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Impact of short-term cadmium treatment on catalase and ascorbate peroxidase activities in barley root tips

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Abstract

The aim of this study was to analyse the relationship between root growth inhibition and alterations in catalase (CAT) and ascorbate peroxidase (APX) content and activities in barley root tips during the recovery after short-term Cd stress. Significant root growth inhibition was observed after a relatively short-term (30 min) exposure of barley roots to low 15 μM Cd concentration. In seedlings treated with 30 μM Cd root growth was renewed 8 - 9 h after Cd treatment. By contrast, seedlings exposed to 60 μM Cd failed to restore root growth. The CAT activity increased after 15 μM Cd treatment, whereas, higher Cd concentrations inhibited CAT activity. However, APX activity was not affected by Cd treatment. The content of APX1 transcript increased while content of APX2 decreased 3 h after short-term treatment in a concentration dependent manner. While the expression of CAT1 was upregulated after 15 and 30 μM Cd treatment, it was strongly downregulated by 60 μM Cd. By contrast, CAT2 was upregulated in a concentration dependent manner. These results suggest that increased CAT activity is crucial for restoration of root growth after moderate Cd stress while at severe Cd stress its inhibition may lead to the irreversible damages.

Additional key words: *Hordeum vulgare*, root growth inhibition, RT-PCR.

Introduction

The most common symptom of stress is the elevated production of reactive oxygen species (ROS) in various compartments of plant cells (Miller *et al*. 2008). The elevated content of ROS activates defence responses. Therefore, ROS production is tightly regulated by different pathways. In addition, redox homeostasis is tightly regulated (Foyer and Noctor 2005).

Hydrogen peroxide is a relatively long-living ROS and is continuously produced in various cell compartments. H_2O_2 as a signal molecule is a key factor in several developmental processes (Neill *et al*. 2002) and stress responses (Hung *et al*. 2005). On the other hand, H_2O_2 in high concentration is detrimental for cells (Dat *et al*. 2000). Therefore, the regulation of its content in cells is very important especially during stress conditions when its considerable generation may occur. The key H_2O_2 detoxifying enzymes are catalase (CAT) in peroxisomes (Willkens *et al*. 1995) and ascorbate peroxidase (APX) in chloroplasts and cytosol (Davletova *et al*. 2005). APX, a heme-containing protein responsible for the reduction of H_2O_2 to water using ascorbate as electron donor has been identified in several isoforms in many plant species (Shigeoka *et al*. 2002).

Heavy metal stress is also undoubtedly connected with increased content of ROS. Due to increased cadmium concentration, both inhibition of antioxidant systems and stimulation of ROS-generating enzymes have been described in various plant species (Gratão *et al*. 2005, Sharma and Dietz 2008). Olmos *et al*. (2003) have reported that the early step in the oxidative burst induced by Cd in culture of tobacco cells is mediated through the activation of NADPH oxidase and superoxide dismutase (SOD) leading to superoxide and H_2O_2 accumulation within some minutes. Function of root and leaf antioxidant enzymes during Cd stress has been reported and their stimulation or inhibition depending on Cd concentration and exposure time was observed (for review see Schützendübel and Polle 2002, Milone *et al*. 2003, Wang *et al*. 2011). In addition, several publications have shown that enhanced antioxidant systems in plants during Cd exposure ameliorate Cd toxicity symptoms (Aravind and Prasad 2005, Chen *et al*. 2010).

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; ROS - reactive oxygen species.
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The aim of this study was to analyse the relation between root growth restoration after short-term Cd stress and alteration in CAT and APX content and activities in barley root tip.

Materials and methods

Barley (*Hordeum vulgare* L. cv. Slaven) seeds (*Hordeum, Ltd.*, Sládkovičovo - Nový Dvor, Slovak Republic) were imbibed in distilled water for 1 h followed by germination between two sheets of filter paper moistened with distilled water in Petri dishes at 25 °C in darkness. The uniformly germinating seeds were arranged into row between two sheets of filter paper moistened with distilled water in rectangle trays. Trays were placed into nearly vertical position to enable downward radical growth. Continuous moisture of filter papers was supplied from the reservoir with distilled water through the filter paper wick. Seedlings, with approximately 4 cm long roots, 60 h after the onset of seed imbibition were used for short-term treatments by immersing roots into 0, 15, 30 or 60 μM $CdCl_2$ for 30 min. After washing in distilled water for 5 min the seedlings were incubated as described above. One, 2, 3, 6 or 9 h after short-term Cd-treatment the individual barley root segments (3 mm in length) were obtained by the gradual cutting of each root from the tip to the base. Under control conditions the first segment contains the meristem and the elongation zone, while the second segment represents the beginning of differentiation zone.

For the determination of root length increment the position of root tips following the treatments was marked on the filter paper. After 6 h, roots were excised at the position of marks and the length of increment was measured after recording with stereomicroscope (*STMPRO BELPhotonics*, Monza, Italy) using *BEL* microimage analyzer. For localization of cell death, intact roots were immersed in the solution of 0.25 % Evans Blue for 15 min at room temperature. After washing with distilled water for 3 × 5 min, roots were photographed with stereomicroscope.

The root segments were homogenized in a pre-cooled mortar with 100 mM potassium phosphate extraction buffer pH 7.0 and centrifuged at 12 000 *g* for 15 min. Proteins from the supernatant were quantified with bovine serum albumin as the calibration standard by the method of Bradford (1976). Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to Nakano and Asada (1981) by following the decrease of absorbance at 290 nm over time period of 1 min. The reaction mixture contained 0.5 mM ascorbate, 0.2 mM H_2O_2 in 0.05 M potassium phosphate (pH 7.0) and 2 μg proteins from root extract. Catalase (CAT; EC 1.11.1.6) activity was determined according to Beers and Sizer (1952) by measuring the decrease in absorbance at 240 nm over a time period of 1 min. The reaction mixture contained 25 mM H_2O_2 in 0.05 M potassium phosphate (pH 7.0) and 20 μg proteins from root extract.

Total RNA was isolated from root segments using *Rneasy* plant kit (*Qiagen*, Germany) and cDNA was synthesized from DNase-treated total RNA with *Omniscript* RT kit (*Qiagen*) using oligo-dT-primer. Primers were prepared according to sequences published by Chen *et al*. (2010) for CAT1 and CAT2 and Bonsager (2010) for APX1 and APX2. Cycle numbers were optimized to assure that the amplification reaction was tested in the exponential phase and the gene product was detectable also in control roots. The expression of ubiquitin was used as an internal positive control. The PCR products were applied to 5 % PAGE and silver stained. After the densitometric analysis of three independent gels the relative transcript amount was expressed as a percentage of control (0 μM Cd represented 100 %).

The experiments were carried out in five independent series with three replicates. The data were analyzed by one-way analysis of variance (*ANOVA*), and the means were separated using Tukey's test.

Results

Significant root growth inhibition representing about one-third of the control root increment was observed after a relatively short-term (30 min) exposure of barley roots to low 15 μM Cd (Table 1). No root growth was observed during the first 6 h after treatment with 30 or 60 μM Cd. In seedlings treated with 30 μM Cd root growth was renewed approximately between 7 and 8 h. By contrast, seedlings exposed to 60 μM Cd failed to restore root growth. In addition to the Cd-induced root growth inhibition visible root swelling with well developed root hairs was detected within 6 h after 15 μM Cd short-term treatment (Fig. 1). Root swelling started immediately behind the root tip (0 - 3 mm) in root part representing meristem and elongation zone. Similarly to the root growth restoration, a longer recovery time was required for the swelling of root tips exposed to 30 μM Cd compared to 15 μM Cd treatment. In seedlings exposed to 60 μM Cd for 30 min the root swelling was not detected up to 9 h after Cd treatment. The analysis of Evans blue uptake by roots (Fig. 1) demonstrated that dispersed cell death was detectable already 6 h after short-term treatment with 30 μM Cd in the elongation zone of root tips and massive cell death was detected after 60 μM Cd treatment in the beginning of elongation zone and it

Table 1. Root length increments 6 and 9 h after short-term treatments with 0, 15, 30 or 60 μM concentration of Cd. Means ± SE, n = 30. Different letters indicate statistical significance according to Tukey's test ($P < 0.05$).

Cd [μM]	0	15	30	60
6 h	5.92 ± 0.42^{a}	1.74 ± 0.18^{b}	0.18 ± 0.08^{c}	0.04 ± 0.05^{c}
9 h	9.20 ± 0.56^{a}	3.76 ± 0.36^{b}	1.62 ± 0.24^{c}	0.08 ± 0.08^{d}

enlarged further with time along the root tips.

While, the activity of APX showed only a slight non-significant increase even 9 h after short-term treatment with 15 μM Cd, CAT activity increased significantly in the first segment behind the root tip containing meristem and elongation zone already 1 h after the end of short-term treatment (Table 2). In contrast to 15 μM Cd treatment, higher Cd concentrations inhibited CAT activity up to 3 h after treatment. However, 6 h after short-term treatment increased CAT activity was observed also in the case of 30 μM Cd, which increased further by time and was detectable also in roots treated with 60 μM Cd. In addition, 9 h after exposure to 60 μM Cd, a significant increase of APX activity was observed along the whole root tip.

Semiquantitative RT-PCR analysis (Fig. 2) revealed that APX1 transcript increased while APX2 decreased 3 h after short-term Cd-treatment in a concentration dependent manner. While the expression of CAT1 was upregulated after 15 and 30 μM Cd treatment, it was strongly downregulated by 60 μM of Cd. By contrast CAT2 was upregulated in both root segments in a concentration dependent manner.

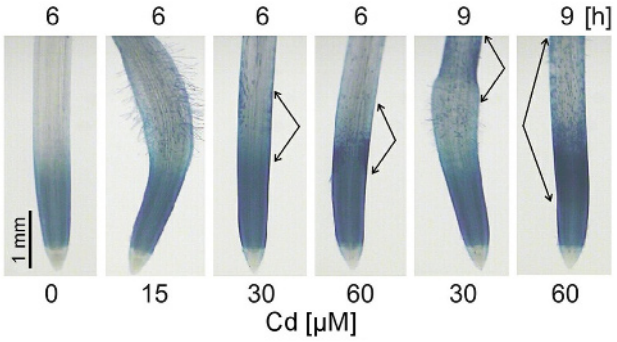

Fig. 1. Evans blue uptake 6 and 9 h after short-term treatment with 0, 15, 30 or 60 μM Cd in barley root tips. *Black arrows* indicate the area of Evans blue-stained cells.

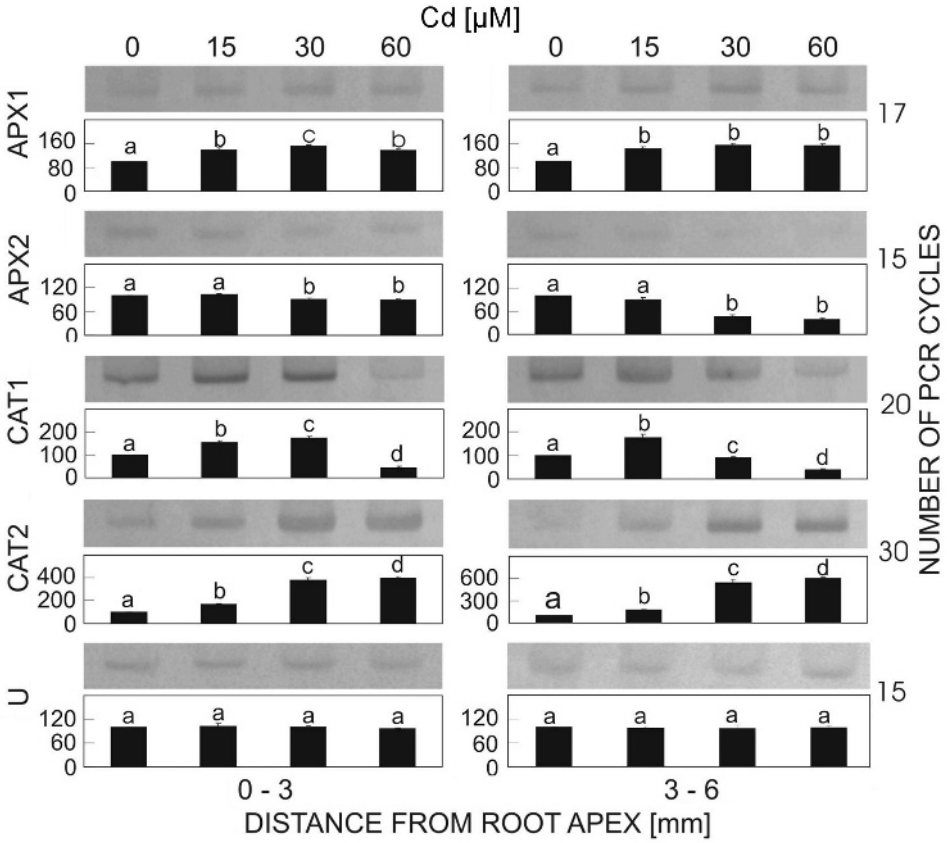

Fig. 2. Expression APX1, APX2, CAT1, CAT2 and ubiquitin (U) 3 h after short-term treatment with 0, 15, 30 or 60 μM Cd in the first and second 3 mm root segments behind the root apex. Representative gel from three independent experiments and their densitometric analysis. Relative transcript amount is expressed as a percentage of control (0 μM Cd) that represents 100 %. Different letters indicate statistical significance according to Tukey's test ($P < 0.05$).

Table 2. APX [ΔA_{290} mg^{-1}(f.m.) min^{-1}] and CAT [ΔA_{240} mg^{-1}(f.m.) min^{-1}] activities 1, 2, 3, 6 and 9 h after short-term treatment with 0, 15, 30 or 60 μM Cd in the first and second 3 mm root segments behind the root apex. Means ± SE, $n = 5$. Different letters indicate statistical significance according to Tukey's test ($P < 0.05$).

Cd [μM]		0		15		30		60	
Root [mm]		0 - 3	3 - 6	0 - 3	3 - 6	0 - 3	3 - 6	0 - 3	3 - 6
APX	1 h	0.208±0.014^{a}	0.107±0.013^{a}	0.209±0.029^{a}	0.097±0.007^{a}	0.225±0.019^{a}	0.105±0.008^{a}	0.193±0.026^{a}	0.096±0.011^{a}
	2 h	0.185±0.020^{a}	0.088±0.017^{a}	0.184±0.014^{a}	0.094±0.015^{a}	0.186±0.016^{a}	0.096±0.011^{a}	0.173±0.015^{a}	0.099±0.014^{a}
	3 h	0.177±0.041^{a}	0.105±0.019^{a}	0.183±0.028^{a}	0.100±0.014^{a}	0.185±0.017^{a}	0.105±0.013^{a}	0.189±0.020^{a}	0.101±0.011^{a}
	6 h	0.191±0.030^{a}	0.107±0.003^{a}	0.202±0.010^{a}	0.101±0.014^{a}	0.177±0.035^{a}	0.110±0.012^{a}	0.182±0.017^{a}	0.128±0.002^{b}
	9 h	0.170±0.006^{a}	0.102±0.012^{a}	0.176±0.006^{a}	0.096±0.007^{a}	0.162±0.009^{a}	0.104±0.014^{a}	0.199±0.011^{b}	0.145±0.008^{b}
CAT	1 h	0.118±0.005^{a}	0.072±0.007^{a}	0.145±0.007^{b}	0.069±0.009^{a}	0.108±0.007^{c}	0.063±0.004^{a}	0.090±0.009^{d}	0.064±0.007^{a}
	2 h	0.104±0.001^{a}	0.065±0.003^{a}	0.112±0.006^{b}	0.072±0.008^{a}	0.096±0.006^{c}	0.078±0.012^{a}	0.093±0.010^{c}	0.064±0.008^{a}
	3 h	0.109±0.014^{a}	0.063±0.008^{a}	0.114±0.010^{a}	0.061±0.001^{a}	0.087±0.011^{b}	0.054±0.009^{a}	0.074±0.008^{b}	0.055±0.007^{a}
	6 h	0.130±0.022^{ac}	0.066±0.002^{a}	0.167±0.008^{b}	0.094±0.009^{b}	0.153±0.015^{a}	0.101±0.011^{b}	0.114±0.019^{c}	0.075±0.013^{a}
	9 h	0.133±0.006^{a}	0.071±0.007^{a}	0.177±0.010^{b}	0.125±0.007^{b}	0.209±0.012^{b}	0.128±0.009^{b}	0.198±0.023^{b}	0.086±0.005^{c}

Discussion

After short-term treatment with 15 μM Cd, root growth restoration was very rapid and approximately after 7 - 8 h lag period root growth restoration was also recorded at 30 μM Cd treatment. This fact indicates that the mitotically active meristematic cells, mainly in the case of 15 μM Cd treatment were not irreversibly affected. Similar results were observed in cowpea roots during the recovery period after various metal treatments (Blamey *et al*. 2010). In some cases the swelling of root apexes with well developed root hairs was also a typical visible symptom indicating cell growth restoration. In contrast to 15 or 30 μM Cd treatments, after 60 μM treatment roots failed to regenerate their growth suggesting that this high Cd concentration caused lethal injuries also in the meristematic cells of root apex.

We have shown previously that the elevated production of both superoxide and H_2O_2 is a general and early response of barley roots to Cd exposure (Tamás *et al*. 2010). It is well known that superoxide radical rapidly inactivates CAT activity *in vitro* (Kono and Fridovich 1982). In pea leaves, high Cd concentration caused the depletion of several enzymes including CAT due to the increased protein oxidation (Romero-Puertas *et al*. 2002). CAT activity in *Arabidopsis thaliana* decreased in leaves with increasing concentration of Cd in nutrient solution (Skórzyńska-Polit *et al*. 2003/4). We showed early stimulation of CAT activity after short-term treatment with 15 μM Cd. This stimulation of CAT activity already 1 h after Cd treatment is crucial for ROS restriction in cells during Cd stress. Also 30 μM Cd induced the stimulation of CAT activity 6 h after short-term treatment, which was followed by the regeneration of root growth during subsequent hours. In contrast, at very high 60 μM Cd concentration this stress-defence reaction was activated only 9 h after Cd treatment. Probably this delayed antioxidant defence activation causing ROS-induced damages and cell death is responsible for root growth arrest or even root death. Similarly to our results, long-term exposure of Scots pine roots to high Cd concentration caused initial inhibition of CAT activity and complete inhibition of root growth in spite of a strong increase of both APX and CAT activity after prolonged incubation (Schützendübel *et al*. 2001). Smeets *et al*. (2008) have reported that 20 μM Cd is too toxic for *Arabidopsis thaliana* and that for the induction of coordinated defence response lower Cd concentration is required.

However, total APX activity was not affected up to 6 h even after the severe Cd treatments. The analysis of APX genes expression revealed that Cd has opposite effect on the APX1 and APX2 expression. Lower amount of APX1 and CAT2 transcripts in comparison to APX2 and CAT1 in unstressed root and their strong up-regulation during stresses suggests that barley APX1 and CAT2 genes have a crucial role mainly during stress conditions in roots. Barley seedlings with CAT deficiency showed necrotic lesions in leaves, reduced viability and incapacity to overcome stress conditions but may grow normally under unstressed condition (Acevedo *et al*. 2001). Barley CAT2 upregulation was also reported after the exposure of seedlings to the high ROS-generating auxin concentration suggesting that CAT1 plays a crucial role during basic antioxidant metabolism, while CAT2 functions in stress-related responses (Guan and Scandalios 2002). The induction of one of the isoforms of APX or CAT and the reduction of other isoforms is common during various stress conditions including heavy metals (Cuypers *et al*. 2011).

In conclusion, these results suggest that the increased CAT activity is crucial for the restoration of root growth after the moderate Cd stress while its inhibition at severe

Cd stress may lead to irreversible damages, that later cannot be corrected by the elevated activity of antioxidant enzymes. In addition we showed that already after short-term treatment the transcriptional activation of specific stress-related APX and CAT genes is a key component of barley root defence response to Cd.

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