Research Article

bHLH104 confers tolerance to cadmium stress in *Arabidopsis thaliana*

**Running title:** bHLH104 is involved in Cd tolerance

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Abstract

Cd is a non-essential heavy metal that is toxic to both plants and animals. Here, we reveal that the transcription factor bHLH104 positively regulates Cd tolerance in *Arabidopsis thaliana*. We found that Fe deficiency-responsive genes were induced by Cd treatment, and that their upregulation was suppressed in *bhlh104* loss-of-function mutants but enhanced upon overexpression of *bHLH104*. Correspondingly, the *bhlh104* mutants displayed sensitivity to Cd stress, whereas plants overexpressing *bHLH104* exhibited enhanced Cd tolerance. Further analysis suggested that bHLH104 positively regulates four heavy metal detoxification-associated genes, *IREG2, MTP3, HMA3* and *NAS4*, which play roles in Cd sequestration and tolerance. The *bHLH104* overexpression plants accumulated high levels of Cd in the root but low levels of Cd in the shoot, which might contribute to the Cd tolerance in those lines. The present study thus points to *bHLH104* as a potentially useful tool for genetic engineering of plants with enhanced Cd tolerance.
INTRODUCTION

Cadmium ( Cd ) has a detrimental effect on living organisms. Human activities have increased Cd contamination of soil, which poses serious threats to human health. Itai-Itai disease is a well-known and typical osteoporotic disease that is a consequence of elevated levels of Cd contamination in the local soil (Kazantzis, 2004). Cd exposure induces production of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen, causing oxidative stress in various plants and animals (Shim et al. 2009; Gallego et al. 2012). Under Cd exposure, plants display Fe deficiency-induced chlorosis in young leaves, inhibited root growth, reduced biomass, and even death (Kahle, 1993; Das et al. 1997; Wu et al. 2012; Chen et al. 2015). Plants have evolved several unique and efficient mechanisms for Cd detoxification and tolerance, such as control of Cd influx, Cd chelation, acceleration of Cd efflux, Cd sequestration and remobilization, and scavenging of Cd-induced ROS (Hall, 2002; Kim et al. 2006; Dalcorso et al. 2010; Lin et al. 2012; Shi et al. 2015).

As a divalent ion, Cd is chemically analogous to iron (Fe) and zinc (Zn). To date, no Cd-specific transporters or channels for absorbing Cd into plant cells have been identified, and the absorption of Cd is attributed to non-specific metal carrier proteins. Therefore, Cd toxicity is partially caused by its competition with essential minerals, especially Fe (Clemens, 2006). IRON-REGULATED TRANSPORTER 1 (IRT1), a member of the ZIP transporter family, is a major Fe transporter that imports Fe from soils to root cells (Vert, 2002). Due to its low substrate specificity, IRT1 also participates in Cd uptake from soils (Lombi et al. 2002; Yoshihara et al. 2006). Cd can compete with Fe during root absorption, which exacerbates Fe starvation and the upregulation of IRT1, leading to an even greater imbalance in uptake of Cd over Fe (Leskova et al. 2017). Recently, OLIGOPEPTIDE TRANSPORTER 3
(OPT3) was found to be involved in the crosstalk between Fe nutrition and Cd allocation. Loss-of-function of OPT3 disrupts Fe homeostasis and results in over-accumulation of Cd in seeds and roots (Mendozacózatl et al. 2014), further emphasizing the biological importance Cd–Fe competition. Although previous studies suggested that Cd exposure aggravates Fe deficiency in plants (Connolly et al. 2002; Lombi et al. 2002; Yoshihara et al. 2006; Besson-Bard et al. 2009), the underlying mechanism is still elusive.

To avoid heavy metal toxicity, plants often upregulate the expression of genes associated with chelation, sequestration and remobilization of heavy metal elements. Nicotianamine (NA), a metal chelator, shows high affinity in vitro for a series of transition metals, such as Fe, Ni, Zn, Co, Mn, and Cu. Overexpression of NICOTIANAMINE SYNTHASE (NAS) genes results in increased NA content, enhanced tolerance to Cd stress, and reduced Cd influx (Koen et al. 2013). The vacuole represents a well-controlled cistern in the plant cell, acting as a storage buffer for mineral elements (Vögelilange et al. 1990). Previous studies have shown that transporters localized in the tonoplast membrane mediate the transport of metal ions into vacuoles. HEAVY METAL ATPASE (HMA), METAL TOLERANCE PROTEIN (MTP) and ABCC-TYPE TRANSPORTER (ABCC) are vacuolar membrane proteins that enhance Cd tolerance and accumulation via vacuolar Cd sequestration in Arabidopsis (Ute, 2005; Arrivault et al. 2006; Morel et al. 2009; Park et al. 2012). Two tonoplast transporters, NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 3 (NRAMP3) and NRAMP4, serve as Fe and Cd transporters and the overexpression of NRAMP3 results in Cd hypersensitivity of Arabidopsis root growth (Thomine et al. 2000; Lanquar et al. 2005; Lanquar et al. 2010). In addition, the plasma membrane-localized ABC transporter PLEIOTROPIC DRUG RESISTANCE 8 (PDR8) and PLANT CADMIUM RESISTANCE 1 (PCR1) enhance Cd resistance via pumping or exporting Cd out of cells to reduce Cd concentrations (Song et al. 2004; Kim et al. 2007).
The BASIC HELIX-LOOP-HELIX (bHLH) IVc subgroup genes bHLH34/104/105/115 are the major regulators of Fe homeostasis in Arabidopsis and function upstream of FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT), bHLH38/39/100/101 and POPEYE (PYE) (Zhang et al., 2015; Li et al., 2016; Liang et al. 2017). FIT controls Fe transport into plant roots, and FIT loss-of-function mutants cannot survive without Fe supplementation (Colangelo and Guerinot 2004; Jakoby et al. 2004; Yuan et al. 2005). FIT interacts with bHLH Ib subgroup proteins, bHLH38/39/100/101, to form heterodimers that directly promote the expression of Fe-uptake genes IRT1 and FERRIC REDUCTION OXIDASE 2 (FRO2) (Yuan et al. 2008; Wang et al. 2013). By contrast, PYE plays a negative role in the Fe-deficiency response in Arabidopsis. PYE is specifically induced in the root pericycle and directly represses the expression of three Fe homeostasis-related genes, NAS4, FRO3 and ZIF1 (Long et al. 2010). Co-overexpression of FIT with bHLH38/39 in Arabidopsis enhances Cd tolerance by increasing Cd sequestration in plant roots and improving Fe homeostasis of shoots (Wu et al. 2012).

In the present study, we explored the functions of bHLH104 in Arabidopsis and found that bHLH104 positively regulates Cd tolerance. We show that overexpression of bHLH104 enhances the expression of Fe deficiency-responsive genes and causes Fe overaccumulation. We also show that bHLH104 overexpression promotes the expression of heavy metal detoxification-associated genes and facilitates Cd sequestration in the roots. OsPRI11, a rice homolog of bHLH104, also facilitates Fe homeostasis in rice (Zhang et al. 2017). Our identification of bHLH104 as a positive regulator of Cd tolerance suggests that it could be used in new strategies to engineer crops for adaptation to Cd stress.

RESULTS
Cd treatment disrupts Fe translocation from roots to shoots
Given that Cd exposure can cause Fe-deficiency symptoms in plants, we tested whether Cd stress affects Fe uptake and accumulation in plants. Wild-type plants were cultivated on normal medium for seven days and then transferred to the same medium containing either 0, 25 or 50 μM CdCl₂ for three days. Subsequently, the shoots and roots were harvested separately and Cd and Fe concentrations were examined by an inductively coupled plasma-optical emission spectrometer (ICP-OES). As expected, the Cd concentration in both the root and shoot increased with the increase of Cd in the medium (Figure 1A, B). Fe concentrations, however, displayed different patterns in roots and shoots. With the increase of Cd concentration in the medium, the root Fe concentration gradually increased, whereas the shoot Fe concentration gradually decreased (Figure 1A, B). These changes are exemplified by an increase in the root/shoot ratio of Fe as Cd concentration in the medium increased (Figure 1C). These data suggest that Cd exposure restricts Fe translocation from roots to shoots.

bHLH104 is required for upregulation of Fe deficiency-responsive genes under Cd stress conditions
We found that Cd treatment interfered with Fe distribution between roots and shoots, which may be one of the reasons Cd exposure causes Fe-deficiency symptoms. Our recent study confirmed that bHLH104 is a key regulator of Fe homeostasis and positively regulates the Fe-deficiency response in Arabidopsis (Li et al. 2016). We examined whether bHLH104 is required for the upregulation of Fe deficiency-responsive genes under Cd stress conditions by comparing the expression of four well-known Fe deficiency-responsive genes in roots of wild type and bHLH104 loss-of-function mutants (bhlh104-1
and bhlh104-2) after transfer to medium containing 0 or 50 μM CdCl₂. The expression of all four genes (IRT1, FRO2, bHLH38 and bHLH39) was significantly upregulated in the roots of both wild type and bhlh104 mutants after treatment with 50 μM CdCl₂; however, their expression was significantly lower in the bhlh104 mutants than in wild type (Figure 2). These data suggest that bHLH104 is required for Cd-mediated upregulation of Fe deficiency-responsive genes.

**Loss-of-function of bHLH104 enhances sensitivity to Cd stress**

To further assess the function of bHLH104 in Cd tolerance, the growth of wild type and the bHLH104 loss-of-function mutants was analyzed under Cd exposure. When grown vertically on normal medium for seven days, no visible differences were observed between wild type and the bhlh104 mutants. However, when grown on medium containing 50 μM CdCl₂, bhlh104 mutants developed significantly shorter roots than wild type (Figure 3A, B). Plants generally show chlorosis in young leaves and have low biomass under Cd stress conditions. Therefore, we also examined chlorophyll concentrations and fresh weight in wild type and bhlh104 mutants. The bhlh104 mutants had lower fresh weight and chlorophyll concentration compared with the wild-type plants under Cd exposure conditions (Figure 3C, D). Similar results were also observed under 25μM CdCl₂ exposure (Figure S1). Together, these results suggest that bHLH104 is required for Cd tolerance.

**Overexpression of bHLH104 promotes expression of Fe deficiency-responsive genes under Cd stress conditions**

Considering that loss of function of bHLH104 resulted in reduced expression of Fe deficiency-responsive genes, we inquired whether overexpression of
*bHLH104* would enhance the Fe-deficiency response under Cd stress conditions. Two *bHLH104* overexpression lines (*OX-4* and *OX-9*; Li et al. 2016) were used for further analysis. After growth on 50 μM CdCl₂ for three days, the expression of Fe deficiency-responsive genes was significantly higher in the *bHLH104* overexpression plants than in wild type (Figure 4). This finding implies that *bHLH104* overexpression enhances the Fe-deficiency response under Cd-treatment conditions.

**Overexpression of *bHLH104* enhances tolerance to Cd stress**

Given the enhanced Fe-deficiency response of the *bHLH104* overexpression plants, we hypothesized that they would also display an enhanced tolerance to Cd stress. To test this notion, we analyzed the phenotypes of *bHLH104* overexpression plants grown on medium with or without Cd. When grown on medium without Cd, the *bHLH104* overexpression plants grew as well as wild type. Under Cd-exposure conditions, the *bHLH104* overexpression plants had longer roots, higher biomass and chlorophyll concentrations than wild-type plants, indicating an enhanced tolerance to Cd stress (Figures 5A-D, S2). Taken together, these results further support a positive role for *bHLH104* in Cd tolerance.

**bHLH104 positively regulates expression of heavy metal detoxification-associated genes**

The three major strategies that plants use for detoxification of heavy metals are sequestration of heavy metals into vacuoles, chelation, and remobilization. Many genes have been characterized to take part in the regulation of the these processes in plants, including *MTP1*, *MTP3*, *IREG2*, *HMA3*, *HMA4*, *NAS4*, *ABCC1*, *ABCC2*, *ABCC3*, *ABCC4*, *NRAMP3*, *NRAMP4*, *PCS1*, and *PCS2* (Thomine et al. 2000, 2003; Arrivault et al. 2006; Schaaf et al. 2006; Morel et al. 2006).
2009; Oomen et al. 2009; Park et al. 2012; Koen et al. 2013; Brunetti et al. 2015; Chen et al. 2016). To test whether the Cd tolerance of bHLH104 overexpression plants is associated with these genes, we analyzed their expression levels. The expression of IREG2, MTP3, HMA3 and NAS4 was markedly upregulated in the bHLH104 overexpression plants and downregulated in the bhlh104 mutants when compared to wild type (Figure 6). By contrast, no significant expression difference was observed for the other genes among wild type, bhlh104 mutants and bHLH104 overexpression plants (Figure S3). These data indicate that bHLH104 promotes the expression of some heavy metal detoxification genes.

Overexpression of bHLH104 represses Cd translocation from roots to shoots

We have confirmed that overexpression of bHLH104 facilitates Cd tolerance. To further examine why the bHLH104 overexpression plants displayed enhanced tolerance to Cd, the Fe/Cd concentrations in roots and shoots were analyzed. Wild-type and bHLH104 overexpression plants were grown on normal medium for seven days and then transferred to the same medium containing 50 μM CdCl₂ for three days. The bHLH104 overexpression roots contained more Cd and Fe than those of wild type (Figure 7A). By contrast, the bHLH104 overexpression shoots had less Cd, but more Fe than wild type (Figure 7B). In addition, we found that the root/shoot ratio of Cd concentration in the bHLH104 overexpression plants was significantly higher than that of wild type (Figure 7C), suggesting the inhibition of Cd transport from roots to shoots in the bHLH104 overexpression plants. The opposite results were obtained from the bhlh104 mutants, in which the shoot Cd concentrations were higher and the root Cd concentrations were lower than in wild type (Figure S4).
DISCUSSION

In plants, Cd detoxification is a complex process that involves multiple physiological and molecular mechanisms, including reduced uptake from soil, cell wall binding, chelation with glutathione (GSH) and phytochelatins (PCs), sequestration and remobilization in the vacuole, and decreased translocation from roots to shoots (Hall 2002; Kim et al. 2006; Dalcorso et al. 2010; Lin et al. 2012). Here, we report multiple lines of evidence showing that bHLH104 participates in Cd detoxification and tolerance in Arabidopsis.

Due to their similar physical properties, Cd can compete with other divalent metal ions, such as Fe and Zn, during uptake by roots from soils (Clemens, 2006). Faced with Cd exposure, plants exhibit some typical Fe-starvation symptoms such as chlorosis in young leaves, shorter roots and reduced biomass (Kahle 1993; Das et al. 1997). Here, we found that Cd treatment restricts Fe translocation from roots to shoots (Figure 1), which may be one reason plants display Fe-deficiency symptoms under Cd exposure. Since Cd competes with Fe to bind chelators such as NA, the long-distance transport of Fe from roots to shoots might be inhibited by the additional Cd supply. In addition to the visible phenotypes, several Fe deficiency-responsive genes were also induced by Cd treatment, in agreement with the elevated Fe accumulation in roots (Figure 1).

Our recent work confirmed that bHLH104 is a key positive regulator of Fe homeostasis in Arabidopsis and is required for the expression of most Fe deficiency-responsive genes (Li et al. 2016). Here, we found that several typical Fe deficiency-responsive genes, such as IRT1, FRO2, bHLH38 and bHLH39, were induced by Cd treatment in wild-type plants. Moreover, their upregulation was dramatically inhibited in the bhlh104 mutants (Figure 2) and enhanced in the bHLH104 overexpression plants (Figure 4). These data suggest that bHLH104 promotes the expression of certain Fe
deficiency-responsive genes under Cd exposure conditions. The loss-of-function of \textit{bHLH104} did not completely block the upregulation of Fe deficiency-responsive genes under Cd exposure, implying that the other factors (possibly other members of the bHLH IVc subgroup such as bHLH34/105/115) may also be involved in the regulation of these genes.

We showed that the loss-of-function of \textit{bHLH104} increased plant sensitivity to Cd exposure, which was mainly reflected in decreased root length, chlorosis in young leaves and reduced biomass (Figure 3). By contrast, the overexpression of \textit{bHLH104} increased Cd tolerance as indicated by high biomass, long roots and elevated chlorophyll concentrations (Figure 5). Under Cd stress conditions plants facilitate Fe uptake to competitively inhibit Cd toxicity (Clemens, 2006). Indeed, Fe application can alleviate Cd toxicity in Arabidopsis and rice (Wu et al., 2012; Sebastian and Prasad, 2016). We also found that additional Fe supply rescued the sensitivity of \textit{bhlh104} mutants to Cd and masked the tolerance of \textit{bHLH104} overexpression plants (Figure S5). This observation suggests that the upregulation of Fe deficiency-responsive genes underlies the Fe increase and Cd tolerance in the \textit{bHLH104} overexpression plants. Therefore, modulating Fe accumulation is one approach for avoiding Cd toxicity in plants.

Accumulation of heavy metals usually depends on at least four processes, including absorption from the soil, loading in the root xylem, unloading from the xylem into leaves, and detoxification in the shoot (Verbruggen et al. 2013). Thus, reducing Cd absorption from soils and restricting Cd transport from roots to shoots represent two basic strategies by which plants could overcome Cd stress. To reduce Cd allocation from roots to shoots, Cd is often sequestered in root vacuoles. We found that, in addition to the Fe deficiency-responsive genes, \textit{bHLH104} positively regulates four heavy metal detoxification-associated genes (\textit{IREG2}, \textit{MTP3}, \textit{HMA3} and \textit{NAS4}) (Figure 6). \textit{IREG2} encodes a tonoplast transport protein involved in iron-dependent nickel
(Ni) detoxification and cobalt (Co) transport in Arabidopsis (Schaaf et al. 2006; Morrissey et al. 2009). Although it is unclear whether IREG2 transports Cd, IREG2 overexpression plants have increased Ni tolerance and accumulate high levels of manganese (Mn), Ni, Copper (Cu) and Zn in the root. Considering that IREG2 is upregulated by Cd treatment, we speculate that IREG2 might also sequester Cd in the vacuoles of roots in plants overexpressing bHLH104, which might contribute to their Cd tolerance. Induction of IREG2 by Fe deficiency is dependent on FIT (Colangelo and Guerinot, 2004; Wu et al. 2012). Therefore, bHLH104 may upregulate IREG2 via the FIT-bHLH38/39 complex.

MTPs are divalent cation transporters that are essential for metal homeostasis and tolerance (Arrivault et al. 2006). Expression analysis indicated that bHLH104 promoted the expression of MTP3 under Cd exposure, implying that MTP3 may be required for the Cd tolerance of bHLH104 overexpression plants. HMA3 is localized to the vacuolar membrane and can transport Cd into the vacuoles (Gravot et al. 2004; Morel et al. 2009). HMA4, which is expressed in vascular tissues and localizes to the plasma membrane, is responsible for transporting Cd from roots to shoots (Verret et al. 2004). HMA3, but not HMA4, was elevated in the bHLH104 overexpression plants. This finding suggests that bHLH104 activates HMA3 to sequester Cd in root vacuoles. NA, a major chelator of divalent metal ions, plays a pivotal role in metal long-distance transport. NAS4 is a major gene required for NA synthesis, which is induced by Fe deficiency (Klatte et al. 2009). In Arabidopsis, NAS4 loss of function causes a decrease in NA content and enhanced sensitivity to Cd stress, whereas its overexpression has the opposite effect (Koen et al. 2013). In agreement with this pattern, NAS4 was markedly upregulated by Cd stress in bHLH104 overexpression plants (Figure 6). The elevated expression of NAS4 might improve the synthesis of NA in these lines, resulting in more Cd being sequestered in the roots. Previous studies indicated that NAS4 is not
regulated by FIT (Colangelo and Guerinot, 2004) and that co-overexpression of FIT and bHLH38/39 has no effect on the expression of NAS4 (Wu et al. 2012). Therefore, it is not clear whether that bHLH104 activates NAS4 directly or regulates it indirectly via unknown transcription factors.

The expression of some heavy metal detoxification genes was unaffected by the overexpression of bHLH104 (Figure S3). NRAMP3 and NRAMP4 mediate inward Cd transport in yeast and the overexpression of NRAMP3 can result in increased sensitivity to Cd stress (Thomine et al. 2000). ABCC1, ABCC2 and ABCC3 encode important vacuolar transporters that confer tolerance to Cd (Park et al. 2012; Brunetti et al. 2015). However, overexpression of bHLH104 did not affect the expression of these genes (Figure S3). Phytochelatin (PC) is one of the most important metabolites sequestering Cd in the vacuoles for detoxification, and PCS1 and PCS2 encode the phytochelatin synthases responsible for PC synthesis (Cobbett and Goldsbrough, 2002; Chen et al., 2016). In accordance with this, we found that the transcript abundances of PCS1 and PCS2 were induced by Cd stress; however, they were not affected by bHLH104 (Figure S3). Therefore, bHLH104 confers Cd tolerance independently of PC synthesis. In agreement with the upregulation of IREG2, MTP3, HMA3 and NAS4 (Figure 6) bHLH104 overexpression plants accumulated more Cd in the root, but less Cd in the shoot, when compared with wild-type plants (Figure 7). Similarly, the downregulation of IREG2, MTP3, HMA3 and NAS4 in the bhlh104 mutants was in accordance with the reduced root Cd and increased shoot Cd in those mutants (Figure S4). Therefore, the restricted Cd translocation from roots to shoots may contribute to the Cd tolerance of the bHLH104 overexpression plants.

Enriching Fe and minimizing Cd in crops is important for human health and nutrition. Our results indicate that the overexpression of bHLH104 enhances the expression of Fe deficiency-responsive genes and heavy metal
detoxification-associated genes, promotes accumulation of Fe, restricts Cd transport from roots to shoots, and results in enhanced Cd tolerance. Our recent work also found that bHLH104 driven by the MYB72 promoter increases Fe concentrations and tolerance to alkaline soils (Wang et al. 2017). Taken together, these findings highlight bHLH104 as an exciting potential tool to generate Fe-rich crops with low Cd content.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

*Arabidopsis thaliana* ecotype Col-0 obtained from the Arabidopsis Biological Resource Center (ABRC; https://abrc.osu.edu/) was used. Seeds were surface-sterilized with 20% commercial bleach for 15 mins and then washed three times with distilled water. After plated on half MS media, seeds were vernalized for 2 d at 4°C before germination in culture room. Normal media are half MS media with 1% sucrose, 0.8% agar, and 0.1 mM Fe-EDTA at pH 5.8. For the experiments of Cd toxicity, media are the same with additional various CdCl$_2$ concentrations. Plates were placed in a culture room at 22 °C under a 16-h light/8-h dark photoperiod. For phenotype analysis, each biological replicate contains three samples and each sample contains fifteen plants. bhlh104-1 and bhlh104-2 mutants were described previously (Zhang et al. 2015; Li et al. 2016). bHLH104 overexpression plants (35S:MYC-bHLH104-GFP) were reported previously (Li et al. 2016).

**Gene expression analysis**

Gene expression analysis was performed as described previously (Ai et al., 2016). Briefly, TRIzol reagent (Invitrogen) was used to extract total RNA. According to the reverse transcription protocol (Takara), cDNA was
synthesized by oligo (dT) 18. On the basis of the manufacturer’s instructions, the SYBR Premix Ex TaqTM kit (TaKaRa) was used as quantitative PCR on a Roche Light Cycler 480 real-time PCR machine. ACTIN2 (ACT2) was used for an internal control, and gene copy number was normalized to that of ACT2. For the quantification of each gene, at least three biological replicates were used. Each biological replicate contained three technical replicates. The statistical significance analysis was performed by student’s t test. The primers for quantitative reverse transcription-PCR were listed in Supplemental Table S1.

**Chlorophyll content analysis**

For determination of chlorophyll contents, leaves from ten-day-old seedlings grown on medium with or without CdCl$_2$ supply were collected and ground to powder in liquid nitrogen. The powder was resuspended in 80% (v/v) acetone on ice and centrifuged at 10,000 g at 4 °C for 5 min. A$_{664}$ and A$_{648}$ was inspected by spectroscopy absorbance measurements, and the chlorophyll a and b contents were calculated according to described previously (Lichtenthaler 1987).

**Measurement of Fe and Cd content**

To inspect Fe and Cd contents, 7-d-old seedlings grown on normal medium were transferred to Cd supply medium for 3 d. The shoots and roots were harvested separately and used for Fe and Cd measurement. Determination of Fe and Cd contents was performed using an inductively coupled plasma spectroscopy.

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AUTHOR CONTRIBUTIONS
G.L. conceived and designed the experiments. X.Y. and Y.C. performed the experiments. X.Y. and G.L. wrote the manuscript. X.Y., Y.C., D.Y. and G.L. revised the manuscript.
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**SUPPORTING INFORMATION**

**Figure S1.** Phenotypes of *bhlh104* loss-of-function mutants grown on media with 25μM CdCl₂

**Figure S2.** Expression of Fe deficiency responsive genes in wild type and *bhlh104* mutants under Cd stress

**Figure S3.** Expression of heavy metal detoxification associated genes in wild type, *bhlh104* mutants and *bHLH104* overexpression plants under Cd stress

**Figure S4** Cd allocation in the roots and shoots of *bhlh104* mutants after Cd treatment

**Figure S5.** Extra Fe supply enhances Cd tolerance

**Table S1.** Primers used in this article
Figure legends

Figure 1. Cd treatment affects Fe translocation from roots to shoots
(A) Fe and Cd concentration in roots. (B) Fe and Cd concentration in shoots. (C) Ratio of root/shoot of Fe concentration. (A–C) Seedlings grown on normal media for 7 days were transferred to the same media with either 0 (Cd0), 25 (Cd25) or 50 μM CdCl$_2$ (Cd50) for 3 days. Three biological replicates of roots and shoots were used for metal analysis. Lower case letters indicate significant differences (one-way ANOVA, Duncan, P<0.05). Error bars indicate SD.

Figure 2. Expression of Fe deficiency responsive genes in wild type and *bhlh104* mutants under Cd stress
Seedlings grown on normal media for 4 days were transferred to the same media with either 0 (Cd0), or 50μM CdCl$_2$ (Cd50) for 3 days. Three biological replicates of root samples were used for qRT-PCR analysis. Each biological replicate contained three technical repeats. Lower case letters indicate significant differences (one-way ANOVA, Duncan, P<0.05). Error bars indicate SD.

Figure 3. *bhlh104* loss-of-function mutants are sensitive to Cd
(A) Representative seedlings grown on media with either Cd0 (CdCl$_2$ free) or Cd50 (50 μM CdCl$_2$). (B) Root length. (C) Fresh weight. (D) Chlorophyll concentration. (A–D) 7-day-old seedlings were used for analysis. Three biological replicates were used for analysis. Lower case letters indicate significant differences (one-way ANOVA, Duncan, P<0.05). Error bars indicate SD.
Figure 4. Expression of Fe deficiency responsive genes in wild type and bHLH104 overexpression plants under Cd stress

Seedlings grown on normal media for 4 days were transferred to the same media with either 0 (Cd0) or 50 μM (Cd50) CdCl₂ for 3 days. Three biological replicates of root samples were used for qRT-PCR analysis. Each biological replicate contained three technical repeats. Lower case letters indicate significant differences (one-way ANOVA, Duncan, P<0.05). Error bars indicate SD.

Figure 5. bHLH104 overexpression plants are tolerant to Cd

(A) Representative seedlings grown on either Cd0 (CdCl₂ free) or Cd50 (50 μM CdCl₂) media. (B) Root length. (C) Fresh weight. (D) Chlorophyll concentration. (A–D) 7-day-old seedlings were used for analysis. Three biological replicates were used for analysis. Lower case letters indicate significant differences (one-way ANOVA, Duncan, P<0.05). Error bars indicate SD.

Figure 6. Expression of IREG2, MTP3, HMA3 and NAS4 in wild type, bhlh104 mutants and bHLH104 overexpression plants under Cd stress

Seedlings grown on normal media for 4 days were transferred to the same media with either 0 (Cd0) or 50 μM (Cd50) CdCl₂ for 3 days. Three biological replicates of root samples were used for qRT-PCR analysis. Each biological replicate contained three technical repeats. Lower case letters indicate significant differences (one-way ANOVA, Duncan, P<0.05). Error bars indicate SD.

Figure 7. Fe and Cd allocation in the root and shoot of bHLH104 overexpression plants after Cd treatment
(A) Fe and Cd concentration in roots. (B) Fe and Cd concentration in shoots. (C) Ratio of root/shoot of Cd concentration. (A–C) Seedlings grown on normal media for 7 days were transferred to the same media with 50 μM CdCl₂ for 3 days. Three biological replicates of roots and shoots were used for metal analysis. Lower case letters indicate significant differences (one-way ANOVA, Duncan, P<0.05). Error bars indicate SD.

Figure. 1
Figure 4

Figure 5
Figure 6
Figure. 7

(A) Fe and Cd concentration (µg/g DW) in WT, OX-4, and OX-9 for Root.

(B) Fe and Cd concentration (µg/g DW) in WT, OX-4, and OX-9 for Shoot.

(C) Ratio of Root:Shoot in WT, OX-4, and OX-9.