Journal of Plant Biology, December 2006, 49(6) : 440-447

Differential Antioxidative Responses of Ascorbate-Glutathione Cycle Enzymes and Metabolites to Chromium Stress in Green Gram (*Vigna radiata* L. Wilczek) Leaves

Thirupathi Karuppanapandian[1]*, Pritam Bala Sinha[1], Abdul Majeeth Kamarul Haniya[2], and Kumariah Manoharan[1]

[1]Department of Plant Sciences, Centre of Potential in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai-625 021, India
[2]Department of Biotechnology, Srimad Andavan Arts and Science College, Thiruvanaikoil, Tiruchirappalli-620 005, India

Chromium-induced antioxidative responses of ascorbate-glutathione cycle enzymes and metabolites in green gram (*Vigna radiata* L. Wilczek) leaves were investigated in both dose and time-dependent manners. Rapid uptake of Cr was observed immediately after the start of treatment. Significant reduction was observed in leaf biomass under 300 μM Cr-treatment. Treatment with 300 μM Cr increases the content of hydrogen peroxide and superoxide dismutase activity upto initial 96 h, and then gradually declined to the basal level. Ascorbate peroxidase and guaiacol peroxidase activities were low in 300 μM Cr-treated leaves during the first 96 h, but significantly increased therefore, suggesting that increased enzyme activities would be responsible for the removal of H_2O_2. Catalase activities were always suppressed under Cr stress. Contents of reduced ascorbate and dehydroascorbate were significantly decreased under 300 μM Cr-treatment. The reduced glutathione content decreased at early stages of Cr-treatment. However, it was restored to the normal level as in controls thereafter. In contrast, the glutathione disulphide content showed a progressive increase during the initial hours of Cr-treatment. The non-protein thiol content was shown to increase during the first several hours, but it declines at later stages. The present results demonstrate that Cr-induced oxidative stress is an important component of the plant's reaction to toxic levels of Cr.

Keywords: antioxidative systems, Cr toxicity, green gram, non-protein thiol, reactive oxygen species

Chromium (Cr), an essential micronutrient, plays a vital role in maintaining normal cell metabolism in higher plants. Cr is involved in a wide range of physiological and biochemical processes. However, at high concentrations it becomes strongly phytotoxic to cells as it causes inhibition of plant growth or even death (Breusegem et al., 2001; Karuppanapandian et al., 2006a, b). Studies with several plant species demonstrated that excess Cr in growth medium induces generation of reactive oxygen species (ROS) in treated-tissues. Cr-induced generation of hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), or other ROS has been directly correlated to the damage of proteins, membrane lipids and nucleic acids (Alscher et al., 1997; Breusegem et al., 2001). Photosynthesis is also sensitive to excessive Cr, and the pigments and protein components of photosynthetic membranes are the target sites (Alscher et al., 1997). In addition, Cr toxicity has been related to disturbances in the uptake of other essential elements. The phenomenon of Cr-induced production of ROS has been described as oxidative stress response (Breusegem et al., 2001). To repair the oxidative damage initiated by ROS, some tolerant plants have established several protective mechanisms. The primary constituents of these protective mechanisms include two scavenging systems: enzymatic and non-enzymatic. Enzymatic scavengers, such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT) and enzymes of the ascorbate-glutathione cycle, are related to the removal of H_2O_2 and the detoxification of peroxy radicals ($O_2^{\cdot -}$) (de Vos et al., 1992; Murphy and Taiz, 1997); whereas, non-enzymatic scavengers, such as ascorbate and glutathione have been interpreted as the key antioxidants for the removal of H_2O_2 in the plant cells, thus reducing the accumulation of the free radicals (de Vos et al., 1992; Foyer, 1993). Evidence from several plant species reveals that Cr caused oxidative stress by mediating the activities of antioxidative enzymes (Karuppanapandian et al., 2006b). Panda and Khan (2004) reported that Cr-treatment triggered distinct oxidative defense mechanisms in *Hydrilla verticillata*. However, the mechanisms in Cr-induced antioxidative responses are not yet fully understood.

The inhibition of plant growth and crop production by excess heavy metals, such as Cu, Cd, Zn and Ni, in contaminated-soil has become an agricultural problem worldwide. Using metal accumulating plants to remove excessive metals from soil and aqueous streams has been proposed as a solution (de Vos et al., 1992; Breusegem et al., 2001). In this regard, understanding of the tolerance mechanism in plants against the heavy metals is important (Shanker et al., 2004). It has been considered that green gram is one of the ideal plant species to remediate heavy metal contamination in soil. However, to date no information has been reported in this species on Cr-induced oxidative stress or antioxidative

*Corresponding author; fax +91-452-2459105
e-mail tkpandian78@yahoo.com

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; Cr, chromium; DHA, dehydroascorbate; GPX, guaiacol peroxidase; GSH, reduced glutathione; GSSG, glutathione disulphide; H_2O_2, hydrogen peroxide; NPT, non-protein thiol; POX, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase

responses. The purpose of present study was to examine the changes in concentrations of H_2O_2 in green gram leaves when exposed to various concentrations of Cr. Additionally activities of several enzymes involved in H_2O_2 metabolism were examined along with antioxidative metabolites like ascorbate and glutathione in the Cr-treated green gram leaves.

MATERIALS AND METHODS

Plant Material and Growth Condition

Green gram (*Vigna radiata* L. Wilczek; obtained from Tamil Nadu Agricultural University, Coimbatore, India) seeds were germinated aseptically after surface sterilization with 70% ethanol, followed by a treatment with 0.1% $HgCl_2$ for 5 min. Subsequently, seeds were thoroughly washed 5 times in sterile double distilled water and germinated in Petri dishes in darkness containing Whatman No.1 filter paper moistened with Hoagland nutrient solution (Hoagland and Arnon, 1950). After 48 h of germination, seedlings were transferred to plastic glasses containing Hoagland nutrient solution at pH 5.8 and kept in a growth chamber with a daily photoperiod of 16 h light of 150 µmol m^{-2} s^{-1} light intensity. Relative humidity was maintained at 35% during the day time and 60% at night. Cr in the form of potassium dichromate ($K_2Cr_2O_7$) was supplemented along with the Hoagland nutrient solution. For the control, seedlings were placed on the filter paper moistened with just double distilled water. Biomass of leaves was determined immediately after harvest.

Measurement of Cr Content

Leaves were separated and oven dried for 3 d at 80°C, and dried leaf samples were ground into fine powder. Conditions used for digestion were according to Davies et al. (2002) that is briefly as follow. Five ml of concentrated HNO_3 was added to 250 mg of dried leaf sample in a 50 ml digestion tube and allowed to stand overnight at room temperature. The digestion tubes were placed in a heating block for 1 h at 150°C, tubes were then removed and allowed to cool and 2 ml of 30% H_2O_2 was added. The contents in the tubes were mixed by swirling, and then heated for 2 h at 150°C. After cooling to room temperature, the solution was diluted to 50 ml in total volume, and the upper clear portion was used for the measurement of Cr content. During dilution, 2% of NH_4Cl or 0.5% of $CaCl_2$ was added to each sample and standard to eliminate interference caused by iron or phosphorus, respectively, during spectrophotometeric analysis. Atomic absorption spectrometer (Spectra AA-220; Varion, USA) with air-acetylene flame at 358 nm and 0.2 mm spectral slit width was used. Cr content was expressed as mg kg^{-1} DW.

Determination of Malondialdehyde (MDA) Content

Peroxidation of lipid was quantified by measuring the amount of MDA as described by Heath and Packer (1968). Five hundred mg of leaves was homogenized with 5% trichloroacetic acid (TCA; w/v). As a blank, 1 ml of 5% TCA and 4 ml of thiobarbituric acid (TBA) reagent (0.5% in 20% TCA) were mixed. For correction of blank, 1 ml of the homogenate was mixed with 4 ml of 20% TCA. For sample, 1 ml of the homogenate and 4 ml of TBA reagent were mixed. After heating at 95°C for 30 min, the mixture was cooled to room temperature and centrifuged at 4000 rpm for 10 min. Absorbance was read at 532 nm. Concentration of MDA was calculated by using an extinction coefficient at 155 $mM^{-1}cm^{-1}$. MDA level is routinely used as an index of lipid peroxidation and was expressed as nmol g^{-1} FW.

Estimation of H_2O_2 Content

H_2O_2 content was determined according to Patterson et al. (1984). Five hundred mg of leaves was ground in 6 ml ice-cold acetone and centrifuged at 8000 rpm at 4°C for 30 min. Five hundred µL of supernatant was mixed with 1.5 ml of mixture of $CHCl_3$ and CCl_4 (1:3, v/v), and 2.5 ml of distilled water was added. The reaction mixture was centrifuged at 1000 rpm for 1 min, and the aqueous phase was collected for H_2O_2 determination. To set controls, 0.1 ml of CAT (0.3 U) was added to the 1 ml of supernatant to remove the H_2O_2. For the treatments, CAT solution was replaced by 100 µL MQ (Millipore, USA) water. The reaction mixtures were incubated at 37°C for 10 min, and 1 ml of 0.2 M phosphate buffered saline (PBS, pH 7.8) and 1 ml of 200 mM 4-(2-pyridylazo) resorcinol were added. The reaction mixtures were incubated at 45°C for 20 min, and the absorbance was read at 508 nm. H_2O_2 content was expressed as µmol g^{-1} FW.

Assays of Antioxidative Enzymes

Five hundred mg of leaves was macerated with 10 ml of 50 mM potassium-PBS (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP) in pre-cooled mortar and pestle and centrifuged at 15000 rpm for 30 min at 4°C. The supernatant was used for enzyme analysis. Protein content was determined according to Lowry et al. (1951) using BSA as a standard. SOD (EC 1.15.1.1) activity was assayed according to Beauchamp and Fridovich (1971). The reaction mixture contained 0.24 mM riboflavin, 2.1 mM methione, 1% Triton X-100, 1.72 mM nitroblue tetrazolium chloride (NBT; in 50 mM potassium-PBS, pH 7.8). SOD activity was expressed as U g^{-1} FW. One unit of SOD is defined as the amount of enzyme required to cause 50% inhibition in the rate of NBT photo-reduction. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to Nakano and Asada (1981). Two hundred mg of leaves was homogenized in 50 mM ice-cold PBS (pH 7.8) containing 2 mM ascorbate and 5 mM EDTA and centrifuged at 10000 rpm at 4°C for 30 min. The reaction mixture contained 2.7 ml of 50 mM PBS (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 100 µL of 0.1 mM H_2O_2 and 50 µL of enzyme extracts. The decrease in absorbance was read at 290 nm. APX activity was expressed as U g^{-1} FW. Guaiacol peroxidase (GPX; EC 1.11.1.7) activity was assayed according to Hammerschmidt et al. (1982). The reaction mixture contained 0.1 M potassium-PBS (pH 6.8), 50 mM guaiacol and 50 µL of enzyme extract. Reaction was initiated by adding 0.2 mM H_2O_2. Absorbance was read at 470 nm. GPX activity was

expressed as U g^{-1} FW, where one U equals the amount of substrate (μmol) transformed by the enzyme in 1 min at 25°C. CAT (EC 1.11.1.6) activity was determined according to Aebi (1984). Two hundred μL of the enzyme extract was added to 1.8 ml of PBS (pH 7.0). Reaction was started with the addition of 1 ml of 30 mM H_2O_2 prepared in PBS (pH 7.0). Absorbance was read at 240 nm. CAT activity was expressed as U g^{-1} FW.

Metabolite Determination

Reduced ascorbate and dehydroascorbate (DHA) contents were measured according to Cakmak and Marschner (1992). Five hundred mg of leaves was macerated in ice-cold extraction buffer [40% (v/v) methanol, 0.75% (w/v) *m*-phosphoric acid, 16.7 mM oxalic acid, and 0.127 mM acetone] and centrifuged at 10000 rpm for 30 min at 4°C. One hundred μL of supernatant was transferred to 900 μL of mobile phase [24.25 mM acetic acid (pH 4.8), 0.1 mM acetone, 0.015% (w/v) *m*-phosphoric acid, 0.04% (w/v) octylamine, and 15% (v/v) methanol] for determination of reduced ascorbate. Amount of DHA was calculated as the difference of total ascorbate and ascorbic acid. Contents of reduced ascorbate and DHA were expressed as μmol g^{-1} FW. GSH and glutathione disulphide (GSSG) contents were determined according to Anderson (1985). Five hundred mg of leaves was homogenized with 5 ml of 0.15% (w/v) sodium ascorbate and centrifuged at 10000 rpm for 15 min at 0°C. Supernatants were incubated at 100°C for 4 min to denature proteins, and centrifuged as described above. One ml of reaction mixture contained 200 μL thylakoid suspension, 0.2 mM NADPH, 0.6 mM 5,5'-dithio-bis(2-nitrobenzoic acid; DTNB), and 50 U of glutathione reductase. Reduction of DTNB was followed at 412 nm for 2 min at 30°C. To measure the amount of GSSG in the supernatant, 2 μM of PVP was added to 100 μL of the supernatant and mixed vigorously for 1 min. Reduction rate of DTNB was measured after 20 and 40 min as in the total glutathione assay. Total glutathione and GSSG were quantified by comparing with the standard curves obtained with the reduced and oxidized forms of glutathione (Sigma, USA). Amount of GSH was calculated by subtracting the amount of GSSG from the total glutathione. Contents of GSH and GSSG were expressed as nmol g^{-1} FW.

Estimation of Non-Protein Thiol (NPT) Content

For estimation of NPT content, 500 mg leaves were macerated in 1 ml ice-cold 5% (w/v) sulfosalicylic acid and centrifuged at 10000 rpm for 30 min at 4°C. NPT content was measured with Ellman's reagent (Ellman, 1959). Three hundred μL of the supernatant was mixed with 1.2 ml of 0.1 M PBS (pH 7.6), and after a stable absorbance reading of 412 nm was obtained, 25 μM DTNB solution (6 mM DTNB dissolved in 5 mM EDTA, 0.1 M PBS, pH 7.6) was added. Increase in absorbance was read at 412 nm. NPT content was expressed as nmol g^{-1} FW.

Statistical Analysis

Mean of at least three replicated treatments was presented. Significance of differences between treatments was statistically evaluated by SD and Student's *t*-test methods.

RESULTS

Figure 1 shows Cr content in green gram leaves over time of treatment with 300 μM Cr. Cr accumulation occurred upon the addition of Cr to the nutrient solution. The increase in Cr uptake lasted upto 96 h. After that, the uptake rate was saturated. The maximum content of Cr in the leaves exposed to 300 μM Cr for 96 h was 895 mg kg^{-1} DW. The biomass of leaves exposed to the various levels of Cr was measured. Treatment of seedlings with 100 μM Cr-induced only a slight increase in leaf dry weight. Seedlings supplied with Cr at 200-400 μM had significantly lower leaf biomass than control (Fig. 2). Treatment with Cr at 300 μM decreased the leaf dry weight by 46.9%. Since a moderate effect of 300 μM Cr on the leaf growth was observed, this concentration was used to examine the biological and physiological responses. Exposure of seedlings to excess Cr led to lipid peroxidation in leaves. The amount of MDA increased with the increasing Cr-treatment (Fig. 3A). A peak was observed with Cr at 300 μM, with the MDA level being 1.7-fold higher than control. A further increase in Cr concentration to 400 μM, however, failed to elevate the production of

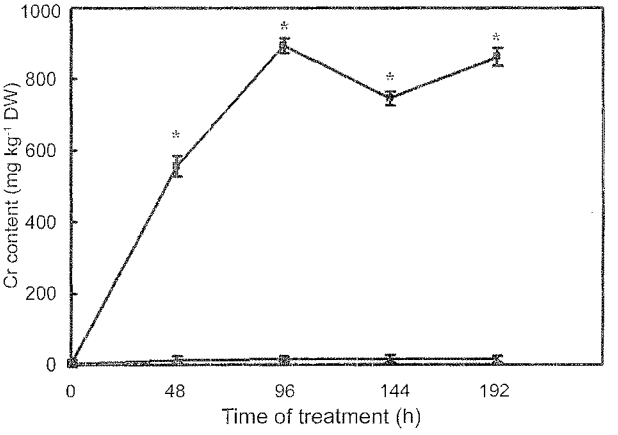

Figure 1. Time-dependent changes in the content of Cr in green gram leaves after 300 μM Cr-treatment (—■—) and control (—▲—). Vertical bars represent SD of the mean (n=4). Asterisks indicate that mean values are significantly different between treatments and control (*P >0.05).

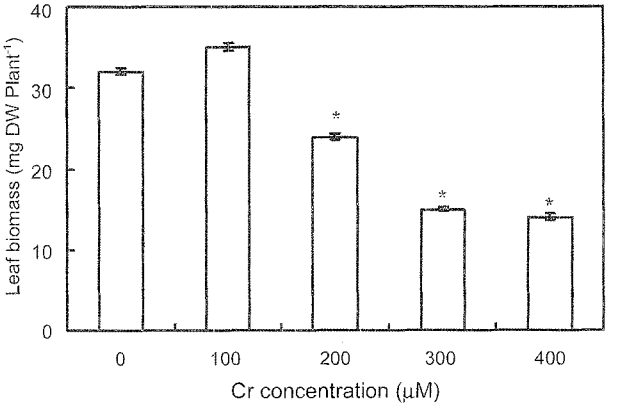

Figure 2. Effect of Cr on leaf biomass of green gram. Seedlings were treated with various concentrations of Cr for 96 h. Vertical bars represent SD of the mean (n=4). Asterisks indicate that mean values are significantly different between treatments and control (*P >0.05).

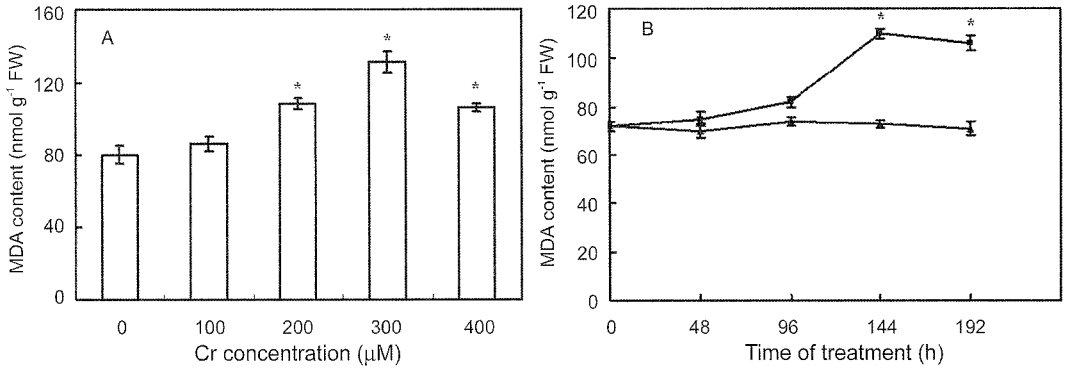

Figure 3. Content of MDA in green gram leaves under Cr stress. Seedlings were treated with various concentrations of Cr for 96 h (**A**) or with 300 μM Cr (—■—) and control (—▲—) for 192 h (**B**). Vertical bars represent SD of the mean (n=4). Asterisks indicate that mean values are significantly different between treatments and control (*P >0.05).

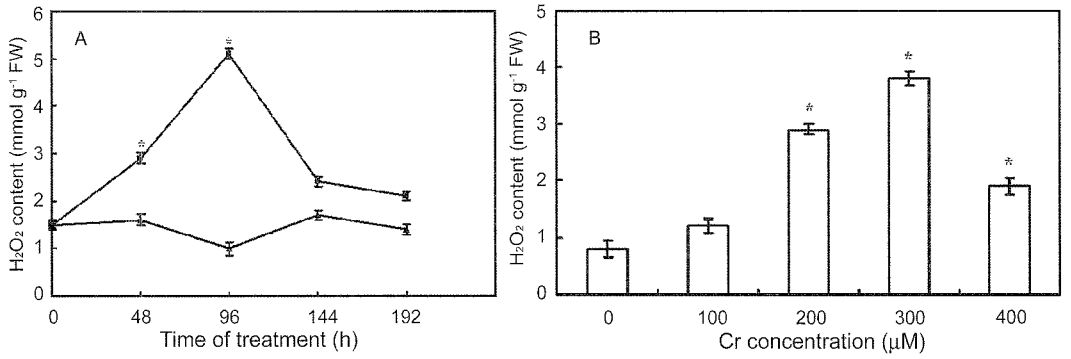

Figure 4. Content of H_2O_2 in green gram leaves under Cr stress. Seedlings were treated with 300 μM Cr (—■—) and control (—▲—) for 192 h (**A**) and various concentrations of Cr for 96 h (**B**). Vertical bars represent SD of the mean (n=5). Asterisks indicate that mean values are significantly different between treatments and control (*P >0.05).

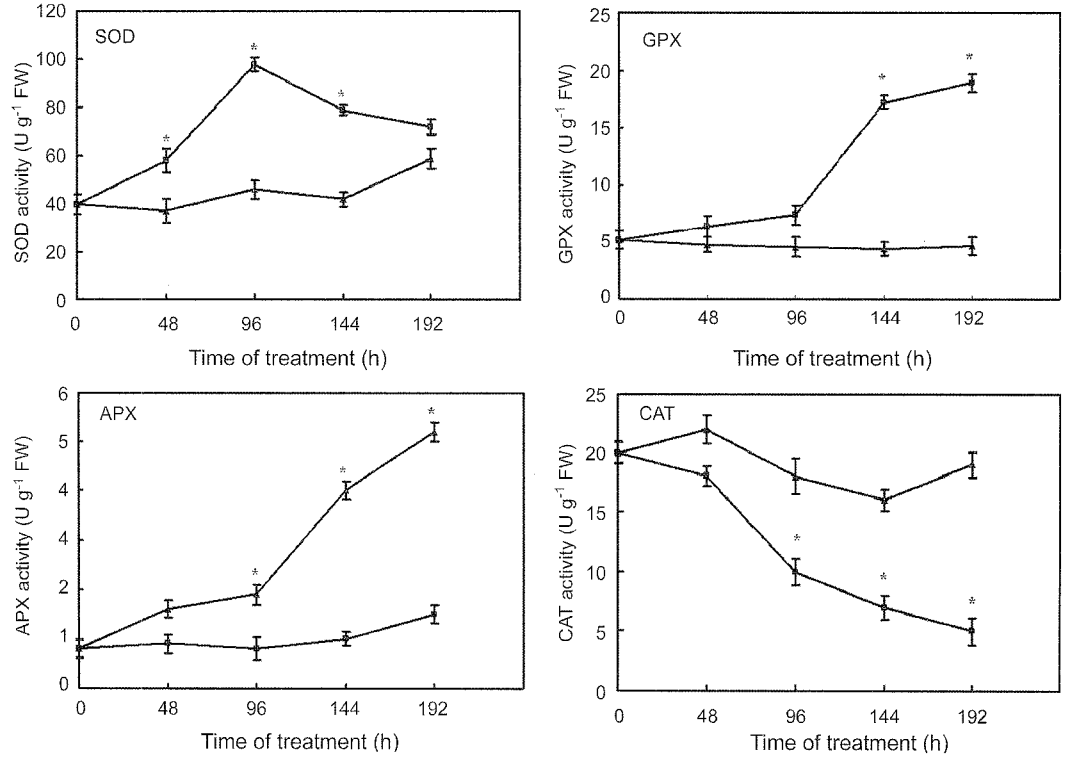

Figure 5. Time-dependent changes in activities of SOD, GPX, APX and CAT in green gram leaves of 300 μM Cr-treatment (—■—) and control (—▲—). Vertical bars represent SD of the mean (n=4). Asterisks indicate that mean values are significantly different between treatment and control (*P >0.05).

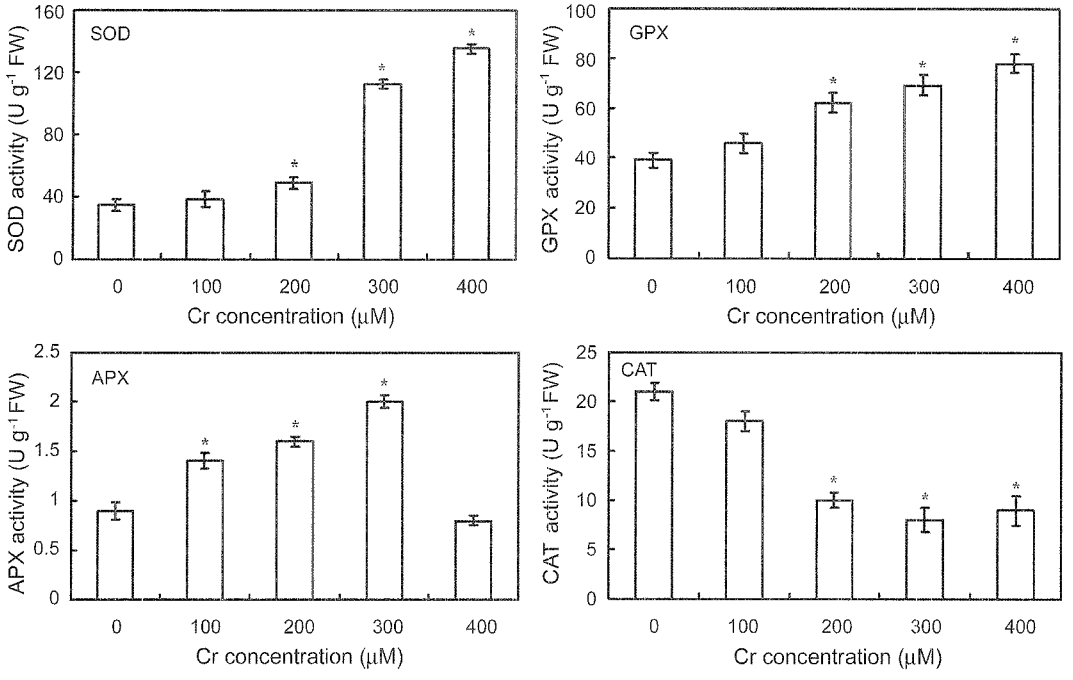

Figure 6. Effect of Cr on activities of SOD, GPX, APX and CAT in green gram leaves. Seedlings were treated with various concentrations of Cr for 96 h. Vertical bars represent SD of the mean (n=4). Asterisks indicate that mean are significantly different treatments and control (*P >0.05).

Table 1. Time-dependent changes in contents of reduced ascorbate, DHA, GSH and GSSG in green gram leaves of 300 μM Cr-treatment and control. Asterisks indicate that the mean values are significantly different between treatments and controls (*P >0.05).

Time (h)	Reduced ascorbate (μmol g^{-1} FW)		DHA (μmol g^{-1} FW)		GSH (nmol g^{-1} FW)		GSSG (nmol g^{-1} FW)	
	Control	Cr	Control	Cr	Control	Cr	Control	Cr
0	8.05 ± 0.65	8.05 ± 0.65	1.48 ± 0.15	1.48 ± 0.15	124 ± 15.0	124 ± 15.0	62.6 ± 5.2	62.6 ± 5.2
48	8.65 ± 0.56	8.92 ± 0.53	1.88 ± 0.20	2.01 ± 0.18*	111 ± 7.5	97 ± 8.9*	52.3 ± 9.2	94.4 ± 8.6*
96	9.87 ± 0.51	5.25 ± 0.48*	1.74 ± 0.21	0.94 ± 0.09*	107 ± 8.5	75 ± 7.8*	69.8 ± 5.6	99.7 ± 10.4*
144	8.69 ± 0.48	5.88 ± 0.58*	1.92 ± 0.18	0.81 ± 0.08*	90 ± 12.0	89 ± 6.7*	60.7 ± 3.9	108.2 ± 9.5*
192	9.48 ± 0.72	6.12 ± 0.49*	1.78 ± 0.09	0.80 ± 0.04*	96 ± 9.1	132 ± 18.1*	74.2 ± 4.5	124.3 ± 19.8*

MDA. Supplementation of 300 μM Cr to the medium induced progressive increases in the lipid peroxidation level (Fig. 3B). Similarly, a significant rise in MDA level was observed at 96 h with 300 μM Cr.

H_2O_2 level was measured to determine whether application of excessive Cr caused oxidative stress in green gram leaves. In a time-course experiment, the H_2O_2 level increased markedly during the 300 μM Cr-treatment. This stimulation reached to a maximum level at 96 h. Then, the H_2O_2 level declined to the level of control (Fig. 4A). Dose-dependent changes in H_2O_2 levels were examined, and it showed that application of Cr at 100-400 μM significantly increased the production of H_2O_2 (Fig. 4B).

Activities of antioxidative enzymes were assayed over a period of 192 h in the Cr-treated leaves. In the control seedlings slight fluctuation in the enzyme activities was observed (Fig. 5). Treatment with 300 μM Cr resulted in 1.6-fold of SOD activity during the initial 48 h, although the response to stimulation lasted for 96 h. Cr at 100-400 μM progressively increased the SOD activity, and 400 μM of Cr was found to be the most effective concentration for inducing increases in SOD activities (Fig. 6). Activities of both APX and GPX in leaves were low during the initial 96 h after exposure of seedlings to 300 μM Cr followed by a significant increase (Fig. 5). However, after 192 h of induction, APX and GPX activities were enhanced by 3.5- and 4-fold, respectively. GPX activities displayed progressive increases and reached to the maximum at 400 μM Cr (Fig. 6). Although APX activities were increased in the presence of 300 μM Cr, 400 μM Cr did not stimulate the enzyme activity (Fig. 6). In contrast, the activities of CAT in the leaves were diminished under Cr stress (Fig. 5, 6).

Three hundred μM Cr had no marked effect on the reduction of ascorbate content in the leaves during the initial 48 h of treatment (Table 1). After that, reduction of ascorbate content decreased significantly to 53.5% at 96 h and then maintained thereafter. In contrast, reduction of ascorbate and DHA contents showed a significant increase during the initial 48 h, and then sharply declined thereafter (Table 1). GSH showed a significant reduction at 96 h to 29.9% of the control. However, the content of GSH was restored after that, and at the end of experiment it exceeded the controls. On the other hand, the GSSG contents showed a progressive increase (Table 1). The total NPT levels were enhanced by 39.8 and 43.5% with Cr at 200 and 300 μM, respectively (Fig. 7A). Neither lower nor

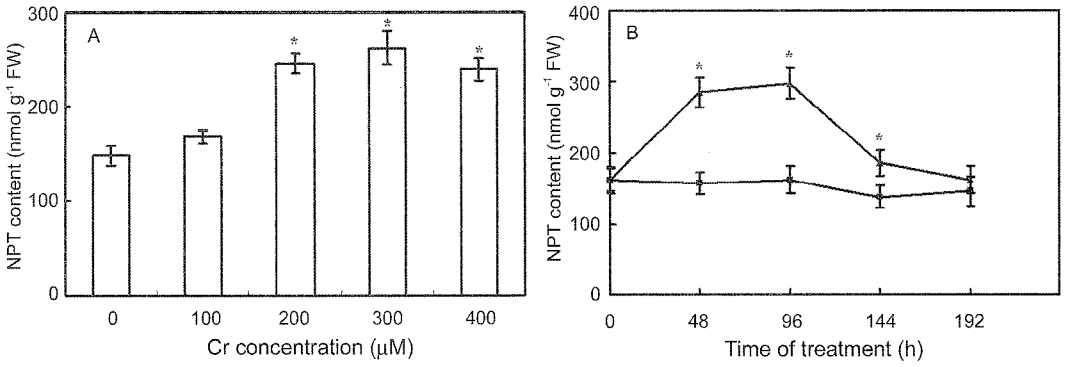

Figure 7. Content of NPT in green gram leaves under Cr stress. Seedlings were treated with various concentrations of Cr for 96 h (**A**) and with 300 μM Cr (—■—) and control (—▲—) for 192 h (**B**). Vertical bars represent SD of the mean (n=5). Asterisks indicate that mean values are significantly different between treatments and control (*P >0.05).

higher concentrations (400 μM) of Cr induced accumulation of thiol compounds. However, time-dependent variations in thiol contents were measured. As shown in Figure 7B, the changes in the total NPT content showed a pattern similar to that of H_2O_2 over the time of 300 μM Cr-treatment.

DISCUSSION

In the present study, we investigated Cr uptake and several biochemical and physiological responses involving the oxidative damage and protection in green gram leaves. Seedlings readily absorbed Cr from the growth medium (Fig. 1). The toxic effects of excess Cr in the leaves probably caused the decrease in biomass (Karuppanapandian et al., 2006b). To approach a situation in which both Cr toxicity and resistance were exhibited, the seedlings were exposed to Cr at a moderate concentration (300 μM), where the leaf biomass decreased compared to the control (Fig. 2).

H_2O_2 level in green gram leaf tissues was progressively increased by Cr-treatment (Fig. 4A, B). In the time-dependent changes in H_2O_2 level indicates that Cr-induced H_2O_2 increase occurred immediately after exposure of seedlings to Cr, this suggests that accumulation of H_2O_2 was an early event. Higher level of H_2O_2 would be the cause of leaf lipid peroxidation. Before the accumulation of Cr in leaves, a significant increase in SOD activities was observed, and at the time the leaves were saturated at 48 h, the SOD activities reached maximum level (Fig. 5). Therefore, Cr-induced increases in SOD activities could be mediated by ROS. ROS like $O_2^{\cdot -}$ and H_2O_2 are considered to be central components of signal transduction, which triggers the defense genes responsible for antioxidant enzymes, including SOD. In contrast, increase in enzyme activities contribute to the removal of $O_2^{\cdot -}$ (Gwozdz et al., 1997; Chatterjee and Chatterjee, 2000). It could be observed that 300 μM Cr-treatment resulted in a 2-fold increase in SOD activities. However, this stimulation lasted only upto 48-96 h of induction (Fig. 5). Later, SOD activities were declined that suggests the degree of oxidative stress alleviated, due to the strong capability of the plants to recover from Cr stress. In fact, after 96 h exposure of seedlings to 300 μM Cr, leaf growth gradually recovered, and no visible difference between the control and Cr-treated leaves could be observed (data not shown). Reduction in H_2O_2 level in the leaves after 96 h with 300 μM Cr-treatment suggests that H_2O_2 scavenging enzymes are involved in the removal of H_2O_2. CAT is one of the key antioxidant enzymes that eliminate H_2O_2. However, CAT activity was suppressed under Cr stress (Fig. 5, 6). Either Cr^{2+} that bound or replaced some components such as Fe^{2+} in the enzyme or due to decrease in CAT activity because of increase in H_2O_2 that in turn inactivated the enzyme (Chatterjee and Chatterjee, 2000), it also has been previously reported that, CAT in green gram more sensitive to excess Cr^{2+} since, it readily bound to thiol groups and thereby inactivated the thiol-containing enzyme (Gwozdz et al., 1997).

APX activities were progressively increased by Cr-treatment (Fig. 5). This suggests that the antioxidative capacity stimulated by Cr could be the involved in conversion of H_2O_2 to H_2O and O_2. APX plays an important role in scavenging H_2O_2. H_2O_2 is reported to be a systemic signal for the induction of APX (Morita et al., 1999). In callus cultures of rice embryos, H_2O_2 transiently induced mRNA for cytosolic APX (Morita et al., 1999). Cr- and Al-induced H_2O_2 accumulations in wheat and green gram seedlings were also found to significantly elevate the APX activity (Sharma and Sharma, 1996; Karuppanapandian et al., 2006a). The low level of APX activity during the initial period of Cr-treatment suggests that plants require a time lag for induction of the enzyme (Fig. 5).

The POX exhibited an inducible activity in the presence of 300 μM Cr. The significant increase in activities of the enzyme at latter stages might greatly contribute to scavenging of H_2O_2 (Fig. 5). This indicated that 300 μM Cr-activated a sufficiently defensive mechanism against oxidative stress by inducing the potent antioxidant enzyme in the green gram leaves. The GPX has been demonstrated to catalyze the oxidation of various organic compounds like phenolics to form lignin or suberin (Quiroga et al., 2000). Schutzendubel et al. (2001) reported that 50 μM Cd-induced increases in GPX activities in pine root tips were accompanied by accumulation of phenolics and lignification. While a variety of reactions were catalyzed by POX for cell wall rigidification, H_2O_2 serve as a substrate for these processes (Lin and Kao, 2001; Schutzendubel et al., 2001). Thus, the endogenous consumption of H_2O_2 might lead to a decrease in Cr-induced oxidative stress in plants.

The two non-enzymatic antioxidants, such as ascorbate and glutathione for plant cells to dispose of H_2O_2 in some cellular compartments (Schutzendubel et al., 2001; del Rio et al., 2002). The reduced ascorbate content in leaves was significantly reduced at 96 h after the start of Cr-treatment (Table 1), suggests the pronounced consumption of reduced ascorbate during scavenging of H_2O_2, a reaction catalyzed by APX. DHA was also shown to be low at latter stages of Cr-treatment, although the amount of DHA increased at the time of 48 h (Table 1). The decreased level of DHA was most likely the result of irreversible hydrolysis to 2,3-diketogulonic acid (Morita et al., 1999) under heavy metal stress.

The NPT compounds are comprised of several acid-soluble sulfhydryl-components, such as cysteine, γ-glutamylcysteine, GSH and phytochelatin (de Vos et al., 1992). The metal-induced depletion of GSH in plants due to phytochelatin synthesis may increase the susceptibility of cells to oxidative stress (de Vos et al., 1992). 300 μM Cr-increased the NPT content during first several hours of Cr-treatment in green gram leaves (Fig. 7A, B). Suggests that Cr-induced increase in the level of thiol compounds represents another possible response to overcome oxidative damage. On the other hand, Cr has been reported to catalyze the oxidation of GSH (Shanker et al., 2004). Our results showed a transient decline of GSH (on 48 h) and progressive increases in GSSG contents (Table 1). Indicating that a large amount of thiol-containing compounds must be consumed to generate GSSG. Despite the possibility that a high level of Cr in the leaves affected the thiol compound synthesis, loss of thiol content at the late hours of experiment did not mean a breakdown of the defensive system against Cr toxicity. Cr-treated species contain induced metallothioneins-like (MTs-like) proteins. These ubiquitous low molecular-weight proteins are rich with cysteine and also have the ability to bind metal ions (Shanker et al., 2004).

ACKNOWLEDGEMENTS

First author (TKP) expresses his sincere thanks to Mr. T. Karuppudurai, Department of Animal Behaviour and Physiology, Mr. R. Kamalakannan and Mrs. A. Vidhyalakshmi Kamalakannan Department of Immunology, SBS, MKU, for their helpful discussion and critical review of this manuscript.

Received May 17, 2006; accepted November 3, 2006.

LITERATURE CITED

Aebi H (1984) Catalase *in vitro*. Methods Enzymol 105: 121-126

Alscher RG, Donahue JL, Cramer CL (1997) Reactive oxygen species and antioxidants: relationships in green cells. Physiol Plant 100: 224-233

Anderson ME (1985) Determination of glutathione and glutathione disulphide in biological samples. Methods Enzymol 113: 548-555

Beauchamp C, Fridovich I (1971) Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal Biochem 44: 276-287

Breusegem FV, James EV, Dat F, Inze D (2001) The role of active oxygen species in plant signal transduction. Plant Sci 161: 423-431

Cakmak I, Marschner H (1992) Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiol 98: 1222-1227

Chatterjee J, Chatterjee C (2000) Phytotoxicity of cobalt, chromium and copper in cauliflower. Environ Poll 109: 69-74

Davies FT, Puryear JD, Newton RJ, Egilla JN, Grossi JAS (2002) Mycorrhizal fungi increase chromium uptake by sunflower plants: Influence on tissue mineral concentration, growth, and gas exchange. J Plant Nutr 25: 2389-2407

de Vos CHR, Vonk MJ, Vooijs R, Schat H (1992) Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. Plant Physiol 98: 853-858

del Rio LA, Sandalio LM, Corpas FJ, Palma JM, Gomez M, Barroso JB (2002) Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. J Exp Bot 53: 1255-1272

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82: 70-77

Foyer CH (1993) Ascorbic acid, *In* RG Alscher, JL Hess, eds, Antioxidants in Higher Plants. CRC Press, Boca Raton, pp 31-58

Gwozdz EA, Przymusinski R, Rucinska R, Deckert J (1997) Plant cell responses to heavy metals: Molecular and physiological aspects. Acta Physiol Plant 19: 459-465

Hammerschmidt R, Nuckles EM, Kuc J (1982) Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Collectotrchum lagenarium*. Physiol Mol Plant Pathol 20: 73-82

Heath RL, Packer K (1968) Leaf senescence: Correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot 32: 93-101

Hoagland DR, Arnon DI (1950) The water culture method for growing plants without soil, California Agricultural Experiment Station Circular 347. University of California, Berkeley, pp 32

Karuppanapandian T, Pritam Bala Sinha, Kamarul Haniya A, Premkumar G, Manoharan K (2006a) Aluminium-induced changes in antioxidative enzyme activities, hydrogen peroxide content and cell wall peroxidase activity in green gram (*Vigna radiata* L. cv. Wilczek) roots. J Plant Biol (India) (In press)

Karuppanapandian T, Pritam Bala Sinha, Premkumar G, Manoharan K (2006b) Chromium toxicity: Correlated with increased in degradation of photosynthetic pigments and total soluble protein and increased peroxidase activity in green gram (*Vigna radiata* L.) seedlings. J Swamy Bot-Cl 23 (In press)

Lin CC, Kao CH (2001) Cell wall peroxidase activity, hydrogen peroxide level and NaCl inhibited root growth of rice seedlings. Plant Soil 230: 135-143

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275

Morita S, Kaminaka H, Masumura T, Yanaka K (1999) Induction of rice cytosolic ascorbate peroxidase mRNA by oxidative stress signaling. Plant Cell Physiol 40: 417-422

Murphy A, Taiz L (1997) Correlation between potassium efflux and copper sensitivity in ten *Arabidopsis* ecotypes. New Phytol 136: 211-222

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22: 867-880

Panda SK, Khan MH (2004) Changes in growth and superoxide dismutase activity in *Hydrilla verticillata* L. under abiotic stress. Braz J Plant Physiol 16: 115-118

Patterson BD, MacRaf EA, Ferguson IB (1984) Estimation of hydrogen peroxide in plant extracts using titanium (IV). Anal Biochem 139: 487-492

Quiroga M, Guerrero C, Botella MA, Barcelo A, Amaya I, Medina MI, Alonso FJ, de Forchetti SM, Tigier H, Valpuesta V (2000) A tomato peroxidase involved in the synthesis of lignin and suberin. Plant Physiol 122: 1119-1127

Schutzendubel A, Schwanz P, Teichmann T, Gross K, Langenfelt-Heyser R, Godbold DL, Polle A (2001) Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in scots pine roots. Plant Physiol 127: 887-898

Shanker AK, Djanaguiraman M, Sudhagar R, Jayaram R, Pathmanabhan G (2004) Expression of metallothionein 3 (MT3) like protein mRNA in Sorghum cultivars under chromium (VI) stress. Curr Sci 86: 901-902

Sharma DC, Sharma CP (1996) Chromium uptake and toxicity effects on growth and metabolic activities in wheat, *Triticum aestivum* L. cv. UP 2003. Indian J Exp Biol 34: 689-691