Hydrogen gas (H$_2$) has a possible signaling role in many developmental and adaptive plant responses, including mitigating the harmful effects of cadmium (Cd) uptake from soil. We used electrophysiological and molecular approaches to understand how H$_2$ ameliorates Cd toxicity in pak choi (Brassica campestris ssp. chinensis). Exposure of pak choi roots to Cd resulted in a rapid increase in the intracellular H$_2$ production. Exogenous application of hydrogen-rich water (HRW) resulted in a Cd-tolerant phenotype, with reduced net Cd uptake and accumulation. We showed that this is dependent upon the transport of calcium ions (Ca$_{2+}$) across the plasma membrane and apoplastic generation of hydrogen peroxide (H$_2$O$_2$) by respiratory burst oxidase homolog (BcRbohD). The reduction in root Cd uptake was associated with the application of exogenous HRW or H$_2$O$_2$. This reduction was abolished in the pretreated with HRW showed decreased expression, H$_2$O$_2$ production, and Cd$_{2+}$ influx inhibition. Collectively, our results suggest that the Cd-protective effect of H$_2$ in plants may be explained by its control of the plasma membrane-based NADPH oxidase encoded by RbohD, which operates upstream of IRT1 and regulates root Cd uptake at both the transcriptional and functional levels. These findings provide a mechanistic explanation for the alleviatory role of H$_2$ in Cd accumulation and toxicity in plants.

Cadmium (Cd) is a toxic heavy metal that is relatively mobile in the soil and has become a serious worldwide environment problem (Chaney, 2015). Cd is easily taken up by plant roots and can be loaded into the xylem for transport to above-ground tissues (Mendoza-Cózatl et al., 2011). Cd accumulation in the shoot inhibits plant growth by causing an array of morphological, physiological, biochemical, and ultrastructural changes (Romero-Puertas et al., 2004; Ali et al., 2015; Gill et al., 2015). Most plants are sensitive to even low (micromolar range) Cd concentrations. Also, even if plants do not show symptoms of toxicity and their growth is not affected, Cd accumulation in the shoot can potentially cause harm to humans through the food chain (Beccaloni et al., 2013). Thus, a better understanding of the mechanisms controlling Cd uptake and transport in plants (especially in edible leafy vegetables), and some practical solutions for minimizing Cd accumulation in above-ground plant parts, is critical to food safety.

Cytosolic free Ca$_{2+}$ is a ubiquitous second messenger mediating a broad array of adaptive responses in plants (Gilroy et al., 2016). Ca$_{2+}$ transport across the plasma membrane (PM) of root cells could be mediated by many ion channels with different gating properties (Demidchik et al., 2018). Cd exposure results in a decrease in Ca content in different plant species due to the competition of binding sites for proteins or transporters (Sandalio et al., 2001). Also, Cd$_{2+}$ influx in plant roots...
could be inhibited by Ca$^{2+}$ channel blockers (Li et al., 2012; Sun et al., 2013; He et al., 2015), suggesting a possible involvement of Ca$^{2+}$-permeable channels in Cd$^{2+}$ uptake. In rice (Oryza sativa), annexin 4 (OsAAN4) and Glu receptor-like 3.4 (OsGLR3.4) channels are the most likely candidates for this role (Chen et al., 2018). Also, elevated Ca levels suppress Cd uptake (Lu et al., 2010) as a result of competition between Ca$^{2+}$ and Cd$^{2+}$ uptake for the same transporter. However, to the best of our knowledge, the role of Ca as a signaling agent in regulating Cd entry into root cells has not been explored.

Other second messengers mediating rapid systemic signaling in response to stress are reactive oxygen species (ROS; Gilroy et al., 2016; Dell’Aglì et al., 2019). The most studied ROS-producing enzymes in plants are respiratory burst oxidase homologs (RBOHs) located at the PM (Torres and Dangl, 2005; Suzuki et al., 2011). Two RBOH isomers, RBOHD and RBOHF, play major roles in plant responses to abiotic stimuli (Kärkönen and Kuchitsu, 2015). The increase in cytosolic Ca$^{2+}$ concentration activates a plethora of Ca-dependent protein kinases that phosphorylate RBOHD and RBOHF (among other substrates) to amplify ROS production (Sierla et al., 2016) via the so-called “ROS-Ca hub” (Demidchik and Shabala, 2018). In its turn, apoplastic ROS production leads to Ca$^{2+}$ influx by activating ROS-sensitive Ca$^{2+}$-influx cation channels. This self-amplification mechanism can increase the duration and amplitude of weak signals (Richards et al., 2014; Demidchik and Shabala, 2018). However, with excessive Ca$^{2+}$, the phosphorylation ability of calcium-dependent protein kinases decreases the production of ROS by RBOH.

Among all ROSs, hydrogen dioxide (H$_2$O$_2$) is often put forward as the most attractive signaling molecule because of its relatively low toxicity, long lifespan, and diffusibility (Cuypers et al., 2016). It has been reported that Cd-induced damage to rice seedlings could be reduced by pretreatment with low concentrations of H$_2$O$_2$ or with heat shock, which is known to increase H$_2$O$_2$ levels (Chao et al., 2009; Bai et al., 2011; Wu et al., 2015b). In the Arabidopsis (Arabidopsis thaliana) Atrboh mutant, Cd accumulation in roots was increased by 2-fold (Gupta et al., 2017), suggesting a possible causal link between NADPH oxidase operation and Cd transport. The important role of NADPH oxidase in regulating ion transport activity is well accepted. For example, RBOHC could regulate arsenic uptake in Arabidopsis plants (Gupta et al., 2013). By interacting with transition metals, RBOH-generated H$_2$O$_2$ can form hydroxyl radicals that in turn directly activate depolarization-activated outward-rectifying K$^+$ (GORK) channels (Demidchik et al., 2014; Wang et al., 2017). H$_2$O$_2$ also activates a range of cation-permeable nonselective cation channels, thus affecting intracellular K$^+$ and Ca$^{2+}$ homeostasis and signaling (Ordonez et al., 2014; Shabala and Pottosin, 2014; Wang et al., 2018). Taken together, it is reasonable to speculate that H$_2$O$_2$ may play an important role in regulating Cd influx. However, the mechanistic basis of this regulation remains to be elucidated.

Hydrogen gas (H$_2$) has recently emerged as a beneficial molecule with multiple bioactive functions (Zeng et al., 2014). It is believed that the main mechanism of H$_2$ action and its modulation of stress tolerance in plants might be related to the preferential scavenging of ROS, thereby reducing the oxidative damage, as reported in alfalfa (Medicago sativa), Arabidopsis, and rice (Jin et al., 2013; Xie et al., 2012, 2015). It is likely that H$_2$ does not act alone but rather interacts with other signaling molecules, such as abscisic acid, H$_2$O$_2$, nitrous oxide, and Ca$^{2+}$, to affect plant physiological activities. It was suggested that exogenous nitric oxide generated by nitric oxide synthase and nitrate reductase might be required for H$_2$-induced adventitious root formation (Zhu et al., 2016). Jin et al. (2016) found that under drought stress, H$_2$ rapidly increased H$_2$O$_2$ and modified the apoplastic pH of leaves in alfalfa via an abscisic acid-based mechanism. Furthermore, incremental IP$_3$-dependent cytosolic Ca$^{2+}$ contributes to H$_2$-promoted anthocyanin biosynthesis under UV-A irradiation in radish sprouts (Zhang et al., 2018).

Until now, alleviation of detrimental effects of Cd by H$_2$ was attributed to enhanced antioxidant defense mechanisms (Wu et al., 2015a; Su et al., 2019). It is also known that H$_2$ decreased Cd accumulation in plants, although specific mechanisms behind this phenomenon were not revealed. Recent evidence indicates that H$_2$ might function as an essential signaling modulator involved in regulation of cation channels or transporter operation. For example, the transcripts of two Na$^+$ exclusion transporters, salt overly sensitivel (SOS1) protein and Arabidopsis H$^+$-ATPase3 (AHA3), were significantly upregulated by H$_2$ pretreatment under salt stress (Xie et al., 2012). Similarly, H$_2$ treatment upregulated the transcript levels of GORK, an outward-rectifying K$^+$ channel in control of stomatal movements (Xie et al., 2014). For Cd$^{2+}$ transport, the expression of BcIRT1 and BcZIP2 (two main transporters in Cd uptake) was found to be significantly repressed by H$_2$ (Wu et al., 2019), suggesting that H$_2$ may reduce Cd accumulation in plants by transcriptional regulation of these transporters. However, the question of how H$_2$ controls the functional activity/operation of iron-regulated transporters (IRTs) remains to be answered.

Leafy vegetables accumulate more Cd than tubers and root vegetables (Wang et al., 2014; Rizwan et al., 2017). One of the most consumed leafy vegetables in China and East Asia, pak choi (Brassica campestris ssp. chinensis), grows rapidly and can readily accumulate Cd (Liu et al., 2012; Yu et al., 2019). Better understanding the mechanism of Cd uptake and developing methods to reduce Cd accumulation in pak choi seedlings are of great significance. In this study, we combined a range of advanced electrophysiological, biochemical, and genetic approaches to elucidate the mechanistic basis of regulation of Cd transport by H$_2$ gas. We show that Cd-triggered rapid H$_2$ production plays an essential role in stress signaling, modulating
Ca\textsuperscript{2+}-dependent H\textsubscript{2}O\textsubscript{2} generation by NADPH oxidase encoded by \textit{RbohD}. The latter operates upstream of IRT1 and regulates root Cd uptake at both transcriptional and functional levels.

**RESULTS**

\textbf{Cd Stimulates H\textsubscript{2} Release and Production}

Effects of Cd on endogenous H\textsubscript{2} production in pak choi seedlings were investigated using a hydrogen needle sensor. These measurements showed a rapid and progressive increase in endogenous H\textsubscript{2} concentration in leaves when roots were treated with 100 \textmu M Cd (Fig. 1A). In comparison with the basal levels of H\textsubscript{2} in control samples, a rapid, significant, and sustained increase of H\textsubscript{2} release was detected that reached a peak value at \textasciitilde 300 s. Cd-triggered H\textsubscript{2} production was further verified by gas chromatography (GC) analysis. As shown in Figure 1B, pak choi seedlings treated with Cd had 2-fold higher H\textsubscript{2} concentration in their shoots following 24 h of Cd exposure, compared with controls.

\textbf{HRW Alleviates Cd Stress-Induced Pak Choi Seedlings Growth Inhibition}

To investigate whether H\textsubscript{2} had any effect on plant growth under Cd stress, phenotyping and viability staining assays were performed. Under normal growth conditions, hydrogen-rich water (HRW) treatment had no effect on the growth of pak choi seedlings (Fig. 2A), whereas 50 \textmu M Cd treatment for 2 d significantly inhibited the root length and fresh weight (by \textasciitilde 33\% and 30\%, respectively). In comparison with the plants challenged with Cd alone, pretreatment of Cd-stressed seedlings with 50% HRW (H\textsubscript{2} concentration 381 \pm 16 \textmu M; Supplemental Fig. S1C) rescued Cd inhibition and increased root elongation and fresh weight by \textasciitilde 27\% and \textasciitilde 19\%, respectively (Fig. 2, C and D). As the O\textsubscript{2} concentration in the 50% HRW treatment was lower than in the control treatment, additional (hypoxic) controls were added to eliminate a possible confounding effect of hypoxia. As shown in Supplemental Figure S2, removing oxygen from the solution by flushing it with N\textsubscript{2} did not alleviate the Cd-induced growth inhibition of pak choi seedlings observed in the HRW treatment, ruling out the above possibility.

At the next step, 2-d-old pak choi roots were exposed to various treatments (HRW, Cd, and diphenyleneiodonium [DPI], alone or in combination) and then double-stained with fluorescein diacetate-propidium iodide (FDA-PI; Fig. 2, B and E). Under the fluorescence microscope, viable cells fluoresced bright green, whereas dead/damaged cells fluoresced bright red. Very few dead/damaged cells were found in the root tips in treatments without Cd stress. Cd exposure for 24 h resulted in a substantial loss of cell viability in the root apex (less green signal and brighter red signal), with \textasciitilde 37\% of the root tip cells damaged. In cells pretreated with DPI (a known NADPH oxidase inhibitor) and exposed to Cd, the percentage of dead cells increased to 48\%. Pretreatment with HRW alleviated Cd-induced cell damage, with only 20\% of cells damaged. However, this positive effect of HRW was offset by DPI cotreatment (34\% of cells damaged).

\textbf{HRW Reduces Cd Influx and Accumulation in Pak Choi Roots}

The microelectrode ion flux estimation (MIFE) technique was used to measure net Cd\textsuperscript{2+} flux from the pak choi roots (Fig. 3). Addition of Cd to the bath solution resulted in a substantial loss of cell viability in the root apex (less green signal and brighter red signal), with \textasciitilde 37\% of the root tip cells damaged. In cells pretreated with DPI (a known NADPH oxidase inhibitor) and exposed to Cd, the percentage of dead cells increased to 48\%. Pretreatment with HRW alleviated Cd-induced cell damage, with only 20\% of cells damaged. However, this positive effect of HRW was offset by DPI cotreatment (34\% of cells damaged).
Cd uptake, with small but significant net Cd\textsuperscript{2+} efflux detected for 24- and 48-h treatments (Fig. 3, C and D).

We next looked at the effect of HRW on Cd accumulation in pak choi tissues. As shown in Figure 4, A and B, Cd content increased in nontreated control roots with increasing duration of exposure to Cd; however, no significant (at $P < 0.05$) difference in tissue Cd content was found between 12- and 24-h Cd exposure and B, Cd content increased in nontreated control roots with increasing duration of exposure to Cd; however, no significant (at $P < 0.05$) difference in tissue Cd content was found between 12- and 24-h Cd exposure
in roots pretreated with HRW. Consistent with the MIFE data, HRW plants showed decreased Cd accumulation in both root and shoot tissues (Fig. 4, A and B) compared with nontreated controls. Recently, yeast assays showed that the role of BcIRT1 and BcZIP2 genes was to confer Cd\textsuperscript{2+} transport activity (Wu et al., 2019). Here we show that under nonstress conditions, HRW pretreatment inhibited BcIRT1 expression and promoted BcZIP2 expression. After 12 h Cd exposure, the expression of BcIRT1 had decreased significantly, but BcZIP2 expression did not change dramatically. Also, Cd-induced BcIRT1 inhibition was further strengthened by the HRW pretreatment. However, the HRW-inducible response was not observed in the BcZIP2 gene, which had a transcript level close to that for the ConCd treatment (Fig. 4, C and D).

### NADPH Oxidase-Generated H\textsubscript{2}O\textsubscript{2} Mediates HRW-Induced Decrease in Cd\textsuperscript{2+} Uptake by Roots

As Rboh-dependent H\textsubscript{2}O\textsubscript{2} generation was suggested to participate in the plant response to Cd stress (Rodriguez-Serrano et al., 2009; Cuypers et al., 2016), we used MIFE technology to examine the effect of HRW on Cd-induced H\textsubscript{2}O\textsubscript{2} efflux (Fig. 5, A and B). No H\textsubscript{2}O\textsubscript{2} flux from the elongation zone of roots was measured under Cd treatment for 30 min (Fig. 5A, white circles), while 15 min of HRW treatment resulted in a substantial increase in H\textsubscript{2}O\textsubscript{2} efflux. This increase reached its maximum value at 20 min (about 2 pmol m\textsuperscript{-2} s\textsuperscript{-1}) and then began to decline (Fig. 5A, blue circles). About 15 min after Cd addition, H\textsubscript{2}O\textsubscript{2} efflux had considerably increased in roots pretreated with HRW compared with control roots (Fig. 5B).

As a next step, H\textsubscript{2}O\textsubscript{2} production under prolonged Cd exposure was quantified (Fig. 5C). Root incubation in HRW resulted in a significantly higher (1.7-fold; \( P < 0.05 \)) H\textsubscript{2}O\textsubscript{2} fluorescent signal compared with control plants (Fig. 5D, time point 0). Exposure to Cd led to a further increase in H\textsubscript{2}O\textsubscript{2} accumulation that reached a peak value at 1 h and then gradually declined. The kinetics of Cd-induced changes in H\textsubscript{2}O\textsubscript{2} accumulation was more drastic in HRW-pretreated plants. The increase in H\textsubscript{2}O\textsubscript{2} induced by HRW treatment was completely offset by DPI cotreatment (Fig. 5D).

We then looked at the effect of Cd on the transcript levels of NADPH oxidase genes. In the family of Brassicaceae, 10 Rboh genes, RbohA to RbohJ, are known to encode NADPH oxidase (Li et al., 2019; Liu et al., 2019). Two prominent members, RbohD and RbohF, have been shown to play an important role in stress-induced H\textsubscript{2}O\textsubscript{2} production (Yang et al., 2018; Jakubowicz et al., 2010). Exposure to Cd resulted in a significant upregulation of BcRbohD transcripts, and this upregulation was much faster in HRW-pretreated roots than in nontreated control roots (peak accumulation after 1 and 3 h, respectively). By contrast, BcRbohD transcription was severely downregulated by HRW and DPI cotreatment (Fig. 5E). Changes in BcRbohF transcripts were not different between HRW-treated and control plants, with both peaking at \( \sim3 \) h after Cd exposure; however, the expression level of BcRbohF under cotreatment of HRW and DPI was much lower at 3 h compared to the other two treatments (Fig. 5F).

Our next aim was to establish a causal link between HRW-stimulated H\textsubscript{2}O\textsubscript{2} generation and HRW-inhibited Cd uptake; this was achieved in a series of pharmacological experiments (Fig. 6). An NADPH oxidase inhibitor, DPI, was used to modulate H\textsubscript{2}O\textsubscript{2} production and reveal its role in HRW-inhibited Cd uptake. Under the control condition, Cd addition induced a fast Cd\textsuperscript{2+} influx from roots, with peak values of \( \sim110 \) and 80 nmol m\textsuperscript{-2} s\textsuperscript{-1} in the elongation and mature zones, respectively. When pretreated with HRW, these peak values were reduced to \( \sim80 \) and 50 nmol m\textsuperscript{-2} s\textsuperscript{-1} (Fig. 6, blue symbols). Addition of DPI eliminated the beneficial effects of HRW (Fig. 6, gray symbols). DPI pretreatment also increased peak Cd\textsuperscript{2+} uptake by non-HRW treated roots in both zones (Supplemental Fig. S3), and exogenous H\textsubscript{2}O\textsubscript{2} application reversed this process (Supplemental Fig. S3). Taken together, these results suggest that apoplastic H\textsubscript{2}O\textsubscript{2} production by
NADPH oxidase mediates HRW-induced decrease of Cd\(^{2+}\) uptake by roots.

To confirm the involvement of NADPH oxidase as a component of the mechanism for HRW-induced decrease in Cd\(^{2+}\) uptake by roots, the above experiments were conducted on Arabidopsis *At* *rbohD* and *At* *rbohF* mutants lacking appropriate functional RBOH isoforms. No phenotypic difference was found between wild-type Col, *At* *rbohD*, and *At* *rbohF* plants grown under normal conditions (Supplemental Fig. S5A). The presence of Cd in the agar media caused inhibition of root elongation in the Rboh mutants, especially *At* *rbohD*, compared to Col (Supplemental Fig. S5B and C). Arabidopsis plants lacking functional RbohD also showed the highest Cd\(^{2+}\) uptake by roots (Supplemental Fig. S5D, E and F). Similar to the results observed for pak choi seedlings, HRW pretreatment reduced Cd uptake by Arabidopsis wild-type (Col-0) roots; this ameliorative effect was absent in *At* *rbohD* and *At* *rbohF* mutants, regardless of the root zone (Fig. 7).

As shown in Figure 4, B and C, HRW treatment downregulated *Bc* *irt1* expression and reduced Cd accumulation in pak choi roots. To reveal a functional role of IRT1 as the downstream target of HRW, the Arabidopsis *At* *irt1* mutant was used in electrophysiological experiments. Consistent with previous findings, both HRW and H\(_2\)O\(_2\) pretreatment lowered net Cd\(^{2+}\) uptake by wild-type roots (in both root zones). Net Cd\(^{2+}\) influx was significantly lower in *At* *irt1* than in wild-type Col-0, peaking at ~20 and 10 nmol m\(^{-2}\) s\(^{-1}\) in the elongation and mature zones, respectively. Neither HRW nor H\(_2\)O\(_2\) pretreatment was able to further reduce Cd\(^{2+}\) influx in the *At* *irt1* mutant (Fig. 8). Taken together, our data are consistent with the model that HRW-induced RBOH-dependent apoplastic H\(_2\)O\(_2\) production operates upstream of IRT1, thus affecting Cd transport.

**Both HRW-Induced H\(_2\)O\(_2\) Generation and Subsequent Inhibition of Cd\(^{2+}\) Influx Are Ca\(^{2+}\) Dependent**

Cytosolic Ca\(^{2+}\) is a ubiquitous second messenger, and changes in the cytosolic free Ca\(^{2+}\) concentration are reported in response to virtually every known environmental stimulus (McAinsh and Pittman, 2009; Dodd et al., 2010). In this study, HRW addition resulted in a rapid Ca\(^{2+}\) influx in both elongation and mature root zones, with peak values of ~50 nmol m\(^{-2}\) s\(^{-1}\) and...
~30 nmol m$^{-2}$ s$^{-1}$, respectively (Fig. 9, A and B). This occurred in parallel with Ca$^{2+}$ accumulation measured with a fluorescent dye, where HRW treatment for 30 min induced a stronger fluorescence signal in both zones (Fig. 9, A and B, insets). Application of Cd$^{2+}$, however, induced a massive leakage of Ca$^{2+}$ from the root, followed by a gradual recovery; this Cd-induced Ca$^{2+}$ efflux was significantly suppressed by HRW pretreatment. The changes in Ca$^{2+}$ flux were consistent with the fluorescent Ca$^{2+}$ signal data (Fig. 9, C and D).

These results imply an involvement of Ca$^{2+}$ in HRW-reduced Cd uptake. H$_2$O$_2$ addition resulted in rapid Ca$^{2+}$ efflux from root elongation zones. This efflux was rather small and recovered after 10 min, with a peak of only ~10 nmol m$^{-2}$ s$^{-1}$ (Fig. 9E). By contrast, in the mature zone, H$_2$O$_2$ addition induced Ca$^{2+}$ influx, from 8 to 25 nmol m$^{-2}$ s$^{-1}$ (Fig. 9F). Consistent with this, H$_2$O$_2$ treatment for 30 min resulted in a slight decrease and increase in fluorescent Ca$^{2+}$ signal in the elongation zone and mature zone, respectively (Fig. 9, E and F, insets).

Two Ca$^{2+}$ channel inhibitors (Gd$^{3+}$ and La$^{3+}$) were used here to further verify the role of Ca$^{2+}$ as a component of HRW signaling to Cd$^{2+}$ transporters in plant roots. Compared with the control, Cd treatment conferred a 30% reduction in Ca concentration (Supplemental Fig. S6). However, the HRW-induced increase in Ca concentration was totally inhibited by Gd$^{3+}$ addition (Supplemental Fig. S6), which suggests activation of Ca$^{2+}$ channels by HRW under Cd exposure. As shown in Figure 10, A and B, the HRW-induced increase in BcRbohD and BcRbohF transcript levels was negated by Gd$^{3+}$ and La$^{3+}$ addition, as was an increase in H$_2$O$_2$ content in roots. Also, amelioration of Cd$^{2+}$ uptake in HRW-treated roots was not observed in plants treated with 0.1 mM Gd$^{3+}$ (Fig. 10C), closely matching the Cd content in roots treated with Cd for 6 and 12 h (Fig. 10D). In parallel with the uptake of Cd, HRW pretreatment-induced downregulation of BcIRT1 expression under Cd stress was totally offset by Gd$^{3+}$ cotreatment (Fig. 10E).

**DISCUSSION**

**IRT1 Operates Downstream of HRW-Regulated Cd$^{2+}$ Uptake by Plant Roots**

Since the first report on the release of H$_2$ in bacteria (Stephenson and Stickland, 1931) and the discovery of hydrogenase in *Clostridium pasteurianum* (Nakos and Mortenson, 1971), research regarding H$_2$ metabolism and hydrogenase in organisms has attracted significant interest due to its multiple biological functions (Ohsawa et al., 2007; Khanna and Lindblad, 2015). In this study, using H$_2$ measurement, we demonstrated that Cd exposure triggered rapid and sustained H$_2$ production (Fig. 1, A and B). Although we did not investigate the enzymatic resource(s) for this process, this observation is consistent with previous findings that H$_2$ production was increased and maintained in plants treated with paraquat and salt stress (Jin et al., 2013; Xie et al., 2012). These results indicated that H$_2$ may play an important role in plant response to abiotic stresses.

The next question was the physiological rationale of Cd-induced H$_2$ production. H$_2$ exhibits a broad range of biological functions, including activation of Ca$^{2+}$ channels and regulation of Cd transporters. However, further studies are needed to clarify the underlying mechanisms.
of biological effects, of which the most common is its antioxidant function (Ohsawa et al., 2007). It is plausible, therefore, that H₂ production in pak choi roots may function uphill of the antioxidant system to inhibit Cd-triggered ROS accumulation reported elsewhere (Cui et al., 2013; Wu et al., 2015b). In addition to that, H₂ also reduced Cd accumulation in pak choi plants (Fig. 4, A and B). There are two possible reasons for the reduction of Cd content in plants. First, lower Cd²⁺ influx in H₂-treated roots (Fig. 3, A and B) could result from lower expression of the BcIRT1 gene conferring the Cd transporter (Fig. 4C) in the HRW treatment. Second, within 12 h of treatment, H₂ induced net Cd²⁺ efflux from roots (Fig. 3C), thus reducing Cd accumulation in plants.

In plants, many transporters of divalent transition metals have Cd²⁺ uptake ability (Verbruggen et al., 2009). In our previous report, BcIRT1 and BcZIP2 were shown to transport Cd²⁺ as well as Fe²⁺ in yeasts and could be regulated by HRW (Wu et al., 2019). Here, only BcIRT1 transcription was significantly inhibited by HRW in pak choi roots (Fig. 4C). In Arabidopsis, the Atirt1 mutant had a significantly smaller net Cd²⁺ influx compared with the wild type, and the ameliorative effects of HRW on root Cd²⁺ uptake were abolished in the Atirt1 mutant (Fig. 8). Taken together, these data suggest that IRT1 may operate downstream of HRW-regulated Cd²⁺ uptake by plant roots.

The cytosolic free Cd²⁺ is the main factor behind negative effects such as membrane peroxidation, disturbance to ion homeostasis, protein cleavage, and even DNA damage in plant tissues (Bashir et al., 2015). Thus, to reduce the damage, plant cells either sequestrate Cd²⁺ in vacuoles or convert it into nontoxic Cd-organic acid complex via chelation (Romero-Puertas et al., 2002; Song et al., 2014; Singh et al., 2016). However, restricting Cd uptake by the root and promoting Cd efflux may be an energetically less costly option for preventing Cd toxicity. The mechanisms controlling Cd²⁺ transport across the root PM, however, remained elusive until now. In this work, we showed that Cd-induced H₂ production may be part of such a mechanism. It is therefore possible that H₂ should be added to the list of early response signals operating upstream of Cd transporters.

H₂O₂ Mediates H₂-Regulated Cd Uptake

One of the interesting observations in our work was a time lag in H₂-induced inhibition of Cd²⁺ influx, with no reduction in Cd²⁺ uptake observed when HRW pretreatment was added together with Cd²⁺ (Fig. 3, A and B). Thus, it appears that effects of H₂ on Cd uptake are indirect and most likely mediated by other components of the signaling pathway.

Consistent with previous reports (Xie et al., 2014; Jin et al., 2016), HRW treatment led to an increase in H₂O₂.
production, as demonstrated by both electrophysiological (Fig. 5, A and B) and fluorescence imaging data (Fig. 5, C and D). Meanwhile, DPI (an NADPH oxidase inhibitor) abolished the beneficial effects of HRW on inhibition of Cd$^{2+}$ uptake by roots (Fig. 6), suggesting that NADPH oxidase H$_2$O$_2$ operates downstream of H$_2$.

RbohD and RbohF are two important members of the Rboh gene family encoding NADPH oxidase (Sagi and Fluhr, 2006). Both have been shown to function in ROS signal amplification and mediation of rapid systemic signaling (Miller et al., 2009). Here we show that RbohD plays a critical role in mediating the beneficial effects of HRW on root Cd$^{2+}$ uptake. Four lines of evidence support this claim. First, there was no disparity between Atrboh mutants and the wild type in H$_2$ release (Supplemental Fig. S4), implying that H$_2$O$_2$ could not impact H$_2$ production. Second, consistent with the H$_2$O$_2$ production data, HRW also upregulated BcRbohD transcript levels (Fig. 5E). Third, H$_2$O$_2$ pretreatment significantly reduced both net Cd$^{2+}$ influx and Cd$^{2+}$ content in pak choi roots, while inhibition of NADPH activity by DPI pretreatment led to opposite results (Supplemental Fig. S3). Fourth, HRW-induced inhibition of Cd$^{2+}$ influx occurred in the Arabidopsis wild type but was absent in AtrbohD mutants (Fig. 7), which had a much more sensitive phenotype (Supplemental Fig. S5). Taken together, these data strongly suggest that Rboh-dependent H$_2$O$_2$ production is essential for HRW-suppressed Cd$^{2+}$ influx, and that IRT1 operates as a downstream factor in this process, as H$_2$O$_2$-induced inhibition of Cd$^{2+}$ influx was abolished in the Atirt1 mutant compared with the wild type (Fig. 8).

The Role of Ca$^{2+}$ in H$_2$-Regulated H$_2$O$_2$ Generation

The H$_2$O$_2$ signal could not be detected until 10 min after HRW treatment (Fig. 5, A and B). One possible explanation for this delay was that H$_2$ regulated H$_2$O$_2$ production indirectly, through an intermediate signal. The instant increase in Ca$^{2+}$ influx after HRW addition (Fig. 9, A and B) prompted a hypothesis that changes in cytosolic Ca$^{2+}$ levels may happen upstream of H$_2$O$_2$ generation. Changes in the cytosolic Ca$^{2+}$ concentration are considered to be one of the earliest cellular responses to all stresses (Marcec et al., 2019), and cytosolic Ca$^{2+}$ elevation is a ubiquitous denominator of the signaling network when plants are exposed to abiotic stresses, including Cd (McAinsh and Pittman, 2009; Bose et al., 2011). Compelling evidence indicates a reciprocal relationship between H$_2$O$_2$ and Ca$^{2+}$, two crucial messengers involved in plant responses to multiple stress conditions (Tuteja and Mahajan, 2007; Mazars et al., 2010; Petrov and Van Breusegem, 2012). The mechanistic basis for this interaction lies in the so-called ‘ROS-Ca$^{2+}$ hub’ at the PM (Demidchik and Shabala 2018; Demidchik et al., 2018), where Ca$^{2+}$-activated NADPH
oxidases work in concert with ROS-activated Ca$^{2+}$-permeable cation channels to generate and amplify stress-induced Ca$^{2+}$ and ROS signals. In this study, Gd$^{3+}$ and La$^{3+}$, two known blockers of Ca$^{2+}$-permeable nonselective cation channels, entirely suppressed HRW-induced changes in BcRbohD expression and H$_2$O$_2$ production (Fig. 10, A and B). Gd$^{3+}$ treatment also markedly abolished the ameliorative effects of HRW treatment on root Cd uptake and accumulation, as well as BcIRT1 expression (Fig. 10, C–E). Ca$^{2+}$ directly binds to EF-hand motifs in the cytosolic N-terminal domain of the NADPH oxidase enzyme, and EF-hands in RBOHD can directly sense Ca$^{2+}$ (Seybold et al., 2014).

The question of how H$_2$ regulates Ca$^{2+}$ transport across the PM remains to be answered in future studies. One of the plausible scenarios may include H$_2$-induced voltage gating of Ca$^{2+}$-permeable PM channels.

Demidchik et al. (2002) proposed that voltage modulation of the coexisting nonselective cation channels and hyperpolarization-activated Ca$^{2+}$ channels by the PM H$^+$-ATPase would be a potent regulator for Ca$^{2+}$ entry into the root cell cytoplasm. In this study, HRW treatment led to increased net H$^+$ efflux from plant roots (Supplemental Fig. S7), indicating the possibility of H$^+$-ATPase activation by H$_2$. The high H$^+$-pumping activity leads to hyperpolarization of the PM and thus increases Ca$^{2+}$ influx through hyperpolarization-activated Ca$^{2+}$ channels.

In summary, the results of this study demonstrate the existence of a new mechanism that explains the ameliorating effect of H$_2$ on Cd toxicity in plants, namely H$_2$ control of the expression level and activity of the PM-based NADPH oxidase encoded by the RbohD gene that operates upstream of IRT1 and regulates root Cd uptake. The timing of events is summarized in the Figure 11.
model in Figure 11: (1) Cd enters into the cytosol and triggers rapid H$_2$ production; (2) an increase in H$_2$ results in Ca$^{2+}$ influx and leads to a rapid elevation in cytosolic free Ca$^{2+}$ levels; (3) the increased cytosolic Ca$^{2+}$ stimulates the activity of NADPH oxidase and subsequently induces H$_2$O$_2$ generation; and (4) H$_2$O$_2$ downregulates IRT1 activity, resulting in inhibition of Cd$^{2+}$ influx. Exogenous application of H$_2$R effectively leads to an increase in intracellular H$_2$ production, thus accelerating and amplifying the above H$_2$ effects.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Treatments

Pak choi (Brassica campestris sep. chinensis ‘Donggang 2’) and Arabidopsis (Arabidopsis thaliana; Col-0, rbohD, rbohF, and iri-1) plants were used in this study. Seeds of pak choi were surface sterilized with 5% (v/v) NaClO solution, extensively rinsed with distilled water, and then soaked in deionized water at room temperature for 3 h. After that, seeds were covered with moist gauze, germinated for 36 h at 23°C, then transferred to plastic chambers containing one-quarter strength Hoagland’s solution (without HRW or CdCl$_2$) as the control. For Arabidopsis, the mutant seeds were kindly donated by Shaojian Shen from Nanjing Agricultural University (AtrbohF, AtrbohD, and Atrbohf; mutants; Xie et al., 2014). For cultivation, seeds were sown on petri dishes with one-half strength Murashige and Skoog basal salt medium supplemented with 2% (w/v) Suc and 0.8% (w/v) agar at pH 5.8. All seedlings were grown in a controlled illuminated chamber at 24 ± 1°C, with a photoperiod of 16 h light/8 h dark and light intensity of 200 ± 5 μmol m$^{-2}$ s$^{-1}$. Uniform 4-d-old pak choi and 6-d-old Arabidopsis seedlings were selected for electrophysiological measurements or pharmacological experiments.

Determination of Cd Concentrations in Plant Tissues

Plant samples were collected, and roots were rinsed in 20 mM EDTA-Na$_2$ solution for 15 min to remove Cd absorbed to the surface. After that, all samples were washed with deionized water and dried at 105°C for 24 h. Dried samples were ground to powder and digested in 2 mL HNO$_3$:HClO$_4$ (87:13 [v/v]) solution overnight. The sample digestion was carried out in a heating block at 121°C for 2 h. After digestion of plant tissues, the residue was filtered through 0.22 μm cellulose acetate membrane. The Cd content in the digest was determined by atomic absorption spectroscopy.

Preparation of HRW

An H$_2$ gas generator (SHC-500; Saikesaisi Hydrogen Energy Co.) was used to produce the purified hydrogen gas (99.99% [v/v]). H$_2$ was bubbled into 1.0 L of one-eighth strength Hoagland’s nutrition solution at a rate of 150 mL min$^{-1}$ for 10 min till 100% saturation. Then, the corresponding HRW was immediately diluted to 50% (v/v) concentration. Under our experimental conditions, the Ca$^{2+}$ concentration in the freshly prepared HRW was 830 ± 10 μM; for 50% HRW this value was 381 ± 16 μM (Supplemental Fig. S1A, A and C). The H$_2$ concentration remained >100 μM for at least 12 h (Supplemental Fig. S1B). To make the HRW and N$_2$ solution, the one-eighth strength Hoagland nutrition solution (Con) was bubbled with H$_2$ and N$_2$ for 5 min at the rate of 150 mL min$^{-1}$.

Measurement of H$_2$ Release and Content

The H$_2$ release was measured using a needle-type hydrogen sensor (DK-8200, Unisense) following the method reported by Xie et al. (2014). The H$_2$-specific electrode was polarized for 4 h before use. Prior to measurement, the pak choi seedlings were placed on 2% agarose gel to avoid damage to the electrode tip. For electrode analysis, the needle was directly stuck into the leaf tissues to a depth of ~200 μm using a micromanipulator. When the basal line of H$_2$ signal was stable, Cd treatment solution was added to immersed roots and the corresponding data were recorded. All manipulations were performed at 25 ± 1°C.

For analysis of endogenous H$_2$ production, GC was used as described in our previous publications (Wu et al., 2013b). Approximately 1.0 g of pak choi seedlings treated with 0 or 50 μM CdCl$_2$ were placed in vials. A pure nitrogen gas was then bubbled into the vial to fully displace the air. Afterward, the vials were immediately capped and incubated at 25 ± 1°C for 12 h to liberate H$_2$ from plant tissues. Nitrogen gas was used as the carrier gas, and the air pressure was 0.5 MPa.

Ion-Selective Microelectrode Preparation

Net K$^+$, Ca$^{2+}$, H$^+$, and Cd$^{2+}$ fluxes were measured noninvasively with ion-selective microelectrodes using the MIFE technique (University of Tasmania) as described by Shabala et al. (1997). Briefly, blank microelectrodes were pulled out of borosilicate glass capillaries (GC130-10; Harvard Apparatus), oven-dried at 225°C overnight, and silanized with chlorotrimethylsilane (tributyl-chlorosilane; catalog no. 282707, Sigma-Aldrich). After cooling, microelectrode tips were flattened to 2 to 3 μm in diameter. The microelectrodes were then back-filled with respective backfilling solutions (200 mM KCl for K$^+$; 500 mM CaCl$_2$ for Ca$^{2+}$; 15 mM NaCl + 40 mM KH$_2$PO$_4$ with pH adjusted to 6.0 using NaOH for H$^+$) and front-filled with respective ion-selective ionophore cocktails (catalog nos. 99311 for K$^+$, 99350 for Ca$^{2+}$, and 95291 for H$^+$, all from Sigma-Alrich). Back-filling solution for Cd$^{2+}$ consisted of 10 mM Cd(NO$_3$)$_2$ plus 100 μM KCl. The Cd-selective ionophore cocktail was freshly prepared with a mixture of % (w/w) cadmium ionophore I (N,N,N,N'-tetraethyl-3,6-dioxaoc- tanedithioic acid); ETH 1062, catalog no. 29009, Sigma-Aldrich), 10% (w/w) sodium tetrakis ([5,5-bis (trifluoromethyl) phenyl] boron sodium; catalog no. 72077, Sigma-Aldrich), and 80% (w/w) 2-nitrophenyl octyl ether (1-nitro-2- octylsolvone; catalog no. 73732, Sigma-Aldrich; Píeres et al., 1998). Prepared microelectrodes were calibrated in respective sets of the standard solutions before and after measurements. Only electrodes with a slope of >50 mV per decade for K$^+$ and H$^+$ and >25 mV for Ca$^{2+}$ and Cd$^{2+}$, and a correlation ≥0.999 were used.

Ion Flux Measurements

Net K$^+$, Ca$^{2+}$, H$^+$, and Cd$^{2+}$ fluxes were measured from the elongation (~350 μm from the root tip) and mature (~1,500 μm from the root tip) root zones of 4-d-old pak choi seedlings. Prior to measurement, roots of intact seedlings were immobilized on a microscopic slide by a paraffin strip. The slide was placed in a measuring chamber containing basic salt medium (0.5 mM KCl and 0.1 mM CaCl$_2$, pH 5.6) for 30 min for adaption, and tips of ion-selective microelectrodes were focoosed and positioned 40 to 50 μm above the root epidermis. During measurements, microelectrodes were moved in a 12-s square-wave cycle by a computer-controlled hydraulic micromanipulator with a travel range of 100 μm. Steady-state ion fluxes were recorded for 5 min, and then the appropriate treatment was administered followed by another 20 to 30 min of measurements. Voltage outputs of electrodes were recorded using CHART software and then converted into net flux data using the MIFEFLUX program (Shabala et al., 2006).

Measurement of Net H$_2$O$_2$ Fluxes

An H$_2$O$_2$-sensitive microelectrode (tip diameter 2–3 μm; XY-DJ-502, Xyue Science and Technology Co.) was used to monitor H$_2$O$_2$ fluxes in the elongation zone of the roots. H$_2$O$_2$ microelectrodes were prepared according to the method described by Zhang et al. (2017) and Twig et al. (2001). Before the measurement, the H$_2$O$_2$ microelectrode was polarized at +0.6 V against an Ag/AgCl reference electrode. Thereafter, the microelectrodes were calibrated in the standard solutions: 0.01, 0.1, and 1 mM H$_2$O$_2$. Roots sampled were immobilized in the measuring solution (0.1 mM NaCl, 0.1 mM MgCl$_2$, 0.1 mM CaCl$_2$, and 0.5 mM KCl with pH adjusted to 5.2 with KOH and HCl) and equilibrated for 30 min. H$_2$O$_2$ flux was measured from the elongation root zone (~350 μm from the root tip) of 4-d-old pak choi seedlings.

Viability Assay

The viability of pak choi root cells was assessed by performing a double staining method using FDA (catalog no. F7378, Sigma-Aldrich) and PI (catalog no. 1,436,480, Cya

H$_2$O$_2$Fluxes

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Microscope (FV1000, Olympus). At least 10 roots were imaged for each 5 min) followed by PI (3
no. P4864, Sigma-Aldrich; Koyama et al., 1995). FDA is permeable through the cell membrane according to the manufacturer, P
ANOVA combined with Duncan’s multiple-range test at a probability of 0.05.
Supplemental Data
The following supplemental materials are available.
Supplemental Figure S1. Changes of H2 and O2 concentration in H2-rich solution as a function of time.
Supplemental Figure S2. Effects of HRW and nitrogen gas (N2) pretreatment on the alleviation of Cd stress-induced growth inhibition in pak choi (Brassica chinensis) seedlings.
Supplemental Figure S3. Net and total Cd2+ fluxes measured from the elongation and mature zones of pak choi seedling roots in response to 50 μM CdCl2.
Supplemental Figure S4. Analysis of H2 release rate from leaves of Arabidopsis seedlings of three different genotypes.
Supplemental Figure S5. Phenotypes and net Cd2+ fluxes of Arabidopsis seedlings of different genotypes treated with 0 (control) or 50 μM CdCl2.
Supplemental Figure S6. Calcium concentration in roots of pak choi seedlings under different treatments.
Supplemental Figure S7. Effects of HRW on net proton (H+ ) flux in the elongation and mature zones of pak choi roots.
Supplemental Table S1. Sequences of primers used for real-time reverse transcription PCR.

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Histochemical Detection of H2O2 and Ca2+
The production of H2O2 in root cells was detected by 2’;7’-dichloro-fluorescein diacetate (H2DCFDA; catalog no. D6883, Sigma-Aldrich) staining method (Bose et al., 2014; Wang et al., 2017). The pak choi roots were collected after treatment with 50 μM CdCl2, washed with 10 mM Tris-HCl buffer, and immersed in 25 μM H2DCFDA for 30 min in the dark. The stained roots were washed thoroughly in distilled water before imaging. Fluorescent signals were visualized using a fluorescent microscope (Leica MZ12; Leica Microsystems) fitted with a high-pressure mercury lamp power (Leica HBO Hg 100 W; Leica Microsystems) and an 13-wavelength filter (Leica Microsystems). The fluores- cein signals were collected with excitation and emission wavelength at 488 to 525 nm for H2DCFDA and analyzed with Image J software.

The calcium accumulation in root cells was measured by the Fluor-3/AM (calcium fluorescent probes; catalog no. 39294, Sigma-Aldrich) based on the method of Yan et al. (2015) and Zhang et al. (2018). Briefly, the root samples were immersed in incubation solution (containing 20 mM Fluor-3/AM, 0.5 % mannitol, 4 mM MES [pH 5.7] and 20 mM KCl) for 30 min at a room temperature. The stained roots were washed thoroughly in distilled water before imaging. The green fluorescence signal was observed using a Laser-Scanning Confocal Microscope (FV1000, Olympus). At least 10 roots were imaged for each treatment.

Reverse Transcription Quantitative PCR Analysis
Total RNA was extracted from the root tissues of treated and untreated seedlings using TRIzol Reagent (catalog no. 15956018, Life Technologies) according to the manufacturer’s protocol from the user guide. The first-strand complementary DNA was synthesized using the SensiFAST cDNA Synthesis Kit (catalog no. BIO-65054, Bioline). RT-qPCR reactions were performed using the SensiFAST SYBR No-ROX Kit (catalog no. BIO-98005, Bioline) and Rotor- Gene Q6000 (Qiagen). Detailed information about gene-specific primers can be found in Supplemental Table S1. The three-step cycling quantitative PCR conditions were as follows: one cycle at 95°C for 2 min followed by 40 cycles of 95°C for 5 s, 65°C for 10 s, and 72°C for 15 s. SYBR-green signals were acquired to detect amplified gene products. Data are averages of three independent bi- ological experiments with three technical replicates for each.

Statistical Analyses
Statistical analysis was performed using SPSS Statistics 20 (IBM). Values are shown as the means ± se of at least three independent experiments with three replicates each. Differences among treatments were analyzed by one-way ANOVA combined with Duncan’s multiple-range test at a probability of P < 0.05.

Accession Numbers
Sequence data from this article can be found in the GenBank database or the Brassica Genome database under the following accession numbers: GU942468.1 (BrRbohD); GU942463.1 (BrRbohF); BRA013419 (BrIRT1); and BRA020314 (ZIP2).
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