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Cadmium activates *Arabidopsis* MPK3 and MPK6 via accumulation of reactive oxygen species

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ABSTRACT

Cadmium (Cd) is a non-essential toxic heavy metal that influences normal growth and development of plants. However, the molecular mechanisms by which plants recognize and respond to Cd remain poorly understood. We show that, in *Arabidopsis*, Cd activates the mitogen-activated protein kinases, MPK3 and MPK6, in a dose-dependent manner. Following treatment with Cd, these two MAPKs exhibited much higher activity in the roots than in the leaves, and pre-treatment with the reactive oxygen species (ROS) scavenger, glutathione, effectively inhibited their activation. These results suggest that the Cd sensing signaling pathway uses a build-up of ROS to trigger activation of *Arabidopsis* MPK3 and MPK6.

1. Introduction

Copper (Cu) and zinc (Zn) are essential heavy metals that participate in many physiological processes in plants. However, cellular damage can be caused when cells are faced with an excess of these essential metal ions, or with non-essential ions such as cadmium (Cd) (Palmer and Guerinot, 2009). Cd is a widespread toxic heavy metal and it is considered to be a serious environmental pollutant. Cd can cause many toxic symptoms in plants. At the morphological level, Cd causes inhibition of root growth, chlorosis of leaves (Wojcik and Tukendorf, 1999; Nocito et al., 2002), inhibition of stomatal opening (Laetitia et al., 2002) and even wilting or death (Barcelo and Poschenrieder, 1990). Cd can also induce a number of physiological changes including inhibition of photosynthesis and transpiration (Nyitrai et al., 2003), induction of oxidative stress (Schutzendubel et al., 2001), changes in enzyme activity (Sanità di Toppi and Gabbrielli, 1999), and modifications to gene expression (Sarry et al., 2006; Herbette et al., 2006). In order to develop heavy metal-tolerant *Arabidopsis* plants, investigators have searched extensively for Cd transporters, which include *IRT*1 (Connolly et al., 2002), *AtATM*3 (Kim et al., 2006) and *AtPDR*8 (Kim et al., 2007), as well as Cd-tolerant genes such as *CDI*19 (Suzuki et al., 2002), *HMA*1 (Moreno et al., 2008) and *HMA*4 (Hanikenne et al., 2008). However, the signaling transduction pathways underlying Cd-mediated regulation of cellular responses and gene expression, remain unclear.

In eukaryotes, the mitogen-activated protein kinase (MAPK) cascade is one of the important pathways by which external stimuli are transduced into cellular responses. At a minimum, the MAPK cascade comprises three kinases: a MAPKKK (MAPK kinase kinase), a MAPKK (MAPK kinase) and a MAPK (Jonak et al., 2002). MAPKKKs are serine/threonine kinases that phosphorylate two amino acids in the *S*/*T*-X$_{3-5}$-*S*/*T* motif of the MAPKK activation loop. MAPKKs are dual-specificity kinases that activate MAPK via double phosphorylation of the *T*-X-*Y* motif in the activation loop. MAPKs are serine/threonine kinases that are able to phosphorylate a wide range of substrates, including other kinases and/ or transcription factors (Colcombet and Hirt, 2008). In this way, information can be transduced via a phosphorylation cascade from

Abbreviations: Cd, cadmium; Zn, zinc; Cu, copper; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; GSH, glutathione; DAB, 3,3-diaminobenzidine; ROS, reactive oxygen species; MBP, myelin basic protein; RT-PCR, reverse transcription/polymerase chain reaction.

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upstream kinases to downstream targets. Such signaling cascades can regulate a variety of cellular responses, including differentiation, cell division and environmental stress responses (Zhang et al., 2006).

In the *Arabidopsis* genome, there are genes encoding 20 MAPKs, 10 MAPKKs and more than 60 MAPKKKs. The MAPKs can be divided into four groups according to their sequences and structures (MAPK group, 2002). Three extensively studied MAPKs, namely MPK3, MPK4 and MPK6, are involved in responses to many biotic and abiotic stresses such as wounding, pathogens, ABA, cold, salt, osmotic and oxidative stresses (Colcombet and Hirt, 2008). Heavy metal-induced MAPK signaling has been investigated in alfalfa and rice (Jonak et al., 2004; Lin et al., 2005; Yeh et al., 2004). Exposure of alfalfa seedlings to either Cu or Cd results in a complex activation pattern of four distinct MAPKs. However, the classes of MAPKs activated by these metals remain to be identified in plants.

Here, we report that two *Arabidopsis* MAPKs are activated by Cd and we demonstrate that these are MPK3 and MPK6. Furthermore, we showed that accumulation of reactive oxygen species (ROS) could be involved in Cd-triggered MAPK activation.

2. Results and discussion

2.1. Cadmium inhibits the growth and development of Arabidopsis

In order to evaluate the toxicity of Cd in *Arabidopsis* plants, a Cd dose response experiment was performed. *Arabidopsis* seeds were germinated and grown for 10 days in media containing different concentrations of Cd. Under these conditions, plant growth was significantly lower than in the Cd unsupplemented medium. As shown in Fig. 1, Cd inhibited the root growth and induced leaf chlorosis. Root growth was significantly reduced as a function of increasing Cd concentration. These results showed that Cd inhibited plant growth in a dose-dependent manner.

2.2. Cd activates myelin basic protein (MBP) kinases in Arabidopsis

To investigate protein phosphorylation during the plant response to Cd stress, Cd-induced protein kinase activities in *Arabidopsis* were measured. *Arabidopsis* plants were treated with $CdCl_2$ as a toxic and non-essential metal, together with either $CuSO_4$ or $ZnSO_4$ as redox-active toxic micronutrient metals. *Arabidopsis* seedlings were challenged with 50 μM $CdCl_2$, 100 μM $CuSO_4$ or 1 mM $ZnSO_4$. An in-gel kinase activity assay was used to analyze changes of protein kinase activities in protein extracts from treated plants. Myelin basic protein (MBP) was used as a general kinase substrate. In response to $CdCl_2$ treatment, two protein kinases (44 and 47 kDa) were activated, with maximum activity observed at 5 min and decreasing thereafter (Fig. 2A). By contrast, $CuSO_4$ (Fig. 2B) and $ZnSO_4$ (Fig. 2C) activated three protein kinases (42, 44 and 47 kDa). With $CdCl_2$ and $ZnSO_4$ treatments, MBP-phosphorylating protein kinases showed maximum activity within 15 min, and then the activity decreased, whereas $CuSO_4$-induced kinase activities were activated within 5 min and continued for 12 h. These results suggested that heavy metal stresses caused different patterns of activation of MBP-phosphorylating protein kinases in *Arabidopsis*.

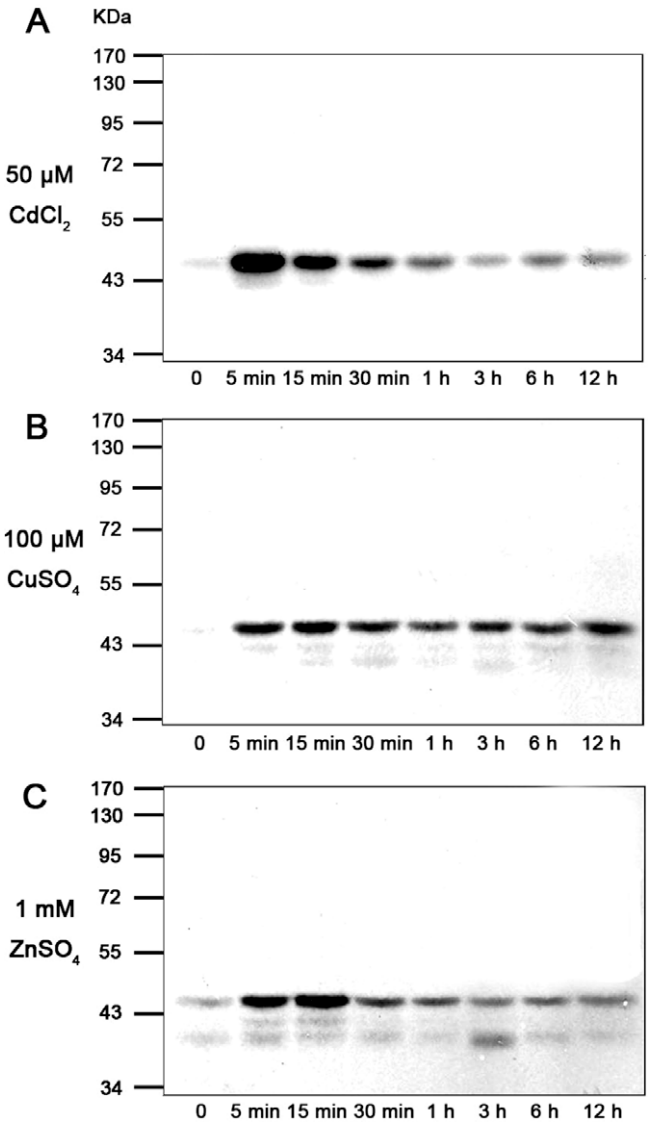

Fig. 2. Different heavy metals activate MBP-phosphorylating protein kinases in *Arabidopsis thaliana*. Whole wild-type plants were grown on MS medium and treated with 50 μM $CdCl_2$ (A), 100 μM $CuSO_4$ (B) and 1 mM $ZnSO_4$ (C) for the times indicated. In-gel kinase activity assays were performed using 30 μg protein extracts from treated plants. MBP was used as a general kinase substrate. The sizes of molecular markers are shown in kDa.

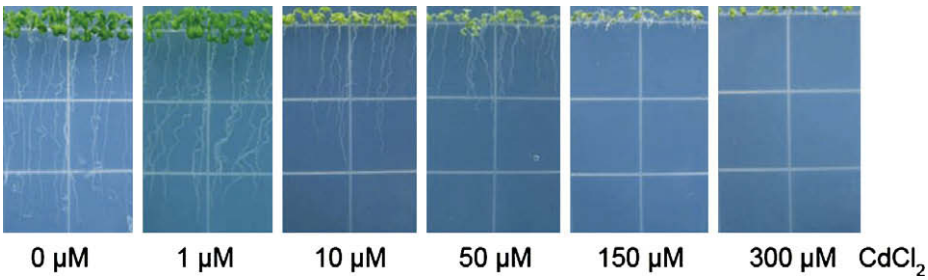

Fig. 1. Inhibition of *Arabidopsis* plant growth by Cd. Wild type seeds were germinated and grown on 1/2 MS agar plates with different concentration of $CdCl_2$. Photographs were taken at 10 days after germination.