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Changes induced by zinc toxicity in the 2-DE protein profile of sugar beet roots

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ABSTRACT

Changes induced by three levels of Zn toxicity in the root proteome from *Beta vulgaris* were studied by two dimensional gel electrophoresis. 320 spots were consistently detected and 5, 5 and 11% of them showed significant changes in relative abundance as a result of the 50, 100 and 300 μM Zn treatments, respectively, when compared to controls (1.2 μM Zn). Forty-four spots had consistent changes between all treatments, and 93% were identified. At low and mild Zn excess, the complex I of the mitochondrial transport chain and the oxidative phosphorylation were mildly impaired, and an effort to compensate this effect by increasing glycolysis was observed. At high Zn excess, a general metabolism shutdown occurred, as denoted by decreases in the aerobic respiration and by an impairment of the defense systems against oxidative stress. Accordingly, lipid peroxidation increased as Zn supply increased. This study suggests that metabolic changes at high Zn supply reflect cell death, while changes at low and mild Zn supplies may rather explain the metabolic reprogramming occurring upon Zn toxicity. Results also suggest that Zn competition with divalent ions including Fe may contribute to many of the Zn toxicity symptoms, especially at low and moderate Zn supplies.

Biological significance
Results in this work provide a comprehensive overview of the effects of Zn toxicity in roots of sugar beet plants. Effects at low and mild Zn excess are similar and reflect changes in the metabolism aimed to overcome this heavy metal stress, whereas effects at high Zn supply indicate a general shutdown of the metabolism and cell death. Our results indicate that Zn toxicity elicits major impairments in the oxidative stress defense systems, possibly due to Zn competition with divalent cations including Fe, in spite that Zn is not a redox active element by itself.

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1. Introduction

Zinc (Zn) is an essential micronutrient for plants, where it is the second most abundant transition metal after Fe. Zinc plays structural and/or catalytic roles in many essential physiological processes, being the only metal present in all enzyme classes [1,2]. However, when present at high concentrations, Zn can be toxic. This usually occurs in polluted acidic soils, where the low soil solution pH increases Zn availability [3,4]. Agricultural soils are often contaminated with heavy metals due to different anthropogenic sources, including waste incineration, industrial processes, fossil fuel combustion and use of fertilizers and sewage sludge [5,6]. In contaminated soils, some crops may take up large amounts of Zn that can be stored in edible parts, therefore posing a threat to food safety and constituting a risk to animal and human health [7].

Efforts have been made in recent years to unveil the mechanisms of Zn homeostasis (see [5,8–11] for reviews). Plant roots usually acquire Zn from the rhizosphere as Zn(II), except for grasses that are also able to take Zn in complexed forms [8]. Once taken up, Zn is distributed throughout the whole plant in a complex series of processes involving different Zn-chelating compounds as well as proteins and metal transporters [12]. Metal transporter families involved in Zn homeostasis include the ZIP (ZRT–IRT-like proteins) and YSL families, with several members participating in Zn import into the cytoplasm, as well as the HMA and MTP families involved in Zn export from the cytoplasm [5,8–10,13]. In *Arabidopsis*, members of the last two families participate in Zn loading into xylem and vacuoles [9,10,13]. A plasma membrane Zn exporter, PCR2, has been proposed to have dual roles in Zn homeostasis by exporting Zn from the cytoplasm, thus avoiding toxicity and facilitating long distance transport [14]. The vacuolar transporter ZIF1 from the major facilitator superfamily is involved in nicotianamine (NA) transport into the vacuole contributing to Zn detoxification [15,16]. Among the different chelators involved in Zn homeostasis, NA has been shown to play significant roles in plant Zn mobilization [8,17]. Nicotianamine is expected to act primarily in the cytoplasm and phloem, but stable Zn–NA complexes may also exist at vacuolar pH [18,19]. In the Zn hyper-accumulator species *Noccaea caerulescens* and *Arabidopsis halleri*, organic acids and histidine are the most abundant chelating agents for Zn [20–22] and therefore may also play a role in non-hyperaccumulators. Proteins are also involved in Zn homeostasis, and two metallothioneins, MT4a and MT4b, are important in Zn storage in seeds [23].

Zinc toxicity in plants is generally associated to increases in reactive oxygen species [24] and/or an uncontrolled displacement from active sites in proteins of essential cofactor metal cations such as Fe and Mn that share similar chemical characteristics. Both mechanisms can cause inactivation and damage of biomolecules. Sugar beet (*Beta vulgaris* L.) is a model plant species that displays a great capacity to accumulate heavy metals such as Cd and Pb [25]. Zinc toxicity in sugar beet causes different effects, depending on the Zn concentration in the nutrient solution, and symptoms include Fe deficiency-induced chlorosis in young leaves, altered plant mineral composition, and root and shoot growth decreases [26]. In *B. vulgaris* shoots, Zn excess induces increases in leaf respiration rates and decreases in photosynthetic rates, stomatal and mesophyll conductances to CO_2, as well as changes in the frequency, morphology and functioning of the stomata, and modifications in mesophyll ultrastructure [27]. In *B. vulgaris* roots, Zn toxicity induces a reprogramming of the carboxylate metabolism similar to that observed in Fe-deficient plants [28].

Proteomic approaches are useful to unravel general effects of stresses on metabolic processes [29]. These approaches have been used, for instance, to study the effects of Fe deficiency in thylakoids and roots [30–32] and Cd toxicity in root metabolism [33]. A recent study combining a proteomic approach and the use of mutant genotypes has found that high Zn toxicity exerts important growth defects, in which cross-talk between Fe and Zn homeostasis and V-ATPase activity might play a central role [34]. Most of the knowledge on Zn homeostasis comes from Zn hyper-accumulating plants or Zn deficiency studies, whereas processes occurring upon Zn toxicity are still largely unknown. The aim of this study was to obtain an overview of the changes induced by mild, moderate and severe Zn toxicity in the root protein profile of the model plant species sugar beet.

2. Materials and methods

2.1. Plant material and growth conditions

Sugar beet (*B. vulgaris* L. cv. Orbis) was grown in a growth chamber with a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} PAR, 80% relative humidity and a photoperiod of 16 h, 23 °C/8 h, 18 °C day/night regime. Seeds were germinated and grown in vermiculite for two weeks. Seedlings were grown for an additional two week period in half-strength Hoagland nutrient solution [35] with 45 μM Fe(III)-EDTA, and then transplanted to 20 L plastic buckets (four plants per bucket) containing half-strength Hoagland nutrient solution with 45 μM Fe(III)-EDTA and different concentrations of Zn. A concentration of 1.2 μM $ZnSO_4$ was used as a control, and excess Zn treatments were 50, 100 and 300 μM $ZnSO_4$. In these solutions Zn was shown to occur mainly as Zn(II) using chemical speciation software [26]. Roots were harvested 9–10 days after imposing the high Zn treatments, frozen in liquid N_2 and stored at −80 °C until analysis.

2.2. Protein extraction

Roots of two plants from the same treatment in a given batch were pooled, approximately 1 g of the pooled material was ground to powder in liquid N_2 using a Retsch M301 mill (Retsch GmbH, Haan, Germany) and then homogenized in 5 mL of phenol saturated with Tris–HCl 0.1 M (pH 8.0), containing 5 mM β-mercaptoethanol, by stirring for 30 min at 4 °C. After incubation, the homogenate was filtered (PVDF, 0.45 μm) and centrifuged at 5000 ×*g* for 15 min. The phenol phase was re-extracted for 30 min with one volume of Tris–HCl 0.1 M (pH 8.0) saturated with phenol containing 5 mM β-mercaptoethanol, and centrifuged as described above. The phenol phase was collected, and proteins were precipitated by adding four volumes of 0.1 M ammonium acetate in cold

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