The Activities of Antioxidant Enzymes in Response to Oxidative Stresses and Hormones in Paraquat-tolerant *Rehmannia glutinosa* Plants

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All members of *R. glutinosa* show the unique characteristic of intrinsic tolerance to paraquat (PQ). Antioxidant enzymes have been proposed to be the primary mechanism of PQ resistance in several plant species. Therefore, the antioxidant enzyme systems of *R. glutinosa* were evaluated by comparatively analyzing cellular antioxidant enzyme levels, and their responses of oxidative stresses and hormones. The levels of ascorbate peroxidase (APX), glutathione reductase (GR), non-specific peroxidase (POX), and superoxide dismutase (SOD) were 7.3-, 4.9-, 2.7- and 1.6-fold higher in PQ-tolerant *R. glutinosa* than in PQ-susceptible soybeans. However, the activity of catalase (CAT) was about 12-fold higher in the soybeans. The activities of antioxidant enzymes reduced after PQ treatment in the two species, with the exception of POX and SOD in *R. glutinosa*, which increased by about 40%. Interestingly, the activities of APX, SOD and POX in *R. glutinosa*, relative to those in soybeans, were further increased by 49, 67 and 93% after PQ treatment. The considerably higher intrinsic levels, and increases in the relative activities of antioxidant enzymes in *R. glutinosa* under oxidative stress support the possible role of these enzymes in the PQ tolerance of *R. glutinosa*. However, the relatively lower levels of SOD versus PQ tolerance, and the mixed responses of antioxidant enzymes to stresses and hormones, suggest a possible alternative mechanism(s) for PQ tolerance in *R. glutinosa*.

Keywords: Abiotic stresses, Paraquat, *Rehmannia glutinosa*, Stress-related hormones

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Introduction

Rehmannia glutinosa (Gaertn.) Ligosch is grown in Asia as a herb, and its roots are used for traditional medicinal purposes. Little is known about the botanical and agronomical characteristics of the species. Recently, it was found that *R. glutinosa* plants exposed to the non-selective herbicide paraquat (PQ; 1,1-dimethyl-4,4-bipyridilium) showed very high levels of tolerance to the herbicide. The plants only suffered 40% phytotoxic injury, even at a rate of 6.0 kg a.i. PQ/ha (Kim and Chun, 1992; Chun *et al.*, 1997).

Six models have been proposed to explain plant resistance to PQ (Fuerst and Vaughn, 1990; Hart and DiTomaso, 1994). Interestingly, none of these models explain the PQ resistance of different plant species. Considerable direct evidence supports the role of antioxidant enzymes in this resistance, which is presumably due to the scavenging of reactive oxygen intermediates (ROIs) (Gressel, 1994; Aono *et al.*, 1995; Allen *et al.*, 1997; Van Breusegen *et al.*, 1999; Gressel, 2000). The sequestration of PQ in vacuoles or other organelles has been observed in PQ-resistant barley (Lasat *et al.*, 1997), and reduced translocation is involved in the PQ resistance of *Arctotheca calendula* (Soar *et al.*, 2003). However, no study to date has indicated that altered target sites, or the detoxification of PQ are primary resistance mechanisms.

The possible involvements of proposed models of PQ-resistance in *R. glutinosa* were examined in an earlier study (Chun *et al.*, 1997), and the results obtained suggested the possibility of PQ metabolism as a primary mechanism of tolerance (Chun *et al.*, 1997). Interestingly, the possible role of antioxidant systems as primary mechanism of tolerance was indicated by the PQ-nullifying activity of acteoside (or verbascoside) isolated from this species (Chun *et al.*, 2002). Acteoside is a potent antioxidant (Gao *et al.*, 1999), and ability to protect against lipid peroxidation has been

demonstrated in animal cells (Liu *et al.*, 2003).

However, not much is known about the antioxidant systems of *R. glutinosa* as the plant has received little interest until recently. Since the role of antioxidants in PQ-tolerance has been demonstrated in the species, we considered that a more detailed characterization of the antioxidant systems in this species would provide information on its PQ-tolerance. Moreover, relative to the considerable evidence on the role of antioxidant enzymes in PQ-resistance, little is known about the antioxidant enzymes of *R. glutinosa.* Furthermore, the antioxidant systems in chloroplasts and cytoplasm are known to play roles in PQ resistance (Donahue *et al.*, 1997; Vartak and Bhargava, 1999). Therefore, in this study we evaluated the cellular antioxidant enzyme systems of *R. glutinosa* by analyzing the responsivenesses of enzymes to oxidative stresses and hormone treatments. Since no PQ-susceptible *R. glutinosa* plants have been reported, comparative analyses were also conducted between the PQ-tolerant *R. glutinosa* and the PQ-susceptible soybeans.

Materials and methods

Chemicals and enzymes All chemicals and enzymes were purchased from Sigma (St. Louis, USA), unless otherwise indicated.

Plant materials *R. glutinosa* (cv. Namwon) and soybean (cv. Danyeopkong) plants were grown under normal growing conditions in a glasshouse at Chonbuk National University, Korea. *R. glutinosa* plants were raised from tubers harvested during the previous growing season. Leaves were collected from healthy *R. glutinosa* and soybean plants grown for 2 months in a glasshouse.

Stress and hormone treatments *R. glutinosa* leaves were collected by cutting the upper part of petioles with a razor blade. Fully expanded trifoliate leaves of the soybean were collected in the same way, and each leaflet was separated with a razor blade. The leaves were then treated with sterile solutions of 5.6 mM paraquat (PQ), 5 mM H_2O_2, 5 mM ethephon (ET) or 5 mM salicylic acid (SA), for 24 h, as previously described, (Chung *et al.*, 2003). Each treatment was performed in triplicate.

Enzyme activity assays Total cellular extracts of each antioxidant enzyme were prepared at 4°C, and used in activity assays. The activities of ascorbate peroxidase (APX; EC 1.11.1.11), non-specific peroxidase (POX; EC1.11.1.7), catalase (CAT; EC 1.11.1.6), glutathione reductase (GR; EC 1.6.4.2), and superoxide dismutase (SOD; EC 1.15.1.1) were determined spectrophotometrically (Beers and Sizer, 1952; Chance and Maehly, 1955; Nakano and Asada, 1981; O'Kane *et al.*, 1996; Baek *et al.*, 2000; Lee, *et al.*, 2003; Yun and Lee, 2003). APX activity was determined after oxidizing ascorbate to dehydroascorbate, as described by Nakano and Asada (1981). The extraction buffer contained 50 mM phosphate, 0.5 mM ascorbate, 0.1 mM EDTA, and 1% (w/v) polyvinylpolypyrrolidone (PVPP), at pH 7.0, and the assay mixture 50 mM phosphate, 0.5 mM ascorbate, 0.1 mM EDTA and 0.1 mM H_2O_2, at pH 7.0. The extraction buffer for POX contained 100 mM

phosphate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% (w/v) PVPP, at pH 7.8, and the assay mixture 50 mM phosphate, 10 mM guaiacol, and 100 mM H_2O_2, at pH 6.4 (Chance and Maehly, 1955). The extraction buffer for the CAT assay contained 100 mM phosphate, 1 mM EDTA, 1 mM PMSF and 1% (w/v) PVPP, at pH 7.8, and the assay mixture 50 mM phosphate and 11.6 mM H_2O_2, at pH 6.9 (Beers and Sizer, 1952). The extraction buffer for GR assay contained 50 mM phosphate, 0.1 mM EDTA, 1 mM ascorbate and 1% (w/v) PVPP, at pH 7.0, and the assay mixture 100 mM Tris-HCl, 2 mM EDTA, 0.05 mM NADPH and 0.5 mM oxidized glutathione, at pH 7.8 (O'Kane *et al.*, 1996). The extraction buffer for SOD contained 100 mM phosphate, 10 mM sodium ascorbate, 5 mM diethylene triaminepentaacetic acid (DTPA), and 1% (w/v) PVPP, at pH 7.0, and the assay mixture 50 mM phosphate, 1.8 mM xanthine, 0.05 mM nitroblue tetrazolium, 1 mM DTPA, 1 unit/ml catalase, and 0.01 unit/ml xanthine oxidase, at pH 7.8 (Obeley and Spitz, 1984). CuZn-SOD activity was determined after extracting enzyme solutions with chloroform/ alcohol (v/v = 37.5 : 63.5) (Nobet *et al.*, 1993), and the protein contents of the enzyme extracts by the Bradford (1979) method. Assays were conducted in triplicate and enzyme activity data were analyzed using SAS (SAS Institute Inc., Cary, USA).

Results

Ascorbate peroxidase activity Specific APX activities showed characteristic responses to stresses and hormones in both plant species. In *R. glutinosa* this activity was reduced by PQ, SA, or ET treatment. No significant changes were observed after H_2O_2 treatment. Conversely, the activity of soybean was increased by adding ET or SA, but reduced by PQ treatment. These increased activity levels of soybean were less than 25% of those of *R. glutinosa*, and activity was decreased by about 50% by PQ treatment (Fig. 1). Comparisons between the two species indicated that the activity of *R. glutinosa* was more than 7-fold that of soybean, and its activity was about 11-fold higher than that of soybean after PQ treatment, but about 2.2- and 1.3-fold higher after ET or SA treatment.

Non-specific peroxidase activity The POX activities of the two species responded differently to all treatments. In *R. glutinosa,* POX activities were increased by H_2O_2 and PQ, but were decreased by ET or SA. The activities in soybean were increased by SA or ET, but were decreased by H_2O_2 or PQ treatment. This decrease in activity was greatest (27%) for PQ (Fig. 2). Comparisons between the two species indicated that the POX activity of *R. glutinosa* was 2.7-fold that of soybean. The activity of *R. glutinosa* was 5.3- or 3.3-fold higher than that of the soybeans after PQ or H_2O_2 treatment, respectively, but 0.1-fold higher after SA treatment.

Superoxide dismutase activity The responses of specific total SOD activities to stresses and hormones were quite different in the two plant species. The activity in *R. glutinosa* was increased by PQ, H_2O_2, ET, or SA. In soybean, SOD
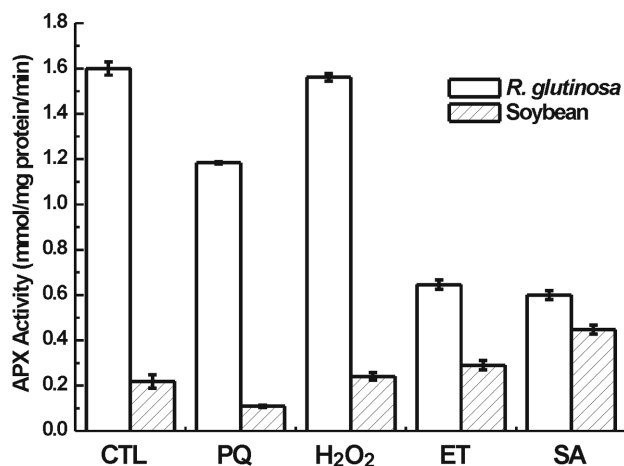
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Fig. 1. The activities of APX in response to oxidative stresses and hormones in *R. glutinosa* and soybeans. CTL, control; PQ, 5.6 mM paraquat; H_2O_2, 5 mM hydrogen peroxide; ET, 5 mM ethephon, and SA, 5 mM salicylic acid. In a pair-wise comparison between species for each treatment. Enzyme activities in *R. glutinosa* and soybean differed significantly for all treatments (p = 0.05). Treatments produced significantly different enzyme activities effects in both species, with the exception of H_2O_2.
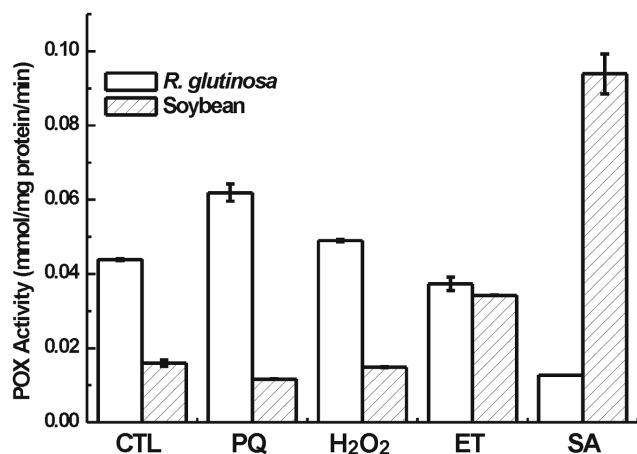

Fig. 2. The POX activities in response to oxidative stresses and hormones in *R. glutinosa* and soybeans. CTL, control; PQ, 5.6 mM paraquat; H_2O_2, 5 mM hydrogen peroxide; ET, 5 mM ethephon and SA, 5 mM salicylic acid. Enzyme activities in both species differed significantly for all treatments (p = 0.05), and each treatment induced significantly different enzyme activities, with the exception of H_2O_2 in soybean.

activity was reduced by PQ, but was increased by SA. Activity remained was unchanged by H_2O_2 or ET (Fig. 3). Total SOD activity in *R. glutinosa* was >1.6-fold greater than in soybean. The total activity of *R. glutinosa* was 2.7-fold higher than that of soybean after PQ, and 2.3-fold was higher after H_2O_2 or ET, and 1.2-fold higher after SA. CuZn-SOD activity increased in the same way as total SOD activity by the treatments in both species. CuZn-SOD activity accounted for 82 and 71% of the total SOD activity of *R. glutinosa* and soybean, respectively.
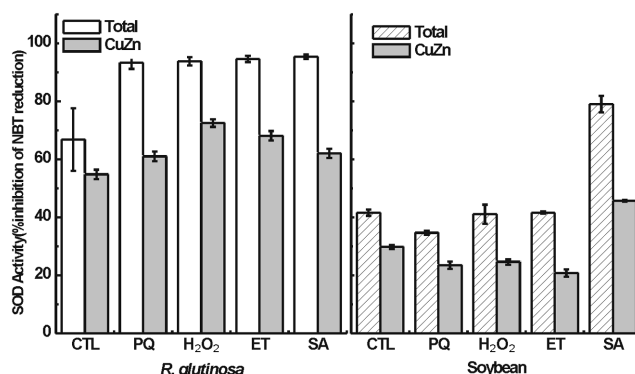

Fig. 3. Total SOD and CuZn-SOD activity in response to oxidative stresses and hormones in *R. glutinosa* and soybeans. CTL, control; PQ, 5.6 mM paraquat; H_2O_2, 5 mM hydrogen peroxide; ET, 5 mM ethephon and SA, 5 mM salicylic acid. Enzyme activities in *R. glutinosa* and soybean differed significantly for all treatments (p = 0.05), and within species, enzyme activities were affected significantly by each treatment, with the exception of H_2O_2 and ET in soybean.

The ratio of CuZn-SOD activity to total activity was decreased by 5 to 16% in *R. glutinosa* and by 3 to 21% in soybean by the treatments (Fig. 3), suggesting that other types of SOD isoforms, like Mn-SOD and Fe-SOD, have an increased role under stress conditions. However, the ratio of CuZn-SOD activity to total activity was higher in *R. glutinosa* than in soybean (Fig. 3). In *R. glutinosa,* the ratio was higher than 65% after all treatments, indicating its major contribution to total superoxide scavenging activity under conditions of stress.

Glutathione reductase activity GR activity responded to stress and hormone treatments in a similar manner in both species. In *R. glutinosa,* GR activity increased dramatically in response to ET or SA, and was reduced by H_2O_2 or PQ. In soybean, GR activity was increased by ET and SA. However, its activity was reduced by H_2O_2 and PQ (Fig. 4), and in *R. glutinosa*, this reduced activity was highest (23%) for PQ. Comparisons of activities between the two species indicated that activity in *R. glutinosa* was 4.9-fold higher than that in soybean. The activity of *R. glutinosa* was 31.8-fold higher than that of soybean treated with ET, but 3.5-fold higher after PQ treatment.

Catalase activity In contrast to the other enzymes, CAT activity was higher in soybean. The response of this enzyme was different for all treatments, except for PQ. In *R. glutinosa,* CAT activity was increased by SA or ET, and was decreased by H_2O_2 or PQ treatment. In soybean, the activity was unchanged by H_2O_2, but reduced by PQ, ET or SA (Fig. 5). Comparisons of activity between the two species indicated that CAT activity in soybean is about 12-fold higher than that of *R. glutinosa*. However, the activity of soybeans was 26-fold higher than that of *R. glutinosa* after PQ or H_2O_2 treatment, but 6.9- or 0.9-fold higher after ET or SA treatment.
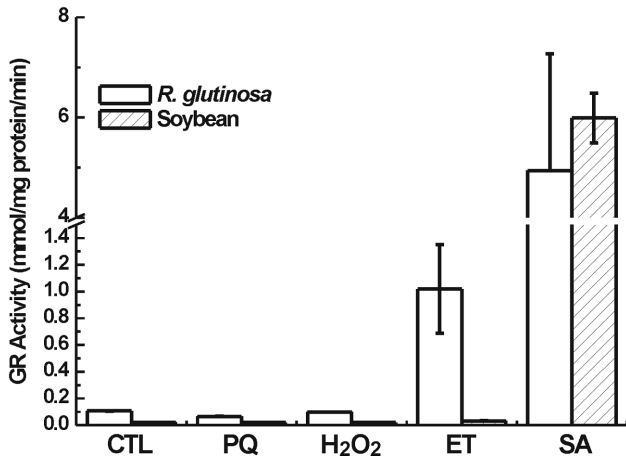
Fig. 4. The GR activities in response to oxidative stresses and hormones in *R. glutinosa* and soybeans. CTL, control; PQ, 5.6 mM paraquat; H₂O₂, 5 mM hydrogen peroxide; ET, 5 mM ethephon and SA, and 5 mM salicylic acid. Enzyme activities differed between species for all treatments, except for SA (p = 0.05). And, within species enzyme activities were changed significantly by ET and SA in *R. glutinosa* and by SA in soybean versus the control.

Fig. 5. CAT activities in response to oxidative stresses and hormones in *R. glutinosa* and soybean. CTL, control; PQ, 5.6 mM paraquat; H₂O₂, 5 mM hydrogen peroxide; ET, 5 mM ethephon and SA, 5 mM salicylic acid. Enzyme activities changes in the two species were significantly different for all treatments, with the exception of SA (p = 0.05). And within species enzyme activities were changed significantly by each treatment, with the exception of H₂O₂ in *R. glutinosa*, and by ET in soybean.

Discussion

It is well established that ROIs and antioxidant enzymes play important roles in the establishment of normoxia in biologic systems and in the resistance to oxidative stress (Mittler, 2002). The dual roles of ROIs, as toxic and signaling molecules, are ensured by the complex but elaborate systems controlling intracellular ROI levels (Mittler, 2002). The ROIs are detoxified by single, or a series of antioxidant enzyme reactions. The superoxide generated by PQ and by other causes, is dismutated to H₂O₂ and oxygen by SOD, and this H₂O₂ is then removed by a family of peroxidases. H₂O₂ is converted to water by APX in concert with the oxidation of ascorbate, and also by GPX with the oxidation of glutathione, in the cytosol and chloroplasts (Asada and Takahashi, 1987). The glutathione cycle is continued by the reduction of oxidized glutathione by GR (Dixon *et al*., 1998). H₂O₂ is also converted to water and oxygen by the two-electron dismutation of CAT in peroxisomes (Willekens *et al*., 1997). Therefore, the capacity of an antioxidant enzyme system is determined by the concerted action of the enzymes involved in the successive steps of conversion and detoxification. The importance of the coordinated induction of a group of antioxidant enzymes in PQ resistance has been demonstrated in *C. bonariensis* (Gressel, 2000).

As expected, the levels of APX, GR, POX, and SOD were all higher, by 7.3-, 4.9-, 2.7- and 1.6-fold, respectively, in PQ-tolerant *R. glutinosa* than in the PQ-susceptible soybean (Figs 1, 2, 3, and 4). However, the activity of CAT was about 12-fold higher in soybean (Fig. 5). Higher levels of PQ resistance have been attributed to elevated levels of antioxidant enzymes
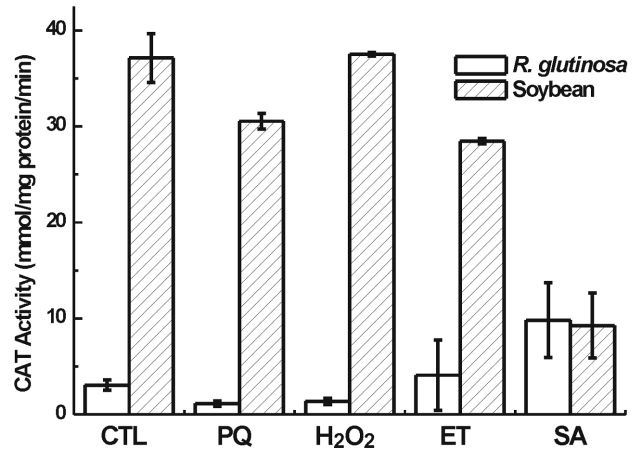
in many plant species. In *C. bonariensis,* the activities of APX, SOD, and GR were found to be about 30 to 50% higher in the total cell extracts of a PQ-resistant biotype than in a PQ-susceptible biotype (Gressel, 2000).

Activities in response to stresses and hormones were different in *R. glutinosa* and the soybean for all enzymes, with the exception of GR, and the activities of these enzymes were reduced by PQ in both plant species, except POX and SOD in *R. glutinosa,* whose activities increased by about 40%. Differential responses of antioxidant enzymes to PQ are often noticed within and among plant species. In *C. bonariensis*, PQ-induced increases in APX, SOD and GR were observed only in the PQ-resistant biotype (Gressel, 2000). Similarly, PQ treatments resulted in increased SOD in maize (Matters and Scandalios, 1986) and wheat seedlings (Okuda *et al*., 1992). In tobacco, however, the responses of SOD expression to PQ differed in the two species. The abundance of Fe-SOD mRNA was increased in response to PQ in *N. plumbaginifolia* (Tsang *et al*., 1991), but reduced in *N. tabacum* (Kurepa *et al*., 1997).

The activities of enzymes in response to H₂O₂ are also different in plant species. In *R. glutinosa*, H₂O₂ caused increases in POX and SOD, little change in APX, but reduced both GR and CAT activity. In soybean, the responses of POX, SOD, and CAT contrasted those in the *R. glutinosa*. It is known that metalloenzymes, such as CuZn-SOD and APX, are inactivated by H₂O₂ in tobacco and spinach (Miyagawa *et al*., 2000; Mano *et al*., 2001). Conversely, H₂O₂ treatment increased both APX and SOD activity in Arabidopsis (Rao *et al*., 1997).

The responses of antioxidant enzymes to ET show enzyme-specific differences, according to plant species. In *R.*

glutinosa, ET reduced both APX and POX activity, but increased SOD, GR, and CAT activity. As in *R. glutinosa*, ET treatment rapidly reduced the level of APX in spinach (Hodges and Forney, 2000), and as was found in soybean, ET increases APX activity in mung beans and peas (Melhorn, 1990), but reduces CAT activity in cut carnations (Sylvestre *et al*., 1989). However, unlike observations in *R. glutinosa* and soybean, ET treatment reduced both SOD and GR activity in cut carnations (Sylvestre *et al*., 1989), but caused no change in SOD activity in spinach (Hodges and Forney, 2000).

Differential responses of antioxidant enzymes to SA are not rare. Acute treatment with SA induced no major influence on foliar CAT activities in Arabidopsis (Summer-Matter *et al*., 1995), but prolonged SA treatment inactivated APX and CAT activities (Rao *et al*., 1997). In rice, however, response to SA was diverse among antioxidant enzymes: the activities of GR, SOD, and POX were increased, unchanged, and inhibited, respectively, by SA treatment (Ganesan and Thomas, 2001). In tobacco, SA treatment induced GR and SOD, but not CAT activity (Fodor *et al*., 1997).

The responses of antioxidant enzymes to stresses and hormones might be related to species characteristic antioxidant systems profiles and hormone levels. Dramatic contrasts were found between APX and CAT activities in *R. glutinosa* and soybean: APX activity was over 7-fold higher in *R. glutinos,* but CAT activity was 12-fold higher in soybean. This suggests that plant species use different enzymes as primary defenses against H_2O_2, and these may require different regulatory systems (Mittler, 2002). The responses of APX and CAT activities to SA and ET in the two species clearly contrasted. The differential responses of antioxidant enzymes to SA treatment in rice and tobacco were attributed to a two orders of magnitude difference in leaf SA concentrations (Ganesan and Thomas, 2001).

Since the herbicidal activity of PQ is related to superoxide production (Preston, 1994), it seems reasonable that plants showing tolerance to PQ should have mechanism(s) capable of reducing the harmful effects of this radical. And, elevated levels of SOD have beeen found to be correlated with higher resistance to PQ in several plant species (Norman *et al*., 1993; Danahue *et al*., 1997; Gressel, 2000). In addition, it has been reported that the tolerance of *R. glutinosa* to PQ is about 9-fold higher than in soybean (Chun *et al*., 1997). Relative to the levels of tolerance of *R. glutinosa* to PQ, the two- to three-fold higher SOD activities in *R. glutinosa* versus soybean, may mitigate against SOD as being a primary player in the removal of PQ-generated superoxide. Nevertheless, considerably higher levels of activity may support a supplementary role for SOD in the PQ tolerance of *R. glutinosa*. In spinach, APX is the primary target of methylvilogen-induced oxidative damage (Mano *et al*., 2001). Therefore, the 7- to 11-fold higher total APX activity exhibited by *R. glutinosa* versus soybean without and with paraquat treatment, has a significant implication with respect to the paraquat tolerance of the species. Since H_2O_2 freely diffuses through membranes (Willekens *et al*., 1997), cytosolic H_2O_2-scavanging enzymes can help protect chloroplasts from high concentrations of H_2O_2. Furthermore, increases in SOD and POX activities, with no inactivation of APX in *R. glutinosa* by H_2O_2 (Figs 1, 2, and 3), may ensure the efficient detoxification of H_2O_2, and thereby contribute to the PQ tolerance of the species. The possible contribution of antioxidant enzymes to PQ tolerance in *R. glutinosa* is further supported by significant increases in the relative activities of antioxidant enzymes in *R. glutinosa* versus soybean after PQ treatment (Figs 1, 2, 3, 4, and 5).

The significance of antioxidant enzymes in PQ resistance has also been demonstrated using transgenic plants. Increased expressions of SOD, APX, or GR, in the cytosol or chloroplast, provide plants with increased PQ resistance (Aono *et al*., 1995; Allen *et al*., 1997; Van Breusegem *et al*., 1999). Interestingly, however, the suppression of APX and CAT activates metabolic and defense genes, which coupled with the inhibition of photosynthetic activity compensate for the lack of APX and CAT, suggesting a plasticity and diversity of responses to different oxidative stresses (Rizhsky *et al*., 2002). Therefore, the possibility of alternative mechanisms of PQ tolerance cannot be ruled out. Together with enzymatic antioxidant defense systems, non-enzymatic systems also contribute to the scavenging of ROIs. Non-enzymatic systems include ascorbate, glutathione, alpha-tocopherol, and phenolic compounds, such as flavonoids, tannins and lignin precursors (Foyer *et al*., 1994; Rice-Evans *et al*., 1997). The PQ-nullifying activity of the phenolic compound acteoside, isolated from *R. glutinosa*, strongly suggests the involvement of non-enzymatic antioxidant systems in the PQ tolerance of *R. glutinosa* (Chun *et al*., 2002). Therefore, the mixed responses of antioxidant enzymes to stresses and hormones may reflect response plasticity to oxidative stress and provides an insight into the complexities of PQ tolerance in *R. glutinosa*.

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