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Cadmium-induced accumulation of hydrogen peroxide in the leaf apoplast of *Phaseolus aureus* and *Vicia sativa* and the roles of different antioxidant enzymes

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ABSTRACT

The effects of cadmium (Cd) on the accumulation of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$) in leaves of *Phaseolus aureus* and *Vicia sativa* were investigated. Cadmium at 100 μM significantly increased the production of $O_2^{\bullet-}$ and H_2O_2, as well as the activities of plasma membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and the symplastic and apoplastic activities of superoxide dismutase and ascorbate peroxidase in the leaves of both species. Apoplastic guaiacol peroxidase activity was significantly induced in the leaves of both species, particularly in *P. aureus* exposed to 100 μM Cd. Experiments with diphenylene iodonium as an inhibitor of NADPH oxidase and NaN_3 as an inhibitor of peroxidase showed that the majority of Cd-induced reactive oxygen species production in the leaves of both species may involve plasma membrane-bound NADPH oxidase and apoplastic peroxidase. Compared to *V. sativa*, increases in Cd-induced production of $O_2^{\bullet-}$ and H_2O_2 and activities of NADPH oxidase and apoplastic peroxidase were more pronounced in *P. aureus*. In contrast, *V. sativa* had higher leaf symplastic superoxide dismutase and ascorbate peroxidase activities than *P. aureus*. The results indicated that *V. sativa* was more tolerant to Cd than *P. aureus*.

1. Introduction

Heavy metal contamination is a serious environmental problem that limits crop production and threatens human health through the food chain. Cadmium (Cd) is one of the most toxic environmental pollutants for plants. Cd can interfere with numerous biochemical and physiological processes including photosynthesis, respiration, nitrogen and protein metabolism, and nutrient uptake [1,2]. However, the mechanisms involved in its toxicity are still not completely understood. Cd toxicity is mediated by the formation of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) even though Cd is not a redox-active metal [3]. ROS are highly toxic and can oxidize biological macromolecules such as lipids, proteins, and nucleic acids, thus causing lipid peroxidation, membrane damage, and inactivation of enzymes.

To avoid the deleterious effects of ROS, plants have evolved antioxidant defense mechanisms. These include enzymatic components such as superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7), and glutathione reductase (GR, EC 1.6.4.2); as well as non-enzymatic components, such as ascorbate (ASC) and glutathione (GSH; [4]). Superoxide anions generated in plants are dismutated to H_2O_2 by the action of SOD. H_2O_2 is scavenged by CAT and the ascorbate–glutathione cycle where APX reduces it to H_2O. GR also plays a key role in the antioxidant defense processes by reducing oxidized glutathione to GSH. These defensive mechanisms against oxidative damage have been specifically observed in plants subjected to Cd stress [5–12]. However, the response to Cd of antioxidant enzymes remains controversial and greatly depends on the plant species, age, duration of treatment, and experimental conditions [3].

Accumulation of ROS may be the consequence of a disruption in the balance between their production and the antioxidant system activity. In plant cells and tissues, ROS can be produced by several enzymatic systems [4]. Plasma membrane (PM)-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are believed to be responsible for $O_2^{\bullet-}$ production under biotic and abiotic stress conditions [13–18]. These enzymes can use cytosolic NADPH to produce $O_2^{\bullet-}$, which is rapidly dismutated to H_2O_2 by SOD. The involvement of NADPH oxidases in Cd-induced production of ROS has also been demonstrated in tobacco cells [19], pea leaves [20], pea roots [21], rice leaves [22], and rice roots [23].

In previous studies, we investigated the effect of different Cd concentrations on growth and the antioxidative metabolism of two plant species, *Phaseolus aureus* and *Vicia sativa* [24,25]. Under these conditions, Cd promoted oxidative stress in the two species by

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increasing the accumulation of $O_2^{\bullet-}$ and H_2O_2. During the 12-day Cd treatment, the activities of SOD, CAT, and APX initially increased and then decreased in the leaves of these plants. Compared to *V. sativa*, production of ROS was higher, while activity of the antioxidant enzymes was lower, in *P. aureus* leaves.

Although many reports have investigated Cd-induced oxidative stress and antioxidant response, relatively little information is available about the ability of heavy metal stress to induce the production of ROS in the apoplast and the roles of apoplastic antioxidant enzymes [18,26,27]. This information is important since in plant cells subjected to heavy metal toxicity, initial events most likely occur externally in the apoplast-cell membrane space. Here we investigated the effect of Cd on the production of ROS in the leaves of *P. aureus* and *V. sativa* using both histochemical and cytochemical methods. We also studied the roles of different antioxidant enzymes in the symplast and apoplast of plant leaves in the defense against harmful effects of Cd stress.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *P. aureus* and *V. sativa* were surface-sterilized with 0.1% $HgCl_2$ and soaked in distilled water at room temperature for 12 (*P. aureus*) or 24 h (*V. sativa*). The seeds were germinated in plastic dishes filled with vermiculite. After 3 d, the plants were transferred to vessels containing Hoagland nutrient solution (1 mM KH_2PO_4, 1 mM KNO_3, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 20 μM Fe–EDTA, 46 μM H_3BO_3, 9 μM $MnCl_2$, 0.77 μM $ZnSO_4$, 0.32 μM $CuSO_4$, 0.11 μM H_2MoO_4) and grown under controlled environmental conditions (14 h day length with photosynthetically active radiation of 400 μmol m^{-2} s^{-1} and 25/20 °C day/night temperatures).

Nine-day-old plants were treated with various regents. In Treatment 1, the plants were pretreated with the full nutrient solution containing 20 μM diphenylene iodonium (DPI, a NADPH oxidase inhibitor), 1 mM NaN_3 (sodium azide, a peroxidase inhibitor), 1 mM $LaCl_3$ (a Ca^{2+} channel inhibitor), 5 μM cantharidin (CANT, a protein-phosphatase inhibitor), or 200 μM 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, a guanylate cyclase inhibitor) for 24 h. The plants were then exposed to a nutrient solution containing 100 μM $CdCl_2$ for 6 d. Twenty leaves (the second youngest) in each treatment were collected for localization of H_2O_2 and $O_2^{\bullet-}$ production *in situ* and cytochemical detection of H_2O_2 and $O_2^{\bullet-}$. Control plants were grown in full nutrient solution without any pretreatment or Cd. In Treatment 2, the plants were grown in full nutrient solution with 100 μM $CdCl_2$ for 6 d, and were then treated with 0, 5, 10, or 20 μM DPI for 24 h. The second youngest leaves were collected for analysis of plasma-membrane NADPH oxidase activities by native polyacrylamide gel electrophoresis (PAGE). Plants grown in full nutrient solution without Cd and DPI served as controls. In Treatment 3, the plants were grown in full nutrient solution without (control) or with 100 μM $CdCl_2$ for 6 d. The second youngest leaves were collected for isolation of apoplastic fluids and the symplast.

The experiment was a completely randomized design with six replicate vessels for each treatment. The pH of the nutrient solution was adjusted to 5.5 with NaOH or HCl, and the nutrient solution was renewed every other day and aerated continuously.

2.2. Superoxide anion and hydrogen peroxide localization in situ

Hydrogen peroxide was visually detected in the leaves using 3,3-diaminobenzidine (DAB) as the substrate [28]. The $O_2^{\bullet-}$ formation in the leaves was visually detected by infiltration with nitroblue tetrazolium (NBT), resulting in visible stains as reported by Romero-Puertas et al. [20].

To check the specificity of DAB-staining for H_2O_2 and NBT-staining for $O_2^{\bullet-}$, the leaves from plants treated with 100 μM $CdCl_2$ for 6 d were immersed in 1 mM ASC (an H_2O_2 scavenger) or 1 mM tetramethylpyrazine (TMP, an $O_2^{\bullet-}$ scavenger) solution for 8 h before the infiltration of DAB or NBT.

H_2O_2 and $O_2^{\bullet-}$ deposits were quantified by measuring the number of pixel of spots by PHOTOSHOP 7.0. Results are expressed as percentage of spot area versus total leaf area in pixels.

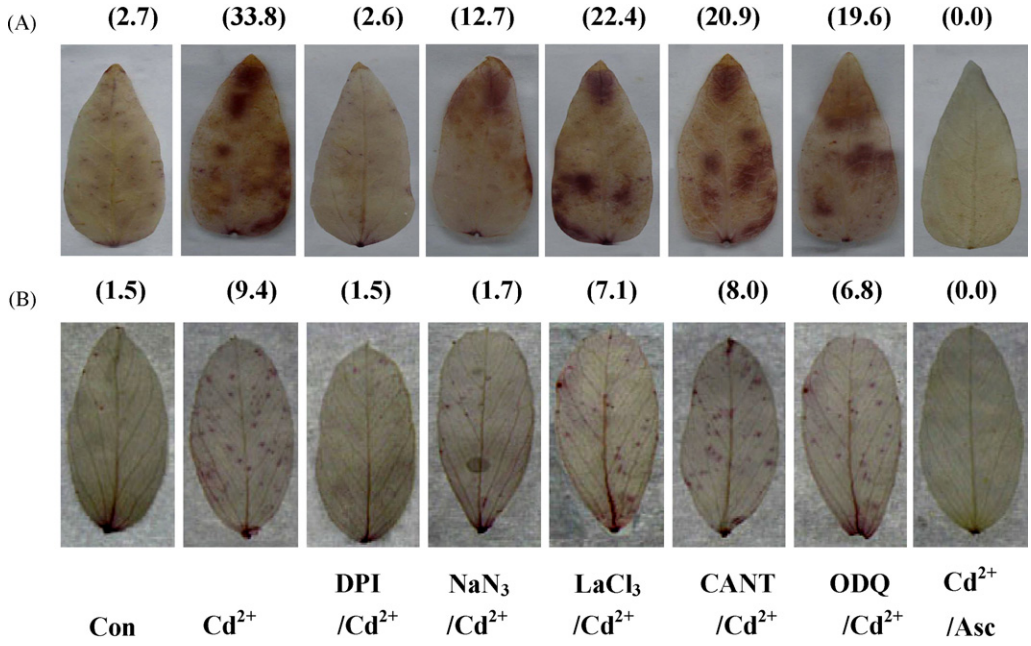

Fig. 1. Effects of inhibitor (DPI, NaN_3, $LaCl_3$, CANT, and ODQ) pretreatment and the H_2O_2 scavenger ASC on the production of Cd-induced H_2O_2 in leaves of *P. aureus* (A) and *V. sativa* (B). The plants were pretreated with 20 μM DPI, 1 mM NaN_3, 1 mM $LaCl_3$, 5 μM CANT, or 200 μM ODQ for 24 h, and then treated with 100 μM $CdCl_2$ for 6 d before infiltrating with DAB. The control plants were grown in full nutrient solution with no pretreatment or Cd. For ASC treatment, the leaves from plants treated with 100 μM $CdCl_2$ for 6 d were immersed in 1 mM ASC solution for 8 h before infiltrating with DAB. H_2O_2 deposits were quantified by measuring the number of pixel of spots by PHOTOSHOP 7.0. Results are expressed as percentage of spot area versus total leaf area in pixels. Experiments were repeated at least three times with similar results.