Journal of Plant Biology, June 2006, 49(3) : 237-246

Polyamines as Antioxidant Protectors against Paraquat Damage in Radish (*Raphanus sativus* L.) Cotyledons

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Pretreatment of radish cotyledons with polyamines (PAs; especially 1 mM spermidine) significantly improved their tolerance to subsequent 50 μM paraquat (PQ)-induced oxidative damage. Symptoms in the cotyledons, e.g., large accumulations of H_2O_2, and losses of fresh weight, chlorophyll, and proteins, were remarkably alleviated. Likewise, analysis of several enzymes belonging to the superoxide dismutase (SOD)/ascorbate-glutathione cycle showed that pretreatment with PAs prevented typical PQ-induced declines in the total activities of SOD, ascorbate peroxidase (APX), and glutathione reductase (GR). Dehydroascorbate reductase (DHAR) activity, which normally decreases sharply under prolonged PQ exposure, was also highly maintained by PA treatment. In a native gel assay, two SOD isozymes (FeSOD and Cu/ZnSOD1), two APX isozymes (APX1 and APX2), and two GSSG-specific isozymes (GR1and GR2) proved to be more responsible for PQ tolerance, as manifested by the strong increases in their activities by spermidine (Spd) pretreatment. In addition, experiments with protein synthesis inhibitors (actinomycin D and cycloheximide) indicated that Spd could stimulate *de novo* synthesis of SOD and APX at the translational level. We can conclude that PAs may function as antioxidant protectors by invoking an efficient SOD/ascorbate-glutathione cycle in radish cotyledons exposed to PQ.

Keywords: antioxidant enzyme, paraquat, polyamine, SOD/ascorbate-glutathione cycle

Polyamines (PAs), such as putrescine (Put), spermidine (Spd), and spermine (Spm), are ubiquitous in plant species (Galston and Sawhney, 1990; Bouchereau et al., 1999). They have been implicated in numerous regulatory processes, including the synthesis of proteins and RNA (Tabor and Tabor, 1985), cell division and organ growth (Smith, 1985; Galston and Sawhney, 1990), and plant senescence (Altman, 1982). Besides their regulatory roles, a close correlation has been suggested between PAs and oxidative stress based on observations that ozone-induced oxidative injury (i.e., leaf necrosis) in tomato and tobacco plants can be prevented by an exogenous supply of Put, Spd, and Spm (Ormrod and Beckerson, 1986; Bors et al., 1989). Interest is growing as to the possible involvement of PAs in plant defense responses to various types of oxidative stresses, e.g., UV radiation (Kramer et al., 1991), salt stress (Aziz et al., 1997), acid rain (Velikova et al., 2000), and heavy metals (Groppa et al., 2001). All of these stresses, which inhibit normal plant growth, lead to the cellular accumulation of activated oxygen species (AOS), such as superoxide radicals and H_2O_2. Drolet et al. (1986) have reported that PAs can function directly as radical scavengers. However, Bors et al. (1989) have discounted this scavenger capability, while confirming the protective influence of these molecules against ozone-induced oxidative damage. In contrast, Velikova et al. (2000) have hypothesized that PAs may prepare the cell to counteract stress by forming a higher potential of cellular antioxidant systems. Thus, the exact mechanism of PA actions for enhancing the defense response in plants still remains unclear despite extensive studies.

It is generally believed that the ability of plants to metabolize oxygen free radicals largely depends on their potential to sustain high superoxide dismutase (SOD) activities and a high redox state of ascorbate and glutathione (Foyer et al., 1994; Lidon and Teixeira, 2000). Operation of the ascorbate-glutathione cycle, which comprises ascorbate peroxidase (APX) and two ascorbate-regenerating enzymes, dehydroascorbate reductase (DHAR) and glutathione reductase (GR), allows a plant to maintain a suitable level of the reduced forms of ascorbate and glutathione in cells (Foyer and Halliwell, 1976; Nakano and Asada, 1981; Foyer et al., 1994). Moreover, this metabolic cycle may directly participate in H_2O_2 scavenging. In contrast, SOD catalyzes the removal of superoxide radicals, producing H_2O_2 as a dismutation product (Lidon and Teixeira, 2000; Martinez et al., 2001). Consequently, the coordination between SOD, which generates H_2O_2, and the ascorbate-glutathione cycle enzymes, which metabolize H_2O_2, can be crucial in preventing the damage induced by oxidative stress. Rao and Ormrod (1995a, b) have suggested that the SOD/ascorbate-glutathione cycle may be an important constituent of the plant antioxidant system by scavenging oxygen free radicals. In this enzymatic system, they have speculated that SOD catalyzes the conversion of superoxide radicals to H_2O_2, which is then metabolized to H_2O. Tepperman and Dunsmuir (1990) have reported that transformed tobacco plants with 50-fold higher than normal SOD activity do not exhibit increased resistance

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to PQ stress. Accumulating evidence relates the oxidative stress tolerance of plants to enhanced enzymes in the SOD/ascorbate-glutathione cycle (Rao et al., 1996). The herbicide paraquat (PQ), a methyl viologen, becomes toxic to leaves by catalyzing the transfer of electrons from PSI in the chloroplast membranes to molecular oxygen for the production of superoxide radicals. Thus, it has been used as a model to study oxidative stress (Bowler et al., 1994; Martinez et al., 2001).

The objectives of our research were to determine (i) whether the prior application of PAs (Put, Spd, and Spm) could protect radish seedlings from subsequent PQ-induced oxidative damage, and (ii) whether this protection might be related to the operation of the SOD/ascorbate-glutathione cycle. Using cotyledons that had been exposed to PQ, we investigated the influence of exogenous PAs on the activities of enzymes in the SOD/ascorbate-glutathione cycle. We also analyzed the mechanism of regulation for these enzyme activities and biochemical changes in their isozyme compositions, and evaluated the protective role of different PAs against PQ toxicity.

MATERIALS AND METHODS

Plant Materials and Treatments

Radish seeds (*Raphanus sativus* L. cv. Taewang) were sterilized in 1% (v/v) NaOCl for 15 min and washed completely with sterile water. They were then sown for germination in plastic containers (115 x 115 x 100 mm) that contained commercial soil (Heung Nong, Korea) moistened with distilled water. The resultant seedlings were reared for 5 d in a growth chamber at 25/21°C (day/night), with 70% RH and a 16-h photoperiod provided by 8000 lux light. For the pretreatments, different PAs (1 mM Put, 1 mM Spd, or 1 mM Spm) in a 0.1% (v/v) Tween 20 solution were applied as sprays to the cotyledons of 4-d-old seedlings (1.0 mL per plant). Control plants were sprayed only with 0.1% (v/v) Tween 20 solution. After the PA pretreatment for 12 h, PQ stress was imposed by spraying the plants with 50 μM PQ in a 0.1% (v/v) Tween 20 solution (1.0 mL per plant). Following their exposure to PQ in the light for 0, 6, 12, or 24 h, the cotyledons were harvested for assay.

Physiological Parameters

To evaluate growth, fresh cotyledons were harvested at various intervals after PQ exposure and immediately weighed to determine their fresh mass. Chlorophyll was extracted in 80% acetone, then quantified based on $[Chl]=27.7A_{652}$ (Arnon, 1949). Protein content was determined by the method of Lowry et al. (1951), with BSA as a standard.

Determination of H_2O_2 Content

Ten pairs of radish cotyledons were homogenized in a mortar on ice with 2.5 mL of 0.1 M sodium phosphate buffer (pH 6.8). The homogenate was centrifuged at 20,000*g* for 20 min and the supernatant was collected for assay. H_2O_2 content was determined according to the method of Bernt and Bergmeyer (1974) using peroxidase. A 0.5-mL aliquot of the supernatant was mixed with 2.5 mL of peroxide reagent [0.12 M sodium phosphate (pH 7.0), 0.005% (w/v) *o*-dianisidine, and 40 μg peroxidase mL^{-1}], then incubated at 30°C for 10 min in a water bath. The reaction was stopped by adding 0.5 mL of 1 N perchloric acid, followed by centrifugation 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 Extractions and Assays

SOD was extracted from five pairs of cotyledon by grinding them in a mortar on ice with 5 mL of a cold extraction medium containing 70 mM potassium phosphate buffer (pH 8.0), 2 mM Na_2-EDTA, and 1% PVPP. The supernatant, recovered at 4°C by centrifugation (20,000*g*, 30 min), was used for the enzyme assay. APX was obtained using 4 mL of 0.1 M potassium phosphate buffer (pH 7.5) that contained 1 mM Na_2-EDTA, 5 mM ascorbate, and 1% PVPP. DHAR and GR were extracted using 4 mL of 0.1 M potassium phosphate buffer (pH 7.5) that contained 2 mM Na_2-EDTA and 1% PVPP. The assay for total 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 was composed of 50 mM potassium phosphate (pH 7.8), 13 mM methionine, 75 μM *p*-nitro blue tetrazolium (NBT), 4 μM riboflavin, 0.1 mM Na_2-EDTA, and 0 to 50 μL of enzyme extracts. Glass tubes containing the reaction mixtures were illuminated with two 20 W fluorescent tubes to start the reaction, which then continued for 15 min before being stopped by turning off the lights. One unit of SOD activity was defined as the amount of enzyme resulting in 50% inhibition of the rate of NBT reduction at 560 nm. Total APX activity was measured according to the procedure described by Asada (1984), in which the rate of ascorbate oxidation was monitored at 290 nm (ε_{290} = 2.8 mM^{-1} cm^{-1}). The 2 mL reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate, 2.5 mM H_2O_2, and the enzyme extracts. A correction was made for the low, nonenzymatic oxidation of ascorbate by H_2O_2. Total DHAR activity was determined by monitoring the formation of ascorbate at 265 nm (ε_{265} = 14 mM^{-1} cm^{-1}), according to the technique of Nakano and Asada (1981), but with a slight modification. The reaction mixture contained 50 mM potassium phosphate (pH 6.5), 0.5 M dehydroascorbate

(DHA), 5 mM GSH, 0.1 mM Na_2-EDTA, and the enzyme extract in a final volume of 2 mL. A correction was made for the nonenzymatic reduction of DHA by GSH. Total GR activity was determined by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH (ε_{340} = .6.2 $mM^{-1}cm^{-1}$). The 2 mL reaction mixture contained 80 mM potassium phosphate (pH 7.8), 2 mM Na_2-EDTA, 0.2 mM NADPH, 0.5 mM GSSG, and the enzyme extract (Schaedle and Bassham, 1977).

Native PAGE and Activity Staining

Equal amounts of protein from cotyledons exposed to different treatments were subjected to native PAGE using the discontinuous buffer system of Laemmli (1970), except that SDS was omitted. SOD isozymes were separated on the 10% separating gel with a 4% stacking gel at 100 V for 6 h at 4°C. The loading samples contained 125 mM Tris-HCl (pH 6.8), 5% glycerol, 0.002% BPB, and the SOD extract (80 μg protein). After electrophoresis, the gels were stained for SOD activity by incubating them for 25 min in a solution containing 2.5 mM NBT, followed by incubation for 20 min in the dark in 50 mM potassium phosphate buffer (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 temperature (Rao et al., 1996). Identification of the SOD isozymes was performed by pre-incubating the gels with either 6 mM KCN or 5 mM H_2O_2 for 30 min prior to activity staining. APX isozymes were separated on the 10% separating gel with a 4% stacking gel at 10 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 (120 μg protein). The gels were pre-run for 30 min to allow the 2 mM ascorbate, present in the tank buffer, to enter the gel prior to applying the samples. After electrophoretic separation, the gels were negatively stained for APX activity as described by Mittler and Zilinskas (1993). The gels were equilibrated for 30 min with a 50 mM potassium phosphate buffer (pH 7.0) supplemented with 2 mM ascorbate. They 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 reaction was continued for 10 min and stopped by a brief wash in distilled water. GR isozymes were separated on a 7% separating gel at 100 V for 7 h at 4°C. The loading samples contained 125 mM Tris-HCl (pH 6.8), 5% glycerol, 0.002% BPB, and the GR extract (100 μg protein). GR activity was detected by incubating the gels under darkness for 1 h in a solution of 0.25 M Tris-HCl (pH 7.8) containing 0.24 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.4 mM NADPH, 0.34 mM 2,6-dichlorophenolindophenol, and 3.6 mM GSSG. Duplicate gels were assayed for GSSG-specific GR activity, one with and one without GSSG (Anderson et al., 1995).

RESULTS

Effect of PA Pretreatment on PQ Injury Symptoms in Radish Cotyledons

We examined whether exogenous PA pretreatment could protect radish seedlings from subsequent PQ-induced oxidative stress. The first visible consequence of paraquat action was cotyledon wilting on the treated plants (Fig. 1). As an indicator of injury, therefore, we monitored the change in cotyledon fresh weights. Our data showed a dramatic weight reduction (Fig. 2A) that paralleled this pronounced wilting following 24 h of exposure to 50 μM PQ alone. These results obviously demonstrated the damage induced by PQ stress.

In addition, damage was manifested by a remarkable decline in chlorophyll and protein contents (Fig. 2B, C). However, these toxic symptoms could be alleviated by PA treatment. Different polyamines led to varying levels of protection, with spermidine being the most expedient (Fig. 1, 2). For plants exposed to 50 μM PQ, 1 mM Spd pretreatment was very effective in preventing losses of chlorophyll, proteins, and fresh weight, whereas 1 mM Put and 1 mM Spm had less significant influences.

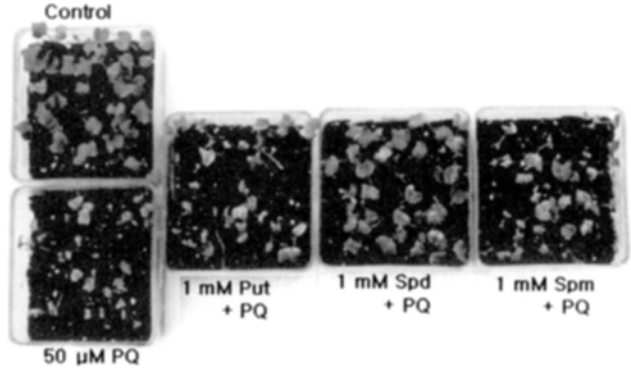

Figure 1. Protection of radish from PQ toxicity by exogenous application of PAs. Four-d-old plants were pretreated for 12 h with 1 mM putrescine, spermidine, or spermine in a 0.1% Tween 20 solution as cotyledon sprays before being exposed for 1 d to 50 μM PQ in a 0.1% Tween 20 solution. Control, plants treated with 0.1% Tween 20 alone; 50 μM PQ, plants treated with 50 μM PQ alone; 1 mM Put+PQ, plants pre-treated with 1 mM putrescine before 50 μM PQ application; 1 mM Spd+PQ, plants pre-treated with 1 mM spermidine before 50 μM PQ application; 1 mM Spm+PQ, plants pre-treated with 1 mM spermine before 50 μM PQ application.

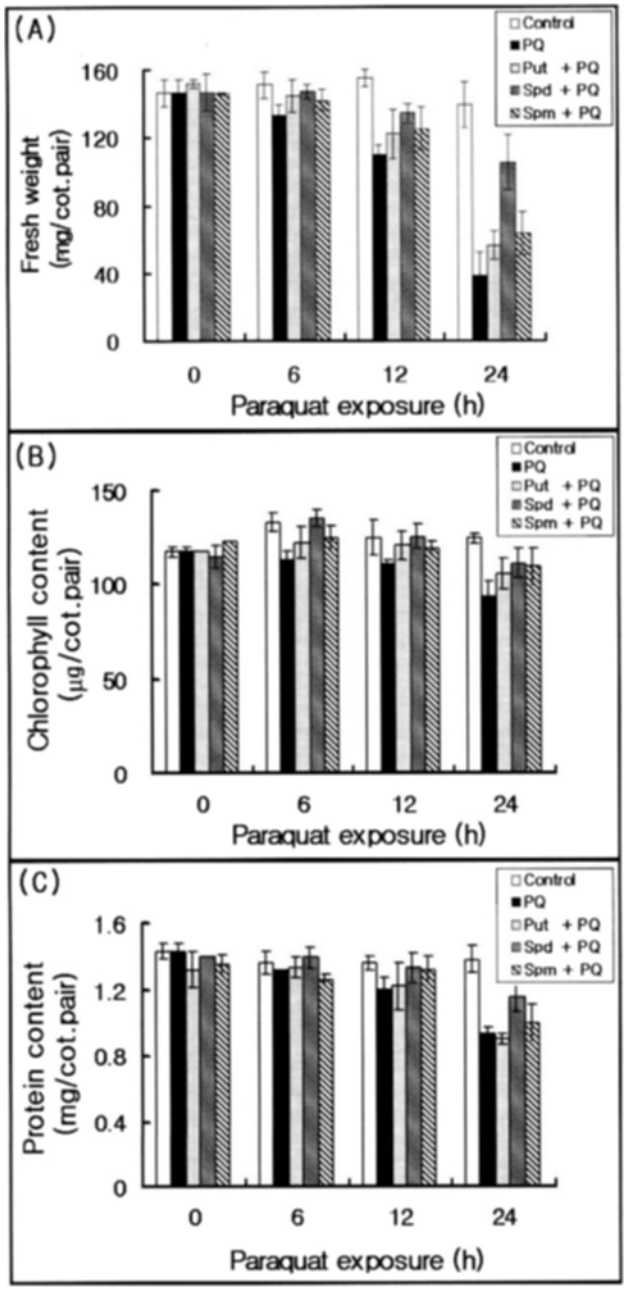

Figure 2. Effects of PA pretreatments on PQ-induced cotyledon-injury symptoms. (**A**) fresh weight, (**B**) chlorophyll content, (**C**) protein content. Four-d-old radish seedlings were pretreated with different PAs as described for Figure 1. Data are means ± SE from three independent experiments. Symbols: Control, plants treated with 0.1% Tween 20 alone; PQ, plants treated with 50 μM PQ alone; Put+PQ, plants pre-treated with 1 mM putrescine before exposure to 50 μM PQ; Spd+PQ, plants pre-treated with 1 mM spermidine before exposure to 50 μM PQ; Spm+PQ, plants pre-treated with 1 mM spermine before exposure to 50 μM PQ.

Effect of PA Pretreatment on Hydrogen Peroxide Content in Cotyledons Exposed to PQ

H_2O_2 content reached about 260% of the original value after 24 h of exposure to 50 μM PQ alone (Fig. 3). In contrast, the non-treated, control cotyledons showed no significant accumulation of H_2O_2 over the entire experimental period. To evaluate the effects of different PA pretreatments, data for H_2O_2 content in the variants (i.e., Put+PQ, Spd+PQ, Spm+PQ) were compared with data for PQ alone.

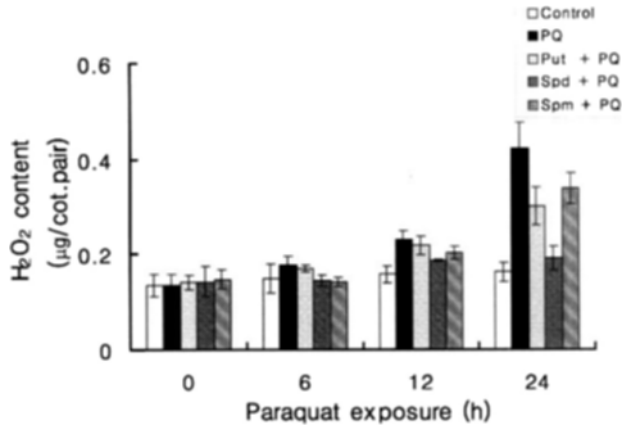

Figure 3. Effects of PA pretreatments on H_2O_2 content in radish cotyledons exposed to 50 μM PQ, as described for Figure 1. Data are means ± SE from three independent experiments. Symbols are the same as those for Figure 2.

Pretreatment with PAs clearly interfered with H_2O_2 accumulation, particularly at the end of the PQ exposure period. Spd was more effective than either Put or Spm. H_2O_2 content in cotyledons pretreated with spermidine was very similar to that of the control.

Influence of PA and PQ on the Activities of Enzymes Related to the SOD/Ascorbate-Glutathione Cycle

SOD, APX, DHAR, and GR enzymes were selected to evaluate the possible involvement of the SOD/ascorbate-glutathione cycle in protecting cotyledons against PQ stress. In the time course of total SOD development (Fig. 4A), enzyme activity remained nearly constant in the control cotyledons over the 24-h experimental period, whereas, within the first 6 h of exposure, 50 μM PQ reduced SOD activity by 73% compared with the control value. However, pretreatment with 1 mM of any PA significantly prevented PQ-induced reductions in activity. Among these, Spd was more effective than Put or Spm; the latter two having similar, but lesser, effects.

In response to PAs and PQ, APX activity was reduced to 40% of the control value after 6 h of exposure to 50 μM PQ alone (Fig. 4B). However, enzyme activity in the controls remained almost unchanged over the 24-h time course. In addition, 1 mM Spd pretreatment was very effective in protecting APX activity against PQ stress. By 24 h after paraquat exposure, activity had been restored up to 89% with Spd compared with the control. In contrast, Put and Spm did not contribute effectively to the prevention of APX inactivation caused by PQ.

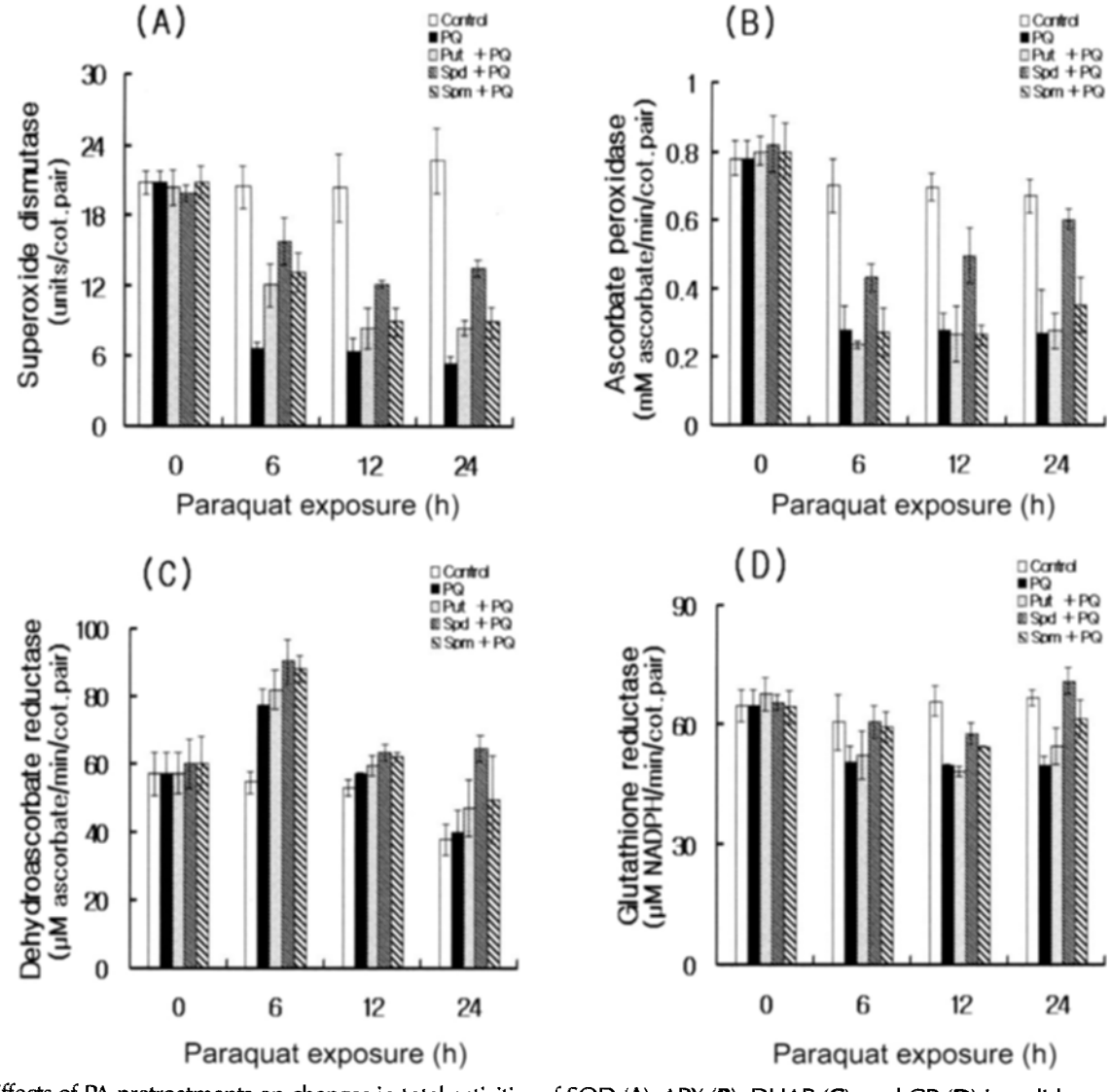

Figure 4. Effects of PA pretreatments on changes in total activities of SOD (**A**), APX (**B**), DHAR (**C**), and GR (**D**) in radish cotyledons exposed to 50 μM PQ, as described for Figure 1. Data are means ± SE from three independent experiments. Symbols are the same as those for Figure 2.

Unlike for SOD and APX, DHAR activity differed in its response to PQ, increasing unexpectedly, to 140% of the control value, at 6 h after exposure. It then rapidly decreased with prolonged exposure. However, PA pretreatment significantly prevented PQ-induced reductions in DHAR activity at the end of the period, with Spd being the most effective in sustaining this enzyme activity (Fig. 4C).

In the case of GR (Fig. 4D), activity in the control remained nearly constant over the entire experimental period, whereas 50 μM PQ reduced GR activity considerably (up to 25% of the control). However, this activity was completely restored by Spd pretreatment at 24 h after PQ exposure. At the same time, levels of GR were restored to 92% with Spm and 82% with Put, compared with the control value (Fig. 4D).

Changes in the Isozyme Compositions of SOD, APX, and GR in Response to PA and PQ

To investigate whether the significantly restored activities of SOD, APX, and GR by PA pretreatment during PQ stress were due to changes in their isozyme composition, we subjected cotyledon protein extracts to native PAGE and stained for their activities. The effects of PAs and PQ on the activities of SOD isozymes are shown in Figure 5.

Five different isozymes were found in the control cotyledons, some being more prominent than others. Based on their sensitivity to specific SOD inhibitors (KCN and H_2O_2), they were identified as two MnSODs (KCN- and H_2O_2-resistant), one FeSOD (H_2O_2-sensitive), and two Cu/ZnSODs (KCN- and H_2O_2-sensitive).

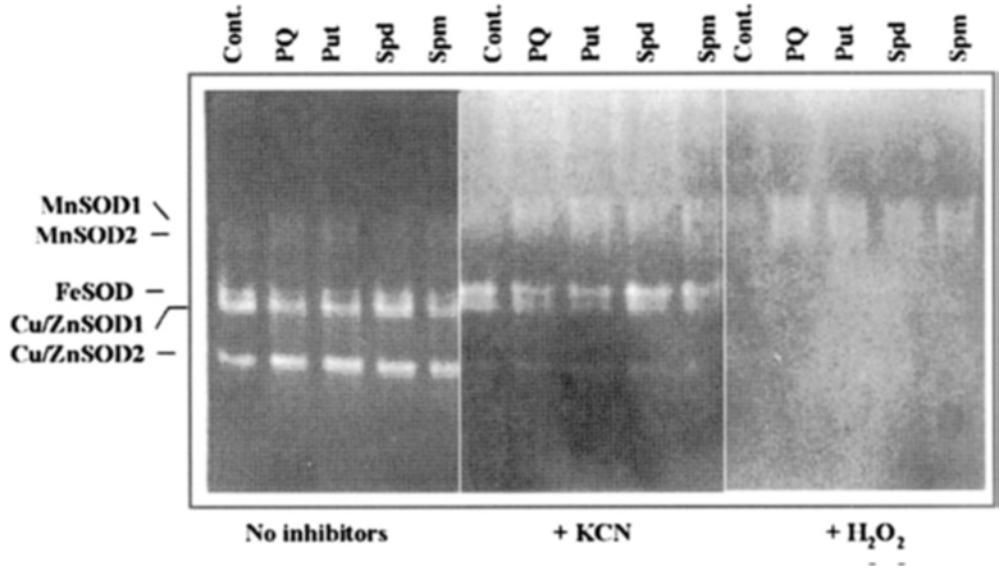

Figure 5. Native PAGE gels stained for SOD isozymes in radish cotyledons. Lane Cont., cotyledons treated with 0.1% Tween 20 alone as control; Lane PQ, cotyledons treated with 50 μM PQ alone for 1 d; Lane Put, cotyledons pre-treated for 12 h with 1 mM putrescine before being exposed for 1 d to 50 μM PQ; Lane Spd, cotyledons pre-treated for 12 h with 1 mM spermidine before being exposed for 1 d to 50 μM PQ; Lane Spm, cotyledons pre-treated for 12 h with 1 mM spermine before being exposed for 1 d to 50 μM PQ. Identification of SOD isozymes was performed by pre-incubating gels with 6 mM KCN or 5 mM H_2O_2 for 30 min prior to activity staining.

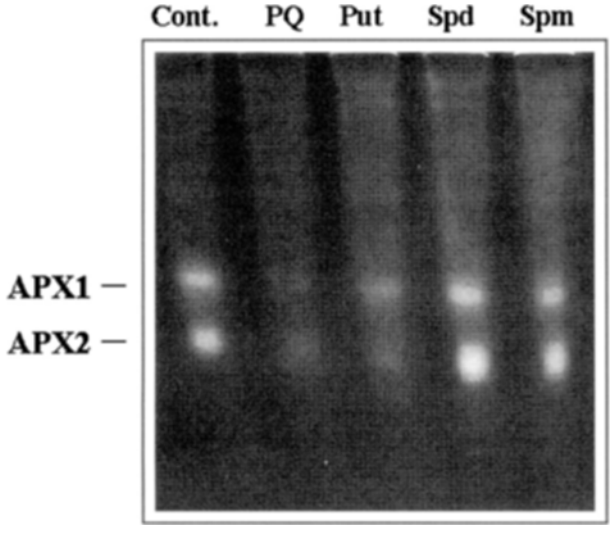

Figure 6. Native PAGE gels stained for APX isozymes in radish cotyledons. Lane Cont., cotyledons treated with 0.1% Tween 20 alone as control; Lane PQ, cotyledons treated with 50 μM PQ alone for 1 d; Lane Put, cotyledons pre-treated for 12 h with 1 mM putrescine before being exposed for 1 d to 50 μM PQ; Lane Spd, cotyledons pre-treated for 12 h with 1 mM spermidine before being exposed for 1 d to 50 μM PQ; Lane Spm, cotyledons pre-treated for 12 h with 1 mM spermine before being exposed for 1 d to 50 μM PQ.

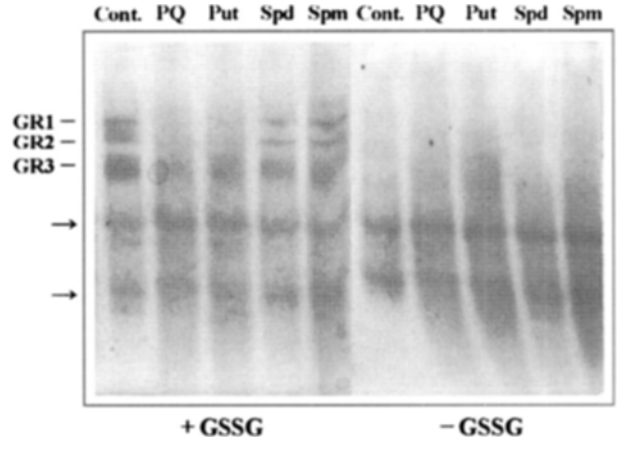

Figure 7. Native PAGE gels stained for GR isozymes in radish cotyledons. Lane Cont., cotyledons treated with 0.1% Tween 20 alone as control; Lane PQ, cotyledons treated with 50 μM PQ alone for 1 d; Lane Put, cotyledons pre-treated for 12 h with 1 mM putrescine before being exposed for 1 d to 50 μM PQ; Lane Spd, cotyledons pre-treated for 12 h with 1 mM spermidine before being exposed for 1 d to 50 μM PQ; Lane Spm, cotyledons pre-treated for 12 h with 1 mM spermine before being exposed for 1 d to 50 μM PQ. Duplicate gels were assayed for GSSG-specific GR activity, one with and one without GSSG. Arrows indicate two GSSG non-specific GR isozyme bands.

Exposing the cotyledons to PQ alone for 24 h caused a remarkable reduction in the staining intensities of FeSOD and Cu/ZnSOD1, whereas the intensities of the other three remained almost unchanged. However, FeSOD and Cu/ZnSOD1 intensities were significantly recovered by Spd pretreatment; Put and Spm were less effective. Gels stained for APX activity revealed two isozymes (APX1 and APX2, in order of their increasing mobility) in the control cotyledons (Fig. 6). According to the isozyme patterns in our results, the staining intensities of both APX1 and APX2 were hardly visible in cotyledons exposed to PQ alone for 24 h, but they reappeared strongly in the cotyledons pretreated with Spd or Spm.

Table 1. Activities of APX and SOD in radish cotyledons, which were pretreated with 1 mM Spd for 12 h and subsequently exposed to 50 μM PQ for 24 h with the addition of either 50 μM CHI or Act. D. "PQ alone" means that cotyledons were exposed to only 50 μM PQ.

Treatment	Ascorbate peroxidase	Superoxide dismutase
	(μM) ascorbate/min/ cot. pair	units/cot. pair
Control	0.682 ± 0.022	23.79 ± 6.08
PQ alone	0.354 ± 0.069	6.71 ± 1.43
Spd+PQ	0.784 ± 0.056	19.11 ± 5.27
Spd+PQ+CHI	0.501 ± 0.072	8.23 ± 1.73
Spd+PQ+Act.D	0.734 ± 0.020	22.60 ± 1.11

Each value is mean ± SE of three independent experiments.

In addition, PA pretreatments differed in the recoverability of intensities, with Put being less effective, and Spm and, especially, Spd, showing pronounced influence.

Three GSSG-specific GR isozymes (GR1, GR2, and GR3, in order of their increasing mobility) were present in the cotyledons with two GSSG-nonspecific isozymes (Fig. 7). According to the isozyme patterns observed for each treatment, the staining intensities of all three isozymes decreased notably in cotyledons exposed to PQ alone for 24 h, whereas they were significantly recovered in PA-pretreated cotyledons. Spd was most efficient in contributing to the recovery of intensities.

Regulation of APX and SOD Activities by Spd

Two inhibitors of protein synthesis (cycloheximide and actinomycin D) were used to analyze the mechanism of Spd action implicated in enzyme regulation. Both APX and SOD activities were decreased remarkably by paraquat alone, but were strongly restored by Spd pretreatment in PQ-treated cotyledons (Table 1).

When Spd was pre-applied in combination with 50 μM cycloheximide (CHI), its enhancement for APX and SOD activities was conspicuously depressed in cotyledons subsequently treated with PQ. However, 50 μM actinomycin D (Act. D) had no significant effect on the action of Spd.

DISCUSSION

Our preliminary research showed that exposing radish seedlings to 50 μM PQ for 24 h caused serious, visible injuries to the cotyledons. Hence, we terminated our experiments at that point to evaluate the plant responses under physiologically relevant conditions. Our observations included pronounced wilting and a dramatic decline in cotyledon fresh weights (Fig. 1, 2A); considerably more reduced contents of chlorophyll and soluble proteins (Fig. 2B, C); and a large accumulation of H_2O_2 (Fig. 3). These results obviously demonstrate the damage incurred by paraquat stress. Likewise, we determined that these toxic symptoms could be significantly alleviated by pretreatment with PAs, although their degree of protection varied, with Spd being more effective than either Spm or Put (Fig. 1, 2A-C, 3). Similar data have been obtained by Zheleva et al. (1994), who demonstrated in pea plants that exogenous Spm and Spd (but not Put) can significantly reverse atrazine herbicide-induced inhibition of growth by preventing the loss of chlorophyll and plant mass. This protective role against PQ stress has also been observed in the germination and growth of *Arabidopsis* plants (Kurepa et al., 1998).

Nevertheless, the exact mechanisms by which spermidine and other polyamines confer oxidative-stress resistance still remain unclear. Much attention has been focused on the following possibilities: 1) PAs could directly or indirectly function as free radical scavengers (Drolet et al., 1986; Bors et al., 1989; Velikova et al., 2000); and 2) PAs might interact with membranes due to their polycationic nature, thereby demonstrating membrane stabilization under stress conditions (Besford et al., 1993; Borrell et al., 1997). It is generally believed that the ability of plants to counteract various oxidative stresses may be related to the scavenging of stress-induced toxic oxygen radicals, e.g., superoxide radicals and H_2O_2. PAs can act as antioxidants by inhibiting lipid peroxidation in several plant species exposed to paraquat or acid rain (Ye et al., 1997; Velikova et al., 2000; Ahn and Jin, 2004). Therefore, we examined whether the antioxidant properties of polyamines are really associated with the protection of radish cotyledons against PQ oxidative stress. The rise in accumulation of H_2O_2 in PQ-treated cotyledons was remarkably inhibited by PA pretreatment, especially 1 mM Spd, and particularly at the end of PQ exposure (Fig. 3). Hydrogen peroxides may give rise to the formation of highly toxic singlet oxygen molecules and hydroxyl radicals, both of which induce lipid peroxidation. Therefore, our results clearly indicated that exogenously applied PAs could function as efficient antioxidants in PQ-stressed radish cotyledons. Our data, together with previous studies, suggest that polyamines might act as oxygen radical scavengers or else adjust the overall antioxidant defense systems in plant cells.

Until now, their precise radical scavenging properties have not been well established. Velikova et al. (2000) have hypothesized that PAs may enable the cell to counteract stress by forming a higher potential in the plant cellular antioxidant system. To eliminate oxygen free radicals, this system (Nakano and Asada, 1981; Foyer et al., 1994) comprises several functionally interrelated enzymes, including SOD, APX, DHAR and GR. SOD catalyzes the removal of superoxide radicals, producing H_2O_2 as a dismutation product, whereas the

others may act in conjunction to metabolize H_2O_2 to H_2O through the metabolic ascorbate-glutathione cycle (Foyer and Halliwell, 1976; Nakano and Asada, 1981; Lidon and Teixeira, 2000). Therefore, increasing the level of SOD alone may not render the plant more resistant to oxidative stress. Rather, greater H_2O_2 scavenging capacity might also be required to enable the removal of H_2O_2 produced by enhanced SOD activity. Thus, coordination between SOD and the ascorbate-glutathione enzymes is needed to prevent the damage induced by oxygen free radicals.

Rao and Ormrod (1995a, b) have suggested that the SOD/ascorbate-glutathione cycle plays a key role in the plant defense response against oxidative stress. This hypothesis is supported by observations that enhanced SOD activity is associated with greater activities of the antioxidant enzymes in that cycle (Foyer et al., 1994; Rao and Ormrod, 1995a, b). Hence, we assayed the impact of exogenous PAs on the expression of major antioxidant enzymes in radish cotyledons exposed to paraquat (Fig. 4). PA-induced cotyledon resistance to PQ toxicity here depended on concerted manipulations between SOD and those enzymes. Pretreatment with 1 mM PAs reversed the PQ-induced decline in SOD, APX, and GR activities. DHAR activity, which decreased sharply as PQ exposure was prolonged, was also highly maintained by polyamines. However, the degree of that protection varied, with Spd being more effective than Put or Spm. For example, the PQ-inactivation of APX and GR enzymes were completely restored only by Spd after 24 h of exposure (Fig. 4B, D). Rao and Dubey (1993) also have shown that enhanced components of the SOD/ascorbate-glutathione cycle can withstand SO_2 stress in an SO_2-tolerant soybean, while Ye and Gressel (1994) have attributed PQ resistance in *Conyza bonariensis* to enhanced levels of SOD, APX, and GR. Based on those reports and our current results, it seems convincing that PAs (especially Spd) were related to the protection of cotyledons against PQ through the coordination of antioxidant enzymes in the SOD/ascorbate-glutathione cycle.

Plants can differentially alter the isozyme composition of their antioxidant enzymes in response to oxidative stress (Bowler et al., 1994; Martinez et al., 2001). Here, we investigated whether this was true for enzyme activities in our radish cotyledons (Fig. 1-4). We also identified which isozymes contributed more to the recovery of total activities. Assays with specific inhibitors revealed three classes of SOD isozymes -- MnSOD, FeSOD, and Cu/ZnSOD. Based on the SOD isozyme patterns observed in Figure 5, we determined that each SOD isozyme was differentially expressed in response to paraquat, depending on whether the cotyledons had received polyamine treatments. The staining intensities of FeSOD and Cu/ZnSOD1 disappeared under PQ-alone exposure, but were significantly restored by Spd. In contrast, no considerable differences were found in the staining intensities of MnSOD1, MnSOD2, or Cu/ZnSOD2 among these treatments. Therefore, we assume that the reappearance of FeSOD and Cu/ZnSOD1 activities was related to the recovery of total SOD activity in PQ-treated cotyledons. Perl et al. (1993) have also demonstrated that the over-production of Cu/ZnSOD in potato chloroplasts protects them against PQ toxicity. In addition, Yu and Rengel (1999) have found that, under moderate drought stress, total SOD activity in lupine plants markedly increases due to the induction of Cu/ZnSOD and FeSOD activities. In particular, expression of the Cu/ZnSOD isozyme, which is broadly localized in the cytosol, chloroplast, and peroxisome (Martinez et al., 2001) elevates the level of APX activity (Sen Gupta et al., 1993).

APX isozymes play a central role in eliminating H_2O_2, and are distributed in distinct cell compartments, i.e., the chloroplast and the cytosol (Asada, 1992; Mittler and Zilinskas, 1993). Here, we searched for any PA-mediated differences among APX activities during PQ stress. Chloroplastic APX isozyme loses all activity within the first minute of aerobic extractions (Nakano and Asada, 1987), but we were able to identify two cytosolic APX isozymes (Fig. 6). The striking decline in total APX activity was due to a drop in both APX1 and APX2. Expression analysis also revealed that Spd pretreatment induced strong recovery in isozyme activities, while Put had very little effect. Our results imply that the remarkable reactivation of both isozymes by spermidine was responsible for maintaining the level of total APX activity in cotyledons exposed to paraquat (Fig. 4B).

Because GR is essential in sustaining the redox state of glutathione and ascorbate, it is also a major antioxidant enzyme that governs the ascorbate-glutathione cycle (Foyer and Halliwell, 1976; Lidon and Teixeira, 2000). Staining our native gels for GR activity with or without GSSG revealed three GSSG-specific (GR1, GR2, GR3) and two GSSG-nonspecific GR isozymes in the radish cotyledons (Fig. 7). The activities of both GR1 and GR2 were preferentially decreased in response to PQ. Pretreatment with Spd also strongly inhibited PQ-induced declines in their activities. These results suggest that this significant isozyme reactivation by Spd was responsible for the recovery of total GR activity during PQ stress.

The SOD and APX enzymes are the main constituents of the SOD/ascorbate-glutathione cycle. Both were strongly reactivated by Spd pretreatment (Fig. 4A, B). Thus, we used two inhibitors of protein synthesis -- CHI and Act. D -- to elucidate the mechanism of Spd action implicated in the regulation of APX and SOD activities (Table 1). CHI acts as a translation inhibitor while Act. D inhibits mRNA formation. When Spd plus 50 μM CHI was pre-applied, the induction of APX and SOD activities by Spd was depressed in cotyledons subsequently treated with PQ. However, Act. D had no significant effect on this Spd action. This suggested that the Spd-

induction of APX and SOD activities could be due to their *de novo* synthesis as controlled at the translational level. This proposal is further supported by the fact that PAs have been shown to regulate protein synthesis (Tabor and Tabor, 1985).

In conclusion, we have found that exogenous feeding with polyamines, especially spermidine, can offer significant levels of protection against paraquat toxicity in radish seedlings. Our results also suggest a role for PAs as antioxidant molecules, invoking the efficient SOD/ascorbate-glutathione cycle. However, more research is necessary to determine how particular polyamines confer different degrees of protection.

ACKNOWLEDGEMENT

This work was supported by Grant No. R05-2001-000-00337-0 from the Basic Research Program of the Korea Science and Engineering Foundation.

Received March 29, 2006; accepted May 4, 2006.

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