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Research article

Cadmium-induced changes in vacuolar aspects of *Arabidopsis thaliana*

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ABSTRACT

We have examined the changes due to Cd treatment in the vacuolar form in root tip cortical cells in *Arabidopsis thaliana* employing a transformant with GFP fused to a tonoplast protein. A Cd-induced enhancement in complexity with general expansion of vacuolar system within 24 h was evident. The changes in the vacuolar form were dependent on the applied Cd concentrations. Concomitantly, as revealed through dithizone staining, Cd accumulated in the seedling roots exhibiting abundance of Cd-dithizone complexes in root tip, root hairs and vasculature. To get insight into the involvement of SNARE protein-mediated vesicle fusion in Cd detoxification, the magnitude of Cd toxicity in a couple of knock out mutants of the vacuolar Q_a-SNARE protein VAM3/SYP22 was compared with that in the wild type. The Cd toxicity appeared to be comparable in the mutants and the wild type. In order to analyze the Cd effects at cellular level, we treated the *Arabidopsis* suspension-cultured cells with Cd. Cd, however, did not induce a change in the vacuolar form in suspension-cultured cells although Cd measured with ICP-MS was obviously taken up into the cell. The V-ATPase activity in the microsomal fractions from vacuoles isolated from *A. thaliana* suspension cultured cells remained unaffected by Cd. Changes in the levels of certain metabolites of Cd-treated cells were also not so distinct except for those of glutathione. The significance of findings is discussed.

1. Introduction

Heavy metal (HM) concentrations have increased in the environment, occasionally to unacceptable levels, owing largely to diverse anthropogenic activities. They adversely affect the plant productivity and eventually pose a threat to human health. Plant cells are equipped with multiple means of HM detoxification; some of these are metal-specific (Hall, 2002; Sharma and Dietz, 2006). The cytoplasmic chelation of HM ions by ligands such as phytochelatins (PCs) and metallothioneins (MTs) constitutes one of the major cellular strategies to keep the HM concentrations below their toxicity threshold levels. Following their synthesis in response to the exposure to HMs, PCs make complexes with HMs that are sequestered into the vacuoles via appropriate tonoplast transporters (Sharma and Dietz, 2006; Sharma et al., 2016). Indeed, the vacuolar compartmentalization plays an important role in HM homeostasis and detoxification. Recently, the molecular and functional aspects of several tonoplast located HM transporters have been characterized (Martinoia et al., 2012; Sharma et al., 2016). A couple of ABC transporters namely, AtABCC1 and AtABCC2, have been demonstrated to transport PC-complexes with As, Cd and Hg into the vacuoles thereby reducing the cytosolic concentrations of the concerned metalloid/metals (Song et al., 2010; Park et al., 2012). Two vacuolar pumps, V-ATPase and V-PPase, are involved in vacuolar compartmentalization via generation of proton motive force necessary for energizing secondary active transport activities and for operation of certain specific metal/proton antiporters (Dietz et al., 2001; Martinoia et al., 2012). In accordance, they have been

Abbreviations: ABC, ATP binding cassette; HM, heavy metal; MT, metallothionein; PC, phytochelatin.

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shown to respond to the exposure to toxic HM ions. For example, the transcript levels of V-ATPase subunits c and E were substantially elevated in the roots of Cd/Fe treated barley seedlings (Sharma et al., 2004). Likewise, in the shoots of a mutant of *A. thaliana, MTP1-1,* that lacks tonoplast Zn transporter MTP-1, elevated VHA-a mRNA levels were observed (Kawachi et al., 2009). However, the transcription of three isoforms of the subunit c of V-ATPase in *Cucumis sativus* (CsVHA-c1, CsVHA-c2, CsVHA-c3) was differentially influenced by Cd, Cu, Ni and Zn (Kabala et al., 2014). V-ATPase activity in *C. sativus* roots was inhibited by Cd but promoted by Cu (Kabala et al., 2013). Obviously, the stated responses exhibit HM- and species-specific variations. Consistent with a role of V-ATPase in HM detoxification, the magnitude of Cd toxicity in barley seedlings in terms of the suppression of root elongation growth was found to be enhanced in the presence of bafilomycin, a specific V-ATPase inhibitor (Dietz et al., 2001).

In parallel with the advancements towards the molecular characterization of V-ATPase and tonoplast HM transporters in the context of HM detoxification and homeostasis (Martinoia et al., 2012; Sharma et al., 2016), HM-induced alterations in vacuolar form and dimension have occasionally been reported, for example, in response to Cd treatment in *Chlamydomonas acidophila* (Nishikawa et al., 2003), *Allium sativum* root cells (Liu and Kottke, 2003) and suspension-cultured *Nicotiana tabacum* cells (Reese et al., 1986). In contrast, no vacuolar change in radish leaves was observed due to Cd treatment (Vitoria et al., 2006). As such, the vacuolar system seems to respond to the cellular HM burden at different levels of organization. The HM-induced changes in vacuolar form need to be assessed particularly in relation to the parameters of cellular conditions.

In the present study, we have monitored the Cd-induced alterations in vacuolar system in the roots of *Arabidopsis thaliana* seedlings and suspension-cultured cells (Deep), as was analyzed in case of high salt treatment by Hamaji et al. (2009). The vacuolar system includes vesicles/prevacuoles and the central vacuole that develops through fusion of the former (Lipka et al., 2007). A comparison of the responses of whole seedlings and suspension-cultured cells to Cd was expected primarily to reveal the dependence, if any, of the Cd-induced change in vacuolar form on certain specific factor(s) associated with the tissue organization. In case of the suspension-cultured cells, we could treat them evenly with Cd compared with the whole tissue. In addition, the Cd localization in the vacuoles was confirmed with dithizone staining (whole tissue) and ICP-MS measurements (suspension-cultured cells). The fusion of prevacuolar vesicles, carrying the toxic ions, into a central vacuole likely represents one of the detoxification mechanisms of salt stress (Hamaji et al., 2009; Leshem et al., 2006). Such a fusion might be functioning for cellular Cd management mediated by the SNARE proteins (Lipka et al., 2007). In order to get insight into the role of vesicle trafficking in cellular management and detoxification of excess Cd concentrations, growth responses of *A. thaliana* mutants which are knock out plants of Q_a-SNARE Vam 3/SYP22 (Uemura et al., 2004; Lipka et al., 2007; Zouhar and Rojo, 2009) were compared with those of the wild type.

2. Materials and methods

2.1. Plant material and culture conditions

Arabidopsis thaliana ecotype Col-0 plants and suspension-cultured cells [Deep: Arabidopsis Col-0 cell suspension supplied courtesy of Dr. Umeda (NAIST)] (Mathur et al., 1998) were used in this study. Plants (*vam 3-1*) transformed with *proVAM3:GFP-VAM3* were developed by Uemura et al. (2010). Seeds of knockout mutants *atvam3-1* (SALK_060946, Ebine et al., 2008) and *atvam3-3* (SALK_075924, Ueda et al., 2006) were donated by the Salk Institute Genome Analysis Laboratory as described in Hamaji et al. (2009).

The surface-sterilized seeds (95% EtOH; 15 min) were sown on modified Murashige and Skoog (MS) medium containing 1% sucrose and 0.2% gellan gum and subjected to 4 °C for 3 d to break the dormancy and synchronize germination. Thereafter, they were cultured for 9–10 d in a growth chamber at 23.5 °C under continuous illumination. The seedlings were washed thoroughly with sterile water to remove the adhered medium and shifted to hydroponic culture on MS medium without sucrose. After 1 d adaptation in the hydroponic system, the nutrient medium was supplemented with the stated Cd concentrations. For microscopic observations of vacuolar changes, 24 h-treated seedlings were studied whereas for Cd toxicity determination growth was measured after 9–10 d of treatment.

Suspension-cultured cells were cultured in MS medium as described in Hamaji et al. (2009). They were also treated with the stated Cd concentrations.

2.2. Cd localization with dithizone staining

Cd was localized in the roots of *A. thaliana* seedlings through dithizone staining method (Seregin and Ivanov, 1997; Clabeaux et al., 2011). Seedlings grown on the nutrient medium as described above were shifted to MS medium without sucrose in a hydroponic system. After 1-d adaptation, the seedlings were shifted to the medium supplemented with 200 μM Cd for 24 h. Thereafter, the seedlings were washed thoroughly with MilliQ water and stained with dithizone solution (4.5 mg dithizone dissolved in 9 ml acetone with addition of 15 μl glacial acetic acid and 3 ml MilliQ water) for 1 h. After thorough washing to ensure the removal of dithizone-Cd stain from the surface, the seedlings were examined under a light microscope (BX51WI, Olympus, Tokyo, Japan) and photographed with an imaging system (DP70, Olympus, Japan).

2.3. Measurement of metals with ICP-MS

Dithizone staining of intracellular Cd was not suitable for suspension-cultured cells. To confirm Cd uptake into cells, Cd and other metal contents in Deep cells were measured with ICP-MS (Agilent 7900, Agilent Technologies, Japan). Samples were prepared according to Kamiya and Fujiwara (2009).

2.4. Microscopic observations

The fluorescent images were obtained with a confocal laser microscope (FV-1000; Olympus) for GFP and propidium iodide (PI) fluorescence as described in Hamaji et al. (2009).

2.5. Isolation of vacuolar membrane-enriched fraction and measurement of H^+-ATPase activity of vacuolar membrane vesicles

A vacuolar membrane-enriched microsomal fraction was collected according to Dietz et al. (1998). Cells were homogenized in a medium composed of 250 mM sucrose, 50 mM Tris, 10 mM EDTA, and 4 mM dithiothreitol, adjusted to pH 8.0 with HCl. The cell homogenate was centrifuged at 8000 *g* twice for 10 min. The supernatant was again centrifuged at 25,000 *g* for 30 min and the pellet solubilized in a medium of 250 mM sucrose, 5 mM PIPES-KOH (pH 7.2), and 0.5 mM dithiothreitol. Samples were layered on top of a two-step sucrose gradient of 30 and 35% solutions supplemented with 250 mM sucrose, 5 mM PIPES-KOH (pH 7.2), and 0.5 mM dithiothreitol. Following centrifugation at 45,000 *g* for 2 h, the vacuolar membrane-enriched fraction was taken from the