

Changes of Thiols and Oxidative Stress in Tomato Seedlings Exposed to Cadmium

Cho, Un-Haing* and Nam-Ho Seo

Department of Biology, Changwon National University, Changwon 641-773, Korea

ABSTRACT: Tomato (*Lycopersicon esculentum* Mill) seedlings exposed to various concentrations of CdCl₂ (0–100 μM) in a nutrient solution for up to 9 days were analyzed with respect to the thiol changes and oxidative stress. The Cd exposure increased total non-protein thiols (NPT) and cysteine in both leaves and roots, total glutathione in leaves, and the ratios of oxidized glutathione (GSSG)/reduced glutathione (GSH) in both leaves and roots, but decreased the ratio of dehydroascorbate (DASA)/ascorbate (ASA) in leaves. Our results suggest that the Cd-induced GSH depletion due to thiol synthesis and oxidation alters the antioxidant activity of seedlings for H₂O₂, and the subsequent H₂O₂ accumulation and oxidative stress result in phytotoxicity.

Key words: Cadmium, Oxidative stress, Thiols, Tomato

INTRODUCTION

High Cd accumulation generally causes growth inhibition and even death of plants due to the reduction of enzyme activity (Ouariti et al. 1997), photosynthesis (Siedlecka and Baszynski 1993), respiration (Kessler and Brand 1995), transpiration (Barcelo and Poschenrieder 1990) and nutrient uptake (Sanita di Toppi and Gabbrielli 1999).

An increasing body of evidence suggests that the metal-induced phytotoxicity can be attributed, at least in part, to oxidative damage (Dixit et al. 2001, Hegedus et al. 2001, Cho and Sohn 2005). Oxidative stress, arising from an imbalance in the regeneration and removal of reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), is a challenge faced by all aerobic organisms. In parallel to metal-induced growth inhibition, alterations in both the activities of antioxidant enzymes and the levels of antioxidants as well as enhanced lipid peroxidation have been observed in plants (Weckx and Clijsters 1997, Chaoui et al. 1997, Cho and Park 2000, Schützendubel et al. 2001, Cho 2004, Cho and Sohn 2005).

Adaptation of the plant cells to metal stress involves chelation, compartmentation or exclusion of metal ions (Mehra and Tripathi 2000). Varying responses to Cd-induced oxidative stress are probably related both to levels of Cd supplied and to concentration of thiols already present, or induced by Cd treatment (Sanita di Toppi and Gabbrielli 1999). Availability of the nonprotein thiols (NPT) including glutathione (γ-glutamylcysteinyl-gly) and cysteine (Cys) in cytosol may be important since Cd induces the synthesis of phytochelatins (PC, [γ-glutamylcysteinyl]_{n=2–11}-glycines), which

bind metals in the cytosol and sequesters them in the vacuole (Grill et al. 1985, Rauser 1995). PCs are the major thiols involved in the physiological mechanism of metal tolerance in various plants (Steffens et al. 1986, Jackson et al. 1987, Salt et al. 1989). The synthesis of the enzymes involved in glutathione formation such as γ-EC synthetase and glutathione synthetase are induced by exposure to Cd and Cu (Xiang et al. 2001), and might be regulated by the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio in the cells (Pilon-Smits et al. 2000, Xiang et al. 2001). However, since over-expression of PC synthase and subsequent high PC synthesis led to hypersensitivity to Cd stress in some situations (Lee et al. 2003), and some plants or cell lines resistant to Cd are not active in synthesis of PC (Jackson et al. 1987, Delhaize et al. 1989, Verkleij et al. 1990, Ernst et al. 2000, Ebbs et al. 2002, Schat et al. 2002, Kupper et al. 2004), the role of PC in Cd resistance is not clearly defined. Therefore, whereas PCs may chelate much of the metal in Cd-tolerant cells, it is necessary to recognize that other mechanisms of complexation and physiological amelioration may prevail in plants growing in Cd-polluted soils.

Although PC synthesis induced by Cd-exposure is associated with a rapid depletion of total glutathione (Delhaize et al. 1989, Scheller et al. 1987, Rauser 1987, Rueggsegger et al. 1990, Tuden-dorf and Rauser 1990), it is still unknown whether the Cd-induced changes in glutathione involve changes in GSH, GSSG, Cys, total NPT and ascorbate. In *Silene cucubalus*, copper caused oxidative stress by depletion of the glutathione due to PC synthesis and a shift in the glutathione redox couple to a more oxidized state (De Vos et al. 1991). However, the Cd ion is unable to catalyze Fenton-Haber-Weiss reactions, which generate ROS. How Cd-exposure

* Corresponding author; Phone: +82-55-279-7445, e-mail: uhcho@sarim.changwon.ac.kr

induces ROS production and oxidative stress is also an interesting subject to investigate.

In this work, the changes of NPTs and ascorbate in tomato seedlings exposed to Cd were investigated to determine if the Cd-induced oxidative stress could be induced by the Cd detoxification process itself, such as PC synthesis and alterations of ROS scavenging systems. PC synthesis and the altered redox status of GSH might have an influence on the GSH-ascorbate cycle and subsequent inefficient quenching of ROS, including H₂O₂, might result in oxidative stress leading to phytotoxicity. This information is also pre-required to understand the response of plants to Cd exposure and to develop Cd-resistant plants. The resistant plants might be used in the phytoremediation of Cd-contaminated environment and the re-establishment of ecosystem.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tomato seedlings (*Lycopersicon esculentum* Mill cv. Seokwang) were germinated in pots containing a perlite:vermiculite (1:1) mixture in a controlled environment chamber at 25°C with 12 h of light (250 $\mu\text{M m}^{-2}\text{s}^{-1}$) and 70~80 % humidity. Two week-old seedlings were transferred to aerated nutrient solutions containing half-strength Hoagland's solution (Hoagland and Arnon 1938) with 0, 10, 50 and 100 μM Cd (as CdCl₂). The solution was changed every 3 days. Plants collected from each treatment after 3, 6 and 9 days were dried for 48 h at 70°C and weighed for biomass, Cd and thiols determination. For measurements of H₂O₂ production, fresh samples were weighed and used.

Determination of Cd Concentration in Seedlings

Leaves were washed twice in deionized water, and the roots of intact plants were washed with ice-cold 5 mM CaCl₂ solution for 10 min to displace extracellular Cd (Rausser 1987). The plant material was dried for 48 h at 70°C, weighed and ground into fine powder before wet ashing in HNO₃ : HClO₄ (3 : 1) solution. Cd was determined directly by atomic absorption spectrophotometry (Varian 200AA equipped with SIPS, Australia) using an air-acetylene flame and Cd hollow-cathode lamp.

Analyses of Total NPTs, Cys, GSH, GSSG, Dehydroascorbic Acid (DASA) and Ascorbic Acid (ASA)

NPTs were extracted by grinding 20 mg lyophilized leaves or roots in 2 mL 5 % (w/v) sulfosalicylic acid + 6.3 mM diethylenetriaminepentaacetic acid (pH < 1) at 0°C and the level of total acid-soluble SH compounds were determined with Ellman's reagent (De Vos et al. 1992). For the determination of GSH and GSSG,

100 mg of samples were powdered in liquid nitrogen, mixed with 1 mL of 5 % (w/v) sulphosalicylic acid and centrifuged (10 min, 4°C, 12,000g), and the supernatant was analyzed according to the method of Fadzilla et al. (1997). The level of cysteine was measured according to the method of Gaitonde (1967).

For the extraction of ascorbate, 100 mg of samples were powdered in liquid nitrogen, mixed with 1 mL of 2 % (w/v) metaphosphoric acid containing 1 mM EDTA and 10 mg polyvinylpyrrolidone, and centrifuged (20 min, 4°C, 12,000g). The supernatant was used for determination of reduced and oxidized ascorbate according to the method of Law et al. (1983). Total ascorbate is determined through a reduction of DASA to ASA by dithiothreitol, and DASA concentrations were deduced from the total ascorbate.

Statistical Analysis

The results are the means \pm SE of five independent replicates. The analyses of variance were computed on statistically significant differences determined by the appropriate F-tests. The mean differences were compared utilizing Duncan's multiple range tests.

RESULTS AND DISCUSSION

Growth and Cd accumulation were monitored for seedlings grown for 9 d in a nutrient solution containing up to 100 μM CdCl₂ (Fig. 1). Compared with the control seedlings, the dry weight of seedlings was significantly reduced in the presence of over 50 μM Cd. The dry weight was reduced by 47 % with 100 μM Cd, so that seedling growth was sensitive to Cd exposure. Meanwhile, the Cd concentration in all seedlings increased in proportion to the level of treatment applied.

The Cd-induced progressive changes of total NPT, Cys, GSH and the ratio of GSSG/GSH in leaves and roots were measured (Figs. 2~5). Both in leaves and roots, NPT content significantly increased with Cd exposure, and the content was much higher in the roots than the leaves (Fig. 2), an indicative of drastic phytochelatin formation for Cd chelation. The content of Cys also increased in both leaves and roots, and the content was higher in leaves than in roots (Fig. 3). Total glutathione content increased significantly only in leaves (Fig. 4). In roots, no significant changes of total glutathione might be induced by the rapid synthesis of thiols including PC. However, an increase in the ratio of GSSG to GSH was invariably observed in both leaves and roots, indicating more oxidation of the glutathione pool under Cd stress (Fig. 5). The large amounts of thiols synthesized in tomato seedlings exposed to Cd have also been observed in a number of other plant species (Scheller et al. 1987, Vogeli-Lange and Wagner 1996).

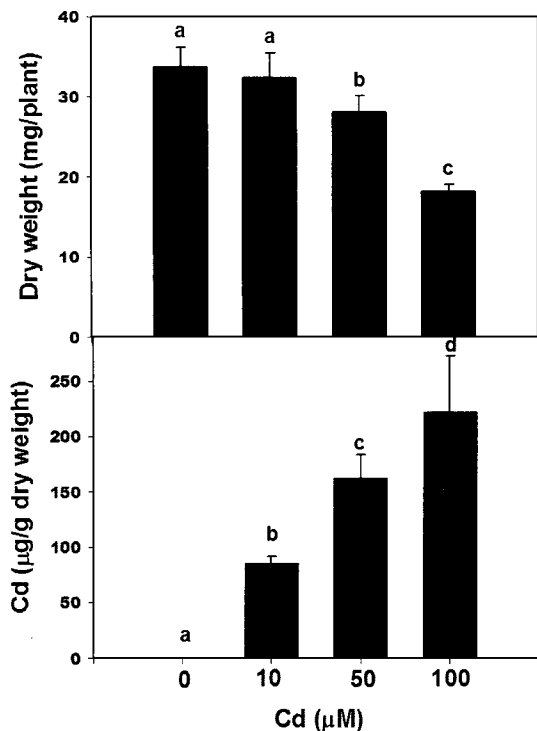


Fig. 1. Dry weight and Cd content in tomato seedlings exposed to Cd for 9 days. Values are mean+SE of five independent replicates. Values with the same letters are not significantly different at the 0.05 levels according to Duncan's multiple range tests.

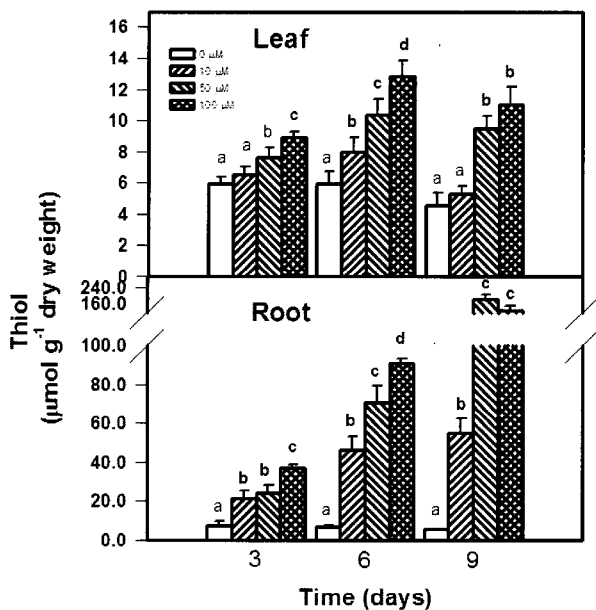


Fig. 2. Concentration of total non-protein thiols in leaves and roots of tomato seedlings exposed to Cd for 9 days. Values are mean+SE of five independent replicates. Values with the same letters are not significantly different at the 0.05 levels according to Duncan's multiple range tests.

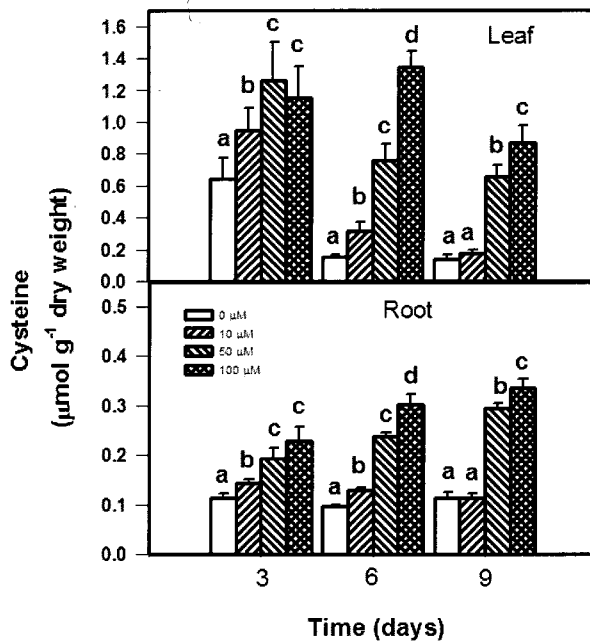


Fig. 3. Concentration of total glutathione in leaves and roots of tomato seedlings exposed to Cd for 9 days. Values are mean+SE of five independent replicates. Values with the same letters are not significantly different at the 0.05 levels according to Duncan's multiple range tests.

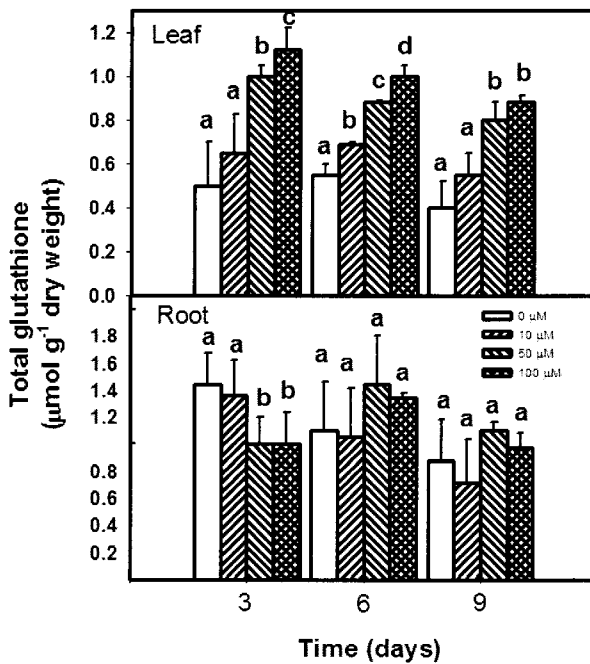


Fig. 4. Concentration of cysteine in leaves and roots of tomato seedlings exposed to Cd for 9 days. Values are mean+SE of five independent replicates. Values with the same letters are not significantly different at the 0.05 levels according to Duncan's multiple range tests.

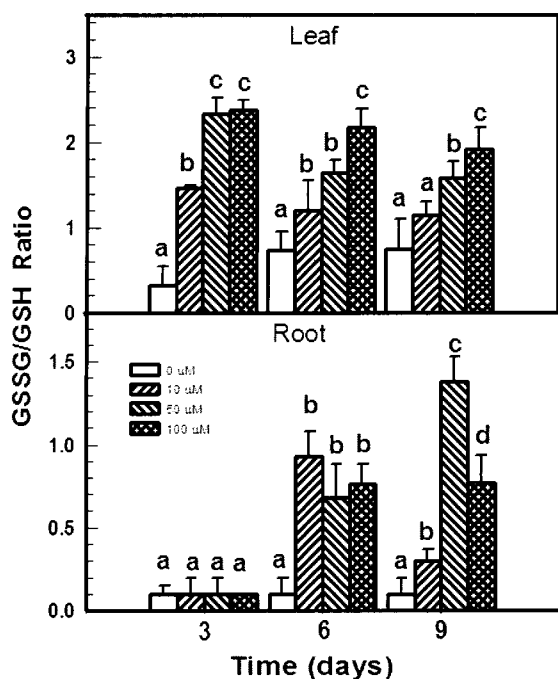


Fig. 5. GSSG/GSH ratio in leaves and roots of tomato seedlings exposed to Cd for 9 days. Values are mean+SE of five independent replicates. Values with the same letters are not significantly different at the 0.05 levels according to Duncan's multiple range tests.

The cellular concentration of GSH declines as thiols are synthesized in response to the addition of Cd to the medium (Figs. 2 and 3 in this study, Jackson et al. 1992, Ruegsegger et al. 1992). Therefore, an increase in total glutathione observed in our experiment might be due to the increase of GSSG. The increase of GSSG could be a result of the increased activity of peroxide scavenging, and Cd-induced PC synthesis might result in the lowered GSH level in cytosol, where PCs are made and have a high affinity for binding with heavy metals particularly Cd and Cu (Grill et al. 1985, Ha et al. 1999).

Glutathione plays a crucial role in controlling and maintaining the intracellular redox state (Noctor and Foyer 1998). The size and redox state of glutathione pool changes in response to various environmental factors including temperature (Wise and Naylor 1987), light (Noctor et al. 1998) and heavy metals (Scheller et al. 1987, Ruegsegger and Brunold 1992, Schneider and Bergmann 1995). GSH synthesis can occur in the chloroplast and the cytosol (Noctor et al. 1998b) and respond to the availability of amino acid substrates, particularly Cys (Buwalda et al. 1990, Strohm et al. 1995, Noctor et al. 1996). Therefore, the increased levels of Cys in response to Cd exposure (Xiang et al. 2001, Fig. 3) might be due to GSH depletion.

A shift in the glutathione redox couple to a more oxidized state and GSH depletion by metal exposure might be related to oxidative stress and metal toxicity. Copper tolerance of *Silene vulgaris* was related to their ability to prevent GSH depletion more than to protective functions of PCs (De Vos et al. 1992) and Indian mustard plants having a 1.5- to 2.5-fold increase in GSH showed some increase in metal resistance (Zhu et al. 1999). Since H_2O_2 level might also be increased by the depletion of GSH and the subsequent inefficient operation of GSH-ascorbate cycle-- a major H_2O_2 scavenging system-- GSH was assumed to be important in metal tolerance, functioning as an antioxidant against free radicals and hydrogen peroxide (Alscher 1989).

The nucleophilic nature of the thiol group is also important in the formation of mercaptide bonds with metals and for reacting with select electrophiles (Xiang et al. 2001).

ASA is widely distributed in both animals and plants, and oxidized DASA occurs in biological materials in relatively low concentrations. DASA is also formed in a redox system in the presence of ascorbic acid. As a result of Cd exposure, the ascorbic acid pool in leaves increased initially but remained unchanged with longer Cd exposure compared to controls (Fig. 6). However, in the same conditions, it increased initially, but decreased progressively later in the leaves. As a result of these changes, the DASA to ASA ratio increased initially but decreased later with Cd exposure.

Thiols also possess strong antioxidative properties and they are consequently able to counteract oxidative stress (Pichner et al. 1993). GSH also acts as an electron acceptor and donor for numerous biological reactions (Noctor and Foyer 1998a), and is an important component of the cell's scavenging system for ROS (Halliwell 1986, Kunert and Foyer 1993). Most of the metabolic

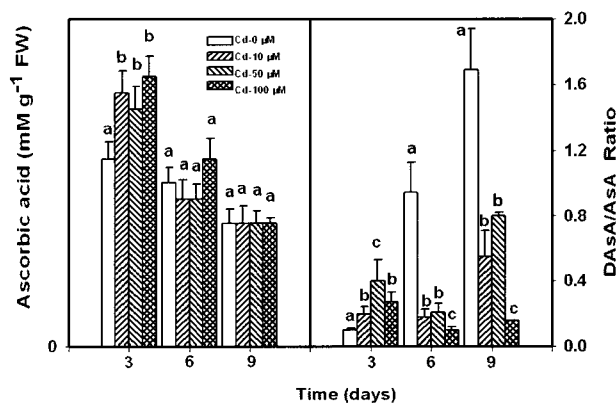


Fig. 6. Concentration of ascorbic acid and DASA/ASA ratio in leaves and roots of tomato seedlings exposed to Cd for 9 days. Values are mean+SE of five independent replicates. Values with the same letters are not significantly different at the 0.05 levels according to Duncan's multiple range tests.

functions of GSH involve the oxidation of reduced GSH to oxidized glutathione disulfide (GSSG). The GSH-ascorbate cycle (Foyer et al. 1994) is essential in removing H₂O₂ (Asada 1999), and the activities of enzymes involved in the cycle depend on the availability of ASA and GSH. In response to various environmental factors including temperature (Wise and Naylor 1987), light (Nocctor et al. 1996) and heavy metals (Scheller et al. 1987, Rueggsegger and Brunold 1992, Schneider and Bergmann 1995), the size or redox state of the GSH pool changes. GSH might be required for both PC synthesis and ROS scavenging and might be a key element for surviving particularly in plants exposed to heavy metals, and the decision of plants to use GSH for PC synthesis to detoxify metals or H₂O₂ scavenging to prevent oxidative stress might be a characteristic response to metal exposure. Therefore, our results might explain the Cd-induced ROS formation and oxidative stress. In fact, heavy metals are known to be involved in many ways in the production of ROS including H₂O₂ (Halliwell and Gutteridge 1984), and it is conceivable to suppose that a decrease of enzymic and non-enzymic free radical scavengers caused by heavy metals (De Vos et al. 1993) may contribute to the shift in the balance of free-radical metabolism towards ROS accumulation. The enhanced production of H₂O₂ by Cd exposure (Cho 2004) could also contribute to the enhanced lipid peroxidation. Further interaction of H₂O₂ and O₂ in the presence of metal ions or metal chelates may produce highly reactive hydroxyl radicals (OH). Since Cd exposure increased TBA-RS, lipid peroxidation products (Cho 2004), it was clear that the decreased growth of these seedlings during Cd exposure could be due to the increased levels of oxidative damage.

The increases in glutathione reductase (GR) activity in both leaves and roots (Cho 2004) appeared to be due to active thiol synthesis and the increased GSSG levels. GR in plastid and cytosol is a highly specific enzyme that utilizes NADPH to reduce GSSG to 2 molecules of GSH (Schaedle 1977) and can maintain a high ratio of GSH/GSSG and by this means plays an important role in the regulation of cell metabolism (Williams 1976). Therefore, over-expression of GR in the plastids showed enhanced Cd tolerance in terms of chlorosis and chlorophyll fluorescence parameters, and the lower Cd stress in the transgenic plants could be the result of increased GSH synthesis and subsequent increase of thiols for metal binding in roots and reduced Cd translocation in shoots (Pilon-Smits et al. 2000). However, the higher GSSG : GSH ratios observed might indicate that GR activity in seedlings exposed to Cd was not sufficient to provide GSH for ROS quenching and thiol synthesis. Meanwhile, the increase of GSSG might be a result of the increased activity of peroxide scavenging and active PC synthesis. The lowered GSH-dependent H₂O₂ quenching pathway due to GSH depletion might lead to increased GSSG accumulation and, perhaps,

lower ASA utilization. This reduced demand for ASA could contribute to the decreased DASA/ASA ratio particularly after day 6. Therefore, depletion of GSH due to synthesis of PC in the presence of heavy metals may result in an increase in oxidative stress (De Vos et al. 1993). Accumulation of GSSG might also be a result of the increased GPX-dependent peroxide scavenging (Roxas et al. 2000). Glutathione peroxidase is capable of rapidly scavenging products of lipid peroxidation generated as a result of enhanced ROS production (Bartling et al. 1993) as well as endogenous reactive products of cellular metabolism (Rushmore and Pickett 1993).

Based on our results, we conclude that the rapid physiological damage as inferred from the reduction of seedling growth could result from oxidative stress. The oxidative stress could be a result of the GSH depletion due to synthesis of thiols such as PC and subsequent inefficient quenching of H₂O₂ by the altered GSH-ascorbate cycle.

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