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Cadmium-Induced Changes in Antioxidative Systems, Hydrogen Peroxide Content, and Lipid Peroxidation in *Arabidopsis thaliana*

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To study the relationship between cadmium (Cd)-induced phytotoxicity and oxidative stress, we grew Cd-sensitive wild-type (WT) and Cd-resistant type (RT) seedlings of *Arabidopsis thaliana* on MS media containing up to 500 μM $CdCl_2$. The resistant seedlings showed higher biomasses and lower hydrogen peroxide and lipid peroxidation levels, the latter expressed in terms of malondialdehyde (MDA) production. These results indicate that RT plants experience lower oxidative stress when exposed to Cd. Furthermore, compared with the WT, RT seedlings have significantly higher activities of superoxide dismutase (SOD) and enzymes related to hydrogen peroxide removal, e.g., guaiacol peroxidase (GPX), ascorbate peroxidase (APX), and glutathione reductase (GR). These differential responses suggest that such phytotoxicity could be induced by oxidative stress, and that lower accumulations of hydrogen peroxide confer Cd tolerance in seedlings.

Keywords: Arabidopsis, cadmium, hydrogen peroxide, oxidative stress

Cadmium (Cd) is one of the most toxic metals in the environment, and is easily taken up by roots and translocated to other plant organs (Baker et al., 1994). High accumulations of Cd generally cause growth inhibition and even death because of reductions in enzyme activity (Schutzendubel et al., 2001), photosynthesis (Clijsters and van Assche, 1985; Cho and Park, 1999), respiration (Kessler and Brand, 1995), transpiration (Barcelo and Poschenrieder, 1990), and nutrient uptake (Sanita di Toppi and Gabbrielli, 1999). Although it is unclear how plants respond to metal stress, researchers suggest that metal-induced phytotoxicity can be attributed, at least in part, to oxidative damage. Parallel to this metal-induced growth inhibition, both the activities of antioxidant enzymes and antioxidant levels (Richards et al., 1988; Dixit et al., 2001; Schutzendubel et al., 2001), as well as lipid peroxidation, can be altered in plants (Cho and Park, 2000; McCarthy et al., 2001; Ali et al., 2002).

Oxidative stress, arising from an imbalance in the generation and removal of reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide, singlet oxygen, and hydroxyl radicals (·OH), is a challenge faced by all aerobic organisms. ROS are highly reactive and, in the absence of protective mechanisms, can damage cell structure and functioning (Elstner, 1991). Susceptibility to this stress depends on the overall balance between factors that increase oxidant generation and those cellular components with antioxidant capabilities (Foyer et al., 1994). The synchronous action of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), ascorbate reductase, and glutathione reductase (GR) is part of an antioxidative system that protects various cell components from ROS (Asada, 1994). The glutathione-ascorbate cycle (Foyer et al., 1994) is essential in removing H_2O_2 and is localized in several subcellular compartments, including the chloroplasts (Mittova et al., 2000), mitochondria, peroxisomes (Jimenez et al., 1998), glyoxisomes (Bunkelmann and Trelease, 1996), and plasma membrane (Berczi and Moller, 1998). The activities of APX and GR are crucial to the operation of this cycle (Asada, 1999).

Plant cells can adapt to metal stress through chelation, compartmentation, or the exclusion of metal ions (Mehra and Tripathi, 2000). The availability of glutathione in the cell may be important because glutathione itself has a relatively high affinity for binding to Cd (Perrin and Watt, 1971). It can also be used to synthesize phytochelatins (PC), which bind to metals in the cytosol and sequester them in the vacuole (Grill et al., 1985). Although PC production is proportional to the degree of Cd incorporation in a cell (Vogeli-Lange and Wagner, 1996), the formation of Cd-PC complexes to reduce the cadmium-free concentration in the cytosol can lead to the depletion of glu-

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tathione content, causing a loss in the cellular antioxidative response (Delhaize et al., 1989; Ruegsegger and Brunold, 1990; Lozano-Rodriguez et al., 1997). Additionally, high PC synthesis leads to hypersensitivity to Cd stress (Lee et al., 2003). Furthermore, some plants or cell lines resistant to Cd do not actively synthesize PC (Delhaize et al., 1989; Ebbs et al., 2002; Kupper et al., 2004). Because glutathione has a role in the antioxidant system that includes the glutathione-ascorbate cycle (Jimenez et al., 1998), inefficient removal of H_2O_2 and its subsequent accumulation due to glutathione status can possibly induce phytotoxicity. It is still unknown whether the Cd ion directly induces ROS (including H_2O_2) because that ion is unable to catalyze Fenton-Haber-Weiss reactions, which generate ROS. Rather, ROS may accumulate indirectly through the Cd-induced inefficient operation of a ROS quenching system that includes the glutathione-ascorbate cycle.

In this work, we used Cd-sensitive and Cd-resistant *Arabidopsis* seedlings to investigate various oxidative symptoms, e.g., H_2O_2 accumulation, lipid peroxidation, and activities of antioxidant enzymes particularly involved in H_2O_2 removal. Our objective was to determine whether cadmium-induced phytotoxicity was caused by oxidative stress that resulted from the over-accumulation of H_2O_2.

MATERIALS AND METHODS

Plant Material

A. thaliana seeds (Col-0; Lehler Seeds, USA) were germinated and grown on MS media containing 0, 300, or 500 μM of $CdCl_2$. Growth chamber conditions included 22°C, 16-h photoperiod (250 μM $m^{-2}s^{-1}$), and 70 to 80% humidity. To obtain Cd-resistant seedlings, wild-type (WT) seeds were treated with 0.3% ethyl methanesulfonate (EMS) according to the method of Buche et al. (2000). These were then germinated and grown in pots containing 1:1 perlite:vermiculite, in a controlled environment chamber at 22°C with 16 h of light (250 μM $m^{-2}s^{-1}$) and 70 to 80% humidity. The resultant seeds were harvested and germinated on MS media containing 300 μM $CdCl_2$ for selection of Cd-resistant mutants. After three weeks, the resistant-type seedlings (RT) were removed and grown in pots until seeds were produced. By repeating those selection procedures, we could eventually obtain seeds of the fifth generation for further experiments. To determine their biomass, 21-d-old seedlings were collected, dried at 80°C for 2 d, and weighed. In addition, fresh, 14-d-old seedlings were weighed and used for measurements of lipid peroxidation, H_2O_2 levels, and enzyme activities.

Determination of Cadmium Concentration in Seedlings

Leaves of 21-d-old seedlings were washed twice in deionized water, and the roots of intact plants were washed with ice-cold 5 mM $CaCl_2$ solution for 10 min to displace extracellular Cd (Rauser, 1987). All plant materials were dried for 48 h at 70°C, weighed, and ground to a fine powder before wet-ashing in a 3:1 HNO_3:$HClO_4$ solution. Cd was detected directly by atomic absorption spectrophotometry (Varian 200AA equipped with SIPS; Australia) using an air-acetylene flame and a Cd hollow-cathode lamp.

Analyses of H_2O_2 and Lipid Peroxidation

Samples of fresh seedlings (500 mg) were ground with liquid nitrogen and suspended in 1.5 mL of 100 mM potassium phosphate buffer (pH 6.8). Afterward, the H_2O_2 contents in the tissues were measured according to the method of Gay and Gebicki (2000). The level of lipid peroxidation in the leaves and roots was defined by the malondialdehyde (MDA) content that resulted from the thiobarbituric acid (TBA) reaction, as described by Dhindsa et al. (1987). MDA concentration was calculated based on A_{532}-A_{600} (=155 $mM^{-1}cm^{-1}$).

Enzyme Assays

All leaf samples (0.2 to 0.5 g fresh weight), except those analyzed for APX activity, were ground with liquid nitrogen and homogenized with an extraction buffer containing 10 mM potassium phosphate buffer (pH 7.8), 0.5% Triton X-100, and 1.0% polyvinylpyrrolidone. To assess APX activity, leaves were homogenized and extracted according to the method of Lee and Lee (2000). After the homogenates were centrifuged at 12000*g* for 20 min at 4°C, the supernatants were immediately evaluated. Protein content was determined according to the method of Bradford (1976), with BSA as the standard. Total SOD activity was defined using one unit of SOD as the amount of xanthine-xanthine oxidase that inhibited the reduction of cytochrome c by 50% (McCord and Fridovich, 1969). CAT activity was assayed based on the decrease in absorbance at 240 nm due to degrada-

tion of H_2O_2 [=39.4 $mM^{-1}cm^{-1}$ (Chance and Maehly, 1955)]. Here, one unit of CAT represented the amount necessary to decompose 1 mmol of H_2O_2 per minute at 25°C. Activity of APX was determined as described by Rao et al. (1995), while guaiacol peroxidase (GPX) activity was assayed according to the modified method of Shah et al. (2001), using an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ for 1 min. Enzyme specific activity was defined as the mmol of H_2O_2 reduced per minute (mg/protein). Our GR assay utilized 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and was based on the method of Smith et al. (1988). All spectrophotometric analyses were conducted on a Bio-20 spectrophotometer (Perkin Elmer, USA).

RESULTS AND DISCUSSION

Growth and Cd accumulation was monitored for seedlings grown for 21 d on media containing up to 500 μM (Fig. 1 and 2). Compared with the control seedlings, the WT biomass decreased by 64% and 94% in the presence of 300 μM and 500 μM of Cd, respectively. In contrast, declines in biomass for RT seedlings were 37% and 84%, respectively, thereby demonstrating that they were more tolerant to Cd exposure.

The Cd concentration in all seedlings increased in proportion to the level of treatment applied. We also found significant differences between WT and RT seedlings (Fig. 2), with the latter showing higher accumulations after exposure to either 300 μM or 500 μM Cd. While survival rates decreased at higher Cd accumulations, the concentration of H_2O_2 in seedlings more than doubled during Cd exposure, and the peroxidation of lipids, expressed as MDA production,

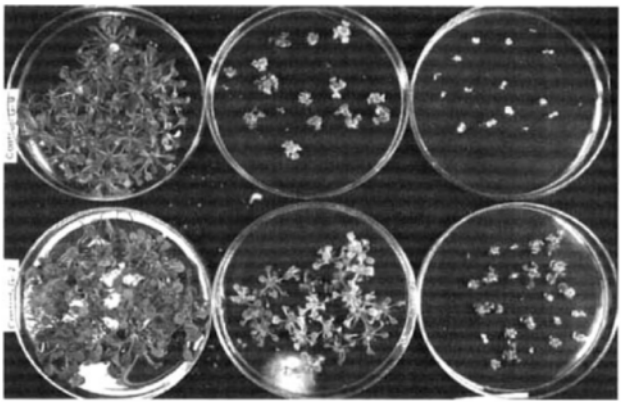

Figure 1. Growth of *Arabidopsis* seedlings exposed to Cd for 21 d.

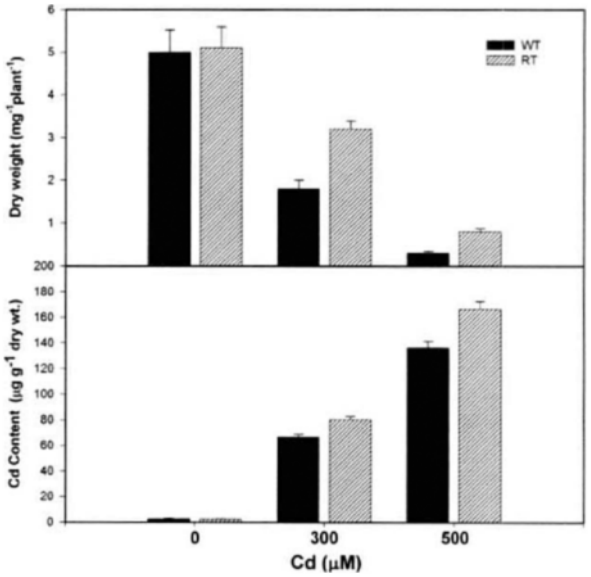

Figure 2. Dry weights and Cd accumulations in *Arabidopsis* seedlings exposed to Cd for 21 d. Values are means ± SE of four independent replicates.

was significantly enhanced (Fig. 3). Because increased MDA concentrations are common symptoms of metal stress, determination of this MDA response served as a non-specific index of Cd-phytotoxicity, which is more reliable than total cadmium content (Buege and Aust, 1978; Pandolfini et al., 1992; De Vos et al., 1993; Lozano-Rodriguez et al., 1997). However, in contrast to the WT values, our resistant seedlings

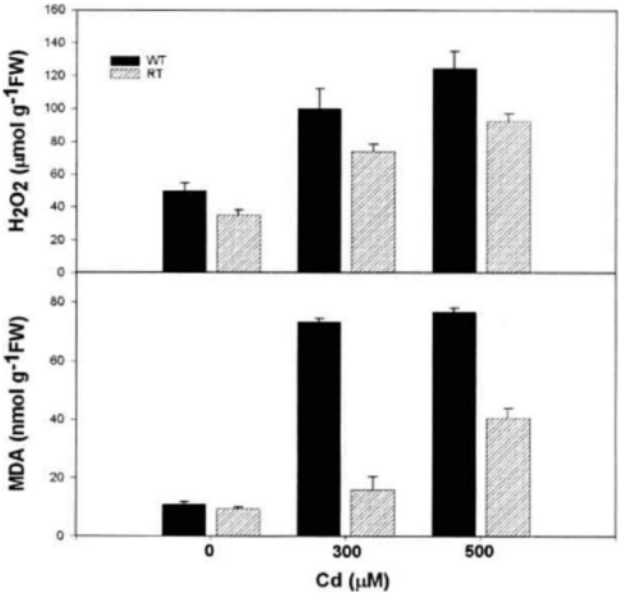

Figure 3. H_2O_2 and MDA contents in *Arabidopsis* seedlings exposed to Cd for 14 d. Values are means ± SE of four independent replicates.

manifested much lower H_2O_2 concentrations and levels of MDA production, indicative of lower oxidative stress. Elstner (1991) has reported that H_2O_2 accumulation can contribute to the suppression of seedling growth. Therefore, despite higher Cd accumulations in those RT seedlings, the decreased level of H_2O_2, together with reduced MDA formation, might explain the enhanced survival of those plants.

Although the mechanism for Cd-induced H_2O_2 formation is unknown, heavy metals are involved in many ways in the production of ROS, including H_2O_2 (Halliwell and Gutterridge, 1984). H_2O_2 accumulations caused by Cd exposure may occur in a similar manner to that found in stressed plants (Prasad et al., 1994). Likewise, it is conceivable that a decrease in enzymatic and non-enzymatic free radical scavengers, caused by heavy metals (De Vos et al., 1993), may contribute to a shift in the balance of free-radical metabolism towards H_2O_2 accumulation. Meanwhile, Cd enhances lipoxygenase activity (Somashekaraiah et al., 1992; Aravind and Prasad, 2003); the products of the lipoxygenase reaction, mainly peroxy, alkoxy, and hydroxyl radicals, are themselves reactive and result in further membrane lipid deterioration that leads to membrane permeability and subsequent growth inhibition (De Vos et al., 1991).

Because H_2O_2 scavenging is accomplished by various antioxidant enzymes and the glutathione-ascorbate cycle in a series of coupled redox reactions (Nakano and Asada, 1981), we measured the activities of those major enzymes in seedlings exposed to cadmium (Fig. 4 and 5). After 14 d of treatment, the activities of SOD, CAT, GPX, and GR were substantially decreased, while APX activity increased in WT seedlings. These changes paralleled the rise in lipid peroxidation and H_2O_2 content (Fig. 3). Although very similar decreases in SOD and CAT activity were observed in RT seedlings, the levels for both were still much lower than those measured in the WT plants (Fig. 4). Although CAT participates in an efficient defense mechanism against metal-induced oxidative stress (Weckx and Clijsters, 1997), we found no relationship between Cd tolerance and CAT activity in the RT seedlings, which had less activity than the WT seedlings. However, the accumulation of H_2O_2 may have prompted lower CAT activity and, subsequently, Cd-induced phytotoxicity. CAT, which is found predominantly in peroxisome dismutase, converts H_2O_2 into H_2O and O_2, whereas peroxidases decompose H_2O_2 by oxidizing co-substrates such as phenolic compounds and/or antioxidants (Sudhakar et al., 2001).

In plants, SOD is a metalloprotein that catalyzes the dismutation of superoxide to H_2O_2 and molecular oxygen in the cytosol, mitochondria, and chloroplasts (Fridovich, 1986; Salin, 1987). Although the dismutation of superoxide radicals produces H_2O_2 in the initial process of ROS removal, enhanced production of H_2O_2 after exposure to metal stress might not be entirely induced by the increased production of

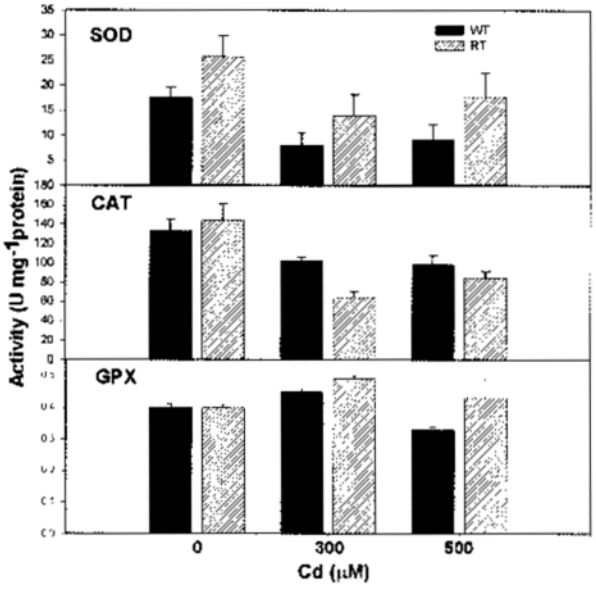

Figure 4. Activities of SOD, CAT, and GPX in *Arabidopsis* seedlings exposed to Cd for 14 d. Values are means ± SE of four independent replicates.

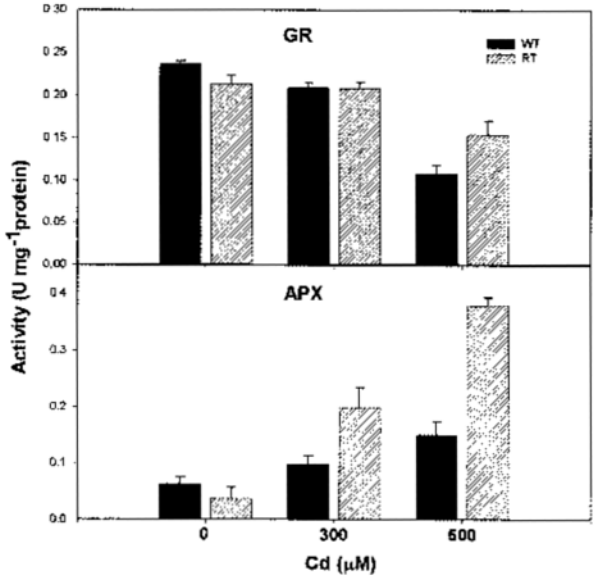

Figure 5. Activities of GR and APX in *Arabidopsis* seedlings exposed to Cd for 14 d. Values are means ± SE of four independent replicates.

superoxide radical (O_2^-) and SOD activity because O_2^- production is not high in other plants exposed to metals (Cakmak and Horst, 1991). In our study, SOD activity decreased after Cd treatment (Fig. 4).

In our WT seedlings, total GPX activity, using guaiacol as a substrate, decreased when plants were exposed to 500 μM, but not 300 μM Cd, thus indicating a lower sensitivity of GPX to cadmium than the other enzymes investigated. However, in the RT seedlings, GPX activity remained stable even at the 500-μM level. This lack of correlation between GPX activities and H_2O_2 contents in both WT and RT plants suggests that GPX might not be related to Cd-induced H_2O_2 accumulation. Nevertheless, the maintenance of higher GPX activity in treated RT seedlings may have contributed to the observed Cd tolerance.

We analyzed two major enzymes, GR and APX, that are involved in the ascorbate-glutathione pathway (Fig. 5). Although Cd exposure decreased GR activity in both the WT and RT seedlings, RT plants maintained much higher activity after treatment with 500 μM Cd. This higher level in the RT samples indicates that GR might also contribute to Cd tolerance. In the glutathione-ascorbate pathway to remove H_2O_2, GR is responsible for the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), a step that is required for the activation of dehydroascorbate peroxidase (Schaedle, 1977). Likewise, the subsequent accumulation of ascorbate enhances APX activity, resulting in the reduction of H_2O_2. The decreases in GR activity observed in treated seedlings were apparently due to active PC synthesis and subsequent GSH depletion (Schutzendubel and Polle, 2002).

Transgenic plants that overexpress GR show lower Cd stress, possibly because of greater glutathione synthesis, a subsequent increase in thiols for metal binding in roots, and reduced Cd translocation into shoots (Pilon-Smits et al., 2000). However, metal tolerance does not always result from increased production of PC (Harmens et al., 1993; De Knecht et al., 1994; Lee et al., 2003). Enhanced levels of GSH may contribute to improved Cd tolerance in plants by protecting their cells from metal-related oxidative stress damage (Weckx and Clijsters, 1997; Zhu et al., 1999; Schutzendubel and Polle, 2002). Therefore, the higher activity of GR observed here might have maintained steady levels of GSH, thereby contributing to Cd tolerance in the resistant seedlings. GSH is also a substrate for the glutathione peroxidases, which reduce H_2O_2 and organic peroxides, thus protecting cell proteins and membranes against oxidation (Navari-Izzo et al., 1997).

APX activity increased in proportion to the level of Cd treatment (Fig. 5), and was much higher in the RT plants. This increase in activity paralleled changes in H_2O_2 accumulation (Fig. 3), demonstrating that APX has a crucial role in Cd-induced oxidative stress and Cd tolerance. APX is located primarily in the chloroplasts and cytosol. As the key enzyme in the glutathione-ascorbate pathway, it eliminates peroxides by converting ascorbic acid to dehydroascorbate (Asada, 1992), making it one of the most important enzymes for eliminating toxic H_2O_2 from plant cells (Foyer et al., 1994). Therefore, the increase in APX activity might have been due to initially higher H_2O_2 production (Fig. 3), and the relatively higher activity could then have contributed to lower accumulations of H_2O_2 in the stress-resistant seedlings. Many researchers have previously reported that APX activity is enhanced following exposure to cadmium (Chaoui et al., 1997; Hegedus et al., 2001; Iannelli et al., 2002). Such a response might result from an imbalance between redox systems and increased H_2O_2 production. Therefore, when comparing the activities of H_2O_2-eliminating enzymes, we believe that APX plays a central role in H_2O_2 detoxification in cells.

Little information is available about the relationship between the activities of antioxidant enzymes and Cd tolerance in plants. Lee et al. (1976) showed that peroxidase activities increases in *Glycine max* treated with Cd, whereas Somashekaraiah et al. (1992) observed that Cd significantly reduces the activity of CAT, GR, and SOD in mung bean plants, thereby causing an increment in lipid peroxidation products. The accumulation of MDA in our experimental seedlings (Fig. 3) could also be explained on that basis -- Cd might be considered an oxidative stress-enhancing factor even though it is not a redox-active cation (Somashekaraiah et al., 1992). In addition, the lower enzymatic activities related to the removal of H_2O_2 and its later accumulation may have primarily contributed to the subsequent oxidative stress and phytotoxicity of Cd. Zhang and Kirkham (1996) have shown that high H_2O_2 concentrations caused by oxidative stress can precipitate the release of peroxidases from membrane structures with which they are normally associated

The increased H_2O_2 content and lipid peroxidation that we observed, and the altered activities of SOD, GPX, GR, and APX, imply that Cd application promoted oxidative stress. Greater lipid peroxidation was probably due to the harmful effect of over-production of H_2O_2 or its poisonous ROS derivatives in various

compartments (Bowler et al., 1992). Excessive levels of ROS can harm cell organelles, including the photosynthetic apparatus, ultimately leading to severe cellular damage and chlorosis of the leaves. H_2O_2 itself is a powerful inhibitor of metabolism, e.g., carbon fixation (Kaiser, 1976), and oxidation-reduction of metal ions by H_2O_2 and O_2^- through the Haber-Weiss reaction, the latter producing the most toxic hydroxyl radical, $^{\cdot}OH$ (Imlay and Linn, 1988).

We propose that the high H_2O_2 concentration resulting from Cd exposure may have been induced by alterations in the GSH-involved H_2O_2 removal systems, such as the glutathione-ascorbate cycle. When exposed to cadmium, plants synthesize PC for Cd detoxification, and GSH levels decrease with PC synthesis. The development of Cd tolerance via GR overexpression, subsequent increases in GSH and thiol levels (Pilon-Smits et al., 2000), and hypersensitivity to Cd stress by the over-expression of phytochelatin synthase (Lee et al., 2003) all support the theory that the depletion of GSH changes the glutathione-ascorbate pathway and H_2O_2 accumulation and, consequently, causes oxidative stress. A shift in the glutathione redox couple to a more oxidized state and GSH depletion due to PC synthesis also stimulate oxidative stress (De Vos et al., 1993). For example, the tolerance of *Silene cucubalus* seedlings to copper can be attributed to their ability to prevent GSH depletion, rather than because of the protective functioning of PCs (De Vos et al., 1992). Moreover, Indian mustard plants with a 1.5- to 2.5-fold increase in GSH show somewhat greater resistance to metal stress (Zhu et al., 1999). Therefore, our EMS-induced resistant seedlings may have possessed a related genetic mutation that alters PC synthesis. Under conditions of less PC synthesis, more GSH might then be available for a GSH-involved H_2O_2 removal system such as the glutathione-ascorbate cycle.

Taking into consideration the differences between our wild-type and resistant seedlings with regard to their levels of oxidative-stress tolerance, H_2O_2 concentrations, and antioxidant enzyme activities, we conclude that H_2O_2 accumulation is the major cause of Cd phytotoxicity in *Arabidopsis* seedlings. We also believe that such accumulations can result from inefficient quenching by the altered activities of particular antioxidant enzymes involved in the glutathione-ascorbate pathway. Cd-resistant plants are tolerant of cadmium stress because they are able to respond to greater ROS production through increased synthesis of antioxidant systems that partially counteract the accumulation of H_2O_2. However, further analysis is required of the redox status of glutathione and ascorbate and the developmental changes in PCs if researchers are to confirm the role of the glutathione-ascorbate pathway in Cd-induced phytotoxicity.

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