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Isoform-Specific Responses of Superoxide Dismutase to Oxidative Stresses and Hormones in Parquat-Tolerant *Rehmannia glutinosa*

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Abstract

All accessions of *Rehmannia glutinosa* show the unique characteristic of intrinsic tolerance to paraquat. The higher level of endogenous superoxide dismutase (SOD) activity and its increase upon paraquat treatment indicated the involvement of SOD in the tolerance mechanism to paraquat in *R. glutinosa*. In this study, we examined the isoform-specific response of SOD to oxidative stresses and hormones. Six SOD isoforms were found in the leaf, and they were identified as two MnSODs (named MnSOD I and MnSOD II, in order of increasing mobility), one FeSOD and three Cu/ZnSODs (named Cu/ZnSOD I, Cu/ZnSOD II, and Cu/ZnSOD III, in order of increasing mobility). MnSOD I, MnSOD II, FeSOD, Cu/ZnSOD I, Cu/ZnSOD II, and Cu/ZnSOD III, contributed to 4, 11, 7, 15, 30, and 32% of the total SOD activity, respectively. Total SOD activity levels in the leaf were increased by 4, 24, and 21% by paraquat, salicylic acid (SA), and yeast extract (YE), respectively, but little by ethephon. Six SOD isoforms responded differentially to these stresses and hormones. The activities of all the isoforms were increased by YE and SA except that of MnSOD I which was decreased by SA. The activities of MnSOD I, FeSOD, and CuZnSOD I were increased by paraquat. These results suggest that amelioration of oxidative stresses by SOD is fine-tuned by the differential expression of isoforms in *R. glutinosa*.

Key words: Ehephon, oxidative stresses, paraquat, *Rehmannia glutinosa*, salicylic acid, superoxide dismutase

Introduction

Reactive oxygen species (ROS) are produced in plants in a number of metabolic processes (Alscher and Hess, 1993). The ROS species play dual roles both as signal transducers and harmful agents to cellular components (Fridovich 1986). Both biotic and abiotic stresses induce the generation of an excess amount of ROS, which may cause membrane rigidification, damage of membrane lipids, protein denaturation, and DNA mutation (Borg and Schaich 1988). In order to prevent oxidation burst due to production of ROS, plants have evolved complex mechanisms to scavenge ROS species, which include both low molecular weight, non-proteinous antioxidants, and proteinous enzymatic components (Howe and Schilmiller 2002). Enzymatic components include superoxide dismutase (SOD; EC 1.15.1.1). SOD is a multimeric metalloprotein that scavenges superoxide (O_{-2}) and dismutates it into H_2O_2. The H_2O_2 is then scavenged by catalase and a variety of peroxidases into H_2O and O_2 (Alscher and Hess 1993).

Plants contain three types of SODs that differ in the metal co-factor in their active site. These are the dinuclear Cu/ZnSOD, mononuclear FeSOD, and MnSOD. SODs are present in most of the subcellular compartments that generate activated oxygen. Cu/ZnSODs are localized in the chloroplast, cytosol, peroxisomes, and glyoxysomes (Bowler et al., 1992). FeSOD is mainly found in prokaryotes and higher plants, mainly in the chloroplast (Bowler et al. 1992). FeSOD was rarely observed in mitochondria during senescence of day-lily petals and wheat leaves (Droillard and Paulin 1990). MnSOD is mainly present in mitochondria and peroxisomes (Scandalious 1993).

In the cell, SODs constitute the first line of defense against ROS. Superoxide is produced at any location where an electron transport chain is present and hence, O_2 activation may occur in different compartments of the cell (Elstner 1991), including mitochondria, chloroplasts, microsomes, glyoxysomes, peroxisomes, apoplasts, and the cytosol. This being the case, it is not surprising to find that SODs are present in all these subcellular locations. While all compartments of the cell are possible sites for O_2- formation, chloroplasts, mitochondria, and peroxisomes are thought to be the most important sites of ROS (Fridovich 1986).

Rehmannia glutinosa is a medicinal plant grown in Asian countries such as China, Japan, and Korea. In addition, it has been reported to exhibit a high level of tolerance to the non-selective herbicide paraquat (Chun et al. 1997). Studies on the paraquat tolerance of *R.*

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glutinosa indicate a partial role of antioxidant enzymes in the tolerance mechanism (Chun et al. 1997; Choi et al. 2004; Moon et al. 2004). SOD activity is more than 9-fold higher in *R. glutinosa* than in paraquat-susceptible soybean, and the activity is further increased by paraquat treatment in *R. glutinosa* (Chun et al. 1997; Choi et al. 2004; Moon et al. 2004).

However, quantitative SOD activity measured by spectrophotometric assays using crude protein extracts is often compounded by interfering enzymes such as cytochrome oxidase and cytochrome peroxidase (Beyer and Fridovich, 1987; Iannelli et al. 1999), making the interpretation of the activity assay results ambiguous. Furthermore, SOD isoforms often respond differentially to various environmental stresses (Mauro et al. 2005), indicating the importance of qualitative nature of SOD system in the scavenging of superoxide radicals (Bowler et al. 1992; Williamson and Scandalios 1992; Guan and Scandalios 1998). Thus, we identified individual SOD isoforms and measured the activity of each isoform by using activity staining gels. Furthermore, we found specific responses of SOD isoforms to stesses and hormones in *R. glutinosa*.

Materials and Methods

Plant materials

R. glutinosa (cv. Namwon) was grown under normal growing conditions in a glasshouse at Chonbuk National University, Korea. *R. glutonosa* plants were raised from tubers harvested from the previous growing season. Leaves were collected from healthy plants and used for SOD activity and isoform analysis.

Stress and hormone treatments

R. glutinosa leaves were collected from healthy plants by cutting the upper parts of petioles with a razor blade. Leaves with petioles were immersed in the following sterile solutions for 24 h: H_2O, 5.6 mM paraquat, 5 mM salicylic acid (SA), 5 mM ethephon (ET), or 25 mg/ml yeast extract (YE) under a 12 h day/night cycle (Chung et al. 2003).

Enzyme extraction and assay

Fresh leaf tissues powdered in liquid nitrogen were homogenized in 50 mM sodium phosphate buffer, pH 7.8, containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM β-mercaptoethanol (Donahue et al. 1997). Polyvinylpolypyrrolidone (PVPP-40) was added at four percent (w/v) during the homogenizing process. The homogenate was centrifuged at 30,000g for 30 min at 4°C. The supernatant was dispensed in aliquots for further analysis. Content of total soluble protein was determined following the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Chloroplast isolation

Chloroplasts were isolated using Percoll step gradient by the method of Mullet and Chua (1983). Fresh leaves (20 g) were homogenized in a pre-chilled blender for 2 min at 25 s intervals in 50 ml of ice cold grinding buffer (GR) that contained 0.33 M sorbitol, 2 mM sodium-EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 mM Hepes-KOH (pH 7.5). Sodium ascorbate (0.05 g/50 ml) was added at the time of grinding. The homogenate was filtered through four layers of cheesecloth and centrifuged at 3,000g for 2 min. The pellet was re-suspended in 4 ml GR buffer, overlaid onto a 40-80% Percoll step gradient, and centrifuged at 9,500g for 6 min. Intact chloroplasts were contained in the lower band of the two green bands generated after centrifugation. Intact chloroplasts were transferred to clean tubes and 30ml of ice-cold GR buffer was added to dilute out Percoll. Tubes were tightly capped with parafilm and mixed by inverting several times. Chloroplasts were collected by centrifugation at 4,300 g for 3 min. The final chloroplast pellet was re-suspended in 4 ml GR buffer and lysed by osmotic shock. The intactness was 80-85% as tested according to the ferricyanide reduction test (Lilley et al. 1975). The thylakoid and stroma were separated by suspending the purified chloroplasts in a hypotonic medium containing 50 mM Hepes:KOH (pH 7.5) and 4 mM ascorbate and followed by centrifugation at 31,000g for 10 min. The supernatant represented stromal fraction and the pellet thylakoidal fraction. Thylakoid membrane fraction was first suspended in 1 ml of 50 mM sodium phosphate buffer (pH 7.8) containing 1% CHAPSO and 1 mM ascorbate was added and the suspension was incubated at 4°C for 1h. After centrifugation at 27,200 g for 30 min, the supernatant was used as the dissolved thylakoidal fraction (Navari-Izzo et al. 1998).

Mitochondria isolation

Mitochondria were isolated following the method of Nishimura et al. (1982). Fresh leaves (20 g) were cut in 100 ml of 10 mM MOPS-KOH buffer, pH 7.2, containing 0.3 M sucrose, 5 mM glycine, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.2% BSA, and 0.6% PVP. The leaves were homogenized in a chilled blender and the homogenate was filtered through eight layers of cheesecloth. The filtrate was centrifuged at 3,000 g for 2 min to eliminate most of the chloroplasts, and the supernatant was centrifuged at 12,000 g for 20 min. The resulting pellet was re-suspended in the wash medium containing 10 mM MOPS-KOH, pH 7.2, 0.3 M sucrose, 5m M glycine, 1 mM EDTA, and 0.1% BSA. The suspension was layered on top of a discontinuous Percoll density gradient. The discontinuous gradient was composed of the following: A, 4.5 ml 60% (v/v) Percoll; B, 6 ml 45% (v/v) Percoll; C, 4 ml 28% (v/v) Percoll; and D, 4 ml 5% (v/v) Percoll containing 0.25 M sucrose, 20 mM MOPS-KOH, pH 7.2, and 0.2% BSA. Centrifugation was carried out at 30,000 g for 30 min. The mitochondrial fraction appeared at the interface between B and C. The fraction was suspended in a wash buffer containing 0.25 M sucrose, 20 mM MOPS-KOH, pH 7.2 and 0.2% BSA and centrifuged at 17,000 g twice, and finally resuspended in a smaller volume of the wash buffer.

SOD isoform identification and activity staining

SOD isoforms were separated on a 15% non-denaturing polyacrylamide (PA) gels at 4°C using a mini protean electrophoresis unit (Bio-Rad, USA). After electrophoresis, the gels were stained for SOD activity by the method of Beyer and Fridovich (1987). Gels were soaked in 50 mM potassium phosphate buffer, pH 7.8, containing 1.25 mM nitro blue tetrazolium (NBT)-2HCl for 30 min in the dark at 25°C, followed by immersing in 50 mM potassium phosphate buffer, pH 7.8, containing 0.1mM EDTA, 30 mM L-methionine and 30 μM riboflavin, which were then exposed under light source (60W, 6 lamps) at room temperature. Isoforms of SOD were differentiated by activity staining of gels previously incubated for 30 min at 25°C in 50 mM potassium phosphate buffer, pH 7.8, containing either 3 mM KCN (inhibitor of Cu/Zn SOD) or 5 mM H_2O_2 (inhibitor of Cu/Zn

SOD and FeSOD) (Salin and Lyon 1983). SOD activities were quantified by converting the stained area and intensity into a relative unit by scanning the gel using a gel documentation system (Kodak, EDAS 120, USA). Thirty micrograms of protein was loaded in each well for activity staining assays using the native gels.

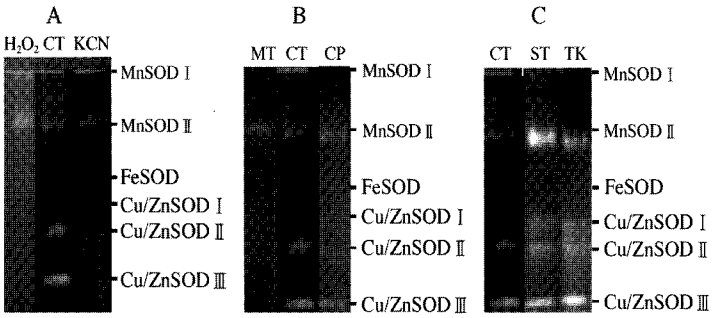

Fig. 1. Identification and localization of SOD isoforms of *R. glutinosa* using native polyacrylamide gel electrophoresis and SOD activity staining. A. Identification of SOD isoforms based on their response to inhibitors, H_2O_2 or KCN. CT, control. B and C. Localization of SOD isoforms using the purified mitochondria (MT) and chloroplasts (CP). ST and TK, stromal and thylakoidal fractions of chloroplasts.

Results

Isoform identification

A total of six SOD isoforms were found in the leaf of *R. glutinosa* (Fig. 1A). As judged by their sensitivity towards KCN and H_2O_2, they were identified as two MnSOD (named MnSOD I and MnSOD II, in order of increasing mobility), one FeSOD and three Cu/ZnSOD (named Cu/ZnSOD I, Cu/ZnSOD II, and Cu/ZnSOD III, in order of increasing mobility) isoforms. In the intact chloroplast of *R. glutinosa* (Fig.1B), four SOD isoforms were found, and they were identified as one MnSOD (named MnSOD II) and three Cu/ZnSOD (named Cu/ZnSOD I, Cu/ZnSOD II, and Cu/ZnSOD III, in order of increasing mobility) isoforms. In stroma and thylakoid, four isoforms were found and were identified as MnSOD II and three Cu/ZnSODs (named Cu/ZnSOD I, Cu/ZnSOD II, and Cu/ZnSOD III, in order of increasing mobility) (Fig. 1C). Only MnSOD II was found in mitochondria.

Effects of oxidative stresses and hormones on isoform expression

Total SOD activity in the leaf of *R. glutinosa* was affected by stresses and hormones. It increased by 4, 24, and 21% by paraquat, SA and YE, respectively, but the SOD activity was unchanged by ethephon treatment (Figs. 2A, B, and C). This result is significantly different from that obtained by the spectrophotometric assay, which detected about 40% increase by these treatments (Choi et al. 2004; Moon et al. 2004). It has been noted that quantitative activity measured by spectrophotometric assays using crude protein extracts could be compounded by interfering enzymes such as cytochrome oxidase and cytochrome peroxidase (Beyer and Fridovich 1987; Iannelli et al. 1999). Total SOD activity was partitioned to each isoform by using the activity-staining gels. Total SOD activity was contributed by MnSOD I, MnSOD II, FeSOD, Cu/ZnSOD I, II, and III, by 4, 11, 7, 15, 30, and 32%, respectively (Figs. 2A, B, and C). Thus, 77% of the total activity was contributed by the three Cu/ZnSODs. It was estimated that 82% of the total activity was contributed by Cu/ZnSODs by the spectrophotometric assay (Choi et al. 2004), indicating the significant over-estimation of Cu/ZnSOD activities by this assay.

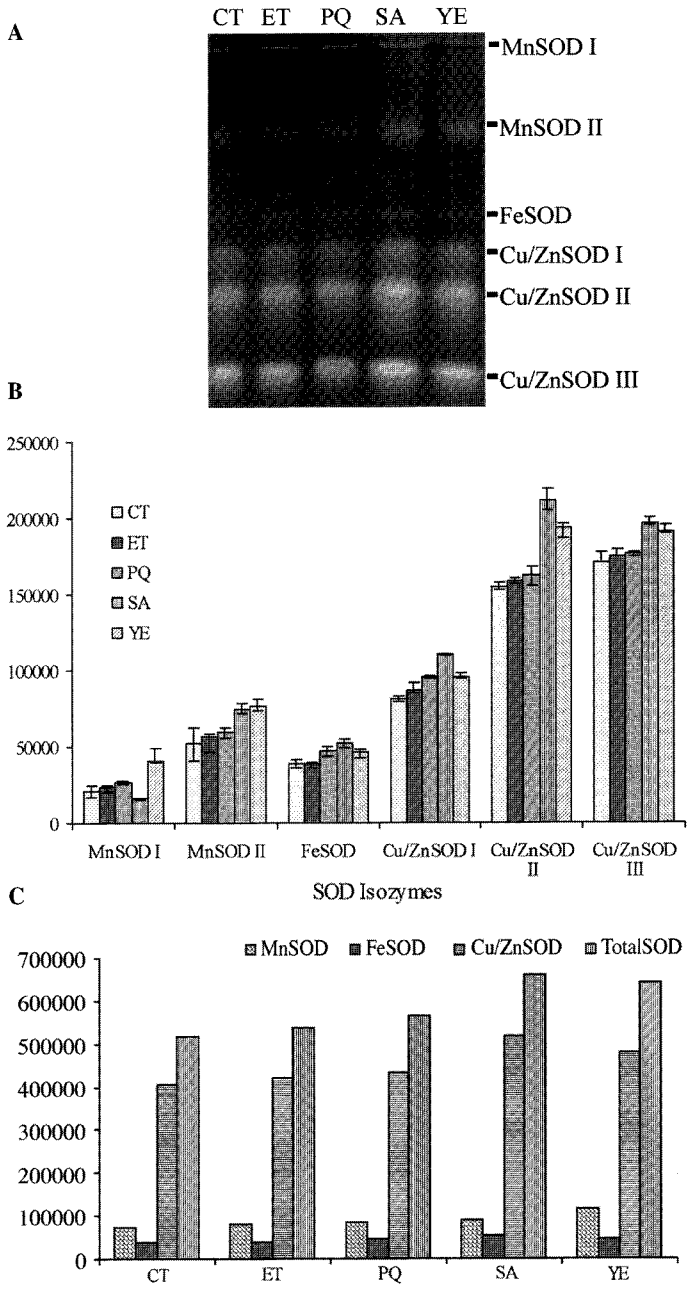

Fig. 2. Effect of oxidative stresses and hormones on the activity of superoxide dismutase isozymes in *R. glutinosa*. A. Activity staining of SOD isoforms in a native polyacrylamide gel. B and C. SOD activity as determined by scanning the gel using gel documentation system, area expressed as relative units. Vertical bars indicate SE (n = 3). CT, ET, PQ, SA, and YE: leaf samples treated with H_2O, ethephon, paraquat, salicylic acid, and yeast extract, respectively.

The expression of each isoform was only quantitatively affected by the stress and hormone treatments as no isoforms were newly induced or repressed by the treatments. Ethephon caused increase in the expression of MnSOD I, MnSOD II, and Cu/Zn SOD I by 12.4, 10.4, and 7.6%, respectively, but little change in the expression of other isoforms compared to that of control, respectively (Figs. 2A, B, and C). Paraquat increased the activity of MnSOD I, MnSOD II, FeSOD, and Cu/ZnSOD I by 28.3, 14.7, 21.2, and 17.9%, respectively, compared to that of control (Figs. 2A, B, and C). Salicylic acid increased the

activity of MnSOD II, FeSOD, Cu/ZnSOD I, Cu/ZnSOD II, and Cu/ZnSOD III, 44.7, 38.4, 35.8, 36.2, and 15.1%, but decreased the activity of MnSOD I by 24.5% compared to that of control, respectively (Figs. 2A, B, and C). Yeast extract induced increase in the activity of MnSOD I, MnSOD II, FeSOD, Cu/ZnSOD I, Cu/ZnSOD II, and Cu/ZnSOD III by 11.9, 93.3, 49.0, 18.6, 17.3, and 24.8%, respectively, compared to that of the control (Figs. 2A, B, and C).

Discussion

SODs play an important defense role against the harmful effect of superoxide in various compartments of the cell. In the leaf of *R. glutinosa*, all the three known SOD isoforms were present and localized in various cellular compartments. MnSOD II was present in both stromal and thylakoidal fractions of chloroplast, and mitochondria. MnSOD I and FeSOD were found only in cytosol (Figs. 1A and B). MnSODs are known to be present in the chloroplast, mitochondria, and peroxisomes (Scandalious 1993; Chopra and Sabarinath 2004). In this study, only the chloroplast and mitochondrial fractions were separated from the extracts of leaf tissues. Therefore, the localization of MnSOD I and FeSOD in cytosol should not exclude localization in other subcellular compartments than the chloroplast and mitochondria (Fig. 1B). Droillard and Paulin (1990) reported the presence of FeSOD in both mitochondria and peroxisomes from carnation petals. FeSOD is usually considered as localized in chloroplasts (Salin and Bridges 1982; Kwiatowski et al. 1985). However, FeSOD has also been identified in subcellular locations other than the chloroplast, such as in the cytoplasm of *Vigna unguiculata* nodules, a non-photosynthetic tissue (Becana et al. 1989). Salin (1988) has suggested that FeSOD might be present in peroxisomes of *Brassica campestris* leaves. Therefore, it appears that FeSOD may be present in wider subcellular locations of plant cells. We found the three Cu/ZnSODs in the chloroplast of *R. glutinosa* (Figs. 1B and C). As the same isoforms were also detected in the roots (data not shown), their presence in the cytosol could not be ruled out before further detailed fractionation study. Cu/ZnSODs have been found in the chloroplast, cytosol, peroxisome, and glyoxysome (Bowler et al. 1992). Specific subcellular localization of SOD isoforms requires their specific affinity to the components and is closely related to the functional integrity of the compartments (Arisi et al. 1998). Therefore, the presence of all the three known SOD isoforms in various subcellular locations could be a chief constituent for the higher SOD activity in *R. glutinosa* (Choi et al. 2004; Moon et al. 2004).

Total SOD activity in the leaf of *R. glutinosa* was increased by paraquat, SA, and YE, but remained unchanged by ethephon. The activity of each isoform also responded differently to the stresses and hormones. By paraquat, the activities of MnSOD I, FeSOD, and Cu/ZnSOD I increased, but that of MnSOD II decreased. By SA, the activities of all the isoforms increased significantly, except that of MnSOD I, which decreased. By YE, however, the activities of all the isoforms increased significantly. Interestingly, while the total SOD increased significantly by paraquat, SA, and YE, the relative contribution of each SOD isoform to the total activity in each treatment remained generally similar with some exceptions. In the paraquat treatment, the relative contribution of MnSOD I, FeSOD, and Cu/ZnSOD I to the total activity increased, but that of MnSOD II decreased. In the SA treatment, the relative contribution of Cu/ZnSOD I and II increased, while that of MnSOD I decreased, though the absolute activity of these isoforms increased significantly. Among these exceptions, the decreased relative contribution of MnSOD I and MnSOD II in the SA and paraquat treatments, respectively, was due to the decreased activity of these isoforms. This result clearly shows that the different response of total SOD activity to each stress factor is closely associated with the differential regulation of each isoform. Similar differential response of Cu/ZnSOD activity to various stresses was also observed through the quantitative analysis using the spectrophotometirc method in *R. glutinosa* (Choi et al. 2004). Differential responses of SOD isoforms were also observed in many plant species under various stress conditions. The protein and mRNA levels of the seven SODs, three FeSODs (FSD1, 2, and 3), three Cu/ZnSODs and one MnSOD, were changed in Arabidopsis plants subjected to a series of oxidative stresses. FSD2 specifically increased by UV irradiation but not by ozone exposure. (Kliebenstein et al. 1998; Alscher et al. 2002). In pea leaves, Cu/ZnSOD and FeSOD activities increased by drought and paraquat treatments but MnSOD activity remained unchanged under both conditions (Iturbe-Ormaetxe et al. 1998). In barley, the activity of Cu/ZnSOD and FeSOD increased, but that of Cu/ZnSOD more significantly, by SA treatment (Ananieva et al. 2004). Differential regulation of isoforms by the stresses and hormones could be related to the specificity and compatibility of the signaling system induced by the stresses and hormones (Bostock 2005).

According to Iturbe-Ormaetxe et al. (1998) the induction of chloroplastic and cytosolic Cu/ZnSOD may be interpreted as a response to increased superoxide generation in both cellular compartments. Protective roles of SOD against oxidative stresses have been demonstrated by various ways including transgenic plants overexpressing genes for SOD isoforms. Plants overexpressing SOD genes showed enhanced tolerance to various oxidative stresses (Allen et al. 1997). Thus, the increased levels of each SOD isoform under stress conditions in various cellular components in *R. glutinosa* plants might enhance the potential to remove the increased superoxide under stress conditions, thereby reducing the toxic effect of superoxide to the cellular components and in the event, conferring higher resistance to various oxidative stresses including paraquat.

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