

Depletion of extracellular calcium increases cadmium toxicity in barley root tip via enhanced Cd uptake-mediated superoxide generation and cell death

Ladislav Tamás¹ · Beáta Bočová¹ · Jana Huttová¹ · Igor Mistrík¹ · Veronika Zelinová¹

Received: 15 June 2016/Revised: 4 January 2017/Accepted: 4 January 2017/Published online: 12 January 2017
© Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków 2017

Abstract Whereas severe Cd stress (150 μM Cd) causes root growth arrest as a consequence of marked superoxide generation leading to extensive cell death in the root tips, mild Cd stress (15 μM Cd) evokes morphogenic responses, such as reduced root elongation and radial root expansion, resulting in shorter and thicker roots. Similar to the low Cd concentration-caused mild stress, treatment of roots with either Ba to remove exchangeable or EDTA to remove both exchangeable and tightly bound cations, including Ca and Mg, from the apoplast, induced root growth inhibition and swelling. However, pre-treatment of roots with Ba had a synergistic effect on the development of these mild Cd stress-induced morphogenic responses, but without the development of any other symptoms in the root tips. In turn, EDTA pre-treatment markedly increased the toxicity of Cd in barley root tips via enhanced Cd uptake-mediated superoxide generation, which evoked extensive cell death in the transition zone of root tips identically to the high Cd concentration-induced severe stress. While the mild stress-induced responses were alleviated by the inhibition of auxin signalling pathway, the severe stress-induced symptoms were prevented by Ca, but not Mg, supplementation or by the inhibition of Cd uptake into the root symplast. Therefore, the appropriate concentration of Ca in the

apoplast is crucial to prevent the rapid accumulation of Cd in the symplast, which above a certain threshold level leads to the huge superoxide generation and cell death.

Keywords Auxin signalling · Cadmium · Extracellular calcium · Reactive oxygen species · Root growth

Abbreviations

CW	Cell wall
GPX	Glutathione peroxidase
LOX	Lipoxygenase
PCIB	<i>p</i> -Chlorophenoxyisobutyric acid
ROS	Reactive oxygen species

Introduction

The main functions of root are anchoring the plant into the substrate and the uptake of water and inorganic nutrients. Some metals, including calcium (Ca), are essential nutrients required in relatively large amounts for all plants. By contrast, large group of metals, e.g., cadmium (Cd), are non-essential for plants since they have no biological function in plant organisms. Except for some soils derived from the specific rocks, the naturally occurring Cd concentration in the soils is very low (lower than 1 mg per kg of soil) and generally up to 2 mg Cd per kg of soil is non-toxic to most cultivated plants (He et al. 2005). However, due to various anthropogenic activities its concentration increases year by year over the world, exceeding several times this non-toxic level also in urban soils.

Cd is one of the most phytotoxic heavy metals affecting several morphogenic and metabolic processes in plants (Gallego et al. 2012). Since Cd enters the cells via essential

Communicated by G Klobus.

Electronic supplementary material The online version of this article (doi:10.1007/s11738-017-2350-8) contains supplementary material, which is available to authorized users.

✉ Ladislav Tamás
Ladislav.Tamas@savba.sk

¹ Institute of Botany, Slovak Academy of Sciences, Dúbravská cesta 9, 84523 Bratislava, Slovak Republic

metal carriers and channels, its uptake by roots is a very rapid process. In protoplasts prepared from wheat root tips, its transport to cytosol through the plasma membranes occurred immediately after the addition of a low concentration of Cd to the medium (Lindberg et al. 2004). In rice roots, less than 10 min was required for the transport of Cd from the medium to the xylem (Fujimaki et al. 2010). Nevertheless, the cell wall (CW) of the peripheral cell layers with rhizosphere as the outermost part of plant roots is the first in contact with the Cd (Lux et al. 2011; Ovečka and Takáč 2014). The role of root apoplast in the uptake of mineral nutrients is well documented (Sattelmacher 2001). Binding of essential cations to the negative charges of CW components leads to their rapid accumulation in the root apoplast. On the other hand, due to the high similarities between essential and some non-essential elements, CW can also rapidly adsorb several toxic metals from the soil. It has been estimated that the contribution of apoplast to the Cd uptake by roots is important just at low Cd concentrations (Redjala et al. 2009).

Ca is an essential macroelement playing a crucial role mainly as a signal messenger in cytosol regulating plant growth and development, as a cross-linker in CW determining its structural rigidity and as a stabilizer of membranes affecting their function and permeability (Hepler 2005). It has long been known that the optimal concentration of Ca in rooting medium is essential for root elongation. In addition, the growth inhibitory effect of various toxic metals may be alleviated by the supplementation of growth medium with a high concentration of Ca (Kinraide 1998). By contrast, Ca deficiency alone caused a marked growth reduction of plants and enhanced their sensitivity to Cd (Cho et al. 2012).

Recently, it has been shown that the Cd-induced inhibition of root growth as a common response of roots to excess of different toxic metals is associated with alteration in reactive oxygen species (ROS) and phytohormones level in roots (Chmielowska-Bak et al. 2014). Previous studies have indicated that the low Cd concentration-induced mild oxidative stress and root growth reorientation are associated with altered IAA level and/or signalling (Tamás et al. 2012). By contrast, the high Cd concentration-induced strong oxidative stress triggered an extensive cell death in the elongation zone of the root tip, causing growth arrest or even death of the whole root (Liptáková et al. 2012).

The aim of this study was to analyze the possible effect of apoplastic Ca depletion, using barium chloride (Ba) to remove exchangeable or EDTA to remove both exchangeable and tightly bound apoplastic cations (including Ca) from the apoplast, on short-term Cd treatment-induced morphogenic changes, IAA content, expression of auxin-regulated gene, enzymes activity, ROS production and cell death in barley root tip.

Materials and methods

Plant material and growth conditions

Growth conditions of germinating seeds and young seedlings between two sheets of filter papers were developed and described in our previous works (Liptáková et al. 2012; Tamás et al. 2012, 2014; Zelinová et al. 2014). Barley seeds (*Hordeum vulgare* L.) cv. Slaven (Plant Breeding Station, Hordeum Ltd., Sládkovičovo-Nový Dvor, Slovakia) were imbibed in distilled water for 15 min. These imbibed seeds were incubated in a horizontal position between two sheets of filter paper (density 110 g/m², Papírna Perštej, Czech Republic) moistened with distilled water in Petri dishes at 25 °C in darkness for 24 h. The uniformly germinating seeds were transferred into rectangle trays and arranged in rows between two sheets of filter paper. The trays were placed in a vertical position and moistened through the filter paper wick from the reservoir with distilled water. For short-term treatment, seedlings with approximately 4 cm long roots were used 60 h after the onset of seed imbibition.

Short-term treatments

The roots of seedlings were immersed into appropriate test solutions (pH 5.5), such as distilled water (dw; control) or 5, 50 or 100 mM BaCl₂ or 10, 20 or 40 mM EDTA, for 5 min with slight agitation followed by two 5 min washes in dw. After these pre-treatments, roots were immersed either into distilled water or into 15 or 150 μM CdCl₂ or 15 μM CdCl₂ containing either 50 μM LaCl₃ or MgCl₂ for 15 min. Following the rinse in dw, the roots were post-treated with immersion into dw or 5 mM CaCl₂ or 5 mM MgCl₂ or 50 μM p-chlorophenoxyisobutyric acid—PCIB (from 10 mM stock in ethanol) or 5 mM CaCl₂ and 50 μM PCIB for 5 min followed by a brief wash in dw (see Supplementary Fig. 1). After these short-term treatments, the seedlings were incubated between two sheets of filter paper saturated with distilled water in the vertically oriented trays as described above.

Root length measurement

Following the short-term treatments, the position of the longest root tip of each seedling was marked on the filter paper. After 6 h of incubation, the roots were excised at the position of marks and the length increment was measured after recording with a stereomicroscope (STM PRO BEL Photonics, Italy) using BEL micro-image analyzer. For root morphology analysis, the roots were stained with

0.05% toluidine blue for 10 min and after washing with distilled water were photographed with stereomicroscope.

Localization of peroxidase-dependent H₂O₂ production

Intact roots 0, 1, 3 and 6 h after the short-term treatments were used for the localization of peroxidase-dependent H₂O₂ production. The roots were immersed into the solution of 10 μM 2,7-dichlorodihydrofluorescein (DCF) diacetate (from 20 mM stock in DMSO) in 20 mM sodium phosphate buffer pH 6.0 for 30 min at 25 °C in darkness. After a brief rinse in distilled water, the fluorescence image of root tips was observed immediately (for no more than 5 min) using a fluorescence stereomicroscope (excitation 500 ± 20 nm; emission 535 ± 30 nm).

Localization of superoxide production

For visualization of superoxide, intact roots were stained with solution containing 1 mM NBT (nitro-blue tetrazolium chloride), 10 mM sodium azide and 20 mM sodium phosphate (pH 6.0) for 20 min in dark at room temperature. Then roots were washed with distilled water and photographed immediately with a stereomicroscope.

Localization of cell death

Intact roots were immersed into a solution of propidium iodide (PI; 3 μg/mL) for 60 min at room temperature. To remove the excess of PI, roots were washed in distilled water and fluorescence was recorded using a fluorescence stereomicroscope (excitation 545 ± 25 nm; emission 606 ± 70 nm).

IAA quantification

IAA from the root tips was extracted 3 h after the short-term treatments in a pre-cooled mortar with ice-cold 80% methanol containing 1 mM butylated hydroxytoluene (80 root tips/mL). After incubation for 30 min at 4 °C the extract was centrifuged at 12,000×g for 10 min. The supernatant was immediately passed through a C18 column (Chromabond, Macherey–Nagel) preconditioned with 80% methanol at 4 °C. After evaporation of methanol, samples were methylated using trimethylsilyldiazomethane in hexane (2/500 μL) at 42 °C for 30 min and diluted in 25 mM sodium phosphate buffer (pH 7.2) containing 15 mM NaCl. Quantification of IAA was performed by competitive enzyme-linked immunosorbent assay (IAA immunoassay kit Olchemin) according to the manufacturer's instructions.

Protein extraction and enzyme assays

The extract was prepared 6 h after the short-term treatments by grinding the root tips (3 mm) in 100 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA in a pre-chilled mortar-pestle. The homogenate was centrifuged at 12,000×g for 10 min and the amount of proteins in the supernatant was determined according to Bradford (1976) using bovine serum albumin as a standard.

Lipoxygenase (LOX, EC 1.13.11.12) activity was assayed according to the method of Anthon and Barrett (2001). The reaction mixture contained (160 μL in a final volume) 5 mM 3-dimethylaminobenzoic acid in 25 mM sodium phosphate buffer (pH 6.0), 1 mM KCN, 0.5 mM linoleic acid (from 25 mM stock solution dissolved in Tween 20) and 2 μg of proteins from the root extract. The mixture was incubated at 30 °C for 15 min then the mix of 20 μL of 1 mM 3-methyl-2-benzothiazolinone and 20 μL of hemoglobin (500 μg/mL) was added. After 5 min incubation at room temperature, the absorbance was recorded at 598 nm. Specific activities were expressed as ΔA₅₉₈/mg/min.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined by the method of Drotar et al. (1985) with some modifications. The reaction mixture (200 μL) contained 2 mM glutathione, 0.5 mM NADPH, 1 mM EDTA, 2 mM *t*-butyl hydroperoxide and 0.5 U of glutathione reductase in 100 mM sodium phosphate buffer, pH 7.0 and 20 μg of extracted proteins. Decrease in the absorbance, due to the NADPH oxidation, was measured at 340 nm for 20 min at 30 °C. Specific activities were expressed as ΔA₃₄₀/mg/min.

Semi-quantitative RT-PCR

The root tips (3 mm) cut off from the two longest roots 3 h after the short-term treatments were used for gene expression analysis. Material was homogenized in liquid nitrogen and total RNA was extracted using RNeasy Plant Kit (Qiagen). Two micrograms of DNase treated total RNA was used for cDNA synthesis [Omniscrypt RT Kit (Qiagen)] using anchored oligo-dT₍₂₃₎ primer. Primers for PCR reactions were prepared according to sequences (HarvEST35 unigene 19159 ABP1F: TCCTGTCGCTCA GAATAGA; ABP1R: CCAGACAAAGGGGAAGTTCA) published by Keisa et al. (2013). Number of cycles was optimized to ensure that the amplification reaction product was tested in the exponential phase. Ubiquitin was used as an internal control. The PCR products were analyzed by electrophoresis on 0.9% agarose gels using HydraGreen staining. Figure shows representative gels from three independent experiments.

Statistical analyses

The values are means of five independent experiments with three replicates (20 root tips for root length measurement, ROS and cell death localization, 40 root tips for enzyme analysis, 80 root tips for IAA and RT-PCR analysis per replicate). The data were analyzed by one-way analysis of variance (ANOVA test), and the means were separated using Tukey’s test.

Results

Transient exposure of roots to a low 15 μM Cd concentration (mild Cd stress) for 15 min markedly reduced, while a high 150 μM Cd concentration (severe Cd stress) completely inhibited root growth measured 6 h after this short-term treatment (Fig. 1a). Mild Cd stress-induced root growth reduction was accompanied by radial cell expansion in the root elongation zone (Fig. 1b). On the contrary, this reorientation of root growth was not observed at 150 μM Cd concentration. Ca post-treatment did not

influence the root growth reduction and radial expansion of 15 μM Cd-treated seedlings but renewed the root growth and caused a large visible root swelling in 150 μM Cd-treated seedlings. By contrast to Ca, the auxin signalling inhibitor *p*-chlorophenoxyisobutyric acid (PCIB) post-treatment strongly alleviated both low Cd concentration-induced root growth inhibition and swelling but did not reverse the high Cd concentration-triggered root growth arrest (Fig. 1). Following the co-treatment of seedlings with Ca and PCIB after the 150 μM Cd treatment, the rate of root growth inhibition was similar to that observed after the Ca post-treatment alone but without the radial expansion of root tips.

Pre-treatment of seedlings with 100 mM Ba, to remove exchangeable cations from the apoplast, caused a similar degree of root growth inhibition and radial root expansion like the application of 15 μM Cd (Fig. 2). Lower 50 mM

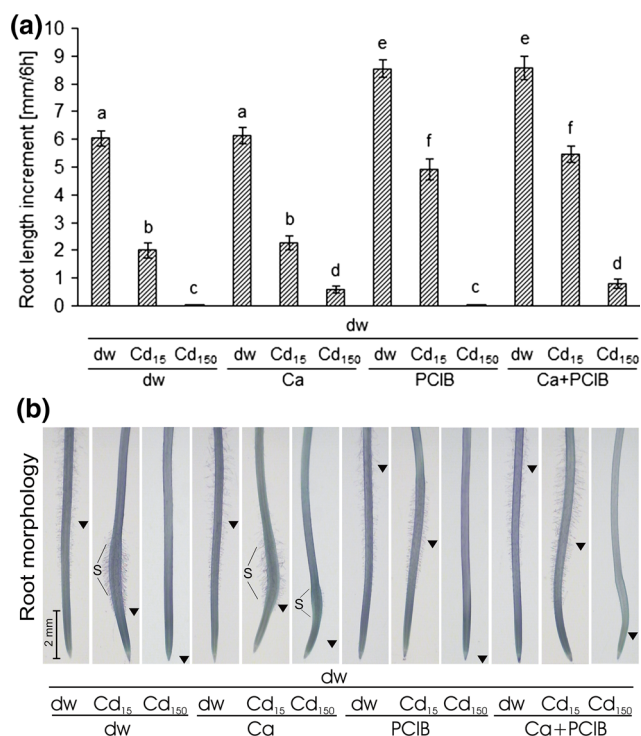


Fig. 1 Root length increments (a) and morphology (b) 6 h after the short-term treatment of roots with distilled water (dw), 15 or 150 μM Cd (15 min) in combination with dw pre-treatment (15 min) and dw, 5 mM Ca, 50 μM PCIB or 5 mM Ca and 50 μM PCIB post-treatment (5 min). The black triangles show the starting position of new root growth after the short-term treatments. S swollen root part. Mean values ± SD (n = 5). Different letters indicate statistical significance according to Tukey’s test (P < 0.05)

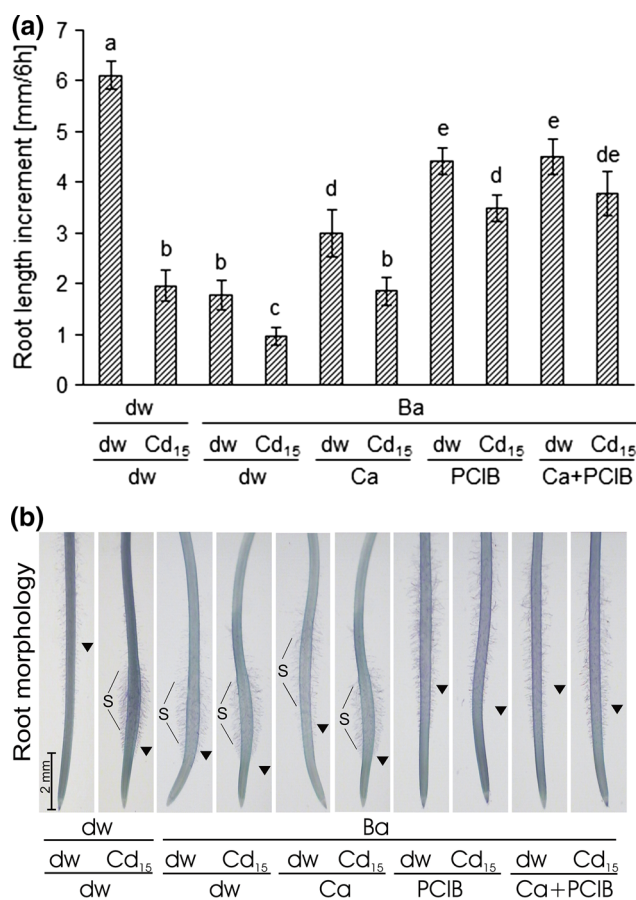


Fig. 2 Root length increments (a) and morphology (b) 6 h after the short-term treatment of roots with distilled water (dw) or 15 μM Cd (15 min) in combination with dw (15 min) or 100 mM Ba (5 min) followed by two 5 min washes in dw) pre-treatment and dw, 5 mM Ca, 50 μM PCIB or 5 mM Ca and 50 μM PCIB post-treatment (5 min). The black triangles show the starting position of new root growth after the short-term treatments. S swollen root part. Mean values ± SD (n = 5). Different letters indicate statistical significance according to Tukey’s test (P < 0.05)

Ba concentration also markedly inhibited root growth (root increment was 3 ± 0.47 mm 6 h after the treatment), while 5 mM Ba affected root growth only slightly (root increment was 5.5 ± 0.62 mm 6 h after the treatment; root increment of control roots was 6.0 ± 0.285 mm/6 h). In addition, Ba pre-treatment had an additive effect on Cd-induced root growth inhibition. In contrast to Cd exposure or to Cd exposure with Ba pre-treatment (where Ca post-treatment had no alleviating effect), Ca post-treatment significantly alleviated the Ba-induced root growth inhibition. However, it did not prevent the radial expansion of root cells. Nevertheless, PCIB post-treatment markedly reduced both Ba and Ba–Cd coupled treatment-induced root growth inhibition and swelling. The effect of Ca and PCIB co-treatment was identical to the post-treatment with PCIB alone.

The 20 mM concentration of EDTA (removes both exchangeable and tightly bound apoplastic cations, including Ca and Mg) was chosen because its short-term application caused a similar degree of root growth inhibition as 15 μ M Cd (Fig. 3a). In comparison, after the short-term treatment of roots with 10 mM EDTA, root growth increment was 3.1 ± 0.54 , while a higher 40 mM EDTA concentration inhibited root growth nearly completely. However, the radial expansion of root tip was not so pronounced as after Cd or Ba treatments (Figs. 2b, 3b). In contrast to the Ba pre-treatment, EDTA pre-treatment resulted in root growth arrest without the swelling of roots (Fig. 3). Ca post-treatment completely reversed this root growth arrest leading to reduced root growth with a typical but a slightly smaller radial cell expansion than after the treatment with 15 μ M Cd alone. In turn, post-treatment with PCIB did not renew the root growth in EDTA pre-treated and 15 μ M Cd-treated roots. Post-treatment with both Ca and PCIB had a similar effect on root growth like Ca alone but without the radial expansion of root tips. In addition, this co-application of Ca and PCIB slightly alleviated the EDTA-induced root growth inhibition (Fig. 3).

Analysis of ROS generation, using DCF (which reacts with the various forms of ROS, including H_2O_2 in the presence of peroxidases), revealed that their production increased immediately after the short-term treatment of roots (0 h) with a high concentration of Cd (150 μ M) or with a low concentration of Cd (15 μ M) coupled with EDTA pre-treatment in the central and proximal elongation and in the beginning of differentiation root zones in comparison with the control roots (Fig. 4a). This enhanced ROS generation was also detectable 1 h, but it decreased 3 h after these short-term treatments. In turn, in low Cd concentration-, Ba-, Ba followed by 15 μ M Cd-, and EDTA-treated roots this enhanced ROS generation was observed only 3 h after short-term treatment in the proximal elongation and in the beginning of differentiation

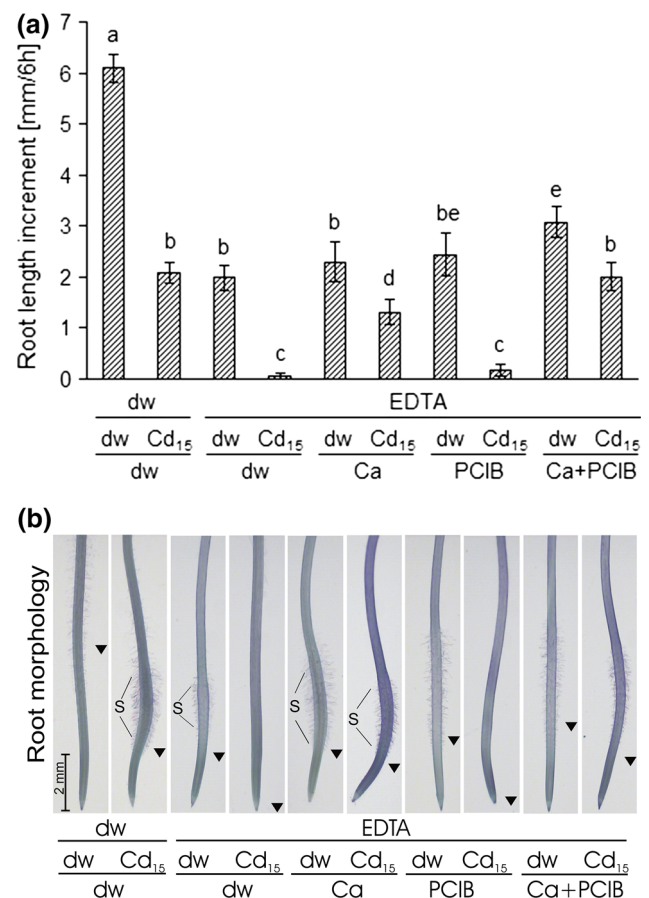


Fig. 3 Root length increments (a) and morphology (b) 6 h after the short-term treatment of roots with distilled water (dw) or 15 μ M Cd (15 min) in combination with dw (15 min) or 20 mM EDTA (5 min followed by two 5 min washes in dw) pre-treatment and dw, 5 mM Ca, 50 μ M PCIB or 5 mM Ca and 50 μ M PCIB post-treatment (5 min). The black triangles show the starting position of new root growth after the short-term treatments. S swollen root part. Mean values \pm SD ($n = 5$). Different letters indicate statistical significance according to Tukey's test ($P < 0.05$)

zones of the root tips. In turn, enhanced superoxide production was observed 1 and 3 h after the short-term treatment of roots with a high concentration of Cd or with a low concentration of Cd in combination with EDTA pre-treatment in the transition zone, approximately between 1 and 1.5 mm behind the root apex (Fig. 4b). This strong superoxide generation was accompanied by an extensive death of cells in this part of the root tip 6 h after these treatments (Fig. 4c).

DCF-detectable ROS generation, immediately (0 h) after the short-term treatment of roots with either a high Cd concentration or a low Cd concentration in combination with EDTA pre-treatment, was not reversed by the post-treatment of roots with Ca (Fig. 5a). By contrast, Ca post-treatment considerably alleviated the superoxide generation (Fig. 5b) and cell death (Fig. 5c) in roots exposed either to a high Cd concentration or to a low Cd

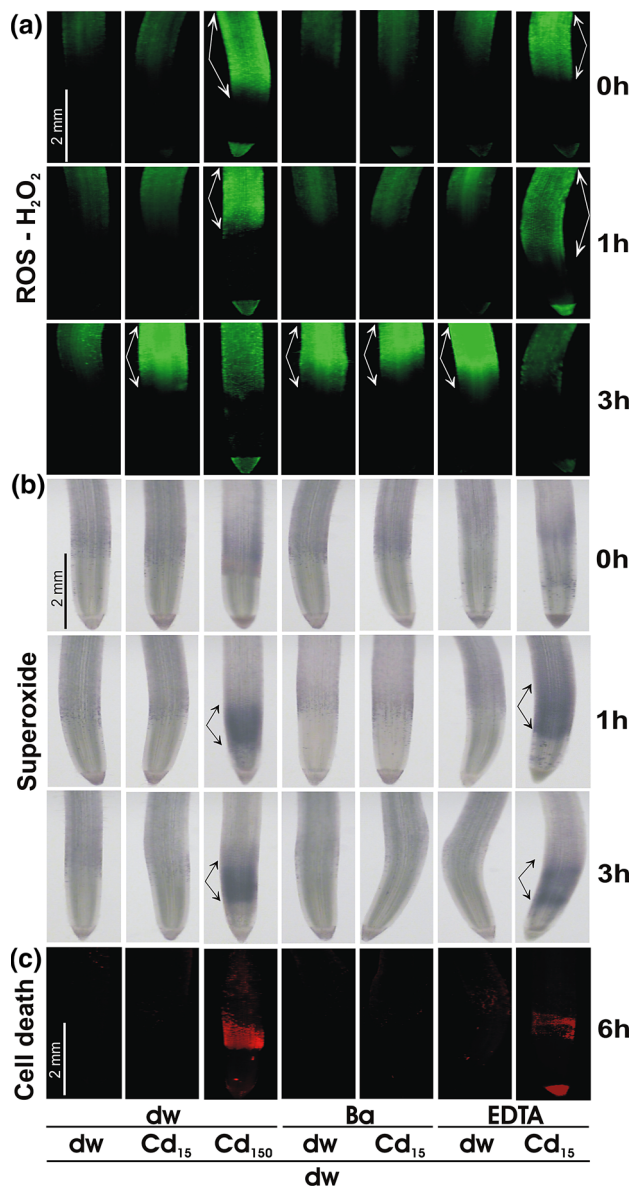


Fig. 4 Localization of ROS/H₂O₂ (a), superoxide (b) production and cell death (c) 0, 1, 3 and 6 h after the short-term treatment of roots with distilled water (dw), 15 or 150 μM Cd (15 min) in combination with dw (15 min), 100 mM Ba or 20 mM EDTA (5 min followed by two 5 min washes in dw) pre-treatment and dw post-treatment (5 min). The *arrows* indicate the area of enhanced ROS/H₂O₂ and superoxide production in comparison with control roots

concentration in combination with EDTA pre-treatment. This alleviating effect was specific to Ca because Mg, another dominant bivalent cation in the apoplast, could not evoke it. Due to this alleviating effect of Ca post-treatment on superoxide generation, an enhanced DCF-detectable ROS generation was observed 3 h after these treatments like in the roots treated with 15 μM Cd alone (without EDTA pre-treatment).

Both lipoxygenase (LOX) and glutathione peroxidase (GPX) activity were considerably increased after the

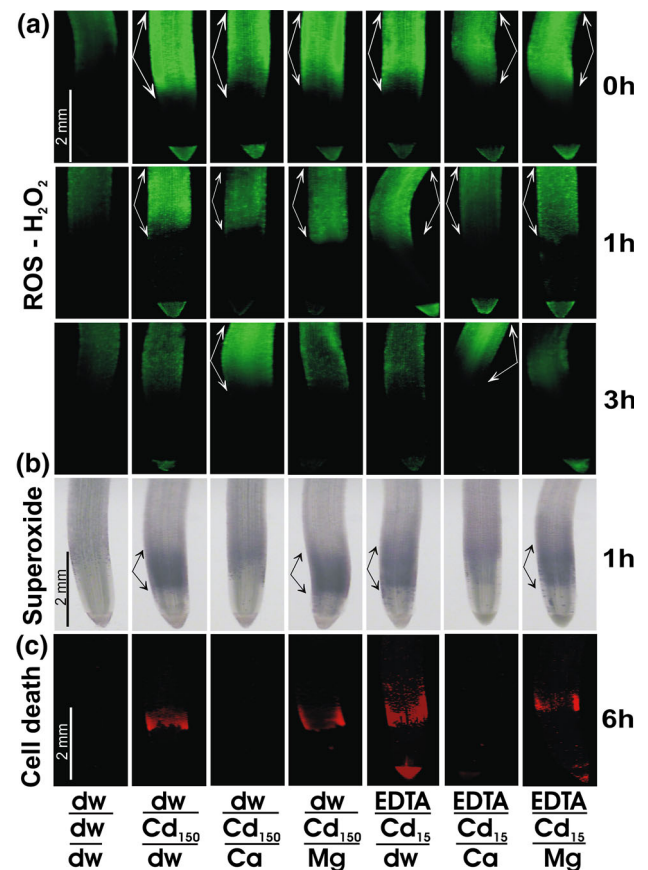


Fig. 5 Localization of ROS/H₂O₂ (a), superoxide (b) production and cell death (c) 0, 1, 3 and 6 h after the short-term treatment of roots with distilled water (dw), 15 or 150 μM Cd (15 min) in combination with dw (15 min) or 20 mM EDTA (5 min followed by two 5 min washes in dw) pre-treatment and dw, 5 mM Ca or 5 mM Mg post-treatment (5 min). The *arrows* indicate the area of enhanced ROS/H₂O₂ and superoxide production in comparison with control roots

transient exposure of roots to 15 μM Cd (Fig. 6). By contrast, Cd at a high concentration did not significantly affect their activity. While Ca post-treatment did not modify the low Cd concentration-induced LOX or GPX activity, it increased their activity in the 150 μM Cd-treated roots. Treatment of roots with Ba alone increased LOX activity but had no effect on GPX activity. In turn, Ba in combination with 15 μM Cd increased both LOX and GPX activity like the Cd treatment alone. Ca post-treatment did not affect their activity in combination with Ba pre-treatment. EDTA slightly increased only LOX activity, while its combination with 15 μM Cd treatment activated neither LOX nor GPX activity. On the other hand, Ca post-treatment markedly increased both LOX and GPX activity in the EDTA pre-treated and 15 μM Cd-treated roots in a similar manner as in the 150 μM Cd-treated roots.

In all cases, the alleviating effect of PCIB post-treatment on root growth inhibition and radial cell expansion (Figs. 2, 3) was associated with the reduction in ROS

generation in the proximal elongation and in the differentiation zones of the root tips to the control level (Fig. 7). Treatment with low Cd concentration and treatment of roots with either Ba or EDTA (also Ba or EDTA pre-treatment of 15 μM Cd-treated roots—data not shown) caused a marked accumulation of IAA in root tips (Fig. 8a). The expression level of gene encoding auxin

binding protein (ABP1) was upregulated under these treatments (Fig. 8b).

Ca channel inhibitor lanthanum (La) completely prevented the EDTA pre-treatment and the low Cd concentration treatment-induced root growth arrest and fully renewed the radial expansion of root tips (Fig. 9a). This effect was specific to La, because the application of Mg at an equal concentration as Ca, did not cause these alterations. Ca post-treatment further increased this alleviating effect of La. However, the most effective was the co-application of La with Ca and PCIB post-treatment resulting in the greatest alleviating effect on root growth inhibition and radial expansion of the low Cd concentration-treated Ca-depleted seedlings (Fig. 9b). This alleviating effect of La, but not Mg, was associated with a strong reduction in superoxide generation (Fig. 10a) and subsequent cell death (Fig. 10b) in the transition zone of these roots.

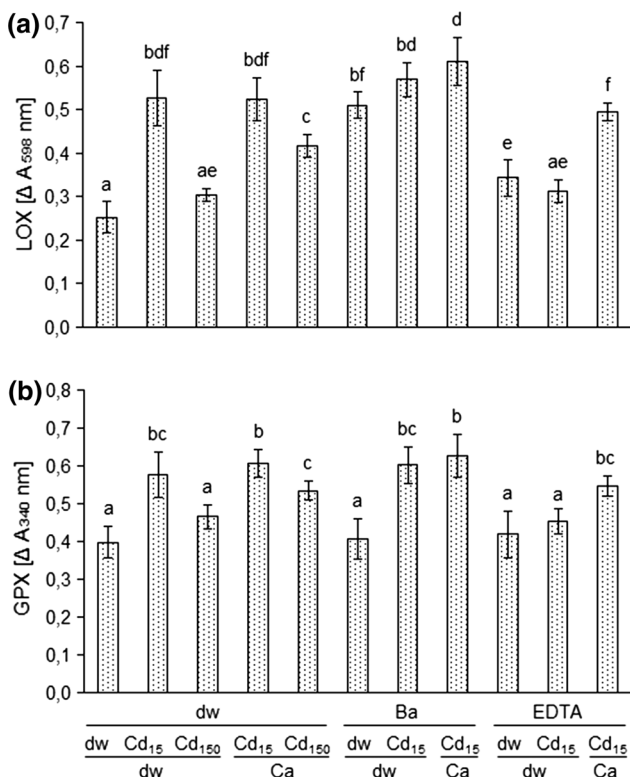


Fig. 6 LOX (a) and GPX (b) activity 6 h after the short-term treatment of roots with distilled water (dw), 15 or 150 μM Cd (15 min) in combination with dw (15 min), 100 mM Ba or 20 mM EDTA (5 min followed by two 5 min washes in dw) pre-treatment and dw or 5 mM Ca post-treatment (5 min). Mean values ± SD (*n* = 5). Different letters indicate statistical significance according to Tukey’s test (*P* < 0.05)

Discussion

Whereas severe Cd stress causes a marked superoxide generation, cell death and root growth arrest, mild Cd stress evokes morphogenic responses, such as reduced root elongation and radial root expansion, resulting in shorter and thicker root in comparison with control. In this study, we showed that root morphogenic responses induced by the treatment of seedlings with Ba to remove exchangeable and EDTA to remove both exchangeable and tightly bound cations, including Ca, from the apoplast were similar to the mild Cd stress-induced responses. However, in contrast to Ba pre-treatment, EDTA pre-treatment markedly increased the Cd toxicity in barley root tips.

These primary mild Cd stress-induced morphogenic responses are not specific to Cd, but they can be induced by various metals at appropriate concentrations as we have shown in our previous work (Zelinová et al. 2014). Increasing evidence indicates that Ca-pectate cycle,

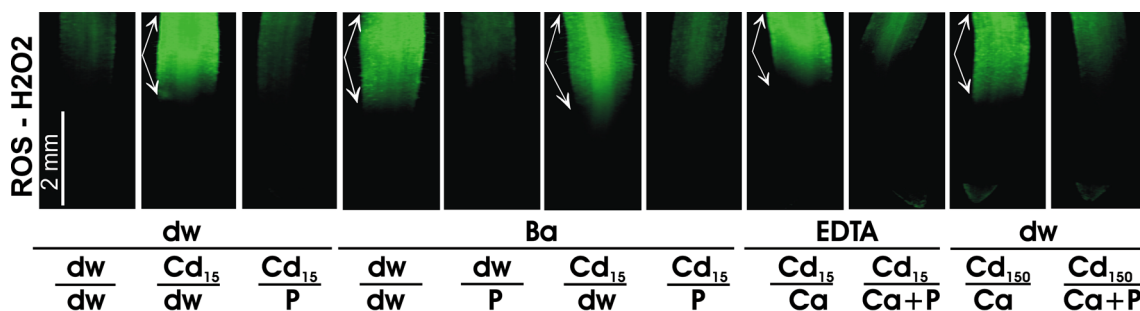


Fig. 7 Localization of ROS/H₂O₂ 3 h after the short-term treatment of roots with distilled water (dw), 15 or 150 μM Cd (15 min) in combination with dw (15 min), 100 mM Ba or 20 mM EDTA (5 min followed by two 5 min washes in dw) pre-treatment and dw, 50 μM

PCIB, 5 mM Ca or 5 mM Ca and 50 μM PCIB (P) post-treatment (5 min). The arrows indicate the area of enhanced ROS/H₂O₂ production in comparison with control roots

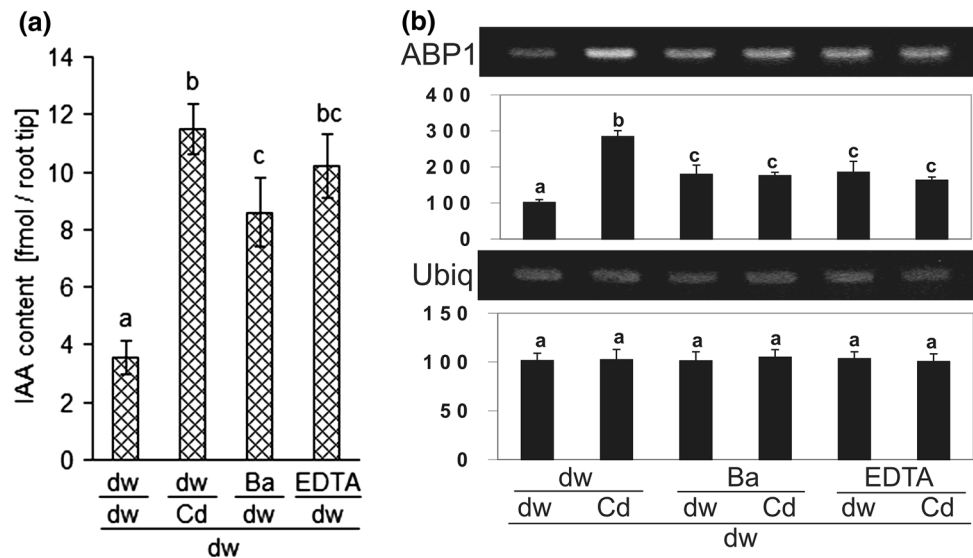


Fig. 8 IAA content (a) and gene expression (b) 3 h after the short-term treatment of roots with distilled water (dw) or 15 μ M Cd (15 min) in combination with dw (15 min), 100 mM Ba or 20 mM EDTA (5 min followed by two 5 min washes in dw) pre-treatment and dw post-treatment (5 min). Representative gel from five

independent experiments and their densitometric analysis. Relative transcript amount is expressed as a percentage of control (dw) that represents 100%. Mean values \pm SD ($n = 5$). Different letters indicate statistical significance according to Tukey's test ($P < 0.05$)

discovered in the green alga, also plays a key role in controlling CW extension rate in higher plants (Boyer 2009). It has been proposed that metal cations have a common toxic mechanism affecting the controlled relaxation of CW during cell elongation and their toxicity at least partially correlated with the strength of their binding to the carboxyl and thiol groups of the pectin matrix (Kopittke et al. 2014). In pectin matrix, strongly bound cations, such as Cu or Al, and also high concentrations of weakly bound cations, such as Ca or Zn, decreased the enzymatic degradability of CW leading to the inhibition of cell elongation (Horst 1995; Wehr et al. 2004). In our experiments, Ba at high concentration evokes similar responses to those observed at a low concentration of Cd. In addition, after Ba pre-treatment Cd at low concentration had only a synergistic effect on the development of morphogenic responses in root tips without the occurrence of other toxic symptoms. This interactive effect of several cations on root elongation, including Ba, was also observed in rice seedlings (Watanabe and Okada 2005). However, in contrast to mild Cd stress, Ba-induced root growth inhibition was significantly alleviated by Ca post-treatment. This suggests that beside the common effect of Cd and Ba on root growth, Cd even at a low concentration had an additional toxic influence on root growth, which was not reversed by Ca post-treatment.

Recently, it has been reported that changes in the pectin structure, including decreased or increased Ca cross-linkage, causing a disruption in the deposition of cellulose microfibrils lead to a marked cell swelling (Yoneda et al.

2010). Our results suggest that the radial expansion of root tips was not mediated directly by the binding of Ba or Cd to pectin or by the Ca deficiency in the case of EDTA treatment, but rather through the changes in the auxin homeostasis induced by these treatments. This hypothesis is supported by the observation that in all cases an elevated auxin level was detected in root tips and PCIB, an auxin signalling inhibitor, reversed the radial cell expansion induced by these treatments. In addition, *ABP1* expression was also upregulated in all treatments where the radial root expansion was observed. It has been recently observed that *ABP1* has a key regulatory function in the auxin-induced remodelling of CW leading to cell expansion (Paque et al. 2014). Numerous pioneer studies have demonstrated the role of Ca in auxin transport and action in plants. In sunflower stem section, the removal of Ca from the tissue by washing in EDTA markedly inhibited the basipetal auxin transport (Dela Fuente and Leopold 1973). Similarly, the application of Cd or Ba markedly inhibited the basipetal auxin transport in the root tip of maize (Hasenstein et al. 1988). In EDTA-treated barley seedlings, the radial expansion of root was less developed in comparison with Ba- or Cd-treated seedlings in spite of the elevated level of auxin; however, it was restored after the post-treatment of roots with Ca. Consistent with this observation, it was previously described in maize roots that rapid auxin action is strongly influenced by the appropriate concentration of extracellular Ca (Hasenstein and Evans 1986). Our recent results suggest that Cd-induced morphogenic responses are not prevented by the co-application of Ca rather at the

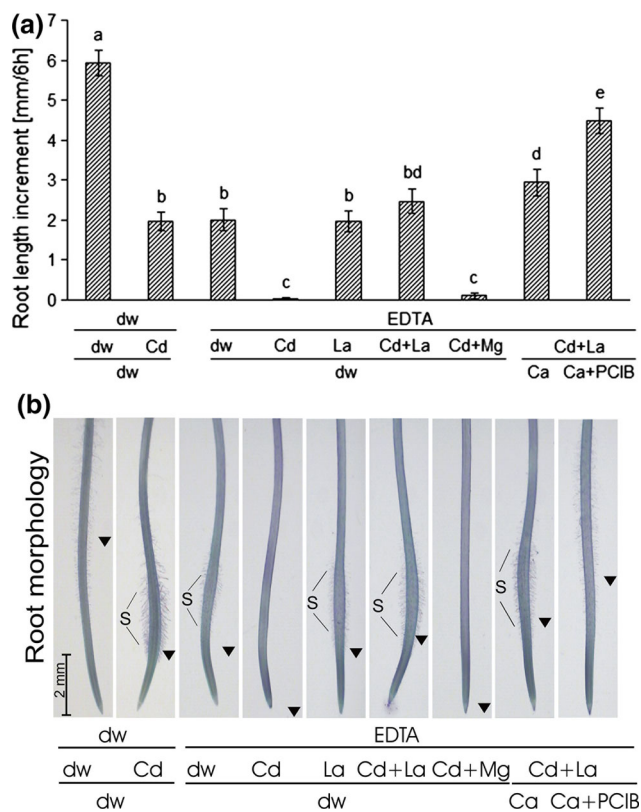


Fig. 9 Root length increments (a) and morphology (b) 6 h after the short-term treatment of roots with distilled water (dw), 15 μ M Cd, 50 μ M La, 15 μ M Cd and 50 μ M La or 15 μ M Cd and 50 μ M Mg (15 min) in combination with dw (15 min) or 20 mM EDTA (5 min followed by two 5 min washes in dw) pre-treatment and dw, 5 mM Ca or 5 mM Ca and 50 μ M PCIB post-treatment (5 min). The *black triangles* show the starting position of new root growth after the short-term treatments. *S* swollen root part. Mean values \pm SD ($n = 5$). Different letters indicate statistical significance according to Tukey's test ($P < 0.05$)

appropriate concentration Ca is required in the apoplast for their development and to prevent the more toxic effect of metals such as oxidative stress and cell death.

Many studies have shown that Cd-induced antioxidant enzymes activity either was alleviated or was not altered by the supplementation of Ca to the medium (Rodríguez-Serrano et al. 2009; Wang and Song 2009; Tian et al. 2011; Srivastava et al. 2015). In turn, in mustard seedlings, the Cd-induced enhanced activity of several antioxidant enzymes was further stimulated by Ca supplementation in spite of the reduced Cd accumulation (Ahmad et al. 2015). We showed that these contradictory results are probably associated with different level of Cd concentration/toxicity. In our experiments, Ca did not affect the low Cd concentration (moderate stress) induced GPX activity. By contrast, under severe stress, evoked either by the high concentration of Cd or by the low Cd concentration in combination with EDTA pre-treatment, Ca supplementation increased the activity of GPX. High concentration of

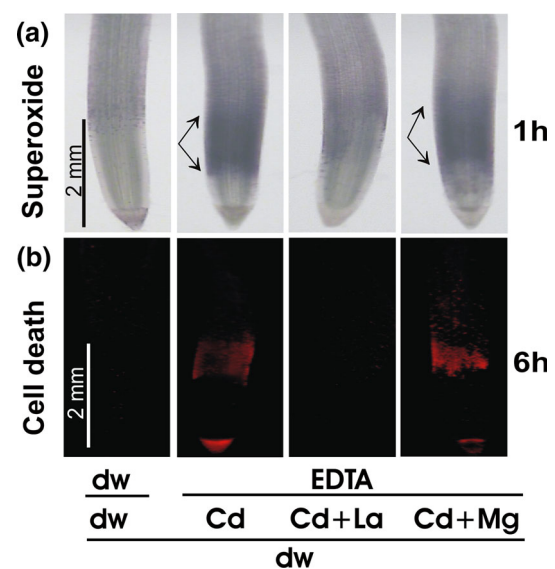


Fig. 10 Localization of superoxide production 1 h (a) and cell death 6 h (b) after the short-term treatment of roots with distilled water (dw), 15 μ M Cd, 15 μ M Cd and 50 μ M La or 15 μ M Cd and 50 μ M Mg (15 min) in combination with dw (15 min) or 20 mM EDTA (5 min followed by two 5 min washes in dw) pre-treatment and dw post-treatment (5 min). The *arrows* indicate the area of enhanced superoxide production in comparison with control roots

Cd caused drastic uncontrolled generation of ROS, which probably disturbs proper defense response, including the activation of antioxidant systems. In our previous work, we showed that alloxan-induced marked ROS production in the root tips blocked the mild Cd stress-induced morphogenic responses (Tamás et al. 2014). Similar to our results, a sublethal concentration of Cd induced radial expansion of *Arabidopsis* root tip cells, while a lethal concentration caused cell death in the elongation zone of root tip (Suzuki 2005).

EDTA pre-treatment removed both exchangeable and tightly bound apoplastic Ca, which had a detrimental effect on cell viability also at low Cd concentration due to the enhanced superoxide generation. Similar toxic symptoms were observed in roots exposed to a very high Cd concentration alone. Cd enters plant cell through the various transporters involved in the uptake of macro- and micronutrients. Plasma membrane Ca channels are permeable to Cd both in root (White 1998) and shoot (Perfus-Barbeoch et al. 2002); therefore, elevated apoplastic Ca may competitively reduce the uptake of Cd into cytoplasm. Alleviating effect of Ca on Cd toxicity, during the long-term co-treatment with the μ M Cd and mM Ca concentrations, is probably due to the reduced uptake of Cd by the root cells (Srivastava et al. 2015). Short-term analysis using radiotracers also confirmed a significant inhibition of Cd influx into the root cells caused by a high concentration of Ca (Lu et al. 2010). Our results showed that already a

low concentration of Cd in combination with EDTA-mediated depletion of Ca from the root apoplast caused a rapid and extensive superoxide generation in the symplasm. However, this high toxicity of low Cd concentration in the Ca-depleted roots was effectively eliminated by the application of Ca channel inhibitor La. These results indicate that the depletion of Ca from the root apoplast markedly accelerated the uptake of Cd into the symplasm. In agreement with these results, the long-term incubation evoked Ca deficiency resulting in an accelerated Cd uptake by root cells in rice seedlings (Cho et al. 2012). Similar results have recently been reported in Cu-treated grapevine seedlings where the long-term treatment of roots with a relative high concentration of Cu at the presence of high concentration of Ca caused a marked root growth inhibition, epidermal CW thickening and root tip swelling due to the cortical cell expansion, whereas at low Ca concentration these responses were restricted and cell deformations and plasmolysis were dominated (Chen et al. 2013). This accelerated Cu uptake in the Ca-depleted roots is associated with observation that in the absence of Ca, the Ca channels are permeable to both monovalent and divalent cations (White 1998). Probably, at the presence of high Cd concentrations, cells are exhausted from the Cd detoxification mechanisms leading to the unregulated ROS generation. In pea leaves, Cd-induced superoxide production was prevented by exogenous Ca, suggesting that enhanced ROS generation in these leaves, at least partially, is associated with the Cd-induced Ca deficiency (Rodríguez-Serrano et al. 2009). All these results suggest that high Cd concentration in the symplasm causes an uncontrolled ROS production, which is highly toxic and leads to cell and root death.

Numerous studies have analyzed the role of CW in the root response to heavy metals either as a target or as a barrier of their toxicity (for review see Parrotta et al. 2015). Our results suggest that the mild Cd stress-induced root growth reorientation is activated already after the binding of Cd to CW probably through the activation of auxin signalling pathway due to the disruption of Ca homeostasis in the apoplast. In bean, Cd-induced radial expansion of cortical cells was observed after the exposure of seedlings to a very low Cd concentration for several days, and Cd was localized mainly into the vacuoles of the outer cortical cells (Vázquez et al. 1992). These results suggest that the mild Cd stress-induced morphogenic and metabolic responses, including radial expansion of cortical cells with large vacuole and activation of antioxidant systems, are components of the defense mechanisms just preventing the accumulation of Cd and disturbance of ROS homeostasis in the symplasm, which above a certain threshold level leads to the huge superoxide generation and cell death. These morphogenic alterations and also the activation of LOX

and GPX were not reversed by Ca post-treatment. In turn, the alleviating effect of Ca post-treatment was evident in all cases where the severe Cd stress-induced superoxide generation and cell death were observed as a toxic symptom of Cd due to the rapid accumulation of Cd in the cytoplasm. Therefore, the appropriate concentration of Ca in the apoplast is crucial to prevent the rapid accumulation of Cd in the symplasm, which above a certain threshold level leads to the huge superoxide generation and cell death.

Author contribution statement VZ and JH carried out the preparation and treatment of seedlings and the analysis of enzymes activity. BB carried out the measurements of root growth and the analysis of IAA content. LT and IM participated in the localization of ROS generation and cell death and in the RT-PCR analysis. LT wrote the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements This work was supported by the Grant Agency VEGA, Project No. 2/0039/16. The authors also thank the anonymous reviewers for their helpful criticisms, which improved the manuscript.

References

- Ahmad P, Sarwat M, Bhat NA, Wani MR, Kazi AG, Tran LP (2015) Alleviation of cadmium toxicity in *Brassica juncea* L. (Czern. & Coss.) by calcium application involves various physiological and biochemical strategies. *PLoS One* 10:e0114571
- Anthony GE, Barrett DM (2001) Colorimetric method for the determination of lipoxygenase activity. *J Agric Food Chem* 49:32–37
- Boyer JS (2009) Cell wall biosynthesis and the molecular mechanism of plant enlargement. *Func Plant Biol* 36:383–394
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Chen P-Y, Lee Y-I, Chen B-C, Juang K-W (2013) Effects of calcium on rhizotoxicity and the accumulation and translocation of copper by grapevines. *Plant Physiol Biochem* 73:375–382
- Chmielowska-Bak J, Gzyl J, Rucińska-Sobkowiak R, Arasimowicz-Jelonek M, Deckert J (2014) The new insights into cadmium sensing. *Front Plant Sci* 5:245
- Cho S-C, Chao Y-Y, Kao CH (2012) Calcium deficiency increases Cd toxicity and Ca is required for heat-shock induced Cd tolerance in rice seedlings. *J Plant Physiol* 169:892–898
- Dela Fuente RK, Leopold AC (1973) A role for calcium in auxin transport. *Plant Physiol* 51:845–847
- Drotar A, Phelps P, Fall R (1985) Evidence for glutathione peroxidase activities in cultured plant cells. *Plant Sci* 42:35–40
- Fujimaki S, Suzui N, Ishioka NS, Kawachi N, Ito S, Chino M, Nakamura S (2010) Tracing cadmium from culture to spikelet: noninvasive imaging and quantitative characterization of absorption, transport, and accumulation of cadmium in an intact rice plant. *Plant Physiol* 152:1796–1806
- Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, Iannone MF, Rosales EP, Zawoznik MS, Groppa MD, Benavides MP (2012) Unravelling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. *Environ Exp Bot* 83:33–46

- Hasenstein K-H, Evans ML (1986) Calcium dependence of rapid auxin action in maize roots. *Plant Physiol* 81:439–443
- Hasenstein K-H, Evans ML, Stinemetz CL, Moore R, Fondren WM, Koon EC, Higby MA, Smucker AJM (1988) Comparative effectiveness of metal ions in inducing curvature of primary roots of *Zea mays*. *Plant Physiol* 86:885–889
- He ZL, Yang XE, Stoffella PJ (2005) Trace elements in agroecosystems and impacts on the environment. *J Trace Elem Med Biol* 19:125–140
- Hepler PK (2005) Calcium: a central regulator of plant growth and development. *Plant Cell* 17:2142–2155
- Horst WJ (1995) The role of the apoplast in aluminium toxicity and resistance of higher plants: a review. *Z Pflanzenernähr Bodenk* 158:419–428
- Keisa A, Nakurte I, Kunga L, Kale L, Rostoks N (2013) Increased auxin content and altered auxin response in barley necrotic mutant *necl1*. In: Zhang G, Li C, Liu X (eds) *Advance in barley sciences. Proceedings of 11th international barley genetics symposium*, Springer, New York, pp 229–241
- Kinraide TB (1998) Three mechanisms for the calcium alleviation of mineral toxicities. *Plant Physiol* 118:513–520
- Kopittke PM, Menzies NW, Wang P, McKenna BA, Wehr JB, Lombi E, Kinraide TB, Blamey FPC (2014) The rhizotoxicity of metal cations is related to their strength of binding to hard ligands. *Environ Toxicol Chem* 33:268–277
- Lindberg S, Landberg T, Greger M (2004) A new method to detect cadmium uptake in protoplasts. *Planta* 219:526–532
- Liptáková L, Bočová B, Huttová J, Mistrík I, Tamás L (2012) Superoxide production induced by short-term exposure of barley roots to cadmium, auxin, alloxan and sodium dodecyl sulfate. *Plant Cell Rep* 31:2189–2197
- Lu L, Tian S, Zhang M, Zhang J, Yang X, Jiang H (2010) The role of Ca pathway in Cd uptake and translocation by the hyperaccumulator *Sedum alfredii*. *J Hazard Mater* 183:22–28
- Lux A, Martinka M, Vaculík M, White PJ (2011) Root responses to cadmium in the rhizosphere: a review. *J Exp Bot* 62:21–37
- Ovečka M, Takáč T (2014) Managing heavy metal toxicity stress in plants: biological and biotechnological tools. *Biotech Adv* 32:73–86
- Paque S, Mouille G, Grandont L, Alabadí D, Gaertner C, Goyallon A, Muller P, Primard-Brisset C, Sormani R, Blázquez MA, Perrot-Rechenmann C (2014) Auxin binding protein 1 links cell wall remodeling, auxin signaling, and cell expansion in *Arabidopsis*. *Plant Cell* 26:280–295
- Parrotta L, Guerriero G, Sergeant K, Cai G, Hausman J-F (2015) Target or barrier? The cell wall of early- and later-diverging plants vs cadmium toxicity: differences in the response mechanisms. *Front Plant Sci* 6:133
- Perfus-Barbeoch L, Leonhardt N, Vavasseur A, Forestier C (2002) Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. *Plant J* 32:539–548
- Redjala T, Sterckeman T, Morel JL (2009) Cadmium uptake by roots: contribution of apoplast and of high- and low-affinity membrane transport systems. *Environ Exp Bot* 67:235–242
- Rodríguez-Serrano M, Romero-Puertas MC, Pazmiño DM, Testillano PS, Risueño MC, del Río LA, Sandalio LM (2009) Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium. *Plant Physiol* 150:229–243
- Sattelmacher B (2001) The apoplast and its significance for plant mineral nutrition. *New Phytol* 149:167–192
- Srivastava RK, Pandey P, Rajpoot R, Rani A, Gautam A, Dubey RS (2015) Exogenous application of calcium and silica alleviates cadmium toxicity by suppressing oxidative damage in rice seedlings. *Protoplasma* 252:959–975
- Suzuki N (2005) Alleviation by calcium of cadmium-induced root growth inhibition in *Arabidopsis* seedlings. *Plant Biotechnol* 22:19–25
- Tamás L, Bočová B, Huttová J, Liptáková L, Mistrík I, Valentovičová K, Zelinová V (2012) Impact of the auxin signaling inhibitor p-chlorophenoxyisobutyric acid on short-term Cd-induced hydrogen peroxide production and growth response in barley root tip. *J Plant Physiol* 169:1375–1381
- Tamás L, Mistrík I, Alemayehu A (2014) Low Cd concentration-activated morphogenic defence responses are inhibited by high Cd concentration-induced toxic superoxide generation in barley root tip. *Planta* 239:1003–1013
- Tian S, Lu L, Zhang J, Wang K, Brown P, He Z, Liang J, Yang X (2011) Calcium protects roots of *Sedum alfredii* H. against cadmium-induced oxidative stress. *Chemosphere* 84:63–69
- Vázquez MD, Poschenrieder Ch, Barceló J (1992) Ultrastructural effects and localization of low cadmium concentrations in bean roots. *New Phytol* 120:215–226
- Wang CQ, Song H (2009) Calcium protects *Trifolium repens* L. seedlings against cadmium stress. *Plant Cell Rep* 28:1341–1349
- Watanabe T, Okada K (2005) Interactive effects of Al, Ca and other cations on root elongation of rice cultivars under low pH. *Ann Bot* 95:379–385
- Wehr JB, Menzies NW, Blamey FPC (2004) Inhibition of cell-wall autolysis and pectin degradation by cations. *Plant Physiol Biochem* 42:485–492
- White PJ (1998) Calcium channels in the plasma membrane of root cells. *Ann Bot* 81:173–183
- Yoneda A, Ito T, Higaki T, Kutsuna N, Saito T, Ishimizu T, Osada H, Hasezawa S, Matsui M, Demura T (2010) Cobtorin target analysis reveals that pectin functions in the deposition of cellulose microfibrils in parallel with cortical microtubules. *Plant J* 64:657–667
- Zelinová V, Alemayehu A, Bočová B, Huttová J, Mistrík I, Tamás L (2014) Primary stress response induced by different elements is mediated through auxin signalling in barley root tip. *Acta Physiol Plant* 36:2935–2946