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Effect of cadmium on H^+ATPase activity of plasma membrane vesicles isolated from roots of different S-supplied maize (*Zea mays* L.) plants

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Abstract

The plasma membrane of root cells constitutes the major membrane barrier between cytoplasm and soil environment and is the first functional site of contact of the root with any ion, so this may have a number of consequences for heavy metal toxicity. In fact, plasma membrane contains potential metal-sensitive enzyme systems, like H^+ATPase. It is well known the role of sulphide in plant responses to Cd and it gives the rationale for studying the interactions between sulphur availability and Cd-exposure. In this work we report on the alteration of non-protein thiols levels and on plasma membrane H^+ATPase activity, by measuring changes in its phosphohydrolytic activity and H^+ pumping capacity, in Cd exposed and differently S-supplied maize roots.
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1. Introduction

Soil contamination with cadmium is a world-wide problem, because the excess of cadmium in biotic systems is a potential hazard for plants and animals as well as humans. Fertilizer impurities, the use of sewage sludge and atmospheric fallout from industrial and urban activities are taken to be major causes for Cd pollution in agricultural soils [1].

Despite being non-essential, cadmium is taken up readily by plants and thus can accumulate in crops. The root is the first site of contact of this ion and the plasma membrane of root cells constitutes the major membrane barrier between cytoplasm and soil environment. Thus, the interactions between cadmium and root cells lead to physiological alterations of plasma membrane properties, that in turn could affect uptake and transport of mineral nutrients.

Several evidences [2–5] suggest that the lipid and protein constituents of membranes, may be injured by the metal and, in particular, we previously demonstrated the involvement of plasmalemma H^+ATPase in the mechanism of Cd toxicity [6].

H^+ATPase is an integral protein associated with the plasma membrane and is located preferentially at the epidermal and cortical cell layers of roots [7]. It is generally accepted that ion transport across the plasmalemma is dependent on the electrochemical gradient generated by the plasma membrane H^+ATPase [8], thus regulation of this enzyme may play an important role in cadmium stress conditions.

It is well known that when exposed to excess heavy metals, plants induce phytochelatins (PCs) and related peptides [9–11]. These compounds are able to bind heavy

Abbreviations: BSA, bovine serum albumine; BTP, bis-tris-propane; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, DL-dithiothreitol; EDTA, ethylene diamine tetracetic acid; EGTA, ethylene glycol tetracetic acid; GSH, glutathione; MDA, malondialdehyde; MES, morpholino ethanesulfonic acid; PCs, phytochelatins; PMSF, phenylmethylsulphonyl fluoride; PVPP, polyvinylpolypyrrolidone; S.D., standard deviation; S.E., standard error; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid

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metals reducing their interactions with important cytosolic proteins [12–15]. The finding that glutathione is the immediate precursor of PCs suggests that high sulphur availability is necessary for the biosynthesis of PCs introducing the possibility that plant S-nutritional status could be implicated in affecting plant ability to cope with heavy metals and thereby in interfering with of heavy metals toxicity.

This idea seems to be of particular interest considering that sulphur content in many areas of western Europe has declined dramatically over the last few years and consequently S-deficiency has become an increasingly problem [16].

In view of the above considerations, our work was initiated to investigate the interaction between sulphur availability and Cd toxicity with emphasis on maize root plasmalemma H^+ATPase responses.

First, we measured in root the content of total sulphate, which is required for the biosynthesis of thiol compounds, and of total non-protein thiols, which in its turn reflects the PCs production. Furthermore, since Cd is known to initiate membrane peroxidation, we determined levels of malondialdehyde in plant roots. Finally, the interaction between sulphur nutrition and Cd toxicity was analysed by preparing plasma membrane enriched fractions from maize roots and by studying ATP hydrolysis and H^+ transport in plasma membrane enriched vesicles; passive H^+ permeability of plasma membrane was also measured.

2. Materials and methods

2.1. Plant material and growing conditions

Maize (*Zea mays* L. cv. Cecilia) seeds were germinated in moistened paper in the dark at 26 °C for 3 days. Seedlings with roots were then transferred in plastic pots (about 18 seedlings in each pots) containing 2 l of nutritive solution [17], continuously aerated and changed every 2 days. Plants were grown into a climate chamber under 200 μE m^{-2} s^{-1} light intensity and 14 h/10 h day/night regime (temperature 27 °C diurnal; 20 °C nocturnal; relative humidity 70/80%).

After 10 days from sowing, half of the plants were transferred in a minus sulphate solution. Sulphate salts (Mg^{2+}, NH_4^+, Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}) were replaced by appropriate amounts of chloride salts (Mg^{2+}, NH_4^+, Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}). $CdSO_4$ or $CdCl_2$ (final concentration 100 μM) was added to half of the S-sufficient (+S) and S-starved (−S) plants, respectively. The roots were harvested after 0, 1, 2, 3 and 4 days from the beginning of the treatment.

2.2. Total sulphur content

To determine total sulphur content, 1 g of root sample, dried at 105 °C, was ashed in a muffle furnace at 600 °C. The ash was dissolved in 10 ml of 3 N HCl and filtered through Whatman No. 42 paper. In contact with $BaCl_2$, a $BaSO_4$ precipitate is formed which is determined turbidimetrically [18].

2.3. Non-protein thiols content

Water soluble non-protein sulphydryl (SH) compounds were determined colorimetrically with DTNB following the procedure described in ref. [19]. Briefly, roots were homogenized in a solution containing 80 mM TCA acid, 1 mM EDTA acid, 0.15% (w/v) ascorbic acid and 10% (w/v) PVPP using 3 ml buffer per gram fresh weight leaves. The DTNB-reactive compounds were measured spectrophotometrically at 415 nm.

2.4. Determination of malondialdehyde content

The level of lipid peroxidation was expressed as malondialdehyde (MDA) content and was determined as TBA reactive metabolites according to Hodges et al. [20] with minor modifications. Plant fresh tissues (0.2 g) were homogenized in 10 ml of 0.25% TBA made in 10% TCA. Extract was heated at 95 °C for 30 min and then quickly cooled on ice. After centrifugation at 10 000 × *g* for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The level of lipid peroxidation was expressed as μmol g^{-1} fresh weight by using an extinction coefficient of 155 mM cm^{-1}.

2.5. Isolation of plasma membrane enriched vesicles

Plasma membrane enriched fractions from maize roots were isolated immediately after root harvesting as previously described by Giannini et al. [21] with minor changes. Briefly, the roots apical segments (8–9 cm) were homogenized in a pre-cooled mortar with 2.5-fold volumes of a freshly prepared medium containing 250 mM sucrose, 2 mM $MgSO_4$, 25 mM BTP, 10 mM glyerol-1-phosphate, 2 mM EGTA, 2 mM EDTA, 10% (v/v) glycerol, 0.5% (w/v) BSA, 6% (w/v) choline-iodide, 1 mM PMSF, 2 mM ATP, 2 mM DTT, 1% (w/v) PVPP, titrated to pH 7.6 with MES.

The brei was filtered through four layers of cheesecloth and then centrifuged in a microcentrifuge at 13 000 × *g* for 3 min; the supernatant was recovered and centrifuged at 13 000 × *g* for 25 min to obtain a microsomal membrane pellet. The last one was gently resuspended in 0.4 ml of homogenization medium, then layered on a discontinuous sucrose gradient consisting of 600 μl of 25% (w/w) sucrose layered over 400 μl of 38% (w/w) sucrose and centrifuged at 13 000 × *g* for 60 min. Finally, the vesicles banding above the sucrose cushion was collected, quickly frozen in liquid N_2 and stored at −80 °C until used for the enzyme assays. All preparation steps were performed at 4 °C.

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