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Protective Role of Exogenous Spermidine against Paraquat Toxicity in Radish Chloroplasts

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When radish chloroplasts were pretreated with 1 mM spermidine (Spd) and then exposed to 30 M paraquat (PQ), they improved their tolerance to subsequent PQ-induced oxidative damages. That included the decreases in the contents of chlorophyll, protein, and ascorbate, as well as the increases in malondialdehyde (MDA) and H_2O_2 levels. Analysis of antioxidant enzymes showed that Spd pretreatment effectively prevented the PQ-induced decreases in the total activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX). In contrast, the normally enhanced activities of dehydroascorbate reductase (DHAR) and glutathione reductase (GR) in PQ-treated chloroplasts were reversed by Spd pretreatment. In a native gel assay, the Cu/ZnSOD isozyme, which disappeared under the PQ alone treatment, was significantly recovered when tissues were pretreated with Spd. The dominant APX4 isozyme activity, which was preferentially decreased in response to PQ alone treatment, was also strongly reactivated by earlier Spd exposure. Therefore, we suggest that Spd could play a substantial role in protecting the radish chloroplasts from PQ stress. Furthermore, the enhancement of the Cu/ZnSOD and APX4 isozymes by Spd pretreatment seems to be responsible for prevention of the PQ-induced decreases in the total activities of SOD and APX, thereby providing a tolerance to PQ toxicity.

Keywords: antioxidant enzyme, chloroplast, paraquat toxicity, radish, spermidine

Oxidative stress in plants is particularly pronounced in the chloroplasts because of their light-harvesting function and the production of oxygen. Under stressful conditions, light-driven electron transport systems in the thylakoid membranes may often divert electrons to molecular oxygen, giving rise to superoxide radicals and hydrogen peroxide in chloroplasts (Lidon and Henriques, 1993). Hence, the chloroplast has received particular attention as a major target organelle of oxidative stress in plants (Munne-Bosch and Alegre, 2002). The chemical herbicide paraquat (PQ) has been used as a model to study this oxidative stress (Bowler et al., 1994; Martinez et al., 2001). PQ transfers electrons from PSI of the chloroplasts to molecular oxygen, forming a superoxide radical that can produce different deleterious oxygen radicals. Such action makes PQ a useful tool for initiating the accumulation of oxidative stress. For instance, PQ-generated superoxide radicals and the decomposition product H_2O_2 can cause the breakdown of cellular components, e.g., membrane lipids, proteins, and pigments, while also inhibiting photosynthesis through the inactivation of several enzymes in the Calvin cycle (Keiser, 1979; Robinson et al., 1980).

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In the meantime, there is an accumulating evidence in the possible involvement of polyamines (PAs) in the defense reaction of plants to various abiotic stresses (Shen et al., 2000). PAs such as spermidine (Spd) and spemine (Spm) are ubiquitously in all plants (Tiburcio et al., 1997; Bouchereau et al., 1999; Shen et al., 2000). Application of PAs decreases the severity of ozone-induced leaf damage in tobacco (Bors et al., 1989) and protects pea against atrazine toxicity (Zheleva et al., 1994). In addition, Spd and Spm protected bean from acid rain-induced oxidative stress (Velikova et al., 2000). Moreover, PAs are reported as efficient antioxidants in some experiments, exerting this effect through the protection of cellular components such as cell membranes, phospholipids and proteins from oxidative damage (Tadolini, 1988; Ye et al., 1997; Velikova et al., 2000). All these reports suggest that PAs may either act directly as oxygen radical scavengers (Drolet et al., 1986), or function by adjusting the antioxidant defense systems in plants (Velikova et al., 2000). Velikova et al. (2000) have hypothesized that PAs may prepare the cell to meet and combat stress by forming a higher potential of cellular antioxidant capacity. In terms of their antistress effects, however, the exact mode of PA actions implicated in these plant defense responses is still unclear. Furthermore, no direct evidence for a role of

PAs in protecting of chloroplasts against PQ stress has yet been reported.

The chloroplasts in higher plants can be protected from toxic oxygen radicals through the action of their antioxidant enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Nakano and Asada, 1981; Gillham and Dodge, 1987; Foyer et al., 1994). Ye and Gressel (1994) also have suggested that even small shifts in antioxidant enzyme activities can greatly affect in stress resistance of plants if the reaction is localized in small cellular compartments such as plastids. In addition, it has been found that activities of some chloroplast enzymes are directly modulated by polyamine binding (Carley et al., 1983).

The aim of this work described here how the exogenous Spd in radish seedlings influences the activities of chloroplast superoxide and H_2O_2 scavenging enzymes. We also examined the biochemical changes in their isozyme compositions during PQ-induced oxidative stress, and evaluated the protective role of Spd against PQ toxicity.

MATERIALS AND METHODS

Plant Materials and Treatments

The surface-sterilized seeds of radish (*Raphanus sativus* L. cv. Taewang) were sown and germinated for 4 to 5 days in plastic pots (115 × 115 × 100 mm) containing commercial soils (Heung Nong, Korea) and moistened with distilled water. The environmental conditions in the growth chamber included the germination pots were 25/21°C (day/night) temperature, 70% RH, and a 8000 lux of light intensity with an 18 h photoperiod. For the Spd pretreatment, 4-d-old seedling plants were sprayed with 1 mM Spd in a 0.1% (v/v) Tween 20 solution (1.0 mL/plant) for 12 h before the application of 30 μM PQ. Control plants were sprayed only with 0.1% Tween 20 solution. After 12 h Spd pretreatment, PQ stress was imposed by spraying the plants with 30 μM PQ in a 0.1% Tween 20 (1.0 mL/plant). Following their exposure to PQ in light for the indicated time intervals (0, 6, 12, and 24 h), the cotyledons were harvested for the chloroplast isolation.

Chloroplast Isolation

Chloroplasts were isolated from harvested radish cotyledons according to the method of Mills and Joy (1980) with slight modifications. Seventy pairs of cotyledons were homogenized at full speed in a Waring blendor for 2 × 5 s in 30 mL of cold chloroplast isolation buffer that contained 50 mM HEPES-KOH (pH 7.6), 0.33 M sorbitol, 2 mM Na_2EDTA and 1 mM $MgCl_2$. The resulting homogenate was immediately filtered through four layers of cotton cloth plus two layers of Miracloth (Calbiochem, USA) into 50 mL centrifuge tubes. After centrifugation at 2,000*g* for 1 min with a swing-out rotor, the supernatant was discarded and the pellet was gently resuspended in 2 mL of the isolation buffer. This resuspended material was carefully layered onto 10 mL of a 30% (w/v) Percoll mixture that had the same composition as the isolation buffer. Intact chloroplasts were sedimented by centrifugation at 4,000*g* for 15 min with a swing-out rotor, but broken chloroplasts failed to pass through the 30% Percoll layer. Finally, the chloroplast pellet was washed and resuspended in the appropriate reaction buffer following removal of the Percoll layer by Pasteur pipette.

Determination of Chlorophyll, Protein and Malondialdehyde (MDA) Contents

Chlorophyll was extracted from our chloroplast preparations in 80% acetone, then quantified as considering that [Chl] (μg mL^{-1}) = 27.7 A_{652nm} (Arnon, 1949). The protein content was determined by the method of Bradford (1976) using BSA as a standard. MDA content was determined by adding an equal aliquot of 0.5% (w/v) TBA in 20% (w/v) trichloroacetic acid to an aliquot of the chloroplast suspensions. The solution was heated at 95°C for 25 min and then rapidly cooled. After centrifuging at 3,000*g* for 10 min, absorbance was measured at 532 nm and was corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968).

Determination of Ascorbate and Dehydroascorbate Contents

Levels of ascorbate and dehydroascorbate were determined by the bipyridyl method of Law et al. (1983). A sample (400 μL; 350 μg chlorophyll) of the chloroplast preparation was added to 200 μL of 10% (w/v) trichloroacetic acid. After thorough mixing, it was allowed to stand on ice for 5 min. NaOH (10 μL, 5 M) was added, mixed and the mixture was centri-

fuged for 2 min in a microcentrifuge. To a 200 μL sample of the supernatant, we added 200 μL of 150 mM NaH_2PO_4 buffer (pH 7.4) and 200 μL of water for the assay only for ascorbate. To determine the amount of total ascorbate (ascorbate + dehydroascorbate), we used another 200 μL of the supernatant sample and added 200 μL of buffer, 100 μL of 10 mM dithiothreitol and, after vortex-mixing and being left at room temperature for 15 min, 100 μL of 0.5% (w/v) N-ethylmaleimide. Both samples were vortex-mixed and incubated at room temperature for 1 min. To each, we added 400 μL of 10% (w/v) trichloroacetic acid, 400 μL of 44% (v/v) H_3PO_4, 400 μL of 4% (w/v) bipyridyl in 70% (v/v) ethanol and 200 μL of 3% (w/v) $FeCl_3$. After vortex-mixing, the samples were incubated at 37°C for 60 min and their absorbances at 525 nm were recorded. The concentration of dehydroascorbate was calculated by subtracting the ascorbate concentration from the total ascorbate content. A standard curve was prepared for ascorbate in the range of 0 to 60 nM.

Determination of H_2O_2 Content

H_2O_2 content was determined according to a modification of the method of Bernt and Bergmeyer (1974), using a peroxidase enzyme. A 0.5 mL sample of the chloroplast preparation was mixed with 2.5 mL of peroxide reagent [83 mM sodium phosphate, pH 7.0, 0.005% (w/v) *o*-dianisidine, and 40 μg peroxidase mL^{-1}], then incubated at 30°C for 20 min in a water bath. The reaction was stopped by adding 0.5 mL of 1N perchloric acid, followed by centrifuging at 10,000*g* for 3 min. The resultant clear supernatant was read at 436 nm and its absorbance was compared with the H_2O_2 standard.

Enzyme Extraction and Total Enzyme Activity Assay

Because chloroplast ascorbate peroxidase (APX) is rapidly inactivated in the absence of ascorbate, we assayed the total APX activity as soon as the isolated intact chloroplasts were ruptured into an assay mixture containing 0.5 mM ascorbate (Nakano and Asada, 1981). Unlike the APX, total activities of superoxide dismutase (SOD), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) were determined in aliquots of the stromal fraction obtained from intact chloroplasts by osmotic lysis and centrifugation (8,000*g*, 10 min) according to the method of Nakano and Asada (1981). APX activity was measured as the decrease in absorbance at 290 nm due to ascorbate oxidation. The 2 mL reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate, 0.5 mM hydrogen peroxide, and 10 μL of chloroplast suspensions. DHAR activity was measured by monitoring the formation of ascorbate at 265 nm, according to Nakano and Asada (1981) with a slight modification. Our reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.5 mM dehydroascorbate (DHA), 5 mM GSH, 0.1 mM EDTA, and the stroma enzyme preparation (100 μL) in a final volume of 2 mL. Correction was made for the non-enzymatic reduction of DHA by GSH. GR activity was defined as the decrease in absorbance at 340 nm due to the oxidation of NADPH, as described by Schaedle and Bassham (1977) with a slight modification. The 2 mL reaction mixture contained 80 mM potassium phosphate (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM GSSG, and the stroma enzyme preparation (150 μL). The measurement of SOD activity was based on the photochemical method of Giannopolitis and Ries (1977), as modified by Dhindsa et al (1981). The 3 mL reaction mixture contained 50 mM potassium phosphate (pH 7.8), 13 mM methionine, 75 μM nitroblue tetrazolum (NBT), 4 μM riboflavin, 0.1 mM EDTA, and the stroma enzyme preparation (0 to 50 μL). Glass tubes containing this 3 mL reaction mixture were illuminated with two 20W fluorescent tubes to start the reaction and allowed to run for 10 min. Thereafter, the reaction was stopped by turning off the lights and the absorbance at 560 nm was read. One unit of SOD activity was defined as the amount of enzyme resulting in 50% inhibition of the rate of NBT reduction.

SOD and APX Isozyme Assay

Equal amounts of stromal protein were subjected to native PAGE using the modified discontinuous buffer system of Laemmli (1970), except that SDS was omitted. SOD isozymes were separated on the 10% separating polyacrylamide gel with a 4% stacking gel at 100V for 5 h at 4°C The loading samples contained 125 mM Tris-HCl (pH 6.8), 5% glycerol, 0.002% BPB, and the SOD extract (160 μg protein). After electrophoresis, the gels were stained for SOD activity by incubating them for 25 min on a solution containing 2.5 mM NBT, followed by incubation for 20 min in the dark in 50 mM potassium phosphate (pH 7.8) containing 28 μM riboflavin and 28 mM TEMED. Thereafter, the gels were placed in distilled water and exposed on a light box for 10 to 15 min at room tem-

perature. SOD activity in those gels was visualized as achromatic bands (Rao et al., 1995). Identification of SOD isozymes was performed by preincubation of gels with 6 mM KCN (inhibitor of the Cu/Zn SOD) for 30 min prior to activity staining. APX isozymes were separated on the 10% separating gel with a 4% stacking gel at 15 mA for 3 h at 4°C The loading samples contained 125 mM Tris-HCl (pH 6.8), 5% glycerol, 0.002% BPB, and the APX extract (150 μg protein). The gels were pre-run for 30 min to allow the 2 mM ascorbate, present in the carrier buffer, to enter the gel prior to the application of the samples (Mittler and Zilinskas, 1993). After electrophoresis, the gels were equilibrated for 30 min with a 50 mM potassium phosphate buffer (pH 7.0) supplemented with 2 mM ascorbate. The gels were then incubated for 20 min in 50 mM potassium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 2 mM H_2O_2. Afterward, they were washed for 1 min with 50 mM potassium phosphate buffer (pH 7.0) and soaked in a solution of 50 mM potassium phosphate buffer (pH 7.8) that contained 28 mM TEMED and 2.45 mM NBT. The APX activity was observed as an achromatic band on a purplish blue background.

RESULTS AND DISCUSSION

Protective Effect of Spd against PQ Toxicity in Radish Chloroplasts

We examined whether exogenous Spd pretreatment could effectively protect radish chloroplasts from subsequent PQ-induced oxidative stress. For the experimental chloroplast preparation, 4-day-old radish seedling plants were pretreated for 12 h with or without 1 mM Spd in 0.1% (v/v) Tween 20 as a spray method (1 mL/plant). Thereafter, PQ stress was subsequently imposed in the light for 0, 6, 12, or 24 h by spraying the plants with 30 μM PQ in 0.1% (v/v) Tween 20. The cotyledons were then harvested for chloroplast isolation from seedling plants after being exposed to PQ for 0, 6, 12 and 24 h, respectively. Changes in the contents of chlorophyll, soluble proteins and MDA (a product of lipid peroxidation) in radish chloroplasts are shown in Figure 1. The 24 h PQ treatment led to a completely inhibited chlorophyll production and caused a decrease of protein levels by 60% in comparison with the control chloroplasts. In contrast, Spd pretreatment significantly counteracted these toxic effects of PQ (Fig. 1A and B). Figure 1C, also illustrates that PQ treatment resulted in a remarkable increase in MDA level, indicating that PQ brings about lipid peroxidation. However, the PQ-induced increase in MDA content was also inhibited by Spd pretreatment.

The PQ stress that accompanies an accumulation of superoxide and H_2O_2 within a cell is widely believed to cause chlorophyll loss, mainly as a consequence of an oxidative damage in the chloroplast membranes (Giardi et al., 1997). Furthermore, active oxygen species (AOS) that involve superoxide and H_2O_2 have also been shown to directly react with proteins containing thiol or heme groups, leading to an oxidative degradation (Thompson et al., 1987). In this sense, the Rubisco protein which is a major soluble protein in the chloroplasts is known to very susceptible to AOS attack (Mehta et al., 1992). Thus, we were able to characterize PQ toxicity in our radish chloroplasts based on in their protein content. Furthermore, lipid peroxidation is also caused by AOS. In fact, AOS-mediated toxicity in plant cells can be partially attributed to induced lipid peroxidation and subsequent membrane damage (Baker and Orlandi, 1995).

Here, we observed severe damage symptoms including decreases in both of chlorophyll synthesis (Fig. 1A) and soluble protein content (Fig. 1B), plus an increase in the MDA level (Fig. 1C) in the chloroplasts treated only with PQ. However, those that had been pretreated with Spd were less affected to PQ toxicity. These results indicate that Spd is certainly involved in protection of radish chloroplasts from PQ-induced oxidative stress.

Influence of Spd and PQ on Total Activities of Chloroplast Antioxidant Enzymes

In the chloroplasts, SOD catalyses the removal of superoxide radicals, producing H_2O_2 as a dismutation product. Hydrogen peroxide that is more toxic to the chloroplast is then scavenged by APX, which uses an ascorbate as a reductant. In this regulatory system, the recycling of the consumed ascorbate is mediated by DHAR and GR (Lidon and Teixeira, 2000). In relation to this, Ye and Gressel (1994) have suggested that even small shifts in the activities of such antioxidant enzymes as SOD, APX, DHAR, and GR can cause great effects in stress resistance of plants if localized in small cellular compartments like plastids.

Thus, we examined the relationship between the Spd-mediated PQ tolerance and the changes in total activities of superoxide and H_2O_2 scavenging enzymes in radish chloroplasts. Figure 2A and B, illustrates that total activities of chloroplast SOD and APX declined

to 68 and 49% of the control values, respectively, at 24 h after 30 μM PQ alone treatment. However, the Spd pretreatment for 12 h before a PQ application effectively prevented PQ-induced decreases in these enzyme activities. Unlike the SOD and APX enzymes, DHAR and GR enzymes responded in the chloroplasts differently with responses to PQ treament. Namely, treatment with PQ alone caused a continuous increase in their activities over the 24 h incubation period. Compared with the controls, their total activities exhibited the maximum inductions of 2.3-(DHAR) and 2.7-fold (GR). Nevertheless, Spd pretreatment significantly reversed the PQ-induced enhancements of these enzymes activities (Fig. 2C and D). Likewise, in the non-treated controls, both DHAR and GR enzymes showed a gradual decreases during the chloroplast development.

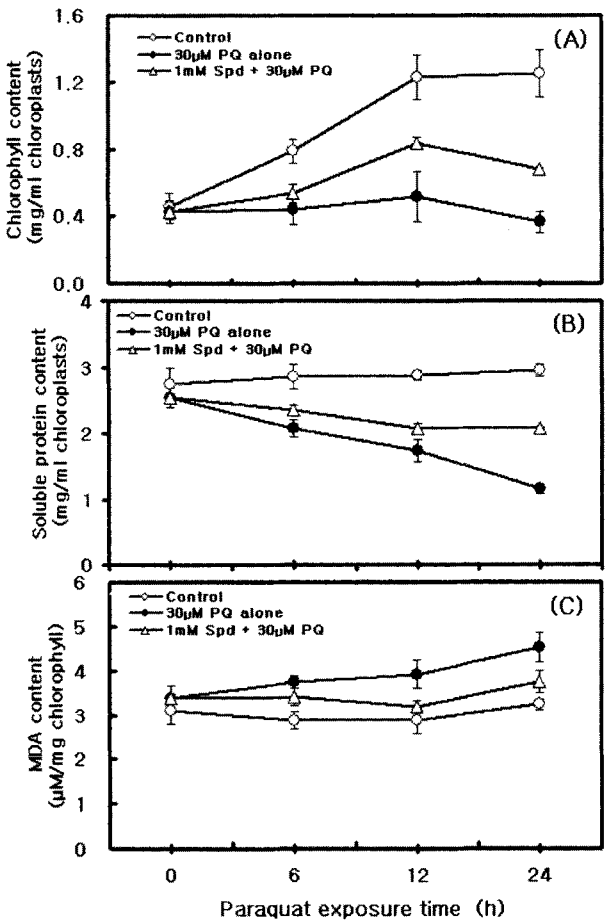

Figure 1. Effects of 1 mM spermidine (Spd) pretreatment on paraquat (PQ)-induced radish chloroplast damage. (A), chlorophyll content; (B), protein content; (C), MDA content. To prepare chloroplasts, 4-d-old radish seedlings were pretreated for 12 h with or without 1 mM Spd in 0.1% (v/v) Tween 20 as a spray (1.0 mL/plant) before the subsequent 30 μM PQ application. Data are means ± SE from three independent experiments.

In this study, APX was rapidly inactivated more than SOD by treatment with PQ alone. This response was accompanied by a decrease in ascorbate content (Fig. 3) and the accumulation of H_2O_2 (Fig. 6). APX is one of the thiol-modulated enzymes most sensitive to H_2O_2 (Shikanai et al., 1998) and is easily inactivated by H_2O_2 in the absence of ascorbate (Hossain and Asada, 1984). Therefore, it is conceivable that the decline in ascorbate level as well as the rise in accumulation of H_2O_2 may bring about the rapid inactivation of APX in the chloroplasts subjected to PQ alone. SOD was also decreased in response to PQ alone, although it was less inactivated than APX. In contrast to SOD and APX, both DHAR and GR were continuously induced over the increasing time of PQ alone treatment. It is unclear why those enzymes in the chloroplasts treated with PQ alone were enhanced. However, we assume that the higher production of H_2O_2 (Fig. 6) and the oxidation of ascorbate (Fig. 3) in the PQ alone-treated chloroplasts induced these increases to meet the greater demand for ascorbate. Because a recycling of the consumed ascorbate is mediated by DHAR and GR. In addition, it has been reported that GR appears to be directly redox-regulated, being partially inhibited by NADPH and activated by H_2O_2 and oxidised glutathione (Lidon and Teixeira, 2000). Our speculation is further supported by the observation that the DHAR and GR responses to PQ were reduced in the presence of Spd (Fig. 2C and D). Furthermore, both enzymes DHAR and GR showed lower activities in the untreated control chloroplasts.

In the meantime, SOD and APX activities were significantly reactivated by Spd pretreatment in subsequent PQ treated chloroplasts (Fig. 2A and B). Sen Gupta et al. (1993a) have demonstrated that tobacco plants over-expressing chloroplast Cu/ZnSOD increased resistance to oxidative stress. In addition, Ye and Gressel (1994) have shown that resistance against PQ in *Conyza bonariensis* can be attributed to enhanced SOD levels. Based on all these reports and our own results, it seems to be likely that the protection of radish chloroplasts from PQ stress was at least partially due to the significant increase in SOD activity by Spd pretreatment. However, there is still a problem on this idea. Considering that the removal of superoxide radical by SOD may cause an accumulation of H_2O_2, SOD alone cannot protect efficiently the chloroplasts against PQ toxicity. Hence, an increase in H_2O_2 -scavenging capacity may also be required for removal of

H_2O_2 produced by enhanced SOD activity (Foyer et al., 1994). In line with this consideration, supplementary APX activity will be expected to adapt to PQ-induced oxidative stress. It has been reported that APX, which is localized in the chloroplasts, scavenges all the H_2O_2 produced there (Nakano and Asada, 1981; Hossain and Asada, 1984). Moreover, Gupta et al. (1993b) have demonstrated that enhanced SOD expression increases APX activity. Thus, both enzymes SOD and APX are now believed to act in conjunction for the protection of chloroplasts from the accumulation of toxic H_2O_2 when exposed to oxidative stress.

In the present work, it is worth noting that our Spd pretreatment prevented the PQ-induced decreases in the activities of both SOD and APX, while also reducing the PQ toxicity. Therefore, we can assume that the considerable rise in those activities in response to Spd pretreatment was responsible for that protection. In this study, however, we could not explain about the exact mechanism of Spd action on the regulation of these enzymes activities. In relation to this, some enzymes whose activities are directly modulated by polyamine binding were observed in chloroplasts (Carley et al., 1983). It has been also reported that putrescine could bind to the SOD during chilling tolerance (Bouchereau et al., 1999).

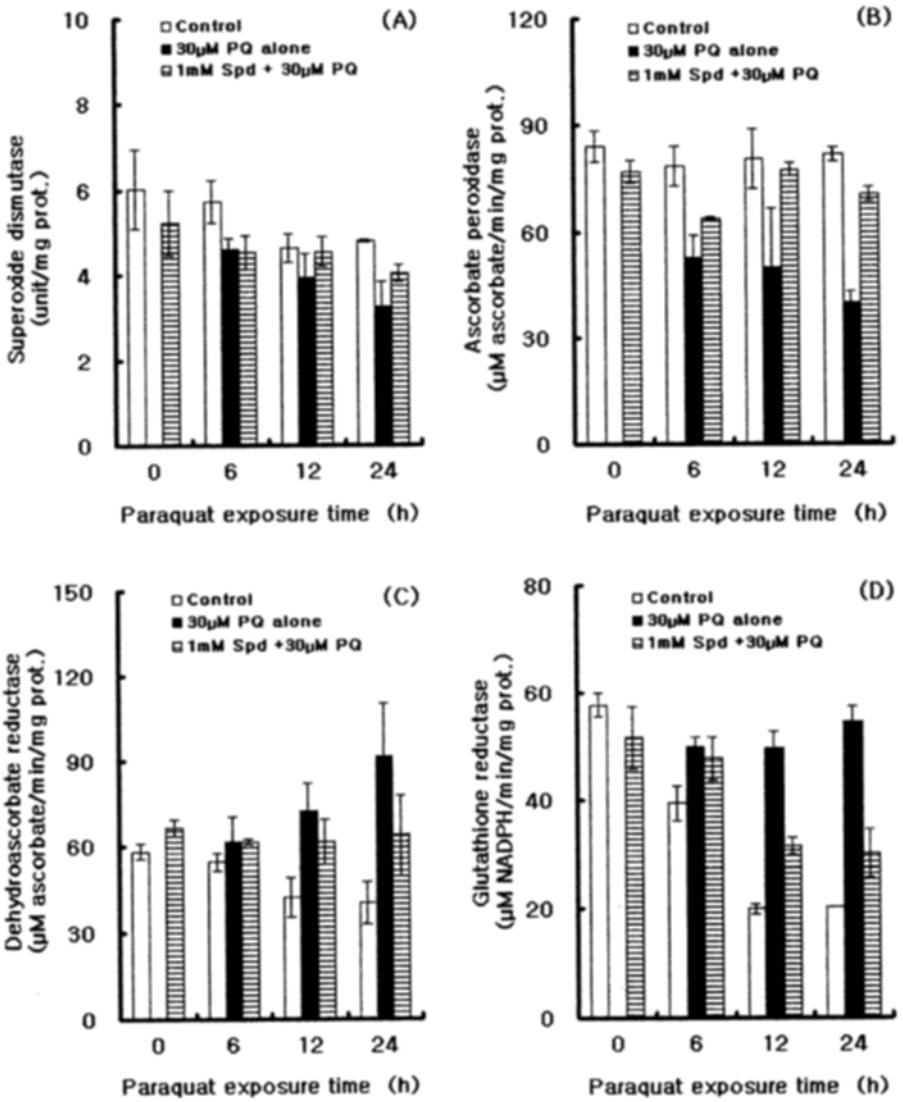

Figure 2. Effects of 1 mM Spd pretreatment on changes in total activities of superoxide dismutase (A), ascorbate peroxidase (B), dehydroascorbate reductase (C) and glutathione reductase (D) in radish chloroplasts treated with 30μM PQ, as described in Figure 1. Data are means ± SE from three independent experiments.

Changes in the Composition of SOD and APX Isozymes in Response to Spd and PQ

Both SOD and APX exist in multiple isozymes within the chloroplasts (Mittler and Zilinskas, 1993; Yoshimura et al., 2000; Martinez et al., 2001). Plants are capable of differentially altering the isozyme composition of their antioxidant enzymes in response to oxidative stress (Bowler et al., 1994; Yoshimura et al., 2000; Borsani et al., 2001; Martinez et al., 2001). In addition, oxidative stress-induced changes in the total activities of antioxidant enzymes are possibly due to changes in their protein content and/or the synthesis of new isozymes (Edwards et al., 1994; Rao et al., 1995).

Because we observed that pretreatment of radish chloroplasts with Spd prevented the PQ-induced decrease in the total activities of SOD and APX with providing a tolerance to PQ (Fig. 1, 2A and B), we first attempted to determine whether these changes in the total activities can be due to the changes in their isozyme compositions. And then, we also tried to identify which isozymes were most important for this protection of chloroplasts by Spd. Thus, chloroplasts extracts were subjected to native PAGE and monitored for SOD and APX activities to identify any changes in their isozyme patterns. In plants, studies of SOD have obtained variable results due to the fact that SOD isozymes can be differentially affected by stress conditions (Bowler et al., 1994; Borsani et al., 2001). Those SOD isozymes are generally divided into three classes according to their metal cofactor, i.e., Cu/ZnSOD, MnSOD, FeSOD (Bowler et al., 1994). Among them, the chloroplast is known to possess FeSOD and Cu/ZnSOD (Martinez et al., 2001). We also detected two different SOD isozymes on our native gels (Fig. 4). Incubation of gels with or without 6 mM KCN before staining for SOD activity revealed the upper band to be FeSOD (KCN-resistant) and the other one was identified as Cu/ZnSOD (KCN-sensitive). Depending on whether the chloroplasts had received Spd pretreatment, each SOD isozyme was differentially expressed in response to PQ (Fig. 4). The band of Cu/ZnSOD disappeared under the PQ alone treatment, but it could be again seen by Spd pretreatment. In contrast to Cu/ZnSOD, there was no remarkable difference in the staining intensities of FeSOD isozymes among different treatments. Based on the SOD isozyme patterns, the overall decrease in the total SOD activity in PQ alone-treated chloroplasts have been largely associated with the suppression of the Cu/ZnSOD isozyme.

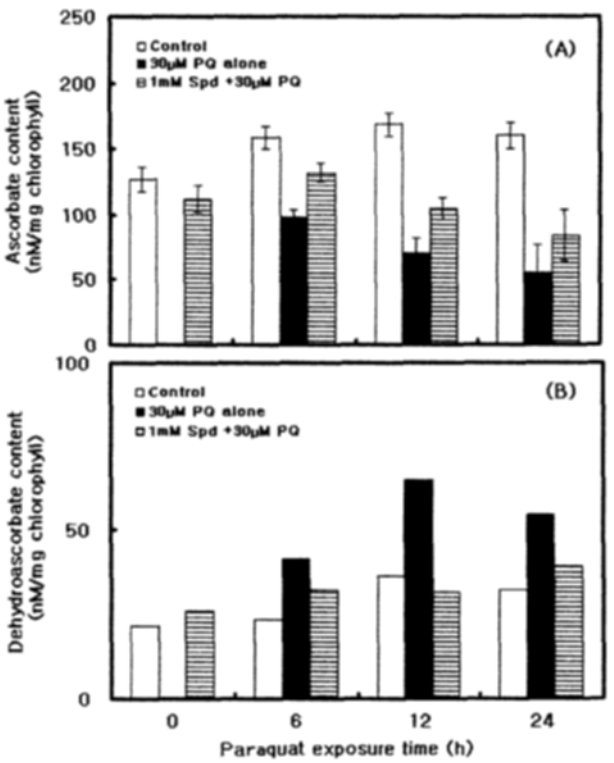

Figure 3. Effects of 1 mM Spd pretreatment on contents of ascorbate (A) and dehydroascorbate (B) in radish chloroplasts treated with 30 μM PQ, as described in Figure 1. Data are means ± SE from three independent experiments.

Therefore, we can assume that the reappearance of this Cu/ZnSOD isozyme have contributed in some degree to the recovery of total SOD activity after PQ-associated suppression. Perl et al. (1993) have demonstrated that overproduction of Cu/ZnSOD in potato chloroplasts is effective in protecting against PQ toxicity. The Cu/ZnSOD isozyme is the most responsive to drought stress, as manifested in the marked change in its activity (Borsani et al., 2001). Furthermore, Yu and Rengel (1999) have found that total SOD activity in lupinus plants markedly increased due to an increase in the activities of Cu/ZnSOD and FeSOD under the moderate drought stress. Based on these findings and our present data, we suggest that the Cu/ZnSOD enhancement by Spd could have been in partly involved in the protecting radish chloroplasts during PQ stress.

APX is believed to act in conjunction with SOD in the chloroplast superoxide and H_2O_2 scavenging systems. Gupta et al. (1993b) have shown that Cu/ZnSOD expression elevates the level of APX activity. Until now, most physiological studies of the APX response to abiotic stresses have not distinguished among the activities of individual isozymes, although

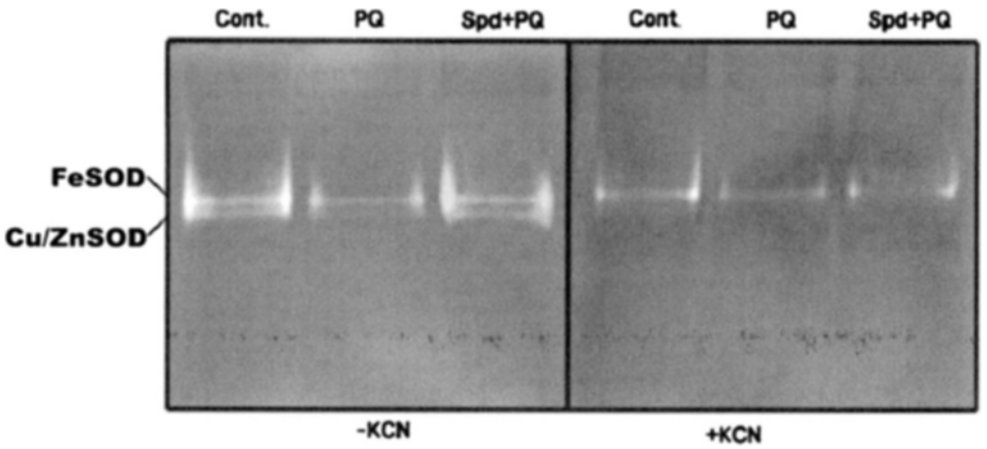

Figure 4. Native PAGE gels stained for superoxide dismutase isozymes in radish chloroplasts. Cont., non-treated chloroplasts as a control; PQ, chloroplasts treated with 30 μM PQ alone for 1 d; Spd+PQ, chloroplasts pretreated for 12 h with 1.0 mM Spd before exposure to 30 μM PQ for 1 d. Identification of SOD isozymes was performed by preincubation of gels with 6 mM KCN for 30 min prior to activity staining.

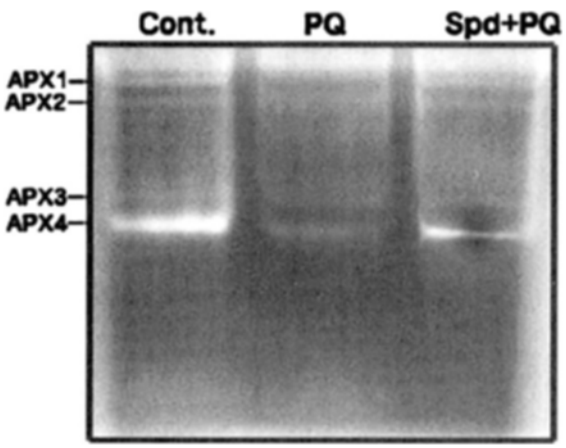

Figure 5. Native PAGE gels stained for ascorbate peroxidase isozymes in radish chloroplasts. Cont., non-treated chloroplasts as a control; PQ, chloroplasts treated with 30 μM PQ alone for 1 d; Spd+PQ, chloroplasts pretreated for 12 h with 1.0 mM Spd before exposure to 30 μM PQ for 1 d.

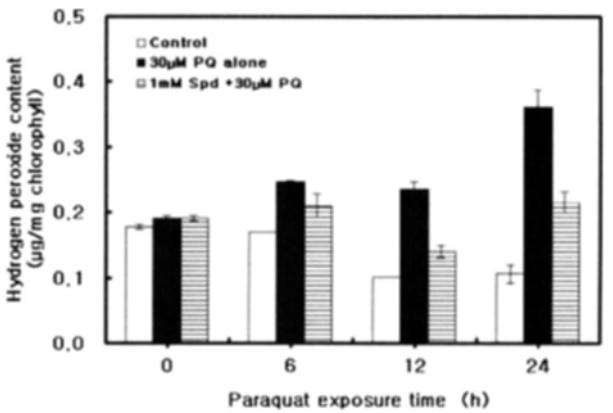

Figure 6. Effects of 1 mM Spd pretreatment on H_2O_2 content in radish chloroplasts treated with 30 μM PQ, as described in Figure 1. Data are means ± SE from three independent experiments.

several APX isozymes specific to the chloroplast have been described (Mittler and Zilinskas, 1993; Yoshimura et al., 2000; Martinez et al., 2001). Therefore, we attempted here to determine whether there are Spd-mediated differences among activities of individual APX isozymes in radish chloroplasts during the PQ stress.

Four isozymes exhibiting APX activity were observed in the chloroplasts; one (APX4) was prominent, the others (APX1, APX2, and APX3) were very faint (Fig. 5). The activity of dominant APX4 isozyme was preferentially and greatly decreased in response to the PQ alone treatment. Based on this, we might conclude that the overall decline in the total APX activity in those chloroplasts may have largely resulted from the inactivation of APX4. Interestingly, we also found that pretreatment with Spd significantly inhibited the PQ-induced decreases in APX4 activity. Regardless of the Spd pretreatment, however, the activities of all other isozymes remained unaffected by PQ. These results imply that a significant reactivation of the APX4 isozyme by Spd could habe been responsible for maintaining the level of total APX activity in the presence of PQ-associated suppression.

In conclusion, our results suggest that Spd plays a substantial role in protecting radish chloroplasts from PQ stress, probably by inhibiting PQ-induced inactivation of chloroplast SOD and APX enzymes. We can

also assume that the enhanced expression of the Cu/ZnSOD and APX4 isozymes by Spd is responsible for preventing the PQ-induced decrease in total activities of SOD and APX, thereby providing a tolerance to PQ toxicity. However, the precise mechanisms for Spd action in the regulation of these enzymes remain unknown.

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