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Scavenger Enzyme Activities in Subcellular Fractions of White Clover (*Trifolium repens* L.) under PEG-induced Water Stress

Chang-Quan Wang · Ye-Feng Zhang · Yong-Bao Zhang

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Abstract Scavenger enzyme activities in subcellular fractions under polyethylene glycol (PEG)-induced water stress in white clover (*Trifolium repens* L.) were studied. Water stress decreased ascorbic acid (AA) content and catalase (CAT) activity and increased the contents of hydrogen peroxide (H_2O_2), thiobarbituric acid reactive substances (TBARS) (measure of lipid peroxidation), and activities of superoxide dismutase (SOD), its various isozymes, ascorbate peroxidase (APOX), and glutathione reductase (GR) in cellular cytosol, chloroplasts, mitochondria, and peroxisomes of *Trifolium repens* leaves. In both the PEG-treated plants and the control, chloroplastic fractions showed the highest total SOD, APOX, and GR activities, followed by mitochondrial fractions in the case of total SOD and GR activities, whereas cytosolic fractions had the second greatest APOX activity. However, CAT activity was the highest in peroxisomes, followed by the cytosol, mitochondria, and chloroplasts in decreasing order. Although Mn-SOD activity was highest in mitochondrial fractions, residual activity was also observed in cytosolic fractions. Cu/Zn-SOD and Fe-SOD were observed in all subcellular fractions; however, the activities were the highest in chloroplastic fractions for both isoforms. Total Cu/Zn-SOD activity, the sum of activities observed in all fractions, was higher than other SOD isoforms. These results suggest that cytosolic and chloroplastic APOX, chloroplastic and mitochondrial GR, mitochondrial Mn-SOD, cytosolic and chloroplastic Cu/Zn-SOD, and chloroplastic Fe-SOD are the major scavenger enzymes, whereas cellular CAT may play a minor role in scavenging of O_2^- and H_2O_2 produced under PEG-induced water stress in *Trifolium repens.*

Keywords Ascorbate peroxidase · Catalase · Glutathione reductase · Superoxide dismutase · *Trifolium repens* L. · Water stress

C.-Q. Wang (✉) · Y.-F. Zhang · Y.-B. Zhang
College of Life Sciences, Shandong University of Technology, Zibo, Shandong 255049, China
e-mail: wangcq@sdut.edu.cn

Introduction

Because of its excellent forage quality and high animal-feed value, *Trifolium repens* L. is an important forage legume and cover crop, extensively grown in temperate and subtropical regions of the world. *T. repens* is agronomically highly valuable because it adds enormous amounts of nitrogen to the degraded soils using nitrogen fixed by the bacterium *Rhizobium trifolii* in the roots.

Water is essential for plant metabolism and any limitation in its availability affects almost all plant functions, including the assimilation and partitioning of carbon (Cabuslay and others 2002; Wu and others 2008). Plants subjected to water stress undergo increased exposure to activated forms of oxygen and changes associated with damage to membranes and buildup of lipid peroxides (Smirnoff 1993). In plant cells chloroplasts, mitochondria, and peroxisomes are important intracellular generators of reactive oxygen species (ROS) (Rich and Bonner 1978; del Rio and others 1991; Salin 1991). ROS produced as a result of various abiotic stresses need to be scavenged for maintenance of normal growth. The primary scavenger is superoxide dismutase (SOD), which converts O_2^- to H_2O_2. This toxic product of the SOD reaction is eliminated by ascorbate peroxidase (APOX) in association with dehydroascorbate reductase and glutathione reductase (GR); the latter two help in regeneration of ascorbic acid (AA) (Asada and Takahashi 1987;

Asada 1994). H_2O_2 is also scavenged by catalase (CAT) (Dhindsa and others 1981; Anderson and others 1995), although the enzyme is less efficient than the APOX-GR system. Water-stress-induced changes in activities of various scavenger enzymes have been reported (Zhang and Kirkham 1994; Hernandez and others 2000). Localization of isozymes of SOD has been reported from various subcellular organelles. Mn-SOD is reported to be located in mitochondria and peroxisomes, Cu/Zn-SOD in chloroplasts, mitochondria, and the cytosol, and Fe-SOD in chloroplasts (Scandalios 1993; Bowler and others 1994). Two isozymes of APOX have been reported, one from chloroplasts and the other from cytosol (Chen and Asada 1989).

Although water-stress responses of *T. repens* at the cellular level have been studied (Bermejo and others 2006; Lee and others 2007), little is known about the effects of water stress on the ROS metabolism in cytosolic, mitochondrial, chloroplastic, and peroxisomal fractions of *T. repens*. This knowledge can supply information on the possible involvement of ROS in the mechanism of damage by water stress, thus allowing insight into the molecular mechanisms of plant tolerance to water-stress-induced oxidative stress. Hence, the objective of the present investigation was to study the effects of short-term PEG-induced water stress on various scavenger enzymes localized in different subcellular fractions and their relevance in terms of water-stress tolerance in *T. repens*.

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 a sand–peat potting mix. When a significant root system had developed, plants were transplanted to plastic jugs of half-strength Hoagland nutrient solution. The ambient temperature 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}. When plants had reached a sufficient size, comprising at least eight to ten mature stolons, water stress was imposed.

For water-stress treatment, *T. repens* plants were cultured in half-strength Hoagland nutrient solution containing polyethylene glycol (PEG) 6000 to create osmotic potentials of −0.5, −1.0, and −1.5 MPa. After 72 h of incubation, *T. repens* leaves were collected for various assays.

H_2O_2, Lipid Peroxidation, and Ascorbic Acid Assay

H_2O_2 estimation was done as we have described previously (Wang and others 2007).

Lipid peroxidation was estimated as thiobarbituric acid reactive substances (TBARS) (Heath and Packer 1968). A fresh leaf sample (0.5 g) or the liquid nitrogen-preserved cell fractions were homogenized in 10 ml of 0.1% trichloracetic acid (TCA), and the homogenate was centrifuged at 15,000 *g* for 15 min. To a 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}.

For ascorbic acid (AA) estimation a fresh leaf sample (0.5 g) or subcellular fractions preserved in liquid nitrogen were extracted with 10 ml of 6% trichloroacetic acid (Mukherjee and Choudhari 1983). Four milliliters of the extract was mixed with 2 ml of 2% dinitrophenylhydrazine (in acidic medium) followed by the addition of 1 drop of 10% thiourea (in 70% ethanol). The mixture was boiled for 15 min in a water bath and after cooling to room temperature, 5 ml of 80% (v/v) H_2SO_4 was added to the mixture at 0°C (in an ice bath). The absorbance was recorded at 530 nm. The concentration of AA was calculated from a standard curve plotted with known concentrations of AA.

Subcellular Fractionation

Cell fractionations were conducted for the purpose of localizing various antioxidant enzymes, and H_2O_2, TBARS, and AA contents in subcellular fractions. Isolation of chloroplasts was as described earlier (Kuźniak and Skłodowska 2001, 2005). The activity of $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9), used as a stromal marker, was assayed as described by Bradbeer (1969). According to Hatch (1978), the activity of fumarase (EC 4.2.1.2) as a specific mitochondrial marker was assayed using the UV method described by Dhindsa and others (1981). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was considered an auxiliary marker for cytosol and was assayed using a Sigma diagnostic kit. Chlorophyll was extracted with 80% acetone and measured according to Porra and others (1989). *T. repens* leaf mitochondria were isolated as described by Kuźniak and Skłodowska (2004, 2005) and purified by a Percoll density-gradient-containing sucrose according to Struglics and others (1993). The mitochondrial fraction located close to the top was identified based on the activity of specific marker enzymes, succinate:cytochrome *c* oxidoreductase (EC 1.6.99.3) and NADH:cytochrome *c* oxidoreductase, both assayed as described by Douce and others (1972), and fumarase. To determine the purity of the mitochondrial fraction,

chlorophyll content and the following marker enzyme activities were assayed: glucose-6-phosphate dehydrogenase for cytosol, $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase for the chloroplast stroma. The intactness of all organelles was determined on the basis of enzyme latency calculated for the activities of specific marker enzymes in the isotonic medium and in the subcellular fractions according to the formula of Burgess and others (1985).

Peroxisomes were purified from *T. repens* leaves by differential and sucrose density-gradient centrifugation (35–60% w/w), as described by López-Huertas and others (1995). All operations were performed at 0–4°C. Peroxisomes purified by this method were essentially free of contamination by other cellular organelles (López-Huertas and others 1995; Distefano and others 1997). Peroxisomal-soluble fractions were obtained by hypotonic shock and ultracentrifugation (López-Huertas and others 1995).

Enzyme Assays

SOD activity in different cell fractions was estimated as we have described previously (Wang and others 2008). To distinguish Cu/Zn-SOD, Fe-SOD, and Mn-SOD, the sensitivity of Cu/Zn-SOD to cyanide (3 mM) and of Cu/Zn-SOD and Fe-SOD to H_2O_2 (5 mM) was used, whereas Mn-SOD was unaffected. The complete reaction mixture plus KCN (3 mM) was used to inhibit Cu/Zn-SOD, whereas the complete reaction mixture plus 3 mM KCN and 5 mM H_2O_2 were used to inhibit both Cu/Zn-SOD and Fe-SOD activities. Separate controls (lacking enzymes) were used for total SOD and inhibitor studies. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme that reduces the absorbance reading to 50% in comparison with tubes lacking enzyme.

APOX was assayed by recording the decrease in optical density due to AA at 290 nm (Nakano and Asada 1981). The 3-ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM AA, 0.1 mM EDTA, 1.5 mM H_2O_2, and 0.1 ml enzyme. The reaction was started with the addition of H_2O_2. Absorbance was measured at 290 nm in a UV-visible spectrophotometer (756B, Shanghai Spectrum Instrumental Company, China), and 0.01 change of absorbance at 290 nm was defined as one unit (U) of APOX activity.

GR was assayed by recording the increase in absorbance in the presence of oxidized glutathione and DTNB (5, 5(-dithiobis-2-nitrobenzoic acid) (Smith and others 1988). The 3-ml reaction mixture contained 200 µmol potassium phosphate buffer (pH 7.5), which contained 1 µmol EDTA, 1.5 µmol DTNB in 0.01 M potassium phosphate buffer (pH 7.5), 0.2 µmol NADPH, 0.1 ml enzyme extract, and distilled water to make up the volume. The reaction was initiated by adding 0.2 µmol GSSG (oxidized glutathione or glutathione disulfide). The increase in absorbance at 412 nm was recorded at 25°C over a period of 5 min spectrophotometrically. A change of absorbance of 0.01 at 412 nm was defined as one unit (U) of GR activity. Each measurement was done in triplicate.

CAT activity was determined by following the consumption of H_2O_2 (extinction coefficient = 39.4 mM cm^{-1}) at 240 nm for 30 s (Aeby 1984). The assay mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2, and 50 µl leaf extract in a 3-ml volume.

The protein content was determined by using bovine serum albumin as the standard according to Bradford (1976).

Results

Hydrogen Peroxide and Thiobarbituric Acid Reactive Substances

H_2O_2 and TBARS contents in different subcellular fractions were proportionally enhanced with the decreasing values of the osmotic potential; the differences between PEG-treated plants and the controls were significant ($p < 0.1$) (Figs. 1 and 2). Although in the control plants the H_2O_2 contents in peroxisomes, cytosol, and chloroplasts were the similar, after different levels of PEG-induced water stress, the highest H_2O_2 accumulation was observed in chloroplasts, followed by peroxisomes, cytosol, and mitochondria in decreasing order. The highest TBARS contents were observed in chloroplastic fractions followed by peroxisomal, mitochondrial, and cytosolic fractions both in the PEG-treated and the control plants.

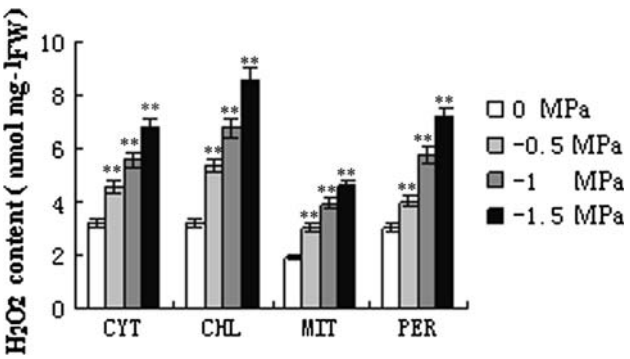

Fig. 1 The H_2O_2 contents in different cell fractions of *T. repens* exposed to different levels of polyethylene glycol-induced water stress and control. CYT: cytosolic fraction; CHL: chloroplastic fraction; MIT: mitochondrial fraction; PER: peroxisomal fractions. Data are mean ± standard error (SE) ($n = 5$), and the differences between PEG-treated plants and controls are indicated by **($p < 0.01$)

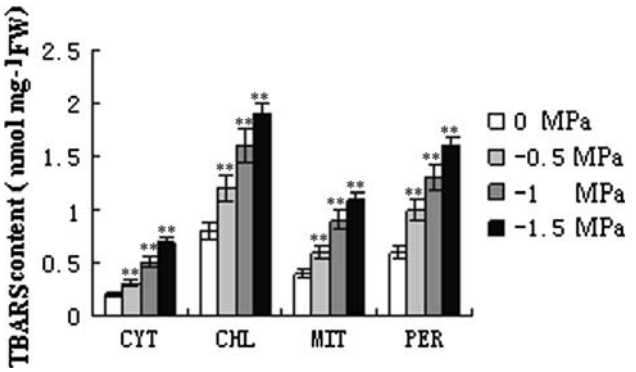

Fig. 2 The TBARS contents in different cell fractions of *T. repens* exposed to different levels of polyethylene glycol-induced water stress and control. CYT: cytosolic fraction; CHL: chloroplastic fraction; MIT: mitochondrial fraction; PER: peroxisomal fractions. Data are mean ± standard error (SE) ($n = 5$), and the differences between PEG-treated plants and control are indicated by **($p < 0.01$)

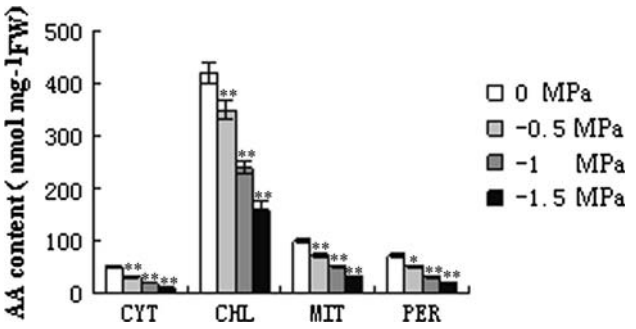

Fig. 3 The AA contents in different cell fractions of *T. repens* exposed to different levels of polyethylene glycol-induced water stress and control. CYT: cytosolic fraction; CHL: chloroplastic fraction; MIT: mitochondrial fraction; PER: peroxisomal fractions. Data are mean ± standard error (SE) ($n = 5$), and the differences between PEG-treated plants and controls are indicated by *($p < 0.05$) and **($p < 0.01$)

Ascorbic Acid

AA contents decreased with decreasing osmotic potentials in all subcellular fractions in *T. repens* leaves (Fig. 3), with significant differences between PEG-treated plants and controls ($p < 0.1$). Chloroplast fractions contained the highest AA content, followed by mitochondrial, peroxisomal, and cytosolic fractions in the PEG-treated and control plants.

Scavenger Enzymes

PEG-induced water stress significantly increased leaf total SOD activity in all subcellular fractions. Total SOD activity was higher in chloroplastic fractions, followed by mitochondrial and peroxisomal fractions, and was lowest in cytosolic fractions in PEG-treated and control plants (Fig. 4a). The activities of three SOD isoforms in all subcellular fractions were promoted at different levels except for Mn-SOD in chloroplasts and Cu/Zn-SOD in mitochondria. Mn-SOD activity was the highest in the mitochondrial fraction, followed by peroxisomal fractions and some marginal activity in cytosolic fractions. No Mn-SOD activity was observed in chloroplasts (Fig. 4b). Cu/Zn-SOD activity was observed in all fractions; however, the highest activity was observed in chloroplastic fractions, followed by cytosolic and mitochondrial fractions, and the lowest activity was in peroxisomal fractions (Fig. 4c). Fe-SOD activity was again highest in chloroplastic fractions, with very low activity in cytosolic, peroxisomal, and mitochondrial fractions (Fig. 4d). Total Cu/Zn-SOD activity, the sum of activities observed in all the fractions, was higher than Mn-SOD and Fe-SOD isoforms.

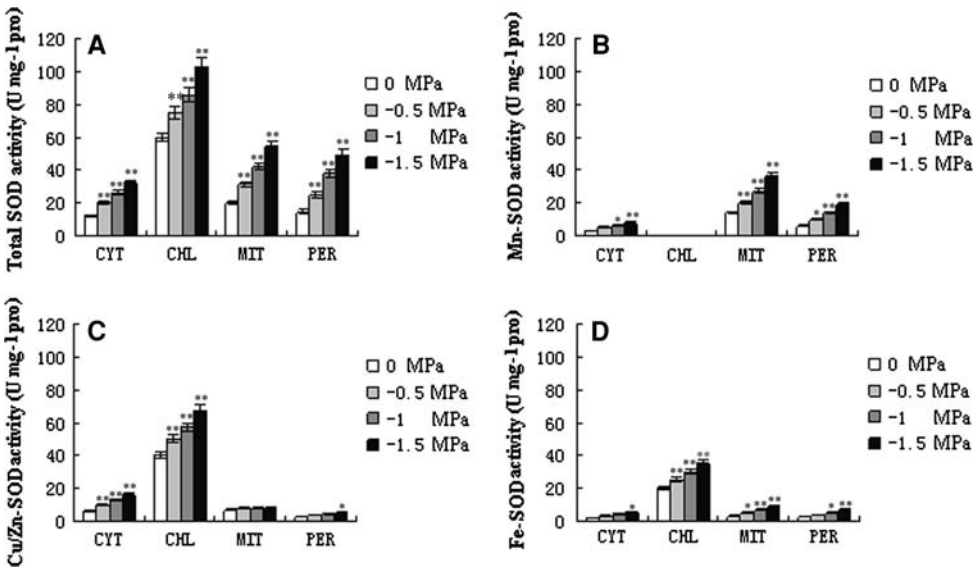

Fig. 4 The total SOD (**a**), Mn-SOD (**b**), Cu/Zn-SOD (**c**), and Fe-SOD (**d**) activities in different cell fractions of *T. repens* exposed to different levels of polyethylene glycol-induced water stress and control. CYT: cytosolic fraction; CHL: chloroplastic fraction; MIT: mitochondrial fraction; PER: peroxisomal fractions. Data are mean ± standard error (SE) ($n = 5$), and the differences between PEG-treated plants and controls are indicated by *($p < 0.05$) and **($p < 0.01$)

APOX and GR activities in all subcellular fractions increased with PEG-induced water stress except that APOX activity in mitochondria was unchanged (Figs. 5 and 6). APOX and GR activities were the highest in chloroplastic fractions, whereas the lowest APOX activity was in peroxisomal fractions and the lowest GR activity was in cytosolic fractions. Even though APOX activities significantly increased in cytosolic, chloroplastic, and peroxisomal fractions during PEG-induced water stress, no increase was detected in the mitochondrial fractions. Furthermore, the increase in chloroplasts was much higher than that in cytosolic and peroxisomal fractions.

In contrast to SOD, APOX, and GR, CAT activity decreased with decreasing osmotic potentials in all subcellular fractions in *T. repens* leaves (Fig. 7). In both the PEG-treated and control plants, the highest CAT activity was found in peroxisomal fractions, followed by cytosolic and mitochondrial fractions; some marginal activity in the chloroplastic fractions was also observed.

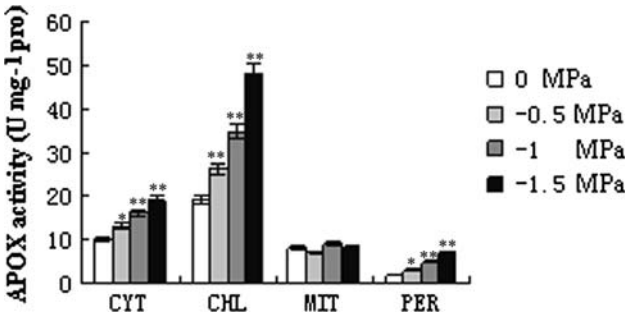

Fig. 5 The APOX activities in different cell fractions of *T. repens* exposed to different levels of polyethylene glycol-induced water stress and control. CYT: cytosolic fraction; CHL: chloroplastic fraction; MIT: mitochondrial fraction; PER: peroxisomal fractions. Data are mean ± standard error (SE) ($n = 5$), and the differences between PEG-treated plants and controls are indicated by *($p < 0.05$) and **($p < 0.01$)

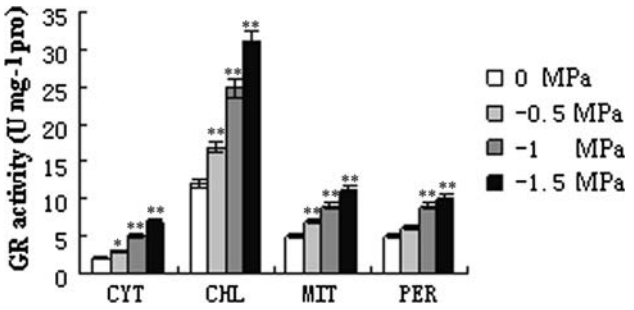

Fig. 6 The GR activities in different cell fractions of *T. repens* exposed to different levels of polyethylene glycol-induced water stress and control. CYT: cytosolic fraction; CHL: chloroplastic fraction; MIT: mitochondrial fraction; PER: peroxisomal fractions. Data are mean ± standard error (SE) ($n = 5$), and the differences between PEG-treated plants and controls are indicated by *($p < 0.05$) and **($p < 0.01$)

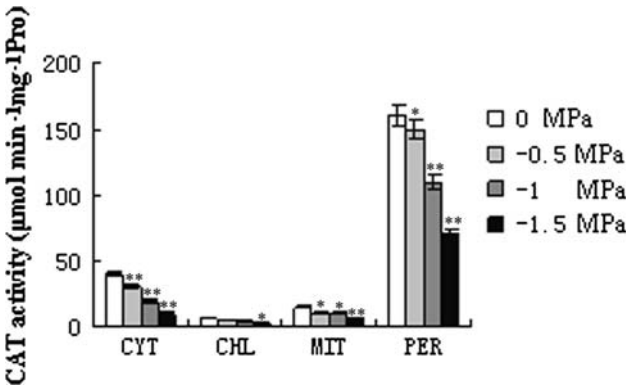

Fig. 7 The CAT activities in different cell fractions of *T. repens* exposed to different levels of polyethylene glycol-induced water stress and control. CYT: cytosolic fraction; CHL: chloroplastic fraction; MIT: mitochondrial fraction; PER: peroxisomal fractions. Data are mean ± standard error (SE) ($n = 5$), and the differences between PEG-treated plants and controls are indicated by *($p < 0.05$) and **($p < 0.01$)

Discussion

H_2O_2 is a toxic ROS and has deleterious effects on plant tissues (Asada and Takahashi 1987; Salin 1988; Sairam and others 1998). Chloroplastic fractions showed higher H_2O_2 accumulation compared with cytosolic, mitochondrial, and peroxisomal fractions (Fig. 1), indicating greater dismutation of O_2^- in chloroplasts resulting in higher H_2O_2 production than cytosolic, mitochondrial, and peroxisomal fractions under PEG-induced water stress. The deleterious effect of H_2O_2 is further reflected in higher lipid peroxidation (estimated as TBARS) in chloroplasts (Fig. 2).

PEG-induced water stress caused a significant decrease in AA content (Fig. 3). AA is an antioxidant that can scavenge O_2^- and H_2O_2 nonenzymatically and also takes part in APOX-mediated scavenging of H_2O_2 (Asada 1992). Higher AA content in chloroplasts, followed by mitochondrial, peroxisomal, and cytosolic fractions, is consistent with the higher antioxidant enzyme activity observed in chloroplasts (Figs. 4–6). However, the comparatively higher levels of AA in mitochondrial and peroxisomal fractions than in cytosolic fractions suggest a role in nonenzymatic scavenging of O_2^- and H_2O_2 in mitochondria and peroxisomes because very low APOX activity was detected in mitochondrial and peroxisomal fractions (Fig. 5). SOD is responsible for scavenging of toxic O_2^- in different cell organelles (Fridovich 1986; Pang and others 2005; Sigaud-Kutner and others 2002; Yu and Rengel 1999). Water-stress-induced increases in SOD activity have been reported in various studies (Foyer and others 1994; Martinez and others 2001). Mn-SOD activity, although highest in mitochondrial fractions, was also detected in cytosolic and peroxisomal fractions but no

Mn-SOD activity was detected in chloroplasts. Mn-SOD activity in all subcellular fractions increased under PEG-induced water stress (Fig. 4b). This is consistent with the results in *Medicago sativa* (Hernandez and others 1994) and *Pisum sativum* (Hernández and others 2000). Cytosolic Mn-SOD activity was very rudimentary, suggesting that cytosolic Mn-SOD has a limited role in scavenging water-stress-induced production of O_2^-. Cu/Zn-SOD was observed in all four fractions but was highest in chloroplasts, followed by mitochondria, cytosol, and peroxisomes (Fig. 4c). It was interesting to note that although water stress increased the Cu/Zn-SOD activity in cytosolic, chloroplastic, and peroxisomal fractions, in mitochondria there was very little or no increase in Cu/Zn-SOD activity in *T. repens*. Fe-SOD was predominantly present in chloroplastic fractions, whereas some residual activity was also detected in cytosolic, mitochondrial, and peroxisomal fractions (Fig. 4d). Furthermore, the increase in Fe-SOD activity in chloroplastic fractions was much more significant than that in cytosolic, mitochondrial, and peroxisomal fractions under PEG-induced water stress.

The presence of APOX, GR, and CAT in chloroplastic, mitochondrial, cytosolic, and peroxisomal fractions has been reported (Becana and others 1986; Creissen and others 1995; Gomez and others 1999; Hernández and others 2006). In the present study, APOX, GR, and CAT, which are responsible for scavenging H_2O_2, were detected in all four subcellular fractions, and the highest activities of APOX and GR were observed in chloroplasts, whereas the lowest APOX activity was in peroxisomal fractions and the lowest GR activity was in cytosolic fractions. APOX and GR activities in all subcellular fractions both increased with PEG-induced water stress except that APOX activity in mitochondria remained unchanged (Figs. 5 and 6). The highest CAT activity was in peroxisomal fractions and the lowest activity was in chloroplastic fractions, and CAT activity decreased with the decreasing osmotic potentials in all subcellular fractions in the PEG-treated and control plants (Fig. 7). Furthermore, the water-stress-induced increases in APOX and GR activities in chloroplasts were much higher than those in the cytosol, peroxisomes, and mitochondria. These results suggest that APOX and GR in chloroplasts rather than CAT play major roles in the scavenging of water-stress-induced H_2O_2. CAT may be the key scavenger enzyme in peroxisomes.

A perusal of the foregoing discussion reveals that cytosolic and chloroplastic APOX, chloroplastic and mitochondrial GR, mitochondrial Mn-SOD, cytosolic and chloroplastic Cu/Zn-SOD, and chloroplastic Fe-SOD are the major scavenger enzymes engaged in the scavenging of O_2^- and H_2O_2 produced in *T. repens* under water stress. It is also evident that in spite of higher AA content and antioxidant activity, chloroplasts are the major site of H_2O_2 production and lipid peroxidation, suggesting that the rate of ROS production in chloroplasts is much higher than the scavenging mechanism. It is also apparent that there is very rapid diffusion of H_2O_2 from chloroplasts and mitochondria into the cytosol, as the lowest SOD activity and significantly higher levels of H_2O_2 were detected in the cytosol.

References

Aeby H (1984) Catalase in vitro. Methods Enzymol 105:121–126

Anderson MD, Prasad TK, Stewart CR (1995) Changes in isozyme profiles of catalase, peroxidase and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. Plant Physiol 109:1247–1257

Asada K (1992) Ascorbate peroxidase—a hydrogen peroxide scavenging enzyme in plants. Physiol Plant 55:235–241

Asada K (1994) Production and action of active oxygen in photosynthetic tissues. In: Foyer CH, Mullineaux PM (eds) Causes of photo-oxidative stress in plants and amelioration of defence system. CRC Press, Boca Raton, FL, pp 77–109

Asada K, Takahashi M (1987) Production and scavenging of active oxygen in photosynthesis. In: Kyde DJ, Osmond CB, Arntun CJ (eds) Photo-inhibition. Elsevier, Amsterdam, pp 227–287

Becana M, Aparicio TP, Irigoyen JJ, Sanchez DM (1986) Some enzymes of hydrogen peroxide metabolism in leaves and root nodules of *Medicago sativa*. Plant Physiol 82:1169–1171

Bermejo R, Irigoyen JJ, Santamaria JM (2006) Short-term drought response of two white clover clones, sensitive and tolerant to O_3. Physiol Plant 127:658–669

Bowler C, Van Camp W, Van Montagu M, Inze D (1994) Superoxide dismutase in plants. Crit Rev Plant Sci 13:199–218

Bradbeer JW (1969) The activities of the photosynthetic carbon cycle enzymes of greening bean leaves. New Phytol 68:233–245

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Burgess N, Beakes GW, Thomas DR (1985) Separation of mitochondria from microbodies of *Pisum sativum* L. cv. Alaska cotyledons. Planta 166:151–155

Cabuslay GS, Ito O, Alejar AA (2002) Physiological evaluation of responses of rice (*Oryza sativa* L.) to water deficit. Plant Sci 163:815–827

Chen GX, Asada K (1989) Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol 30:987–998

Creissen G, Reynolds H, Xue YB, Mullineaux P (1995) Simultaneous targeting of pea glutathione reductase and of a bacterial fusion protein to chloroplasts and mitochondria in transgenic tobacco. Plant J 8:167–175

del Rio LA, Sevilla F, Sandalio LM, Palma JM (1991) Nutritional effects and expression of superoxide dismutase: induction and gene expression, diagnostics, prospective protection against oxygen toxicity. Free Radic Res Commun 12–13:819–828

Dhindsa RA, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot 126:93–101

Distefano S, Palma JM, Gómez M, del Río LA (1997) Characterization of endoproteases from plant peroxisomes. Biochem J 327:399–405

Douce R, Christensen EL, Bonner WD Jr (1972) Preparation of intact plant mitochondria. Biochim Biophys Acta 275:148–160

Foyer CH, Descourvieres P, Kunert KJ (1994) Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. Plant Cell Environ 17:507–523

Fridovich I (1986) Biological effects of superoxide radical. Arch Biochem Biophys 247:1–11

Gomez JM, Hernandez JA, Jimenez A, del Rio LA, Sevilla F (1999) Differential response of antioxidative enzymes of chloroplasts and mitochondria to long-term NaCl stress of pea plant. Free Radic Res 31((Suppl)):11–18

Hatch MD (1978) A simple spectrophotometric assay for fumarate hydratase in crude tissue extracts. Anal Biochem 85:271–275

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Hernández JA, del Rio LA, Sevilla F (1994) Salt stress induced changes in superoxide dismutase isozyme in leaves and mesophyll protoplast from *Vigna unguiculata* (L) Walp. New Phytol 126:37–44

Hernández JA, Jimenez A, Mullineaux P, Sevilla F (2000) Tolerance of pea (*Pisum sativum* L.) to long term salt stress is associated with induction of antioxidant defences. Plant Cell Environ 23:853–862

Hernández JA, Díaz-Vivancos P, Manuel Rubio, Olmos E, Ros-Barceló A, Martínez-Gómez P (2006) Long-term plum pox virus infection produces an oxidative stress in a susceptible apricot, *Prunus armeniaca*, cultivar but not in a resistant cultivar. Physiol Plant 126:140–152

Kuźniak E, Skłodowska M (2001) Ascorbate, glutathione and related enzymes in chloroplasts of tomato leaves infected by *Botrytis cinerea*. Plant Sci 160:723–731

Kuźniak E, Skłodowska M (2004) The effect of *Botrytis cinerea* infection on the antioxidant profile of mitochondria from tomato leaves. J Exp Bot 397:605–612

Kuźniak E, Skłodowska M (2005) Compartment-specific role of the ascorbate-glutathione cycle in the response of tomato leaf cells to *Botrytis cinerea* infection. J Exp Bot 56:921–933

Lee BR, Kim KY, Jung WJ, Avice JC, Ourry A, Kim TH (2007) Peroxidases and lignification in relation to the intensity of water-deficit stress in white clover (*Trifolium repens* L.). J Exp Bot 58:1271–1279

López-Huertas E, Sandalio LM, del Río LA (1995) Integral membrane polypeptides of pea leaf peroxisomes: characterization and response to plant stress. Plant Physiol Biochem 33:295–302

Martinez CA, Loureiro ME, Oliva MA, Maestri M (2001) Differential responses of superoxide dismutase in freezing resistant *Solanum curtilobum* and freezing sensitive *Solanum tuberosum* subjected to oxidative and water stress. Plant Sci 160:505–515

Mukherjee SP, Choudhari MA (1983) Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in vigna seedlings. Physiol Plant 58:166–170

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880

Pang CH, Zhang SJ, Gong ZZ, Wang BS (2005) NaCl treatment markedly enhances H_2O_2-scavenging system in leaves of halophyte *Suaeda salsa*. Physiol Plant 125:490–499

Porra JR, Thompson WA, Kiedermann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta 975:384–394

Rich PR, Bonner WD (1978) The sites of superoxide anion generation in higher plant mitochondria. Arch Biochem Biophys 188:206–213

Sairam RK, Deshmukh PS, Saxena DC (1998) Role of antioxidant system in wheat genotypes tolerance to water stress. Biol Plant 41:387–394

Salin ML (1988) Toxic oxygen species and protective system of the chloroplasts. Physiol Plant 72:681–689

Salin ML (1991) Chloroplast and mitochondrial mechanism for protection against oxygen toxicity. Free Radic Res Commun 12–13:851–858

Scandalios JG (1993) Oxygen stress and superoxide dismutases. Plant Physiol 101:1–7

Sigaud-Kutner TCS, Pinto E, Okamoto OK, Latorre LR, Colepicolo P (2002) Changes in superoxide dismutase activity and photosynthetic pigment content during growth of marine phytoplankters in batch-cultures. Physiol Plant 114:566–571

Smirnoff N (1993) The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol 125:27–58

Smith IK, Vierheller TL, Thorne CA (1988) Assay of glutathione reductase in crude tissue homogenates using 5, 5(-dithiobis (2-nitrobenzoic acid). Anal Biochem 175:408–413

Struglics A, Fredlund KM, Rasmusson AG, Møller IM (1993) The presence of a short redox chain in the membrane of intact potato tuber peroxisomes and the association of malate dehydrogenase with peroxisomal membrane. Physiol Plant 88:19–28

Wang CQ, Chen M, Wang BS (2007) Betacyanin accumulation in the leaves of C_3 halophyte *Suaeda salsa* L. is induced by watering roots with H_2O_2. Plant Sci 172:1–7

Wang CQ, Xu C, Wei JG, Wang HB, Wang SH (2008) Enhanced tonoplast H^+-ATPase activity and superoxide dismutase activity in the halophyte *Suaeda salsa* containing high level of betacyanin. J Plant Growth Regul 27:58–67

Wu FZ, Bao WK, Li FL, Wu N (2008) Effects of drought stress and N supply on the growth, biomass partitioning and water-use efficiency of *Sophora davidii* seedlings. Environ Exp Bot 63:248–255

Yu Q, Rengel Z (1999) Drought and salinity differentially influence activities of superoxide dismutase in narrow-leafed lupins. Plant Sci 142:1–11

Zhang J, Kirkham MB (1994) Drought-stress-induced changes in activities of superoxide dismutase, catalase and peroxidase in wheat species. Plant Cell Physiol 35:785–791