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Chemosphere 64 (2006) 1695–1703

CHEMOSPHERE

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Uptake and toxicity of Cr(III) in celery seedlings

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Received 14 September 2005; received in revised form 22 December 2005; accepted 2 January 2006
Available online 14 February 2006

Abstract

The present study shows that in celery Cr(III) induces deleterious effects on seedling development and morphology, and a number of metabolic responses related to stress. Exogenous $CrCl_3$ from 0.01 to 1 mM increasingly inhibited seed germination and hypocotyl elongation, or completely blocked it (10 mM), while the root apparatus was dramatically damaged even at the lowest dose. Seedlings took up exogenous Cr(III) in a dose-dependent manner, roots being the site of major metal accumulation; translocation towards the hypocotyl and cotyledonary leaves was also detected. Either total or chlorophyll *a* content was significantly reduced by chromium as low as 0.01 mM. A large accumulation of free and, to a lesser extent, conjugated polyamines occurred in all segments of treated plants. A dose-dependent relationship linking actual amounts of Cr(III) recovered in the entire seedling or organ and the respective polyamine titre was evidenced. Free putrescine, in particular, was the polyamine exhibiting the highest rate of increase, and cotyledonary leaves the organ where the major response occurred. A marked increase in ubiquitin–protein conjugates after Cr(III) treatment was also observed, particularly in roots. Thus, the study suggests for the first time a possible relationship between ubiquitination and Cr(III)-stress. The putative function of polyamines as a stress response, and the recruitment of the ubiquitin pathway to remove damaged or aberrant proteins which might have been produced in metal-treated seedlings are discussed.

Keywords: Celery; Chromium; Polyamines; Stress; Ubiquitin

1. Introduction

Heavy metal contamination is one of the major environmental stresses that affect plant metabolism, and their toxic levels in soils are the result of heavy traffic, mining, industrial and agricultural activity. Due to its widespread industrial use, chromium (Cr) has become a serious pollutant of soil and aquatic bodies (Zayed and Terry, 2003) where is released mainly from leather tanning, textile and electroplating industries (Stern, 1982). Although it is able to exist in several oxidation states, the most stable and common forms are the trivalent Cr(III) and hexavalent Cr(VI) species, which display quite different chemical properties (Katz and Salem, 1994). Both forms are taken up by plants: Cr(VI) is taken up actively by the sulfate carrier and immediately converted to Cr(III) in roots, possibly by the Fe(III) reductase enzyme (Zayed et al., 1998). Using X-ray absorption spectroscopy, in fact, only the trivalent form of chromium was detected in roots and shoots of several crop plants (including celery) treated with CrO_4^{2-} or Cr^{3+} (Zayed et al., 1998). In contrast, Cr(III) is taken up passively, being retained by the cation-exchange sites of the cell walls (Skeffington et al., 1976; Marschner, 1995). Chromium interferes with several metabolic processes, causing toxicity to plants as exhibited by reduced root growth and biomass, chlorosis, photosynthetic impairing and, finally, plant death (Sharma et al., 2003; Panda and Choudhury, 2005;

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0045-6535/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2006.01.005

Shanker et al., 2005). Furthermore, changes in the levels of free polyamines as a consequence of chromium exposure were observed in oat, barley and rape seedlings (Wettlaufer et al., 1991; Hauschild, 1993). Polyamines (PAs), spermidine (Spd), spermine (Spm) and their diamine precursor putrescine (Put) are widespread polycations that act as regulators of cell proliferation and differentiation (Bagni and Torrigiani, 1992). In plants, they have been implicated in a wide range of biological processes, including growth, development and stress responses (Flores, 1991). The cationic nature of free polyamines at physiological pH allows them to interact with negatively charged molecules, such as nucleic acids, phospholipids and proteins, and to protect them from metal-induced oxidative damage (Roberts et al., 1986). PAs have been also suggested to function as metal chelators (Lovaas, 1996), and as free radical scavengers (Schraudner et al., 1996). In particular, Put has been shown to accumulate in plant cells following many different types of stress (drought, deficient mineral nutrition, acid stress, phytotoxic metals), and therefore it can be considered as a stress marker (Hauschild and Jacobsen, 1990). In plant cells, PAs do not only occur as free molecular bases but can be also covalently linked to phenolic acids (soluble conjugated PAs), as well as to high molecular-mass substances like hemicelluloses and lignins, and in small amounts also to proteins (insoluble conjugated PAs). In recent years, attention has been focused on possible roles of conjugated forms of PAs in plants exposed to unfavorable environmental conditions (Bouchereau et al., 1999). In the present paper we investigated the possible relationship between uptake and distribution of Cr(III) in celery seedlings and the endogenous levels of free and conjugated polyamines. The toxic effects of Cr(III) on plant development and chlorophyll content were also evaluated. In addition, since heavy metals are known to contribute to the accumulation of aberrant or damaged proteins whose removal is essential for maintaining cellular integrity, the involvement of the ubiquitin system in $CrCl_3$-stress response was also investigated. Indeed, although there is increasing evidence for the involvement of the ubiquitin pathway in plant response to stress (Belknap and Garbarino, 1996), so far very little or nothing is known about chromium effects on this master proteolytic pathway.

2. Material and methods

Seeds of celery (*Apium graveolens* L.) var. Pascal, kindly provided by Anseme, Cesena, Italy, were soaked for 30 min in tap water and then sterilized in a 5% sodium hypochlorite for 10 min under vacuum, washed three times with sterile water and sown in glass pots on Heller medium (1953) supplemented with Fe-EDTA (0.1 mM ferric-sodium EDTA complex), 10 g l^{-1} sucrose and 0.8% agar with or without (controls) 0.01, 0.1 or 1 mM $CrCl_3$. Seeds were allowed to germinate at 24 ± 1 °C under a 16-h photoperiod with cool white fluorescent lamps (Philips TLD 36W/33; irradiance, 50 μmol m^{-2} s^{-1}). Fifteen-day-old seedlings were harvested, washed with distilled water and subdivided into roots, hypocotyls, and cotyledonary leaves which were pooled within each treatment, weighed, and immediately used or frozen in liquid nitrogen and kept at −80 °C until use.

Leaves, hypocotyls and roots were digested with 8 ml HNO_3 (Suprapur® 65%, Carlo Erba)–H_2O_2 (Suprapur® 30%, Merck), 3:1, v/v, according to Campanella et al. (2001) using an oven (CEM MDS-2100) equipped with a microwave power system with an operator selectable output of 0–950 ± 50 W in 1% increments. Chromium analyses were performed by GFAAS under STPF conditions. Standards were prepared with serial dilutions of stock solution (standard Cr(III) calibration solution AA Certipur® 1000 mg/l, Merck) out of the linear range of the metal. The atomic absorption spectrometer utilized was a Perkin–Elmer AA-300, equipped with a graphite furnace assembly (Model HGA-800), an autosampler (Model AS-72) and a deuterium arc lamp background correction system. The detection limits, determined as three times the standard deviation of ten replicates of blank, was 0.5 μg/l. The sample preparation method used was checked by spiking samples with calibration solutions; mean recovery was 83%. The precision of Cr(III) determination, based on variation in replicate analyses ($n = 2$) on the same sample, was 15% lower.

Chlorophyll content (total, *a* and *b*) in acetone extracts was determined according to Arnon (1949) at 663 and 645 nm using a V-530 Jasco spectrophotometer. The concentration was expressed as milligram chlorophyll per gram fresh weight.

For polyamine determination, about 0.5 g of fresh tissue were homogenized in a mortar with 10 volumes of 4% perchloric acid (PCA), and centrifuged at 12 000*g* for 30 min at 4 °C. The pellets were washed twice by resuspension in PCA, recentrifuged and resuspended in the original volume of PCA. Aliquots (0.4 ml) of the resuspended pellet (PCA-insoluble fraction) and of the supernatant (PCA-soluble fraction) were subjected to acid hydrolysis (6 N HCl at 110 °C overnight) in order to release free, di- and polyamines from their PCA-insoluble and -soluble conjugates, respectively. Aliquots (0.4 ml) of the supernatant, and of the hydrolysed supernatant and hydrolysed resuspended pellet were benzoylated (Redmond and Tseng, 1979), and extracted in 2 ml anhydrous diethyl ether. After 5 min centrifugation at 1500*g*, 1 ml of the ether phase was evaporated to dryness, redissolved in 0.1 ml methanol and analysed by HPLC according to Flores and Galston (1982). Standard polyamines were subjected to the same procedure. Putrescine, spermidine and spermine were analysed by HPLC on a reverse phase C_{18} column (Kromasil column, 5-μm particle diameter, 4.6 × 250 mm, Akzo Nobel). The system utilized two pumps (PU-880 Jasco). The solvent system consisted of water (solvent A) and acetonitrile (solvent B). The starting gradient was composed of 48% solvent A (isochratic for 15 min), then the proportion of solvent A decreased linearly down to 0% (10 min), and