Journal of Plant Physiology 175 (2015) 174–182

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

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

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ARTICLE INFO

Article history:
Received 29 May 2014
Received in revised form
25 September 2014
Accepted 28 September 2014
Available online 18 November 2014

Keywords:
Chinese cabbage
Hydrogen-rich water
Cadmium tolerance
Cadmium uptake
Oxidative stress

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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http://dx.doi.org/10.1016/j.jplph.2014.09.017
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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 μmol 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 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 → 0Cd, (II) HRW → 0Cd, (III) H_2O → 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.

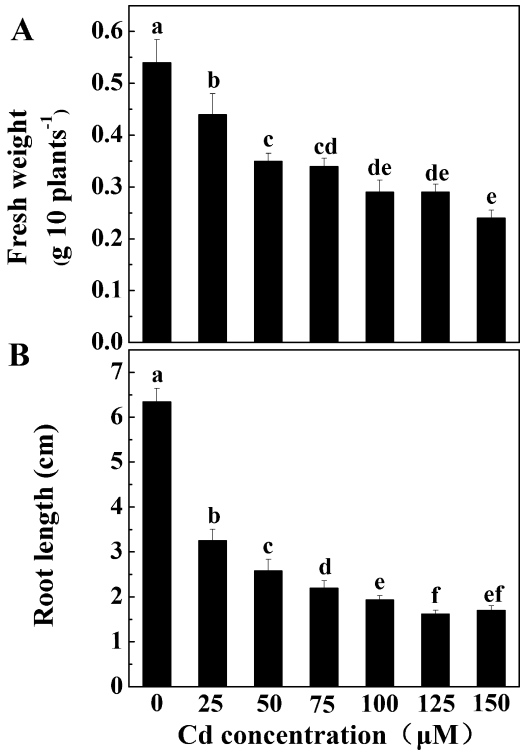

Fig. 1. Effects of varying concentrations of Cd on fresh weight (A) and root length (B) of Chinese cabbage. Seedlings were incubated in the solutions containing different concentrations of Cd and elongation was measured after 48 h incubation. Data are means ± SE of three independent experiments. Bars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

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

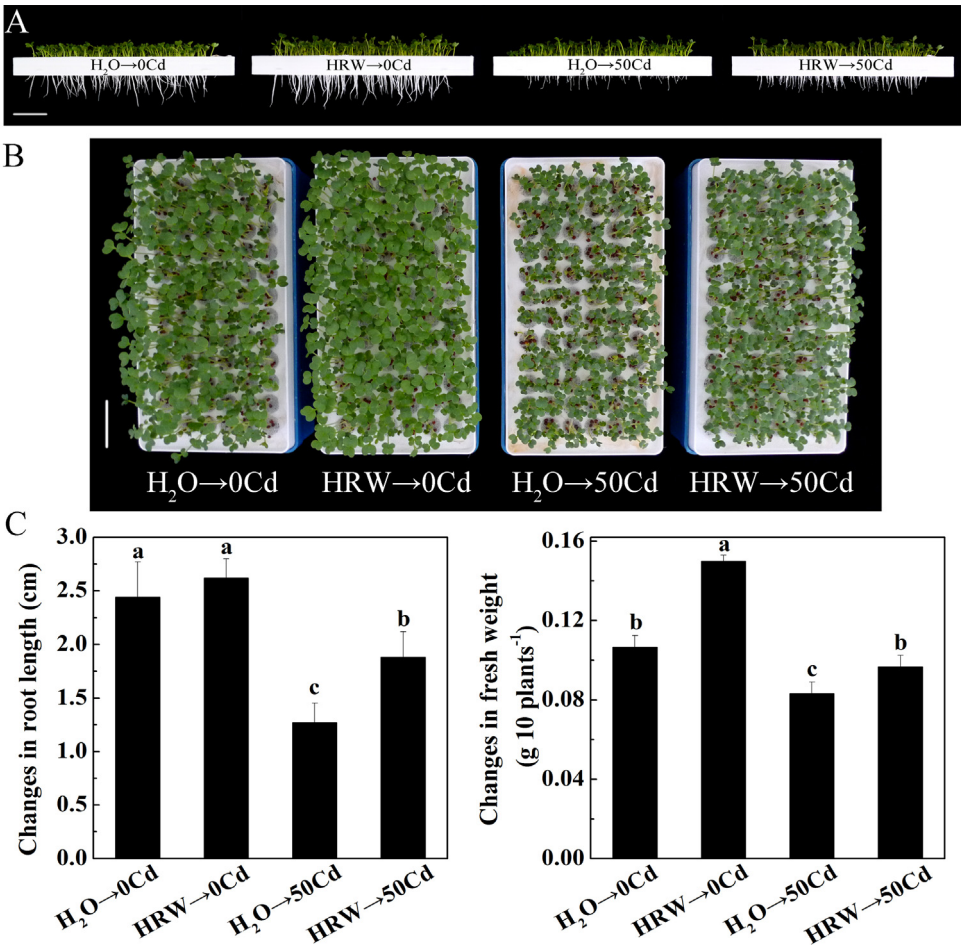

Fig. 2. HRW pretreatment alleviated Cd stress-induced Chinese cabbage seedlings growth inhibition. The seedlings in solution were supplemented with 0 or 50% saturation of HRW for 48 h, followed by another 24 h incubation in 0 or 50 μM $CdCl_2$. Bar = 3 cm (A). Changes in root length (B) and fresh weight (C) during the 24 h treated with 0 or 50 μM $CdCl_2$. Data are means ± SE from three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

3,3′-diaminobenzidine (DAB) solution (0.1%, w/v, pH 3.8) for 1 h (Lv et al., 2011). After washing extensively, all the decolorized roots were observed under a light microscope (model Stemi 2000-C; Carl Zeiss, Jena, Germany) and photographed on a color film (Powershot A620; Canon Photo Film, Tokyo, Japan).

Analysis of TBARS concentration and root vitality

Lipid peroxidation based on thiobarbituric acid reactive substances (TBARS) reported in malondialdehyde (MDA) equivalents was used as an indicator of oxidant stress in Cd-exposed roots. It was estimated by measuring the concentration of TBARS as described by Jin et al. (2013a). Briefly, fresh root tissues (0.5 g) were homogenized in a mortar with 5 ml solution containing 0.25% 2-thiobarbituric acid (TBA) and 10% trichloroacetic acid (TCA). The mixture was heated at 95 °C for 30 min, and then quickly cooled in an ice bath and centrifuged at 10,000 × *g* for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The blank was 0.25% TBA in 10% TCA. Root activity was analyzed as described previously (Kerley, 2000).

Antioxidant enzyme activity assays

Fresh root tissues (0.3 g) were homogenized in 5 ml of 50 mM cool phosphate buffer (pH 7.0), containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) polyvinylpolypyrrolidone (PVP) for assays of superoxide dismutase (SOD), guaiacol peroxidase (POD) and catalase (CAT), or combinations with the addition of 1 mM ascorbic acid (AsA) in the case of ascorbate peroxidase (APX) determination. The homogenates were centrifuged at 12,000 × *g* for 20 min at 4 °C, and the supernatants were used for assays of the enzyme activity.

SOD and POD activities were analyzed using the methods as described previously (Han et al., 2008). Total SOD activity was measured on the basis of its ability to reduce nitroblue tetrazolium (NBT) by the superoxide anion generated by the riboflavin system under illumination. One unit of SOD (U) was defined as the amount of the crude enzyme extract required to inhibit the reduction rate of NBT by 50%. POD was determined by measuring the oxidation of guaiacol (extinction coefficient 26.6 mM^{-1} cm^{-1}) at 470 nm. APX activity was measured by monitoring the decrease in absorbance at 290 nm as AsA was oxidized (ε = 2.8 mM^{-1} cm^{-1}) for at least 1 min in 3 ml reaction mixture, as described by Jin et al. (2013a). CAT activity was spectrophotometrically measured by monitoring the consumption of hydrogen peroxide (H_2O_2; ε = 39.4 mM^{-1} cm^{-1}) at 240 nm for 3 min (Huang et al., 2006).

Determination of Cd concentrations in plant tissues

At harvest, 100 plant samples of each treatment were collected and separated into roots, stems and leaves. They were then carefully

washed with deionized water after rinsing with 20 mM EDTA–Na solution for about 30 min, dried at 105 °C for 20 min and then at 70 °C in an oven until completely dried (Wei and Zhou, 2004). The dried plant samples were ground to powder and digested in a solution containing 87:13 HNO_3:$HClO_4$ solution. The concentrations of heavy metals were determined using atomic absorption spectrophotometry (180-80 Hitachi, Tokyo, Japan) as described by Liu et al. (2008).

Cd localization at root

To characterize Cd localization at the tissue level, intact tissues of fresh samples of fine roots were rinsed in deionized water, subsequently exposed to a staining solution (30 mg diphenylthiocarbazone in 60 ml acetone, 20 ml water, and 100 ml glacial acetic acid) for 1 h, and then briefly rinsed in deionized water as suggested by Clabeaux et al. (2011). Some of the samples were fixed with fixative and cut into pieces with a freezing microtome. The microsections and intact roots with cadmium–dithizone precipitates displaying red–black were photographed under a light microscope (Axio Imager, Olympus) with a CCD (DS-Fi1, Canon) connected to a computer.

Real-time quantitative RT-PCR analysis

Total RNA was isolated from root tissues using Trizol extraction reagent (Invitrogen, Gaithersburg, MD, USA) and the RNA purity was verified by the ratio (>1.9) of 260/280 nm absorbance. DNA-free total RNA (5 μg) from different treatments was used for first-strand cDNA synthesis in a 20 μl reaction volume (Thermo Scientific, MD, Lithuania) according to the manufacturer's instructions. Real-time quantitative PCR reactions were performed using a Mastercycler® ep realplex real-time PCR system (ABI7500, MD, USA) with Bestar® SybrGreen qPCR mastermix (DBI, Bioscience Inc., Germany) in a 20 μl reaction volume according to the user manual.

PCR primers targeting *IRT1* (accession number AY087095.1), *IRT2* (accession number BT025714.1), *Nramp1* (accession number AF165125.1), *HMA2* (accession number NM119157.3), *HMA3* (accession number DQ446885.1) and *HMA4* (accession number AY096796) were designed using Primer Express® version3.0 (Applied Biosystems), and *actin* (accession number AF111812) was followed as described by Xiao et al. (2012). All primers (Supplementary Table 1) were synthesized by Genewiz Bio-engineering Ltd. Company (Suzhou, China). The relative expression level was presented relative to that of corresponding control sample at the indicated time, after normalization to *actin* transcript levels.

Supplementary Table S1 can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2014.09.017.

Data presentation and statistical analysis

Values are shown as the means ± SE of three independent experiments with three replicates each. Differences among treatments were analyzed by one-way analysis of variance (ANOVA) combined with Duncan's multiple range test, taking $P < 0.05$ as the thresholds.

Results

HRW alleviated the inhibitory effects of Cd on seedling growth

To evaluate the sensitivity of Chinese cabbage to Cd, seedlings were exposed to hydroponic solution supplemented with different concentrations of $CdCl_2$ (25, 50, 75 and 100 μM Cd) for 48 h. The results of Fig. 1 revealed that the growth of seedlings was markedly inhibited by $CdCl_2$ as evaluated by a significant decrease in both fresh weight and root length, especially at Cd concentration

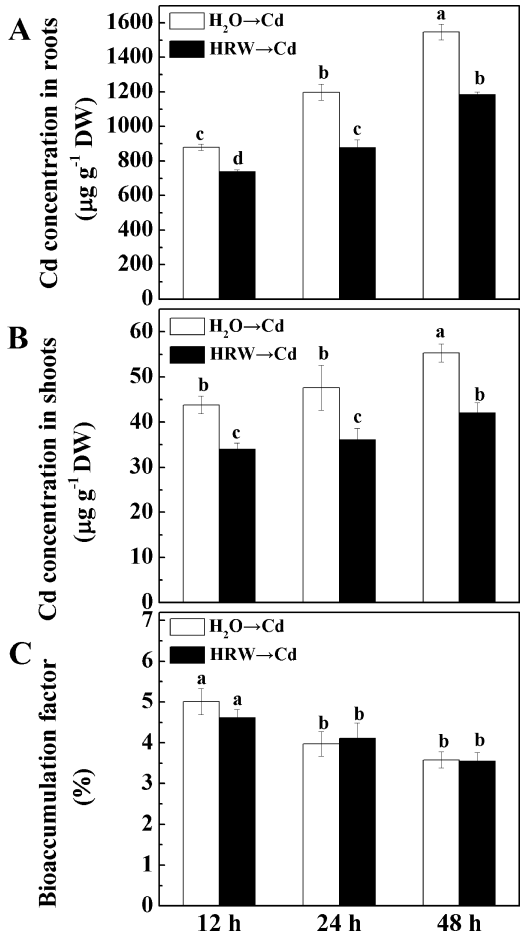

Fig. 3. Effects of HRW pretreatment on Cd concentration in the seedlings of Chinese cabbage upon Cd stress. The roots in solution were supplemented with 0 or 50% saturation of HRW for 48 h, followed by another 12 h, 24 h, 48 h incubation in 50 μM $CdCl_2$. Afterwards, Cd concentration of root (A) and shoot (B) parts of 100 seedlings were determined using atomic absorption spectrophotometry respectively. The bioaccumulation factor (%) presents the ratio of the Cd concentration in shoots to that in roots. Data are means ± SE from three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

>50 μM. Thus, 50 μM Cd was subsequently used to investigate the role of HRW in the alleviation of Cd-induced inhibition of seedling growth.

Further experiments showed that pretreatment with different concentrations of HRW (1, 10, 50 and 100%) differentially alleviated inhibitory effects of Cd on the growth of seedlings. A maximal inducible response was observed in 50% HRW-pretreated plants, which was manifested as a significant increase in both fresh weight and root length (in particular), and a decrease in TBARS compared with the HRW-free control samples (Fig. S1). Therefore, we used 50% HRW to further investigate the role of HRW in the regulation of Cd tolerance.

Supplementary Fig. S1 can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2014.09.017.

Subsequently, the effects of HRW on growth parameters were observed in the presence or absence of Cd. Results of Fig. 2 showed that, compared with the control samples ($H_2O \rightarrow 0Cd$), a significant increase in fresh weight by HRW was observed under non-stress conditions, but not in root elongation. In comparison with the plants challenged with Cd alone, a pretreatment of Cd-stressed seedlings with 50% HRW increased root elongation and fresh weight by about 48% and 16% after 24 h Cd treatment, respectively (Fig. 2C and D). These results clearly revealed that HRW was

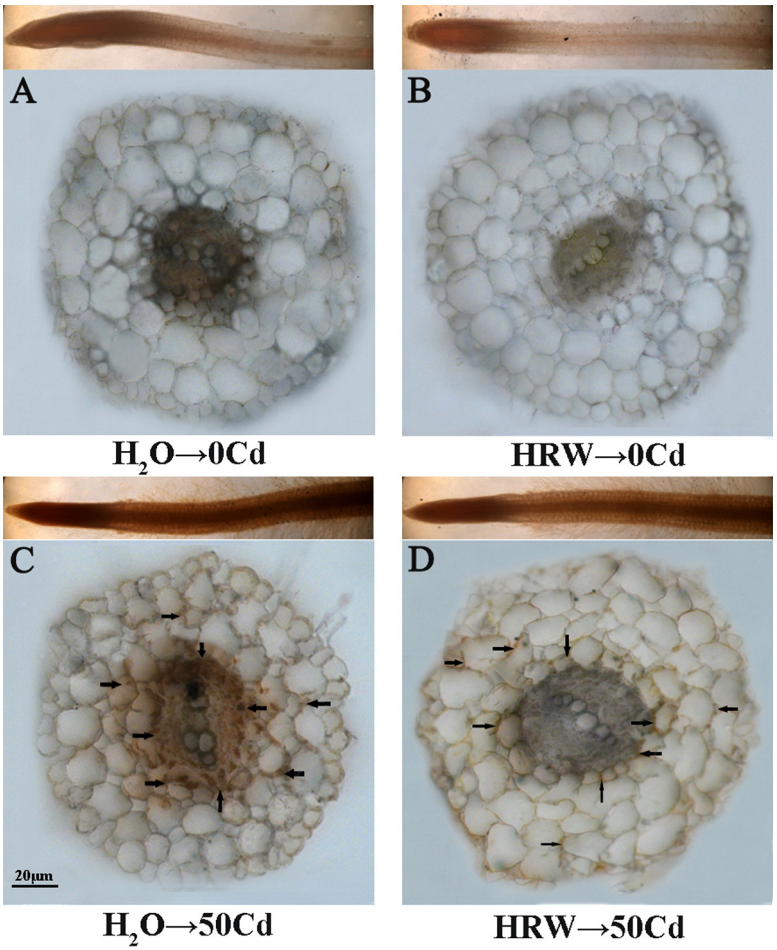

Fig. 4. Cadmium localization in root tissues of Chinese cabbage. The roots were pretreatment with 0 or 50% saturation of HRW solution for 48 h, followed by exposure to 0 or 50 μM $CdCl_2$ for another 24 h. Bar = 20 μm. Arrows point to precipitates of cadmium–dithizone.

beneficial to increase fresh weight and attenuate the Cd-induced inhibition of root elongation in Chinese cabbage seedlings.

HRW decreased Cd accumulation

As shown in Fig. 3, HRW pretreatment inhibited Cd uptake of seedlings at different time points, manifested as a significant decrease of Cd concentration in root and shoot parts. For example, Cd concentrations in roots at 12, 24 and 48 h after treatments were reduced by 16.0, 36.6 and 23.5%, respectively, when pretreated with 50% HRW *versus* Cd treatment alone (Fig. 3A) as compared to 22.4, 24.3 and 24.1% in shoots (Fig. 3B). These results revealed that the pretreatment of 50% HRW significantly decreased the accumulation of Cd. Additionally, the bioaccumulation factor, which reflects the ratio of Cd concentration in shoots to that in roots, showed no notable difference between H_2O and HRW treatments (Fig. 3C).

Cadmium–dithizone staining was widely used to monitor Cd localization in plants (Clabeaux et al., 2011; He et al., 2013). The alleviation of Cd accumulation in root apexes by HRW pretreatment was also supported by histochemical staining results (Fig. 4). Notably, stronger cadmium–dithizone staining was observed in Cd treatments, with formation of distinct granules in the apoplast of root cortical cells (Fig. 4C and D). Furthermore, the roots treated with Cd alone were stained extensively, while those pretreated with HRW displayed less dithizone-stained. Interestingly, in root cross sections, much more granules of cadmium–dithizone staining were observed in endodermis cells near stele in the Cd treatment alone, but granules were observed mainly in intercellular spaces of cortical cells in the co-treatment with HRW.

Expression of genes responsible for heavy metal homeostasis

To confirm the above results, expression of genes related to Cd uptake or heavy metal detoxification in roots was investigated. As shown in Fig. 5, under non-stress conditions, pretreatment of HRW increased the transcript level of *IRT1* at the time points of 24 and 48 h and decreased the transcript level of *HMA4* as compared with the HRW-free control samples. Under Cd-stressed conditions, the expression of most of these genes was dramatically changed in roots. For instance, *IRT1*, *Nramp1* and *HMA2* transcript levels were increased in plants treated with Cd alone and these increases in *IRT1*, *HMA2* were significant, all of which were significantly blocked by HRW (Fig. 5A, C and D). Additionally, at the time points of 12 and 24 h, Cd caused an increase in the transcript level of *IRT2* and *HMA3* (Fig. 5B and E), which was strengthened by pretreatment with HRW. The opposite inducible responses were observed in *HMA4* gene with its transcript level being lower in plants pretreated with HRW (Fig. 5F).

HRW regulated lipid peroxidation and ROS homeostasis and improved root vitality

The effect of HRW on the Cd-induced ROS overproduction was investigated by histochemical staining method. The results showed that low production of O_2^{-} was detected (NBT staining)

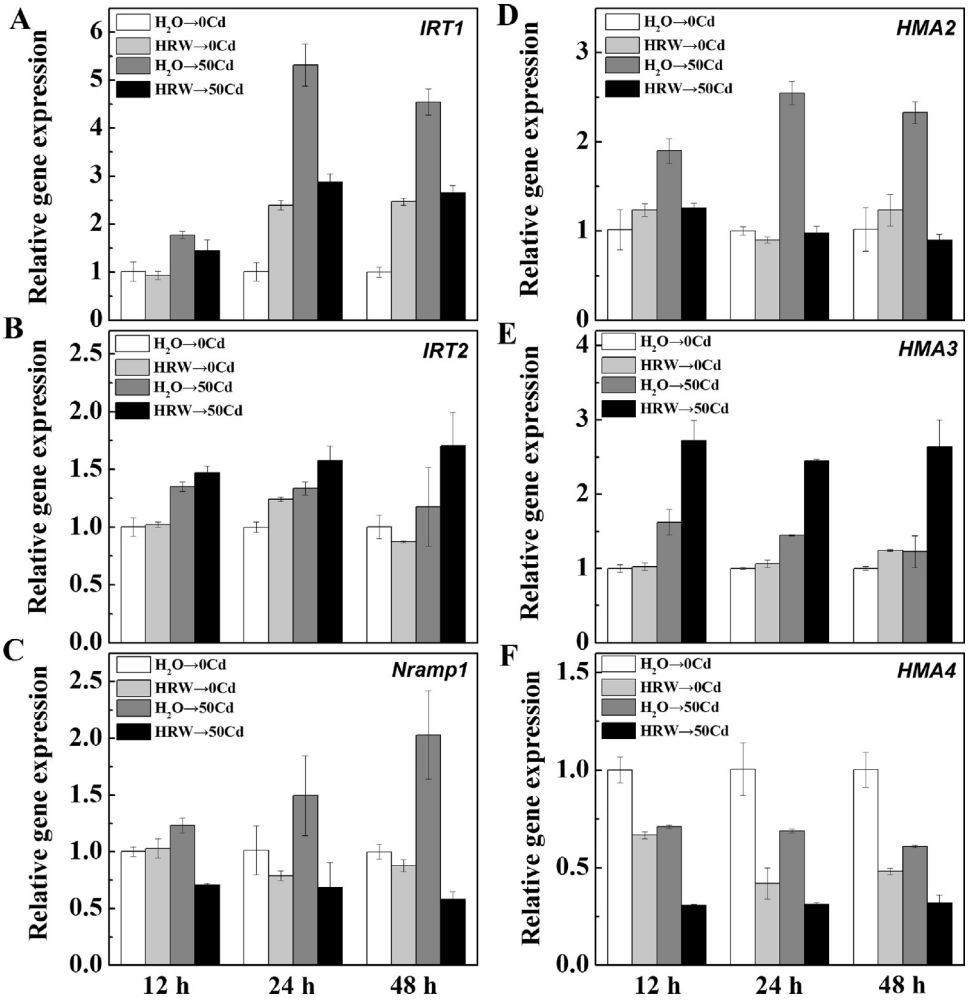

Fig. 5. Effects of HRW pretreatment on gene expression of *IRT1*, *IRT2*, *Nramp1*, *HAM2*, *HAM3* and *HAM4* in the roots of Chinese cabbage upon Cd stress with time course. Expression levels of corresponding genes are presented relative to the control samples, with normalized against expression of two internal reference genes in each sample. Data are means ± SE from three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

and H_2O_2 (DAB staining) was hardly detected in Cd-free control plants (Fig. 6A and B). As expected, root tissues of Chinese cabbage seedlings exhibited marked blue and brown coloration after Cd treatment, suggesting more ROS accumulation compared with Cd-free control plants. Meanwhile, the roots treated with Cd alone were stained extensively, but those pretreated with 50% HRW had only slight staining. Comparatively, the changes of TBARS contents were consistent with the corresponding histochemical staining. For example, the results of Fig. 6C revealed that in co-treatment with HRW and Cd, TBARS concentration was 14.9% lower than that in Cd-stress alone (Fig. 6C). We also noticed that the root vitality of Cd-stressed plants was significantly higher in the presence of HRW than in the absence of HRW (Fig. 6D).

HRW activated antioxidant enzyme activities in roots

Activities of SOD, POD and APX in roots were 51.6, 39.9 and 40.3% lower in the Cd treatment than in the Cd-free control treatment, respectively (Fig. 7A–C). However, the higher SOD, POD and APX activities were noted in the co-treatment with HRW than in the Cd treatment alone. Although Cd alone could clearly stimulate the CAT activity, the Cd-induced CAT activity was further enhanced significantly by HRW pretreatment (Fig. 7D).

Discussion

HRW alleviated Cd accumulation by modulating genes related to Cd uptake and transport

Cd tolerance in Chinese cabbage was boosted by HRW pretreatment. By combining Cd test with cadmium–dithizone staining analysis, we first showed that Cd concentration in both root and shoot parts was decreased, which was consistent with the previous work in alfalfa, showing that H_2-indcued plant tolerance to Cd is due to less toxic-metal absorption (Cui et al., 2013).

Most importantly, we showed that the decreased Cd accumulation in shoot and root parts was related to genes which regulate Cd uptake and transport. Previous investigations suggested that *IRT1* and *Nramp1* play important roles in Cd uptake in plants (Lin and Aarts, 2012; Yang et al., 2010; Varotto et al., 2002). The present results indicate that HRW significantly inhibited the Cd-induced up-regulation of the transcript levels of *IRT1* and *Nramp1* at different time points (Fig. 5A and C). This observation was in accordance with the decreased Cd accumulation in both root and shoot tissues (Fig. 3A and B). Genes of *HMAs* regulate metal homeostasis in plant cells, which was mainly manifested by *HMA2* and *HMA4* mediating the Cd loading into the xylem and *HMA3* regulating Cd sequestration into the root vacuoles (Barabasz et al.,

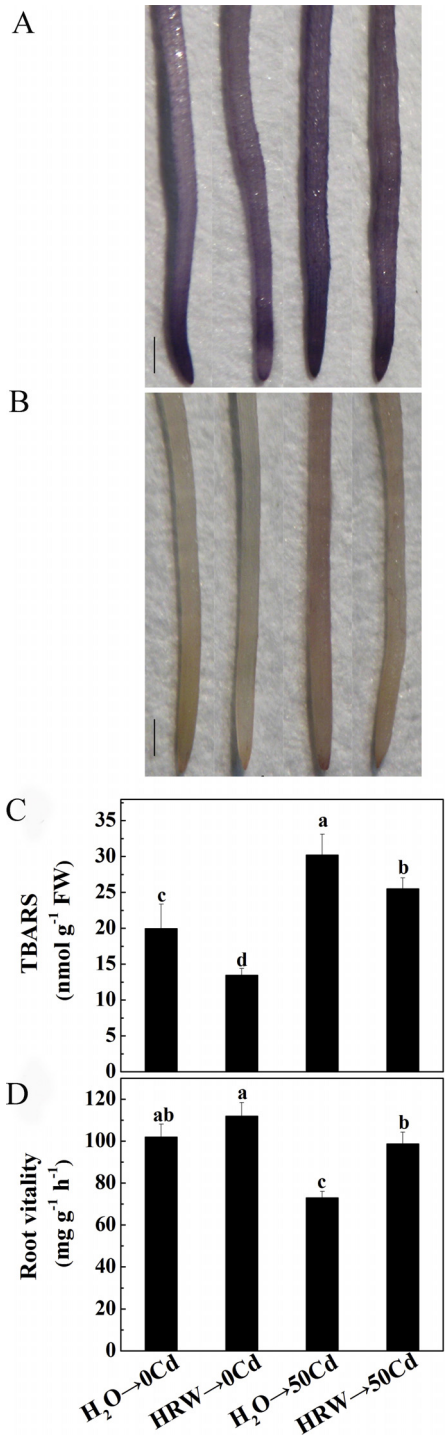

Fig. 6. Effects of HRW pretreatment on loss of plasma membrane integrity (A), reactive oxygen species (ROS) localization (B), TBARS (C), and root vitality (D) in the roots of Chinese cabbage under Cd stress. The seedlings in solution were supplemented with 0 or 50% saturation of HRW for 48 h, followed by another 24–h-incubation in 0 or 50 μM $CdCl_2$. Bar = 1 mm (a, and b). Data are means ± SE from three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

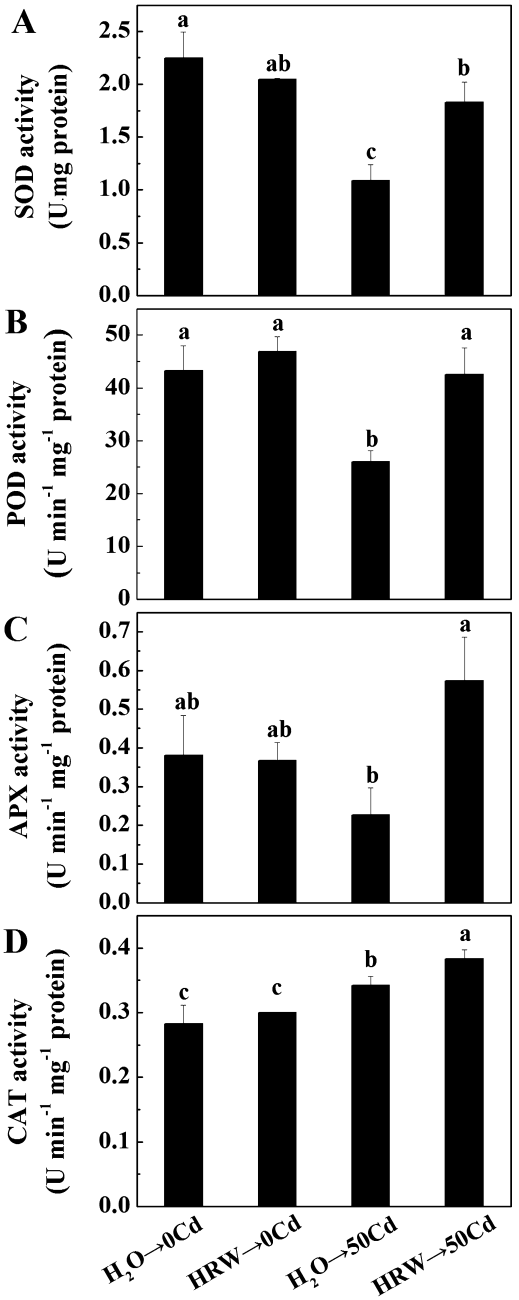

Fig. 7. Effects of HRW pretreatment on SOD (A), POD (B), APX (C) and CAT (D) activities in the roots of Chinese cabbage upon Cd stress. The roots in solution were supplemented with 0 or 50% saturation of HRW for 48 h, followed by another 24-h-incubation in 0 or 50 μM $CdCl_2$. Data are means ± SE from three independent experiments. Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

2013; Hanikenne et al., 2008). Therefore, increased transcript level of *HMA3* in HRW-pretreated seedlings could favor to sequestrate more Cd into vacuoles, resulting in alleviating Cd toxic effect. Meanwhile, pretreatment with HRW also decreased *HMA2* and *HMA4* transcript levels (Fig. 5D and F). Thus, it is possible that HRW decreases Cd transport to xylem tissue of roots, which leads to lower Cd concentration in shoots (Fig. 3B) and less granules of cadmium–dithizone staining (Fig. 4D). However, the ratio of Cd concentration in shoot/root had no significant differences (Fig. 3C), which was not consistent with the results shown above. Therefore, the mechanisms underlying need to be further explored. *IRT2*, which is a gene with a minor function in Fe uptake (Cheng et al., 2007; Vert et al., 2001), was up-regulated to make plants absorb more Fe from the Fe-deficient soil (Vert et al., 2009). In the present study, pretreatment with HRW increased Cd-induced transcript level of *IRT2* (Fig. 5B), suggesting that HRW may increase Fe uptake by Chinese cabbage seedlings. Previous reports showed that addition of Fe significantly alleviated Cd stress by reducing Cd uptake

(Astolfi et al., 2012; Muneer et al., 2012). Although *IRT1* and *IRT2* belong to the same gene family and have similar function in Fe uptake, inconsistent expression patterns of *IRT1* and *IRT2* genes responsive to HRW under Cd-stress were observed. The Fe uptake may be blocked by the suppression of IRT1 induced by HRW treatment under Cd stress. In order to reestablish Fe^{2+} homeostasis, *IRT2* was up-regulated as a compensation effect to promote Fe absorption.

HRW-enhanced antioxidant defense capacity: a possible link between Cd tolerance and the amelioration of oxidative damage

Numerous experimental studies have shown that Cd may cause oxidative stress by inducing production of ROS (Romero-puertas et al., 2003; Cho and Seo, 2005 Rodríguez-Serrano et al., 2009). Our results demonstrated that treatment with 50 μM Cd enhanced TBARS levels in cabbage seedlings (Fig. 6C), which is an index of lipid peroxidation and, therefore, of oxidative stress as well. Similarly, considerable H_2O_2 and O_2^{-O} formation was observed in the Cd-treated roots of cabbage seedlings compared with the control (Fig. 6A and B). Under most conditions, H_2O_2 in plants can be efficiently scavenged by CAT, POD and APX (Foyer and Noctor, 2005). In the present study, Cd treatment significantly decreased POD and APX activities in cabbage seedlings, suggesting that the high level of H_2O_2 in Cd-stressed plants was caused in part, at least, by a decreased capacity to detoxify H_2O_2. This result was consistent with the previous reports (Uraguchi et al., 2006; Metwally et al., 2005; Shah et al., 2001).

In recent years, HRW has been found to enhance the antioxidant capacities inducing plant tolerance to salinity stress (Xie et al., 2012; Xu et al., 2013), Cd toxicity (Cui et al., 2013) and paraquat-induced oxidative stress (Jin et al., 2013a). Our results also demonstrate that HRW treatment may ameliorate damaging effects of Cd in Chinese cabbage. This HRW-induced Cd tolerance in plants may be attributed to both H_2-enhancement of antioxidant defense activities (Fig. 7) and H_2-regulation of Cd uptake, transport, and distribution in plant organs (Fig. 3). Pretreatment with HRW led to a decrease in oxidative injuries caused by Cd (as measured by TBARS) in Chinese cabbage seedlings. This result was in good agreement with the decreased accumulation of ROS and increase in activities of antioxidant enzymes (SOD, CAT, APX, and POD), suggesting that HRW may decrease lipid peroxidation in cabbage seedlings under Cd stress by activating antioxidant enzymes. Similar induction of antioxidant enzymes has been observed in Arabidopsis (Xie et al., 2012), rice (Xu et al., 2013) and alfalfa (Jin et al., 2013a). One of the possible roles of HRW may be that H_2 can readily permeate the cell membrane thereby increasing gene expression of antioxidant genes encoding for SOD, CAT, POD and APX (Cui et al., 2013). Moreover, it is possible for H_2 to directly reduce ROS *in vivo* in the same manner as in animals (Ohsawa et al., 2007). It is observed in an *in vitro* experiment that HRW was able to directly quench H_2O_2, but not singlet oxygen radical (Xie et al., 2012). Electrolyzed-reduced water, which dissolved large amounts of H_2, could scavenge ROS and protect pBluescript II plasmid DNA from oxidative damage (Shirahata et al., 1997). Some other non-enzymatic antioxidant materials were also found to affect Cd uptake; for instance, overexpression of γ-glutamylcysteine synthetase was found to be bound up with the Cd accumulation in Indian mustard (Zhu et al., 1999) and exogenous addition of AsA or *N*-acetyl-L-cysteine also played a part in the reduction of Cd uptake (Jin et al., 2013b; Deng et al., 2010). Whether the reduction of Cd accumulation of the seedlings by HRW in this study was related to the change of non-enzymatic or enzymatic antioxidants also needs to be further explored.

Taken together, evidence from this work shows that pretreatment with HRW could effectively alleviate the growth inhibition and oxidative damage triggered by Cd stress in Chinese cabbage. Reduction of Cd concentration in roots and shoots, regulated by the genes responsible for Cd absorption, could be one of the main reasons why the HRW pretreatment improved Cd tolerance. Additionally, HRW-attenuated inhibition of the seedling growth induced by Cd was partially due to the reduced lipid peroxidation by enhancing antioxidant defense capacities. However, the detailed mechanisms by which HRW executes function in Cd tolerance responses should be investigated by genetic and molecular means in the future.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (31171998) and the Fundamental Research Funds for the Central Universities (KYZ201316).

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