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Enhanced Antioxidant Enzymes Are Associated with Reduced Hydrogen Peroxide in Barley Roots under Saline Stress

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Antioxidant enzymes are related to the resistance to various abiotic stresses including salinity. Barley is relatively tolerant to saline stress among crop plants, but little information is available on barley antioxidant enzymes under salinity stress. We investigated temporal and spatial responses of activities and isoform profiles of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), non-specific peroxidase (POX), and glutathione reductase (GR) to saline stress in barley seedlings treated with 200 mM NaCl for 0, 1, 2, 5 days, respectively. In the control plant, hydrogen peroxide content was about 2-fold higher in the root than in the shoot. Under saline stress, hydrogen peroxide content was decreased drastically by 70% at 2 d after NaCl treatment (DAT) in the root. In the leaf, however, the content was remained unchanged by 2 DAT and increased about 14 % at 5 DAT. In general, the activities of antioxidant enzymes were increased in the root and shoot under saline stress. But the increase was more significant and consistent in the root. The activities of SOD, CAT, APX, POX, and GR were increased significantly in the root within 1 DAT, and various elevated levels were maintained by 5 DAT. Among the antioxidant enzymes, CAT activity was increased the most drastically. The significant increase in the activities of SOD, CAT, APX, POX, and GR in the NaCl-stressed barley root was highly correlated with the increased expression of the constitutive isoforms as well as the induced ones. The hydrogen peroxide content in the root was most highly correlated with the CAT activity, indicating an increased role of CAT in hydrogen peroxide detoxification under salinity stress. In addition, the results suggest the significance of temporal and spatial regulation of each antioxidant isoform in determining the competence of the antioxidant capacity under saline stress.

Keywords: Antioxidant enzymes, Barley, Hydrogen peroxide, Salt stress

Introduction

Increased use of fertile agricultural lands for human activities other than crop production pushes crop cultivation to less productive lands, including saline areas. Plants in saline areas are easily exposed to multiple abiotic stresses. Among these stresses, high salinity is the most severe factor limiting plant growth in the areas.

It is well documented that abiotic stresses exert at least in part of their effects by causing oxidative damage (Smimoff, 1995). Oxidative damages are caused by reactive oxygen species (ROS) and excess amounts of ROS are harmful to many cellular components, including membrane lipids. ROS cause peroxidation of polyunsaturated fatty acids in the membranes (Smirnoff, 1995). Production of ROS is increased under saline conditions (Greenway and Munns, 1980; Hasegawa *et al.*, 2000) and ROS-mediated membrane damage has been demonstrated to be a major cause of the cellular toxicity by salinity in rice, tomato and citrus (Gueta-Dahan *et al.*, 1997; Dionisio-Sese and Tobita, 1998; Mittova *et al.,* 2004).

However, ROS are inevitable byproducts from the essential aerobic metabolisms, and they need to be maintained under sublethal levels for normal plant growth. Hence, plants are

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equipped with an array of enzymatic and non-enzymatic antioxidant molecules to alleviate cellular damage caused by ROS (Foyer and Noctor, 2000; Apel and Hirt, 2004). Multiple antioxidant enzymes systems are involved in the enzymatic scavenging of ROS. Superoxide dismutases (SOD, EC 1.15.1.1) react with the superoxide radical to produce H_2O_2. Hydrogen peroxide is scavenged by catalases (CAT, EC 1.11.1.6) and peroxidases (POX, EC 1.11.1.7). Among peroxidases, ascorbate peroxidases (APX, EC 1.11.1.11) and glutathione peroxidase (GPX, EC 1.11.1.9) which use ascorbate and glutathione as electron donors, respectively, are well known for their role in H_2O_2 detoxification in plants. Glutathione reductase (GR, EC 1.6.4.2) is responsible for the reduction of oxidized glutathione for the chain reactions of scavenging H_2O_2 by APX and GPX to be completed and continued (Mittler, 2002; Apel and Hirt, 2004).

A large body of evidence has shown that the antioxidant enzyme systems are altered under abiotic stresses, including salinity. The quantitative and qualitative aspects of changes are often related to the levels of resistance to salinity. In rice, the salt-tolerant varieties have higher SOD activity and lower lipid peroxidation than the salt-sensitive varieties (Dionisio-Sese and Tobita, 1998). In tomato and citrus, salt-tolerance is attributed to the increased activities of SOD, APX, and CAT (Gueta-Dahan *et al.*, 1997; Mittova *et al*., 2004). Further supporting evidence on the involvement of antioxidant enzymes in salt tolerance has been provided by transgenic plants with a reduced or an increased expression of antioxidant enzymes. The antisense plants with reduced CAT activity are hypersensitive to salt and other oxidative stresses (Willekens *et al.,* 1997). Increased protection to salt stress has been demonstrated by the overexpression of cytosolic APX (Torsethaugen *et al.,* 1997). Enhanced oxidative stress tolerance was also observed in the plants overexpressing Fe-SOD (Van Camp *et al.,* 1996).

Barley is relatively tolerant to salt stress compared to other crop plants and considered to be used at the early stage of cultivation trial of newly acclimated tideland. Even though much supporting evidence on the role of antioxidant enzymes in salt tolerance is available, there is little information on barley antioxidant enzymes under salinity stress. Here, we report that the increases in the expression of activities and isoforms of SOD, CAT, APX, POX and GR were associated with decrease in hydrogen peroxide in the salt-stressed root.

Materials and Methods

Chemicals and plant materials All chemicals and enzymes were purchased from Sigma (St. Louis, USA), unless otherwise indicated. Barley seeds (*Hordeum vulgare* cv. Saessalbori) were surface-sterilized in 0.5% sodium hypochloride solution for 20 min and germinated at 20°C. Uniform seedlings were transferred to and grown in a hydrophonic system as previously described (Park *et al.,* 2003). At the 3rd leaf stage, the seedlings were subjected to treatment with 200 mM NaCl for 0, 1, 3, and 5 d, respectively. Samples of roots and shoots were collected at the treatment intervals, ground into fine powder under liquid nitrogen, and used for enzyme assays.

Hydrogen peroxide assay Hydrogen peroxide content was determined by measuring the absorbance of titanium-hydroperoxide complex (Mukherjee and Choudhari, 1983). The acetone extract of the tissue was reacted with titanium reagent and ammonium to form hydroperoxide-titanium complex. The complex was dissolved in 1 M sulfuric acid and absorbance of the supernatant was measured at 415 nm against blank. Concentration of hydrogen peroxide was determined using the standard curve plotted with known concentration of hydrogen peroxide.

Enzyme activity assay and isozyme analysis Essentially the same protein extraction and electrophoresis methods were used as described (Baek *et al.,* 2000; Choi *et al.,* 2004). Total cellular extracts, prepared at 4°C were used for activity assays and isozyme analysis for each antioxidant enzyme. The activities of APX, POX, CAT, GR, and SOD were determined spectrophotometrically. APX activity was determined following the oxidation of ascorbate to dehydroascorbate, as described by Nakano and Asada (1981). Non-specific POX activity was determined by measuring peroxidation of hydrogen peroxide with guaiacol as an electron donor (Chance and Maehly, 1955). APX isoforms were detected on nondenaturing polyacrylamide gels (7% acrylamide, 3% bis-acrylamide) containing 10% glycerol according to Mittler and Zilinskas (1993). POX isoforms were detected on ultrathin-layer IEF gels according to Wendel (1987). CAT activity was assayed by measuring the conversion rate of hydrogen peroxide to water and oxygen molecules (Beers and Sizer, 1952). CAT isoforms were detected on nondenaturing polyacrylamide gels (7% acrylamide, 3% bis-acrylamide) containing 10% glycerol as described by Woodbury *et al.* (1971). Dithiotreitol (60 mM) was added in the sample loading buffer for CAT isoform detection. GR activity was determined by measuring the reduction kinetics of oxidized glutathione (OKane *et al.,* 1996). GR isoforms were detected on nondenaturing polyacrylamide gels (7% acrylamide, 3% bis-acrylamide) containing 10% glycerol according to Anderson *et al.* (1995). SOD activity was assayed by determining the inhibition rate of nitroblue tetrazolium reduction with xanthine oxidase as a hydrogen peroxide generating agent (Obeley and Spitz, 1984; Kang, 2004). SOD isoforms were detected on nondenaturing polyacrylamide gels (10% acrylamide, 3% bis-acrylamide) as described by Beauchamp and Fridovich (1971). The protein contents of the enzyme extracts were determined by using the Bradford (1979) method. Assays were conducted for the three-replicated treatments and the enzyme activity data were analyzed using the SAS program (SAS Institute Inc., Cary, USA).

Results

Hydrogen peroxide content Hydrogen peroxide content was 2.2-fold higher in the root than in the shoot of unstressed plants as 5.6 μmol/g fresh weight. In the root, the content was

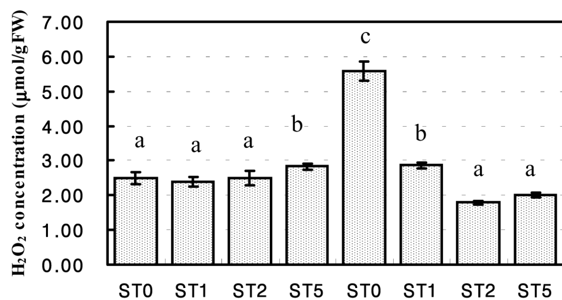


Fig. 1. The hydrogen peroxidase content in the NaCl-treated barley shoots (ST) and roots (RT). Barley seedlings at the 3rd leaf stage were treated with 200 mM NaCl for 0, 1, 2 and 5 d, respectively. The treatments indicated by the same letters are not significantly different ($p \leq 0.05$) within the tissue.

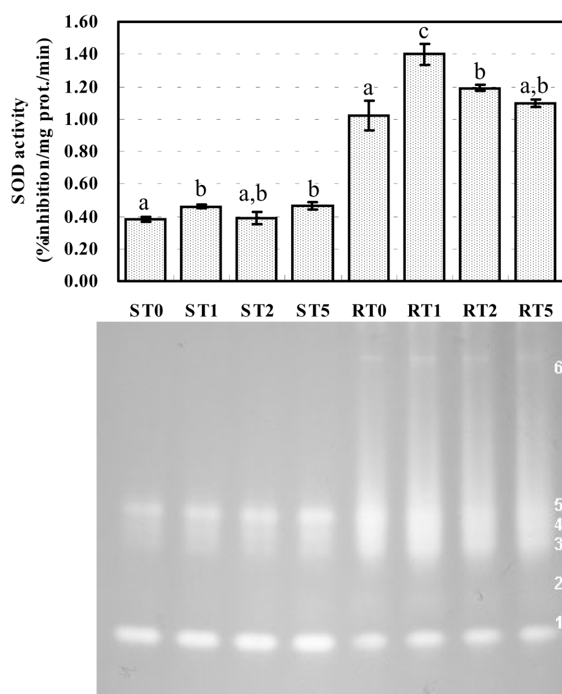
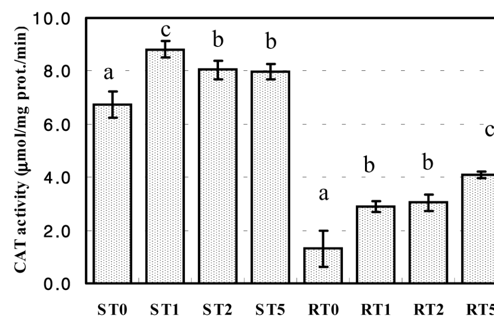


Fig. 2. The responses of SOD activities and isoforms in the NaCl-treated barley shoots (ST) and roots (RT). Barley seedlings at the 3rd leaf stage were treated with 200 mM NaCl for 0, 1, 2 and 5 d, respectively. The treatments indicated by the same letters are not significantly different ($p \leq 0.05$) within the tissue. SOD isoforms were detected on nondenaturing polyacrylamide gels (10% acrylamide, 3% bis-acrylamide) as described by Beauchamp and Fridovich (1971).

decreased to about 50 and 30% of the control by 1 and 2 days after 200 mM NaCl treatment (DAT). However, the content in the shoot was remained unchanged by 2 DAT, and increased 12% in 5 DAT (Fig. 1). Hydrogen peroxide content is generally increased in the leaf of salt-stressed plant (Sairam *et al*., 2002; Fedina *et al*., 2003) showing little association with salt-tolerance. However, hydrogen peroxide content in the root is often related to the salt tolerance. Decreased hydrogen



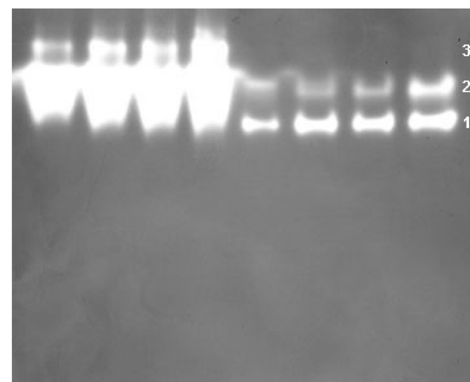


Fig. 3. The responses of CAT activities and isoforms in the NaCl-treated barley shoots (ST) and roots (RT). Barley seedlings at the 3rd leaf stage were treated with 200 mM NaCl for 0, 1, 2 and 5 d, respectively. The treatments indicated by the same letters are not significantly different ($p \leq 0.05$) within the tissue. CAT isoforms were detected on nondenaturing polyacrylamide gels (7% acrylamide, 3% bis-acrylamide) containing 10% glycerol as described by Woodbury *et al.* (1971).

peroxide content in the root coincides with salt tolerance in rice (Dionisio-Sese and Tobita, 1998) and tomato (Mittova *et al*., 2004).

Superoxide dismutase The specific SOD activity was increased in the root and shoot under salt stress, but its response patterns were variable in the tissues. In the root, SOD activity was increased the most, by about 37% 1 DAT, but the increase was reduced gradually by 5 DAT. In the shoot, the activity was increased by about 21% at 1 DAT and the increase alternated at variable levels. The activity in the root was about three-times higher than that in the shoot (Fig. 2).

Increase in enzyme activity coincided with a variable increase in the individual isoform expression. At least five or six isoforms were detected in the shoot and root with SOD1 and SOD5 being the major ones. The isoforms showing concomitant increase with the total enzyme activity were SOD3 and SOD4 in the root and SOD1 and SOD5 in the shoot (Fig. 2). Isoform SOD2, which is expressed only in the cellular space of freeze-stressed barley leaves (Baek *et al*., 2000), was expressed a little in the saline-stressed leaves, indicating stress-specific expression of barley SOD2 isoform.

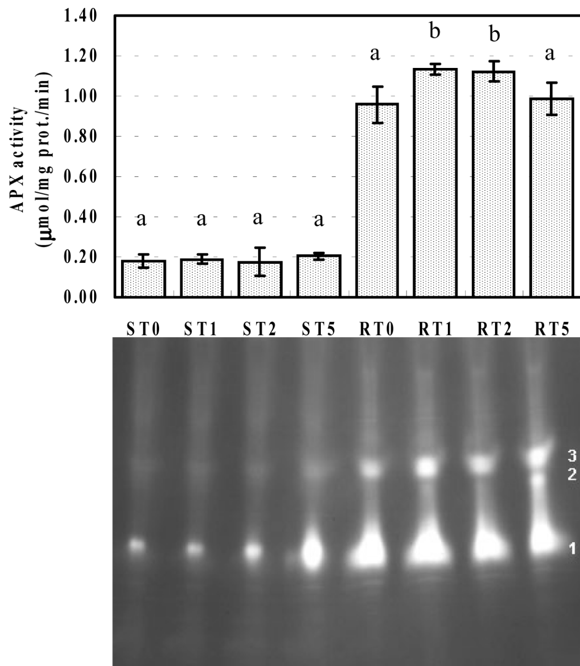


Fig. 4. The responses of APX activities and isoforms in the NaCl-treated barley shoots (ST) and roots (RT). Barley seedlings at the 3rd leaf stage were treated with 200 mM NaCl for 0, 1, 2 and 5 d, respectively. The treatments indicated by the same letters are not significantly different ($p \leq 0.05$) within the tissue. APX isoforms were detected on nondenaturing polyacrylamide gels (7% acrylamide, 3% bis-acrylamide) containing 10% glycerol according to Mittler and Zilinskas (1993).

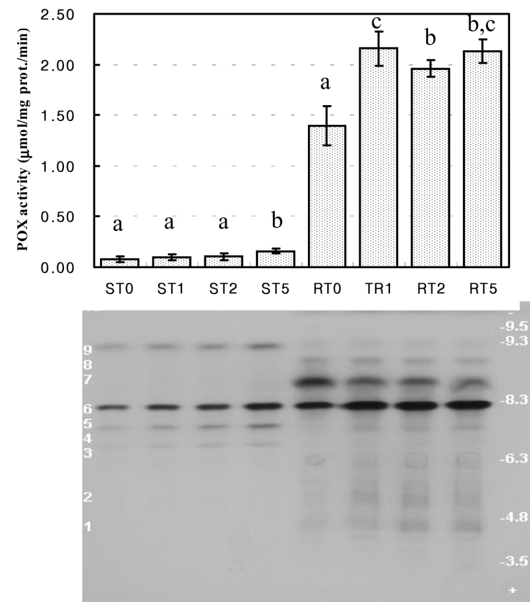


Fig. 5. The responses of POX activities and isoforms in the NaCl-treated barley shoots (ST) and roots (RT). Barley seedlings at the 3rd leaf stage were treated with 200 mM NaCl for 0, 1, 2 and 5 d, respectively. The treatments indicated by the same letters are not significantly different ($p \leq 0.05$) within the tissue. POX isoforms were detected on ultrathin-layer IEF gels according to Wendel (1987).

Catalase The specific CAT activity was increased in the root and shoot by the NaCl treatment. In the root, CAT activity was increased drastically from 1 DAT, reaching to about a 316% increase at 5 DAT. In the shoot, the activity was increased by about 31% 1 DAT and the increase was reduced by about 20% by 5 DAT. The activity in the shoot was about five-times higher than that in the root (Fig. 3).

Two or three isoforms were detected in the root and shoot with CAT1 and CAT2 as the major ones in the root and shoot, respectively. In the root, CAT1 expression was increased consistently from 1 to 5 DAT, while CAT2 expression was only at 5 DAT. In the shoot, CAT1 was induced upon the NaCl treatment and its expression was increased with treatment time, as was the expression of CAT2 and CAT3 (Fig. 3).

Ascorbate peroxidase The specific APX activity was only increased significantly in the root at 1 and 3 DAT. In the shoot, APX activity was slightly increased only at 5 DAT but with no statistical significance. The activity in the root was about five-times higher than that in the shoot (Fig. 4).

Two and three isoforms were detected in the root and shoot with APX1 as a major one. In the root, the APX1 and APX3 expression showed a concomitant increase with the total APX activity. APX2 expression was induced at 5 DAT only in the root. A slight increase in APX activity in the shoot coincided with an increase in the APX1 expression (Fig. 4).

Non-specific peroxide The specific POX activity was increased significantly in the root and shoot by NaCl treatment. In the root, the activity was increased by about 50% from 1 DAT to 5 DAT. In the shoot, the activity was increased by about 25% at 1 and 3 DAT and by about 100% at 5 DAT. The activity in the root was about 18-times higher than that in the shoot (Fig. 5).

At least ten POX isoforms were detected with POX6 as a major one. Increase in root enzyme activity coincided with the increased expression of POX6. Increase in shoot enzyme activity correlated with the enhanced expression of POX5, POX6, and POX9 (Fig. 5). The neutral and basic POX isoforms expressed in saline-stressed leaves are also expressed in the freeze-stressed leaves, but the major isoform POX6 in the saline-stressed leaves is only a minor isoform in the freeze-stressed leaves (Baek *et al.*, 2000). This indicates a difference in the quantitative isoform expression patterns in the two stresses.

Glutathione reductase GR activity was only increased significantly in the root in response to the NaCl treatment. The activity was increased by about 47% at 1 DAT, and was maintained at the increased levels until 5 DAT. The activity in the root was about three-times higher than that in the shoot (Fig. 6).

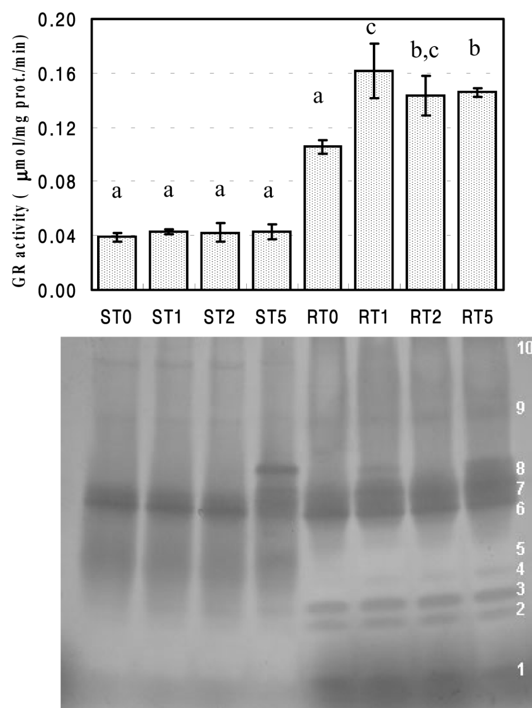


Fig. 6. The responses of GR activities and isoforms in the NaCl-treated barley shoots (ST) and roots (RT). Barley seedlings at the 3rd leaf stage were treated with 200 mM NaCl for 0, 1, 2 and 5 d, respectively. The treatments indicated by the same letters are not significantly different ($p \leq 0.05$) within the tissue. GR isoforms were detected on nondenaturing polyacrylamide gels (7% acrylamide, 3% bis-acrylamide) containing 10% glycerol according to Anderson *et al.* (1995).

A total of ten GR isoforms were detected with GR6 as the major one. Increase in root enzyme activity coincided with the increased expression of GR7 and GR8. In the shoot, the expression of GR8 was induced, but that of GR6 was suppressed at 5 DAT (Fig. 6).

Discussion

The activities of barley antioxidant enzymes were increased in the root and shoot under the NaCl stress. But the increase was more significant and consistent in the root. The activities of SOD, CAT, APX, POX, and GR were increased significantly in the root within 24 hs of the NaCl treatment, indicating rapid responses of antioxidant enzymes to salt stress in barley roots (Figs. 2, 3, 4, 5, and 6). The increased antioxidant enzyme activities were correlated with decreased hydrogen peroxide in the salt-stressed roots (Fig. 1). Among the enzymes, CAT showed the highest negative relation with hydrogen peroxide content. In glycophytes, the root is the primary site of salt stress and the ability to maintain ion homeostasis and redox potential is critical for the normal root growth and function under saline stress, and often related to salinity resistance (Greenway and Munns, 1980; Hasegawa *et al.*, 2000). The observations also strongly imply a possibility that antioxidant enzyme systems are also utilized in barley to alleviate oxidative stress caused by salinity, thus protecting the cells from oxidative damage. Detoxification of excess ROS produced during stress is important to reduce ROS-induced membrane lipid peroxidation, enzyme inhibition, and nucleic acid damage (Mittler, 2002). Enzymatic scavenging of ROS could be efficiently achieved through the complex, but elaborate coordination among the enzymes involved (Foyer *et al.*, 1994; Foyer and Noctor, 2000; Apel and Hirt, 2004). The increased activities of the antioxidant enzymes upon salt stress are often related to the enhanced tolerance to salt stress (Gueta-Dahan *et al.*, 1997; Mittova *et al*, 2004). Thus, the highest negative correlation of the CAT with hydrogen peroxide content in the salt-stressed root suggests an increased role of CAT in hydrogen peroxide detoxification under salt stress.

Furthermore, each enzyme showed specific quantitative and qualitative responses under salt stress. The major ROS-scavenging mechanisms of plants include SOD, APX and CAT (Mittler, 2002). Increase of APX activity was relatively low compared with that of SOD and CAT in the NaCl-treated barley root (Figs. 2, 3 and 4). A rapid and continued increase in CAT activity might indicate that CAT is a major enzyme detoxifying hydrogen peroxide in barley under salt stress. Since ROS are produced through the multiple pathways including the SOD reaction under salt stress, over 2-fold higher increase in CAT activity than in SOD could better contribute in maintaining steady-state levels of cellular hydrogen peroxide. Also, direct evidence from CAT-deficient mutant barley demonstrates the essential role of CAT in stress resistance of barley. The CAT-deficient barley plants develop severe necrotic lesions and barely survive field stress conditions (Acevedo *et al.*, 2001).

The utilization of multiple isoforms of enzymes is one of the primary control mechanisms of cellular metabolism in plants. However, little data is available on the regulation of antioxidant enzymes isoforms expression under salt stress (Gueta-Dahan *et al.*, 1997). Therefore, the isoform specific responses of antioxidant enzymes to salt stress in barley deserve to have a considerable significance. The temporal increase of constitutive isoforms was generally observed in SOD, CAT, POX, and GR in the NaCl-treated barley root and shoot (Figs. 2, 3, 5, and 6). However, the temporal decrease of a specific isoform was only observed in POX in the root. Moreover, the induction of new isoforms was also observed in CAT in the shoot, APX in the root, and GR in the root and shoot, respectively (Figs. 3, 4, and 6). Freezing stress also induces drastic changes in the temporal and spatial expression of antioxidant isoforms in overwintering barley leaves (Baek *et al*, 2000). The limited success with the single gene transgenic approach to improve abiotic stress tolerance via enhancing antioxidant enzyme capacity indicates the significance of fine-tuning temporal and spatial regulation of

isoform expression (Bohnert and Sheveleva, 1998; Smimoff, 1998).

In summary, the significant increase in the activities of SOD, CAT, APX, POX, and GR in the NaCl-stressed barley root was highly correlated with the temporal regulation of the constitutive isoforms as well as the induction of new isoforms. Together with a relative importance of CAT in hydrogen peroxide detoxification, the results suggest the significance of temporal and spatial regulation of each antioxidant isoform in determining the competence of the antioxidant enzyme capacity under oxidative stress.

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