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Enhanced lipoxygenase activity is involved in the stress response but not in the harmful lipid peroxidation and cell death of short-term cadmium-treated barley root tip

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ABSTRACT

Root growth inhibition and radial root swelling were the characteristic symptoms of barley root tips after the short-term exposure of roots to 15 and 30 μM Cd. Higher Cd concentrations caused extensive cell death and root growth arrest. Enhanced lipid peroxidation was observed as early as 1 h after the short-term treatment in a Cd concentration-dependent manner. In contrast to lipid peroxidation, the induction of lipoxygenase activity was detected only 3 h after the exposure of roots to 15 or 30 μM Cd. In addition, it was not observed in 60 μM Cd-treated root tips. The highest lipoxygenase activity was detected 6 h after 15 μM Cd treatment in the meristematic and elongation zone of root tip and was probably associated with the radial expansion of cells. Our results indicate that the upregulation of lipoxygenase is an important component of stress response in barley roots to toxic Cd. It is probably involved in the morphological stress response of root tips or/and in the alleviation of Cd-induced toxic alterations in plant cell membranes, but it is not responsible for the Cd-induced harmful lipid peroxidation and cell death.

Introduction

Heavy metals are natural constituents of the Earth's crust including the biosphere. Some of them are essential for life and toxic only when a certain threshold of internal concentrations is exceeded, while others are non-essential and toxic even at very low concentrations (Appenroth, 2010). Due to the various anthropogenic activities, mainly the agriculture and chemical industries, soil pollution by heavy metals, including cadmium (Cd), increases year by year all over the world, affecting not only crop production, but also breeding and human health (Pan et al., 2010).

Cd is a non-essential metal for plants with the exception of marine diatoms, organisms highly specialized to low zinc concentrations in marine environments, where the Cd-containing enzyme carbonic anhydrase was characterized (Lane and Morel, 2000). It is one of the most toxic heavy metals due to its great solubility in water and high toxicity even at very low concentrations in many plant species. Cd has been reported to interfere with several processes both at the cellular and tissue levels, but a growing body of evidence indicates that the Cd-induced elevated generation of reactive oxygen species (ROS) in cells plays a pivotal role in the development of Cd toxicity symptoms, including root growth inhibition (Benavides et al., 2005). Root growth inhibition is the most general syndrome of various heavy metal-induced responses. In addition, only a few minute-long exposures of roots to Cd were sufficient for inhibition of root growth in bean and barley (Maksymiec, 2011; Bočová et al., 2012). Mildly elevated ROS levels signal unfavorable conditions for cells and activate different antioxidant defense responses, while marked ROS accumulation causes cell damage such as protein oxidation and lipid peroxidation, leading even to cell death (Sharma and Dietz, 2009). Cd as a non-redox-active metal causes enhanced accumulation of ROS indirectly, either through the inhibition of antioxidant systems or through the activation of ROS-generating enzymes. Cd-induced activation of NADPH oxidase, peroxidase and oxalate oxidase as ROS-generating enzymes is well documented in barley and also in other plant species (Tamás et al., 2010; Cuypers et al., 2011). In addition, many studies have also reported the connection between the increased activity of lipoxygenase (LOX) and enhanced lipid peroxidation in the presence of excess heavy metals in plant tissues (Aravind and Prasad, 2003; Zhou et al., 2008; Smeets et al., 2009).

Lipoxygenases are ubiquitously distributed enzymes among eukaryotic organisms that catalyze the oxygenation of polyunsaturated fatty acids (Hildebrand, 1989; Brash, 1999). LOX catalyzes the first reaction in the synthesis of compounds derived from polyunsaturated fatty acids collectively called oxylipins (Feussner and Wasternack, 2002). In barley, three isoforms have been described,

Abbreviations: LOX, lipoxygenase; ROS, reactive oxygen species.

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including LOX-1, which is present in quiescent grains. LOX-2 is a germination associated LOX isoform. However, in mature plants, its distribution is similar to LOX-1, with the highest activity in leaves and roots (Holtman et al., 1996). The LOX-3 isoform was detected only after germination as with LOX-2, but in mature vegetative tissues, LOX-3 was present only at a low level (van Mechelen et al., 1999). LOX activity has been detected in all plant developmental processes, including seed development, germination, vegetative growth, wounding and senescence (Porta and Rocha-Sosa, 2002). In addition, increased LOX activity in different tissues during various stress conditions suggests its function in the stress response of plants (Leone et al., 2001).

The aim of the present study was to examine the possible role of LOX activity along the longitudinal axis of barley root tips during Cd treatment and its association with Cd-induced enhanced lipid peroxidation, root growth inhibition, root swelling and cell death.

Materials and methods

Plant material and growth conditions

Barley seeds (*Hordeum vulgare* L.) cv. Slaven (Plant Breeding Station – Hordeum Ltd Sládkovičovo-Nový Dvor) were imbibed in distilled water for 15 min followed by germination between two sheets of filter paper moistened with distilled water in Petri dishes at 25 °C in darkness. 24 h after the onset of seed imbibition, the uniformly germinating seeds were arranged in a row between two sheets of filter paper moistened with distilled water in rectangle trays. Trays were placed into a nearly vertical position to enable downward radical growth. Continuous moisture of filter papers was supplied from a reservoir with distilled water through the filter paper wick. 60 h after the onset of seed imbibition, seedlings, with approximately 4 cm long roots, were used for treatments. Roots of seedlings were immersed in distilled water (control); 15, 30 or 60 μM $CdCl_2$ for 30 min. After washing in distilled water for 5 min, the seedlings were incubated between two sheets of filter paper moistened with distilled water as described above. After 1, 3, 6 or 9 h of incubation after short-term treatments, the individual barley root segments were obtained by the gradual cutting of each root from the tip to the base. Under control conditions, the first segment represents the meristem, the second root segment the elongation zone, and the third segment the beginning of differentiation zone (0–1, 1–3 and 3–6 mm) behind the root apex. The whole root tip represents the root segment from 0 to 6 mm behind the root apex.

Root length measurement

For the determination of root length, the position of root tips following the treatments was marked on the filter paper. After 6 or 9 h, roots were excised at the position of marks and the length was measured after recording with stereomicroscope (STMPRO BELPhotonics, Italy) using a BEL micro image analyzer.

Protein extraction and sample preparation

The root segments were homogenized in a pre-cooled mortar with 100 mM potassium phosphate extraction buffer pH 7.8 containing 1 mM EDTA. After centrifugation at 12,000 × *g* for 10 min, supernatants were desalted and concentrated by ultrafiltration using cellulose membrane (Amicon Ultra, USA). Proteins were quantified with bovine serum albumin as the calibration standard by the method of Bradford (1976).

Enzyme assays

Lipoxygenase – LOX (EC 1.13.11.12) activity was measured using the colorimetric method according to Anthon and Barrett (2001). The reaction mixture contained in a final volume of 110 μL 5 mM 3-dimethylaminobenzoic acid in 25 mM sodium phosphate buffer pH 6.0, 0.5 mM linoleic or linolenic acid (from 25 mM stock solution dissolved in Tween 20) and 2 μg of proteins from the root extract in the case of linoleic or 5 μg of proteins from the root extract in the case of linolenic acid. The mixture was incubated at 30 °C for 15 or 30 min (for linoleic or linolenic acid respectively), 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 measured at 598 nm.

Gel electrophoresis and LOX activity staining

The proteins (50 μg in the case of whole root tips or 100 μg in the case of root segments containing meristematic and elongation zones respectively) were separated under non-denaturing conditions on 6% slab polyacrylamide gels using the discontinuous buffer system (Laemmli, 1970). After brief washing in distilled water, the gels were immersed into the cold 100 mM sodium phosphate buffer pH 5.8 containing 1 mM KCN for 20 min. The LOX activity was detected by the incubation of gel in the mix of 40 ml of 100 mM sodium phosphate buffer pH 5.8 containing 1.0 mM KCN and 0.5 mM linoleic or linolenic acid (from 25 mM stock solution dissolved in Tween 20) and 10 mg o-dianisidine in 10 mL of ethanol overnight at 30 °C (Park and Polacco, 1989). Light opaque yellow bands were intensified by immersing the gel into 0.005% toluidine blue for 1–2 min, obtaining dark blue bands.

Determination of lipid peroxidation

Lipid peroxidation was determined by measuring the amount of malondialdehyde using TBA – thiobarbituric acid. Barley root segments (80 pieces/500 μL) were excised into 10% trichloroacetic acid containing 0.5% TBA and 0.5% Triton X100 and homogenized using a cold pestle and mortar. After incubation for 30 min at 95 °C, the homogenate was quickly cooled on ice and centrifuged at 12,000 × *g* for 5 min in a cold rotor. The absorbance of the supernatant (250 μL) was measured at 532 and 600 nm for correction.

Determination of cell viability

The loss of cell viability was evaluated using the Evans blue staining method. Freshly harvested roots were stained with 0.25% (v/v) aqueous solution of Evans blue for 15 min. After washing with distilled water (3 × 5 min), root segments (20 pieces/500 μL) were excised and homogenized in extraction solution containing 50% methanol and 1% SDS. After incubation at 80 °C for 15 min, extracts were centrifuged at 12,000 × *g* for 5 min. The optical density of released Evans blue was measured in the supernatant (250 μL) spectrophotometrically at 600 nm.

Statistical analyses

The experiments were carried out in five independent series. The significance of differences between control and heavy metal treatments was analyzed using the Student's *t*-test.

Results

Considerable root growth inhibition was observed 6 h after a relatively short-term (30 min) exposure of barley roots to a low 15 μM Cd concentration in comparison with the control roots (Fig. 1).