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Hydrogen-rich water enhances cadmium tolerance in Chinese cabbage by reducing cadmium uptake and increasing antioxidant capacities

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ABSTRACT

The aim of the present paper was to understand the specific mechanism of hydrogen-rich water (HRW) in alleviating cadmium (Cd) toxicity in Chinese cabbage (*Brassica campestris* spp. *chinensis* L.). Our results showed that the addition of 50% saturation HRW significantly alleviated the Cd toxic symptoms, including the improvement of both root elongation and seedling growth inhibition. These responses were consistent with a significant decrease of Cd accumulation in roots and shoots, which was further confirmed by the histochemical staining. Molecular evidence illustrated that Cd-induced up-regulations of *IRT1* and *Nramp1* genes, responsible for Cd absorption, were blocked by HRW. By contrast, Cd-induced up-regulation of the *HMA3* gene, which regulates Cd sequestration into the root vacuoles, was substantially strengthened by HRW. Furthermore, compared with those in Cd stress alone, the expressions of *HMA2* and *HMA4*, which function in the transportation of Cd to xylem, were repressed by co-treatment with HRW. HRW enhanced the activities of antioxidant enzymes, including superoxide dismutase, guaiacol peroxidase, catalase and ascorbate peroxidase. These results were further confirmed by the alleviation of oxidative damage, as indicated by the decrease of thiobarbituric acid reactive substances (TBARS) and reactive oxygen species (ROS) production. Taken together, these results suggest that the improvement of Cd tolerance by HRW was associated with reduced Cd uptake and increased antioxidant defense capacities. Therefore, the application of HRW may be a promising strategy to improve Cd tolerance of Chinese cabbage.

Introduction

Cadmium (Cd) is a highly toxic trace element, and is of particular concern to human health as it can be readily absorbed by plant roots, and be concentrated or accumulated by many cereals, potatoes, vegetables and fruits, *etc.* (Muchuweti et al., 2006; Magdalena et al., 2011). In plants, numerous biochemical and physiological processes, such as photosynthesis, respiration, nitrogen and protein metabolism, and nutrient uptake, are altered by Cd (Cho and Seo, 2005; Clemens, 2006). In addition, secondary oxidative stress usually appears, indicated by an increase of reactive oxygen species (ROS) and interference of cellular antioxidant systems.

Depending on the species, plants have evolved several mechanisms for metal detoxification, including exclusion, compartmentalization, chelation, and binding to organic ligands such as organic acids, amino acids, phytochelatins (PCs), and metallothioneins (MTs) (Cobbett and Goldsbrough, 2002; Pomponi et al., 2006). Plants with exclusion mechanisms, avoiding excessive metal uptake and restricting metal translocation from root to shoot, would be more appropriate for human consumption than metal-accumulating species. Therefore, it is very important to decrease the accumulation of toxic metals in the edible parts of vegetables by regulating uptake and transport of metals in plants.

Plants are presumed to take up Cd from the soils through their root systems, load it to the xylem and then transport it to the aerial parts (Clemens, 2006). Previous results have confirmed that several genes are involved in these transport processes controlling Cd accumulation in plants (Mendoza-Cózatl et al., 2011). The uptake of Cd, at the root level, is mediated by iron-regulated transporter 1 (IRT1) and natural resistance-associated macrophage protein

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; Cd, cadmium; HRW, hydrogen-rich water; POD, guaiacol peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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1 (Nramp1) (Kerkeb et al., 2008; Guerinot, 2010). Heavy metal ATPase2 (HMA2) and heavy metal ATPase4 (HMA4) promote root-to-shoot Cd translocation by loading Cd into the xylem (Haydon and Cobbett, 2007; Verret et al., 2005). In contrast, the heavy metal ATPase3 (HMA3) transporter, located at the tonoplast of root cells, limits root-to-shoot Cd translocation through selectively segregating Cd into the root vacuoles (Yang et al., 2010; Oomen et al., 2009).

Hydrogen gas, with the molecular formula H_2, is a colorless, odorless, tasteless and highly combustible diatomic gas that has been known for many years (Huang et al., 2010). Since H_2 was found to be potentially a “novel” antioxidant in preventive and therapeutic applications (Ohsawa et al., 2007), beneficial effects of H_2 have been reported in 38 diseases and physiological states, including hepatic ischemia, glaucoma, atherosclerosis, and Parkinson disease *etc.* (Ohta, 2012). Hydrogen gas was previously found to be released in plants (Renwick et al., 1964). For example, evolution of H_2 by seedlings, excised embryos, roots and hypocotyls in several higher plant species was reported previously (Renwick et al., 1964; Torres et al., 1984, 1986). Despite long knowing of H_2 releasing in plants, relatively few studies have focused on H_2 biology in plant systems in the last century. Meanwhile, the enzymes responsible for H_2 production in higher plants remain elusive.

More recently, increasing interest has shown in studies focusing on abiotic stress acclimation in plants by exogenously applied hydrogen-rich water (HRW), including Cd and aluminum (Al) tolerance (Cui et al., 2013; Chen et al., 2014), salt tolerance (Xie et al., 2012), and drought and freezing tolerance (Jin et al., 2013a). Although HRW was found to decrease Cd uptake in alfalfa seedling root tissues (Cui et al., 2013), the specific mechanism is unclear. In addition, H_2 has the ability to delay postharvest ripening and senescence of kiwifruit (Hu et al., 2014), regulate adventitious root development of cucumber (Lin et al., 2014), promote anthocyanin synthesis of radish sprouts (Su et al., 2014) and accelerate shoot and root growth of mung bean (Zeng et al., 2013).

Chinese cabbage (*Brassica campestris* spp. *chinensis* L.) is one of the most widely grown vegetables in China, and its productivity and quality are considerably decreased due to abiotic stresses, including Cd exposure. Therefore, the main aim of this study was to provide more convincing evidence showing how HRW decreased Cd accumulation at both the structural and gene levels. In addition, the link between the HRW-triggered improvement of seedling growth inhibition and the amelioration of oxidative damage in Chinese cabbage seedling challenged with Cd was preliminarily illustrated. These results suggest a positive role of HRW in reducing pollutant residues for food safety in the fields.

Materials and methods

Plant materials, growth conditions, experimental design and growth analysis

The seeds of Chinese cabbage (*Brassica campestris* spp. *chinensis* L., Dongfang 2), kindly supplied by Jiangsu Agricultural Institutes (Jiangsu Province, China), were soaked in different concentrations of HRW for 3 h, and then germinated for 1 day at 23 °C in the darkness. Uniform-sized seeds were selected and transferred to the plastic chambers containing quarter-strength Hoagland’s solution. The nutrient solutions were prepared with different concentrations of HRW and the initial pH was adjusted to 6.0 by using dilute NaOH or HCl. All treatment solutions were renewed every 12 h to maintain constant concentrations. Seedlings were grown in the illuminating incubator (12 h light with a light intensity of 200 $\mu mol\ m^{-2}\ s^{-1}$, 25 ± 1 °C, and 12 h dark, 23 ± 1 °C). After having grown in solution supplemented with 0 or 50% saturation of HRW for 48 h, seedlings were transferred into solutions with 0 or

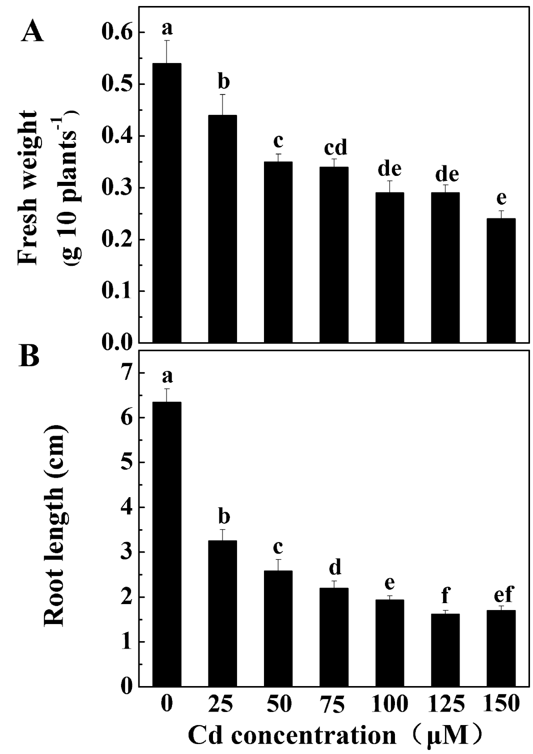

Fig. 1. Effects of varying concentrations of Cd on fresh weight (A) and root length (B) of Chinese cabbage. Seedlings were incubated in the solutions containing different concentrations of Cd and elongation was measured after 48 h incubation. Data are means ± SE of three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan’s multiple range test.

50 μM $CdCl_2$ and incubated for another 24 h. Seedlings without HRW pretreatment were used as the control (H_2O). We described these different treatments as (I) $H_2O \rightarrow$ 0Cd, (II) HRW → 0Cd, (III) $H_2O \rightarrow$ 50Cd and (IV) HRW → 50Cd. After various treatments, the seedlings were sampled and growth parameters were determined. Root tissues were used immediately or frozen in liquid nitrogen for further analysis.

Growth tests were carried out on three replicates of 10 plants each. The measurements of root length and fresh weight were used by ruler (0.1 cm) and electronic balance (0.0001 g) after various treatments. The changes in root length and fresh weigh were obtained by calculating the difference value between pre- or post-treatment with 0 or 50 μM $CdCl_2$ for 24 h.

Preparation of HRW

Purified H_2 gas (99.99%, v/v) was generated using a H_2-producing apparatus (SCH-300, Saikesaisi Hydrogen Energy Co Ltd., Shandong, China). It was bubbled into 4000 ml distilled water (pH 6.0, 25 °C) at a rate of 160 ml min^{-1} for 1 h, a sufficient duration to saturate the solution with H_2 (Cui et al., 2013). Then, the corresponding HRW was immediately diluted to the required concentrations [1, 10 and 50% concentration, (v/v)].

Histochemical detection of H_2O_2 and $O_2{}^{-O}$

Stress-induced generation of $O_2{}^{-O}$ *in situ* was detected by nitroblue tetrazolium (NBT) staining (Sung and Hong, 2010). Seedling roots were immersed with 0.1% solution of NBT in 10 mM potassium phosphate buffer (pH 7.8) containing 10 mM sodium azide NaN_3, and then incubated in the darkness at 22 °C for 10 min until a purple-blue color became visible. Hydrogen peroxide (H_2O_2) production was detected with freshly prepared