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BRIEF COMMUNICATION

Antioxidant defense system in *Phragmites communis* Trin. ecotypes

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Abstract

The antioxidant defense system in three ecotypes of reed (*Phragmites communis* Trin.), swamp reed (SR), dune reed (DR), and heavy salt meadow reed (HSMR), from northwest China were investigated. The HSMR possessed the highest ratio of ascorbate (ASC)/dehydroascorbate (DHA) and activities of superoxide dismutase (SOD) and catalase among the three reed ecotypes, whereas, the DR exhibited the highest ratio of glutathione/glutathione disulfide and activities of ASC peroxidase (APX) and DHA reductase. Malondialdehyde and hydrogen peroxide contents were highest in HSMR, intermediate in SR, and lowest in DR. In addition, different isoenzymes of glutathion reductase, APX, SOD and DHA were also observed in three reed ecotypes.

Additional key words: antioxidative enzymes, ascorbate, drought-prone and saline habitats, glutathione, reed.

Plants possess a complex antioxidant system, which consists of ascorbic acid, glutathione and enzymes that protect the plant against oxidative damage induced by environmental stresses (Noctor and Foyer 1998, Polle 2001, Arbona *et al*. 2003, Reddy *et al*. 2004, Kim *et al*. 2005). Ascorbate (ASC), a ubiquitous soluble antioxidant in photosynthetic organisms, is the most important reducing substrate for hydrogen peroxide detoxification (Smirnoff 1996, Horemans *et al.* 2000). Another soluble antioxidant, reduced glutathione (GSH), is a disulfide reductant that protects thiols of enzymes. It can regenerate ASC and react with singlet oxygen, hydrogen peroxide and hydroxyl radical (Noctor and Foyer 1998, Polle 2001). Changes in ASC and GSH pools including the increases in ASC and GSH level, ASC/dehydroascorbate (DHA) and GSH/oxidized glutathione (GSSG) ratios, and activities of the enzymes related to their biosynthesis and metabolism, are tightly related to the responses of plants to a wide range of stresses (May *et al*. 1998, Noctor *et al*. 1998, Horemans *et al*. 2000, Balestrasse *et al*. 2001, Arbona *et al*. 2003, Chaparzadeh *et al*. 2004, Mittova *et al*. 2004, Kim *et al*. 2005). Levels and redox status of ASC and GSH also regulate expression of genes and activities of the redox sensitive transcription factors and enzymes (Sen 1999, Davey *et al*. 2000, Cnubben *et al*. 2001, Pastori and Foyer 2002).

Reed (*Phragmites communis* Trin.) is a hydrophytic species whose typical habitats are the fresh and brackish water areas of swamps, riversides and lakesides. However, reed plants have adapted to terrestrial habitats and various ecotypes have evolved with resistance to drought, salinity and low temperature. In addition to swamp reed (SR), there are three other terrestrial reed ecotypes, heavy salt meadow reed (HSMR), light salt meadow reed (LSMR) and dune reed (DR), growing in the desert regions of northwest China (Zheng *et al*. 2000). These four reed ecotypes have shown some stable variations of morphological, physiological and genetic characteristics

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Abbreviations: APX - ascorbate peroxidase (EC 1.11.1.11); ASC - ascorbate; CAT - catalase (EC 1.11.1.6); DHA - dehydroascorbate, DHAR - dehydroascorbate reductase (EC 1.8.5.1); DR - dune reed; DTT - dithiothteitol; EDTA - ethylenediaminetetraacetic acid; GR - glutathione reductase (EC 1.6.4.2); GSH - reduced glutathione; GSSG - glutathione disulfide; HSMR - heavy salt meadow reed; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase (EC 1.6.5.4); NBT - nitroblue tetrazolium; PAGE - polyacrylamide gel electrophoresis; POX - peroxidase (EC 1.11.1.7); PVP - polyvinylpolypyrrolidone; SOD - superoxide dismutase (EC 1.15.1.1); SR - swamp reed.
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in response to drought and salinity (Wang *et al*. 1998, Zheng *et al*. 2000, Cheng *et al*. 2001, Zhu *et al*. 2001, Chen *et al*. 2003, 2004a). However, little information is available on antioxidant defense system of these reed ecotypes. In the present study, three reed ecotypes referred to as swamp reed (SR), dune reed (DR), and heavy salt meadow reed (HSMR) according to the traits of their respective habitats as previously described (Wang *et al*. 1998, Zheng *et al*. 2000, Chen *et al*. 2003), were considered for clarifying the mechanism of antioxidant defense system in the plant responding to natural long-term environmental stresses such as drought and salinity.

During June 20 to 25 in 2001, the second leaves from the top of the three reed ecotypes were simultaneously collected at midday and frozen in liquid N_2 until analysis was performed. For determination of glutathione, the fresh leaves were collected at midday, on July 2, 2002. Glutathione contents were assayed as described by Nagalakshmi and Prasad (2001). Ascorbate and dehydroascorbate contents were determined as described by Fryer *et al*. (1998). Antioxidant enzymes were extracted as described by Biemelt *et al*. (1998). Leaf tissue samples (approximately 0.5 g) were ground to a powder in liquid N_2 and homogenized in 3 cm^3 of ice-cold extraction buffer: for SOD, POX, CAT, APX and GR, the buffer contained 50 mM potassium phosphate buffer, pH 7.8, 5 mM ASC, 5 mM DTT, 100 mM NaCl, 5 mM EDTA, and 2 % PVP-40; and for MDHAR and DHAR, the buffer contained 50 mM potassium phosphate buffer, pH 7.8, 0.2 mM EDTA, 10 mM β-mercaptoethanol, and 2.5 % PVP-40. The extracts were centrifuged at 15 000 *g* for 15 min at 0 to 4 °C, and the supernatants were used for the enzyme activity assays. SOD was assayed using the NBT method described by Dhindsa *et al*. (1980). POX, CAT and DHAR were measured using the method of Cakmak *et al*. (1993), whereas APX was measured using the method of Asada (1984). GR and MDHAR were assayed following the method of Fryer *et al*. (1998). Protein concentration was determined using a Coomassie brilliant blue with bovine serum albumin as the standard (Bradford 1976). Leaf MDA contents were assayed as indicators of the extent of lipid peroxidation in leaf tissue by the method of Sudhakar *et al*. (2001). Leaf hydrogen peroxide contents were determined by the method of Patterson *et al*. (1984). For native-PAGE assays, about 100 μg of total protein in each lane were loaded for analysis of each enzyme from the leaves of the different reed ecotypes. Determination of DHA and APX isozymes were performed as described by Tommasi *et al*. (2001). Electrophoresis of GR was performed according to the method of Ye *et al*. (1997). Native-PAGE of SOD was carried out as described by González *et al*. (1998). Data from three independent experiments with 4 to 6 replicates were statistically analyzed by a one-way analysis of variance, combined with Fisher's PLSD test with the *DSTP*-statistical package. The differences were considered significant when $P \leq 0.05$.

The HSMR exhibited the highest H_2O_2 and MDA contents among three reed ecotypes, whereas the DR possessed the lowest H_2O_2 and MDA contents (Table 1). Total ASC and reduced ASC contents were higher in the HSMR than those in SR and DR, whereas DHA contents were almost similar among three reed ecotypes (Table 1). The ASC/DHA ratio was about 46 % higher in HSMR than in the other reed ecotypes. The HSMR possessed the highest total GSH and reduced GSH contents among three reed ecotypes, while the SR and DR shared similar values of total GSH and reduced GSH contents, respectively. GSSG contents, however, were highest in HSMR, followed by SR and DR. In addition, the GSH/GSSG ratio was about 22 % higher in DR than in the SR. SOD activity was highest in HSMR, followed by DR and SR, whereas CAT activity was highest in HSMR, intermediate in SR, and lowest in DR. POX activity was lower in DR than that in the others while GR activity showed similar values in three ecotypes. APX activity was higher in DR and HSMR than in the SR. Although the activity of DHAR was much higher than that of MDHAR, the activities of two enzymes were markedly higher in DR and HSMR than those in SR.

A total of seven activity bands for GR isoenzymes, designated as GR1-GR7, were observed in the three reed ecotypes; three, six and six bands were found in SR, DR and HSMR, respectively (Fig. 1). The highest activity for GR2 and GR3 was found in HSMR. GR4 and GR6 were present in both HSMR and DR, whereas GR5 was found only in HSMR and GR7 only in DR. A total of four APX isoenzymes were found in the three ecotypes and the HSMR possessed the highest activity in APX2. It should be noticed that the migration rate of APX4 was different in three reed ecotypes. It was highest in SR, less in DR, and lowest in HSMR. Three different bands of activity associated with DHA were observed. DHA2 activity was much higher in HSMR, but was absent in DR. Seven bands of SOD were found. It seemed that no new band appeared in either of the two terrestrial reed ecotypes, DR and HSMR. However, the amount of activity for each band offered different between ecotypes.

As an indicator of the extent of lipid peroxidation, MDA content has been found to be increased when plants were subjected to a serial of environmental stresses (Iturbe-Ormaetxe *et al*. 1998, Sudhakar *et al*. 2001, Sairam and Srivastava 2002). Many studies have shown that high antioxidant contents and defense enzyme activity are associated with low MDA content (Li and van Staden 1998, Mittova *et al*. 2004). In this work, content of MDA was the highest in HSMR and the lowest in DR among the three reed ecotypes (Table 1), indicating that the rate of lipid peroxidation in leaves is different in the reed ecotypes adapted to their respective habitats. Probably, DR maintained integrated membrane systems, whereas HSMR showed more injury in it. This might be connected with the different contents of the leaf H_2O_2 in the two reed ecotypes (Table 1).

Changes in activities of antioxidant enzymes have been found in many species when plants were subjected to a wide range of stresses such as drought and salinity (Noctor and Foyer 1998, Polle 2001, Reddy *et al*. 2004, Kim *et al*. 2005). Most studies show that the higher

activities of the enzymes were positively correlated with the plant stress-resistance (Li and Van Staden 1998, Sairam and Srivastava 2002, Kim *et al*. 2005). Changes of the enzyme activities also depend on plant genotypes and stress intensity (Li and Van Staden 1998; Jain *et al*. 2006; Zlatev *et al*. 2006). In the present work, except POX and CAT exhibited lower activities in leaves of DR, the activities of other antioxidant enzymes including SOD, GR, APX, DHAR, and MDHAR were obviously higher in DR and HSMR than those in SR (Table 1), implying the presence of oxidative stress in the two terrestrial reed ecotypes. The fact that the DR exhibited lower POX and CAT activities might be explained by its lower H_2O_2 content. Considering the lower water contents and water potential of leaves in the two terrestrial reed ecotypes (Chen *et al*. 2003), these data indicate that antioxidant enzymatic system functions in the adaptation of the reed plants to their long-term drought and salinity in these habitats.

Together with glutathione, ascorbate contributes to

Table 1. H_2O_2, MDA, ASC, DHA, GSH and GSSG contents and activities of antioxidant enzymes in leaves of the three reed ecotypes. Means ± SE; values with different letters are significantly different at $P \leq 0.05$. SR - swamp reed, DR - dune reed, HSMR - heavy salt meadow reed.

Reed ecotypes	SR	DR	HSMR
H_2O_2 [μmol g^{-1}(d.m.)]	195.20 ± 20.90a	96.40 ± 18.10b	245.10 ± 22.00c
MDA [μmol g^{-1}(d.m.)]	154.50 ± 8.00a	126.00 ± 5.30b	181.30 ± 8.80c
Total ASC [μmol g^{-1}(d.m.)]	13.69 ± 0.71a	13.21 ± 0.38a	18.40 ± 0.70b
ASC [μmol g^{-1}(d.m.)]	8.91 ± 0.71a	8.73 ± 0.38a	13.45 ± 0.70b
DHA [μmol g^{-1}(d.m.)]	4.78 ± 0.23a	4.48 ± 0.25a	4.95 ± 0.07a
ASC/DHA	1.86 ± 0.06a	1.95 ± 0.04a	2.71 ± 0.13b
Total GSH [μmol g^{-1}(d.m.)]	5.23 ± 0.21a	5.13 ± 0.15a	6.56 ± 0.34b
GSH [μmol g^{-1}(d.m.)]	3.57 ± 0.07a	3.73 ± 0.21a	4.57 ± 0.5b
GSSG [μmol g^{-1}(d.m.)]	1.65 ± 0.15a	1.41 ± 0.08a	1.96 ± 0.22b
GSH/GSSG	2.17 ± 0.09a	2.64 ± 0.31b	2.32 ± 0.23a
SOD [U mg^{-1}(protein) min^{-1}]	0.28 ± 0.09a	0.88 ± 0.24b	1.30 ± 0.11c
POX [μmol(tetraguaiacol) g^{-1}(protein) min^{-1}]	0.21 ± 0.01a	0.12 ± 0.01b	0.20 ± 0.00a
CAT [μmol(H_2O_2) g^{-1}(protein) min^{-1}]	71.60 ± 7.20a	33.60 ± 5.00b	106.80 ± 9.80c
GR [μmol(NADPH) mg^{-1}(protein) min^{-1}]	0.10 ± 0.01a	0.09 ± 0.02a	0.10 ± 0.01a
APX [μmol(ASC) mg^{-1}(protein) min^{-1}]	0.64 ± 0.06a	0.77 ± 0.05b	0.63 ± 0.06a
DHAR [μmol(ASC) mg^{-1}(protein) min^{-1}]	0.60 ± 0.10a	1.37 ± 0.15b	1.34 ± 0.10b
MDHAR [μmol(NADH) mg^{-1}(protein) min^{-1}]	0.27 ± 0.02a	0.33 ± 0.07b	0.33 ± 0.02b

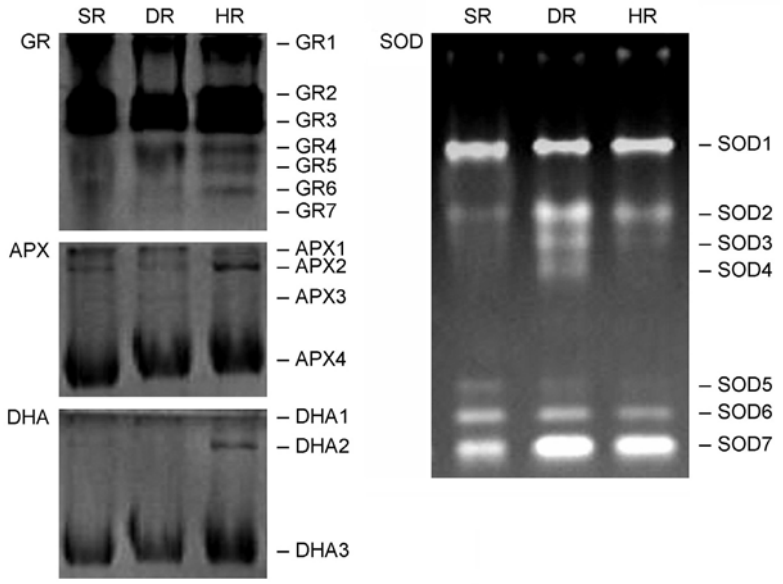

Fig. 1. Isozyme patterns of GR, APX, DHA reducing proteins and SOD in the leaves of the three reed ecotypes. In each lane, 100 μg of proteins were loaded for each enzyme determination. The bands of activity of isozymes were represented as GR1 - GR7, APX1 - APX4, DHA1 - DHA3 and SOD1 - SOD7, respectively. SR - swamp reed; DR - dune reed; HR - heavy salt meadow reed.

maintain the redox homeostasis and to protect plant tissues by direct scavenging active oxygen species (May *et al*. 1998, Noctor and Foyer 1998, Arbona *et al*. 2003, Chaparzadeh *et al*. 2004, Mittova *et al*. 2004). The function of ASC in the detoxification of hydrogen peroxide is depended on APX and GSH, and the pools of ASC and GSH in plant intracellular compartments are largely reduced, except under conditions of oxidative stress (Noctor *et al*. 1998, Horemans *et al*. 2000). As the two key redox couples in plant cells, the ratios of ASC/DHA and GSH/GSSG and their redox cycling can modulate expression of genes and activities of the redox sensitive transcription factors and enzymes (Sen 1999, Davey *et al*. 2000, Cnubben *et al*. 2001, Pastori and Foyer 2002). It has been found that ASC regenerated from DHA by two enzymic-dependant steps (catalyzed by DHAR and MDHAR, respectively) is essential for protecting the plant from oxidative damages (Fryer *et al*. 1998, Balestrasse *et al*. 2001). The activity of GR, which is responsible for the conversion of GSSG to GSH, increases when plants are subjected to environmental stresses (Noctor *et al*. 1998). In the present work, activities of DHAR and MDHAR and APX (another important enzyme in ASC metabolism) were obviously higher in DR and HSMR than those in SR (Table 1), indicating that the capacity of redox cycling of ASC was increased in the two terrestrial reed ecotypes. Considering the higher ratios of ASC/DHA and GSH/GSSG in DR and HSMR (Table 1), we can conclude that high ratios of ASC/DHA and GSH/GSSG and their redox cycling might be much essential for adaptation of the terrestrial reed ecotypes to their long-term drought or saline habitats by maintaining a more reduction state of redox status in the plant cells. In fact, the high ratios of ASC/DHA and GSH/GSSG and their redox cycling capacity were found to be closely related with the high reduction redox status of cells in other studies when plants were under stressed conditions (May *et al*. 1998, Chen *et al*. 2003, Chen *et al*. 2004b).

Development of isozymes was also involved in adaptation of the three reed ecotypes to their respective habitats. Isozyme patterns and varying specificity of SOD and APX have been reported in many studies that found the isozymes induced might be involved in the stress tolerance such as salt, drought, heavy metals, high light, and infections of pathogens (González *et al*. 1998, Kwon and Anderson 2001, Synková and Valcke 2001, Sairam and Srivastava 2002). Foyer *et al*. (1994) found that several isozymes of APX present in plant cells under oxidative stress. Some are located in the chloroplast, and others are thought to occur in the cytosol. Isoforms of GR and DHA have also been found in *Lupinus albus* and *Allium cepa* (Paciolla *et al*. 2001). In the present study, we found that not only new isozymes were present (such as in GR) and some isozymes strongly expressed in intensity (such as in SOD and APX), but also some isozymes vanished (such as in DHA reducing proteins) (Fig. 1), associated with the adaptation of the two terrestrial reed ecotypes to their extreme habitats, drought and salinity.

In summary, although the DR and HSMR exhibited many similar changes in the trends of both enzymatic and non-enzymatic antioxidants, some profound differences also existed between the two terrestrial reed ecotypes. For example, the DR had the highest ratio of GSH/GSSG and highest activities of the leaf APX and DHAR, whereas the HSMR had the highest ratio of ASC/DHA and highest activities of the leaf SOD and CAT among the three reed ecotypes. The isozyme pattern was also different in different ecotypes. Although the regulatory mechanisms of these changes between the reed ecotypes are not well known, these data suggest that the activity of antioxidant defense system changed when hydrophytic reed plants translocated their habitat from water to drought-prone dunes or salinity, thereby contributing to resistance/ tolerance of the plants to these long-term environments.

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