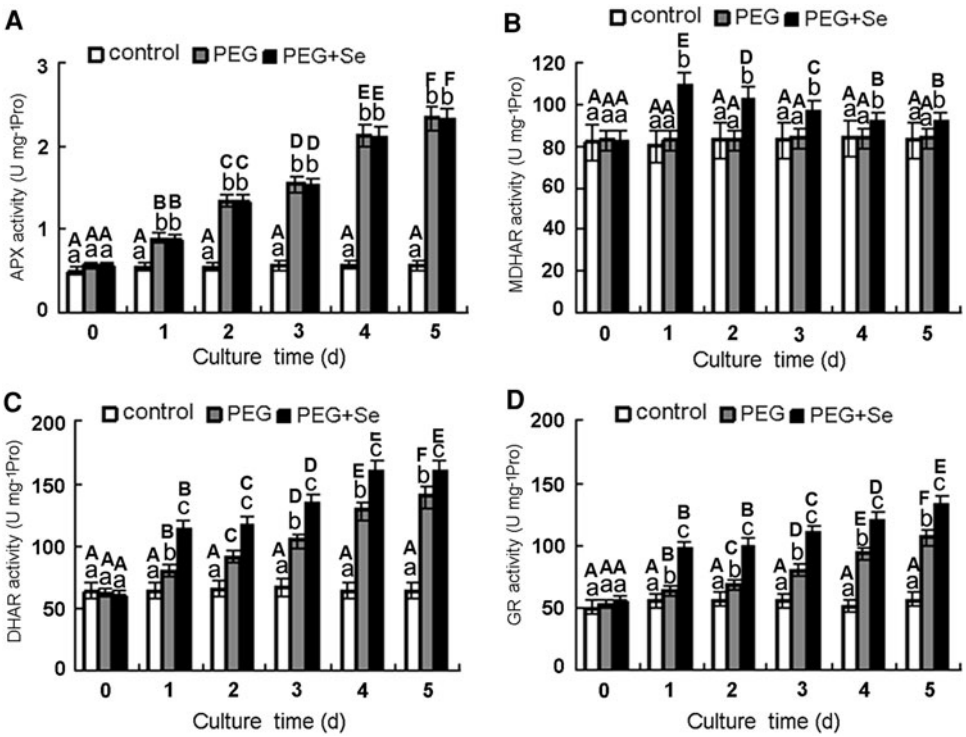

Fig. 4 The activities of APX (**a**), MDHAR (**b**), DHAR (**c**), and GR (**d**) in leaves of *T. repens* during the PEG-induced water-deficit period. The differences among different treatments and culture days were both analyzed; each value is the mean ± SE of five independent measurements. *Bar values* not sharing a common superscript (a–c) at the same day differ significantly at $p \leq 0.05$ among PEG + Se-treated, PEG-treated, and the control plants, whereas bar values not sharing a common superscript (A–F) in the same treatment differ significantly at $p \leq 0.05$ among culture days

activity increased gradually in PEG-treated seedlings (Fig. 4d), the content of GSH remained unchanged (Fig. 3c). Thus, the constant level of GSH may be due not only to the limitation of GR activity, it may also be due to its utilization as an electron donor in the synthesis of ASC. Se addition further increased the GR activity significantly, resulting in an increase in GSH content and a decrease in GSSG content, as well as an increase in the GSH/GSSG ratio in PEG + Se-treated seedlings compared to PEG-treated seedlings (Fig. 3c, d, f). Moreover, the increased GSH provides reducing substrates for the reduction of DHA to ASC by DHAR, then the produced ASC is used by APX to directly detoxify H_2O_2.

Se application is reported to reduce the oxidative damage in biological organisms by stimulating the activity of selenoenzymes that prevent the accumulation of hydrogen peroxide and lipid peroxides in organs and tissues (Hartikainen 2005; Djanaguiraman and others 2010). Due to its chemical similarity to S, Se can nonspecifically replace it in compounds containing sulfhydryl groups, such as sulfur amino acids, glutathione peroxidase, and coenzyme A. Enzymes of the oxidoreductase class can be the most Se sensitive because of the antioxidant properties of the element. When replacing sulfur in enzymes, Se may result in S–S bonds being replaced by the less stable Se–Se bonds thereby leading to changes in their structure and biological properties (Brown and Shrift 1982; Fu and others 2002; Valkama and others 2003). Our results presented in this article revealed significant changes in the activities of oxidoreductase in the ASC–GSH cycle in response to Se. Although the detailed mechanisms responsible for the effect of Se on antioxidant enzymes involved in the ASC–GSH cycle can not be clarified with the results of this experiment, it is noteworthy that Se regulated ASC–GSH metabolism through changing the enzyme activities in *T. repens*. On the other hand, Hartikainen and others (2000) found that the antioxidative effect of Se was associated with α-tocopherol synthesis but not with enzyme activity increase. Blokhina and others (2003) have also suggested the possible role of nonenzymatic reactions in removing oxidative radicals. In the present study, such reduction patterns of oxygen species could be explained by the positive role of Se in upregulation of GSH and ASC, which are two crucial nonenzymatic compounds involved in defense against oxidative stress. Moreover, the protective role of Se in stabilization of the DNA methylation patterns has been reported in both animals and plants (Davis and others 2000; Filek and others 2008). Se ions can be bound specifically to DNA cytosine and change their methylation. The protective role of Se in the changes in DNA methylation pattern may also be connected to the removal of oxygen species in situ produced by stress, as participation of oxygen radicals in nucleic acid fragmentation has been shown in many stress conditions (Filek and others 2008). The above three mechanisms may all possibly contribute to the protective role of Se in *T. repens* under water deficit.

In conclusion, the redox pairs ASC/DHA and GSH/GSSG and their redox enzymes were affected by

PEG-induced water deficit. Constant decreases in the ASC/DHA and GSH/GSSG ratios and increases in the activities of APX, DHAR, and GR were observed in the presence of PEG. Addition of Se not only improved the activities of MDHAR, DHAR and GR, it also changed the ASC/DHA and GSH/GSSG ratios toward reduced states. Therefore, the fluctuations of ASC–GSH metabolism resulting from Se may have a positive effect on drought stress mitigation, and the regulation in the ASC–GSH cycle can be mainly attributed to GR and DHAR in PEG + Se-treated seedlings of *T. repens*. Further studies are needed in a true "drought stress" environment with different water regimes rather than a simulated condition (via PEG) to confirm the role of Se in drought stress tolerance.

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