Expression of the Tobacco Non-symbiotic Class 1 Hemoglobin Gene Hb1 Reduces Cadmium Levels by Modulating Cd Transporter Expression Through Decreasing Nitric Oxide and ROS Level in Arabidopsis

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Hemoglobin (Hb) proteins are ubiquitous in plants, and non-symbiotic class 1 hemoglobin (Hb1) is involved in various biotic and abiotic stress responses. Here, the expression of the tobacco (Nicotiana tabacum) hemoglobin gene NtHb1 in Arabidopsis (Arabidopsis thaliana) showed higher cadmium (Cd) tolerance and lower accumulations of Cd, nitric oxide (NO), and reactive oxygen species (ROS) like hydrogen peroxide (H$_2$O$_2$). NtHb1-expressing Arabidopsis exhibited a reduced induction of NO levels in response to Cd, suggesting scavenging of NO by Hb1. In addition, transgenic plants had reduced accumulation of ROS and increased activities of antioxidative enzymes (catalase, superoxide dismutase, and glutathione reductase) in response to Cd. While the expression of the Cd exporters ABC transporter (PDR8) and Ca$_2^+$/H$_+$ exchangers (CAXs) was increased, that of the Cd importers iron responsive transporter 1 (IRT1) and P-type 2B Ca$_2^+$ ATPase (ACA10) was reduced in response to Cd. When Col-0 plants were treated with the NO donor sodium nitroprusside (SNP) and H$_2$O$_2$, the expression pattern of Cd transporters (PDR8, CAX3, IRT1, and ACA10) was reversed, suggesting that NtHb1 expression decreased the Cd level by regulating the expression of Cd transporters via decreased NO and ROS. Correspondingly, NtHb1-expressing Arabidopsis showed increased Cd export. In summary, the expression of NtHb1 reduces Cd levels by regulating Cd transporter expression via decreased NO and ROS levels in Arabidopsis.

Keywords: ACA10, cadmium, CAX, hemoglobin, IRT1, nitric oxide, PDR8, ROS

Abbreviations: ANOVA, analysis of variance; CaCl$_2$, calcium chloride; CAT, catalase; CM-H2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; cPTIO, 2-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxid; DAF-2DA, 4,5-diaminofluorescein; DMSO, Dimethyl sulfoxide; DTNB, 2-nitrobenzoic acid; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GR, glutathione reductase; GSSG, glutathione disulphide; H$_2$O$_2$, hydrogen peroxide; ICP, inductively coupled plasma, MS, Murashige and Skoog; NBT, nitroblue tetrazolium; NO, nitric oxide; qRT-PCR, quantitative real-time PCR; RNS, reactive Nitrogen Species; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase; TNB, 2-nitro-5-thiobenzoate; WT, Wild type.
INTRODUCTION

Cadmium is a non-essential metal and a major hazardous environmental pollutant because it has toxic effects even at low concentrations. Cadmium negatively influences several aspects of plant metabolism and development, such as growth, transpiration, photosynthesis, respiration, and nutrient distribution (Das et al., 1997; Sanita et al., 1999; Zanella et al., 2016; Ronzan et al., 2017). Moreover, Cd toxicity causes various alterations in plants at genetic, biochemical, and physiological levels and results in phytotoxicity (Wahid et al., 2010). Due to similar oxidation states, Cd can be exchanged with Fe$^{2+}$, Zn$^{2+}$, and Ca$^{2+}$ in some protein structures, leading to protein malfunction (Goyer, 1997). In addition, at excess concentrations, Cd induces the formation of ROS, leading to lipid peroxidation and DNA damage, as well as calcium homeostasis modification (Stohs and Bagchi, 1995). The mechanism of Cd uptake by plants is significantly affected by various factors, such as pH, temperature, aeration, Cd concentration in the environment, and concentration of other micro and macro elements (McLaughlin et al., 2008). Uptake of Cd by plant roots occurs through divalent cation transport systems, including those for iron, zinc, and calcium (Verbruggen et al., 2009).

Several Cd transporters involved in the uptake, efflux, and sequestration of Cd have been identified. Among these proteins, plasma membrane-localized transporters, such as AtIRT1 (Connolly et al., 2002), OsIRT1 (Lee and An, 2009), AhIRT1 (Chen C. et al., 2017), AtNRAMP1 (Thomine et al., 2000), AhNRAMP1 (Chen C. et al., 2017), NnNRAMP1 (Sano et al., 2012), OsNRAMP5 (Sasaki et al., 2012), HvNramp5 (Wu et al., 2016), OsMTP1 (Yuan et al., 2012), OsZIP1 (Ramesh et al., 2003), and SaNramp6 (Chen S. S. et al., 2017), have been implicated in Cd uptake. Furthermore, members of the HMA family, including AhHMA2 (Mills et al., 2003), AhHMA4 (Hussain et al., 2004; Verret et al., 2004), and OsHMA2 (Takahashi et al., 2012), as well as the ATP-binding cassette transporter AtPDR8 (Kim et al., 2007), are located at the plasma membrane and are involved in Cd efflux. In addition, vacuolar transporters, including AtCAX1 (Wu et al., 2011), AtCAX2 (Hirschi et al., 2000), AtCAX4 (Korenkov et al., 2007), AtHMA3 (Morel et al., 2009), TcHMA3 (Ueno et al., 2011), FHMA3 (Guo et al., 2017), AtABCC1/AtABCC2 (Park et al., 2012), and AtABCC3 (Brunetti et al., 2015), play considerable roles in Cd transport into the vacuole.

Calcium (Ca$^{2+}$) is a vital nutrient and signaling molecule that is implicated in various metabolic and signal transduction pathways (Yang and Poovaiyah, 2003; Demidchik et al., 2018). It has been reported that Ca interferes with Cd uptake and transport by regulating Cd transporter expressions (Kim et al., 2002; Zeng et al., 2017). Moreover, due to their similar ionic radii, Ca and Cd may compete with each other for uptake and transport into plant cells. It was shown that Cd treatment repressed the activity of Ca$^{2+}$ channels (Li S. et al., 2012). So far, several genes have been found to be involved in Ca$^{2+}$ transport as well as Cd tolerance. Over-expression of OsACA6 encoding P-type 2B Ca$^{2+}$ ATPase improved Cd tolerance by mediating Cd distribution (enhanced and decreased Cd levels in roots and shoots, respectively) and lowering oxidative stress in tobacco (Shukla et al., 2014). In addition, plant cadmium resistance 1 (PCR1) protein is able to transport Ca$^{2+}$ (Song et al., 2011) and promotes Cd tolerance by reducing Cd accumulation in yeasts and Arabidopsis protoplasts (Song et al., 2004).

Nitric oxide is a short-lived free-radical reactive gas that functions in a wide range of physiological processes in plants, such as growth and development, iron homeostasis, and responses to biotic and abiotic stresses (Ramirez et al., 2011). Production of NO in plants is affected by biotic and abiotic stresses (Lamattina et al., 2003; Leitner et al., 2009). NO production in plants under Cd stress conditions can act as either an enhancer or reducer of Cd toxicity. Cd-induced NO production primarily contributes to Cd toxicity through elevating oxidative stress by enhancing ROS, RNS and lipid peroxidation, as well as by repressing the activity of antioxidant enzymes (Groppa et al., 2008; De Michele et al., 2009; Arasimowicz-Jelonek et al., 2012; Kulik et al., 2012). By contrast, NO protects plants against Cd-induced oxidative stress by enhancing antioxidant enzyme activity and reducing Cd accumulation (Verma et al., 2013; Perez-Chaca et al., 2014; Yang et al., 2016). Therefore, a decrease in NO by Cd results in higher ROS levels and toxicity (Rodriguez-Serrano et al., 2009; Ortega-Galisteo et al., 2012; Gupta et al., 2017). Moreover, treatment with exogenous NO donors, such as SNP, mitigates Cd-induced oxidative stress by enhancing the activities of antioxidant enzymes (Kopyra and Gwozdz, 2003; Hsu and Kao, 2004; Li L. et al., 2012; He et al., 2014). In addition, NO accumulation is involved in programmed cell death under Cd toxicity conditions (Ye et al., 2013). NO can also mediate the induction or inhibition of Cd toxicity by increasing Cd accumulation (Ma et al., 2010; Arasimowicz-Jelonek et al., 2012; Chmielowska- Baş and Deckert, 2013; Han et al., 2014), potentially by enhancing Cd uptake (Besson-Bard et al., 2009; Luo et al., 2012; Zhu et al., 2012) or by decreasing Cd accumulation (Li L. et al., 2012; Zhang et al., 2012; He et al., 2014).

Hemoglobin (Hb), an ubiquitous protein in plants, was first identified in the root nodules of soybean (Glycine max) plants and implicated in oxygen binding and transport (Appleby, 1992). There are two classes of plant non-symbiotic Hb genes (class 1 and 2), which have over 50% sequence identity, but are distinct in terms of their phylogenetic characteristics, gene expression, and oxygen binding features (Kundu et al., 2003; Igamberdiev et al., 2011). Several lines of evidence indicate a significant role for non-symbiotic class 1 hemoglobin (Hb1) in NO detoxification (Dordas et al., 2003; Igamberdiev et al., 2004; Perazzolli et al., 2004; Hebelstrup et al., 2006). Therefore, Hb1 can participate in plant responses to biotic and abiotic stress by modulating the level of NO. Transgenic Arabidopsis plants that overexpress AthHb1 have higher tolerance to hypoxia stress (Hunt et al., 2002). In addition, tolerance to submergence, salinity, and osmotic stresses are increased by ZmtHb expression in tobacco (Zhao et al., 2008). Furthermore, heterologous expression of MsHb1 in tobacco and Arabidopsis (Seregelyes et al., 2004; Maassen and Hennig, 2011), GhHb1 in Arabidopsis (Qu et al., 2006), and AtHb1 in barley (Hebelstrup et al., 2014) improves defense responses against pathogen attack. Down-regulation of cold-responsive genes, as well as mitigated oxidative stress, has
also been observed in transgenic Arabidopsis expressing AtHb1 (Cantrel et al., 2011; Thiel et al., 2011). Previous studies have revealed that the growth responses and development of plants are mediated by Hb1, as its expression delays bolting in Arabidopsis (Hebelstrup and Jensen, 2008) and reduces plant growth and development in barley (Hebelstrup et al., 2014). Recently, we also showed that over-expression of NtHb1 enhances Cd tolerance by reducing the NO and Cd levels in transgenic tobacco (Lee and NtHb1 showed that over-expression of Arabidopsis revealed that the growth responses and development of plants are (Cantrel et al., 2011; Thiel et al., 2011). Previous studies have µ on 1/2 MS agar plates supplemented with 0 and 50 µmol/L NaCl, and osmotic stresses (Bai et al., 2016). Moreover, AtHb1-overexpressing Arabidopsis plants fumigated with NO showed improved growth parameters and NO fixation ability compared with the WT plants (Kuruthukulangarakool et al., 2017). In this study, to understand a mechanism for Hb1-induced Cd tolerance in Arabidopsis, we over-expressed NtHb1 in Arabidopsis and examined concentrations of Cd, NO, and ROS and expression levels of the Cd transporters Ca2+/H+ exchangers (CAXs), ABC transporter (PDR8), and iron responsive transporter 1 (IRT1).

MATERIALS AND METHODS

Plasmid Construction and Transformation of Arabidopsis

The coding sequence of NtHb1 (GenBank Access No. KJ808726.1) was amplified by RT-PCR using gene specific primers flanked by XbaI and BamHI restriction sites and sub-cloned into the pBS vector. After sequencing the insert, the XbaI-BamHI fragment was cloned into the binary vector pBI121. The pBI121 vector harboring NtHb1 was transformed into Agrobacterium tumefaciens strain GV3103 by the freeze–thaw method, and plant transformation was achieved by the floral dipping method (Clough and Bent, 1998). To generate pAtHb1::GUS-expressing Arabidopsis, the promoter region of AtHb1 (1-kb upstream of the start codon) was amplified using primers with HindIII and XbaI restriction sites and cloned into the binary vector pBI121. The primer pairs used for these constructs are shown in Supplementary Table S1.

Quantitative RT-PCR (Real-Time PCR)

Total RNA was isolated from transgenic Arabidopsis seedlings (T3 homozygous) grown on 1/2 MS agar plates for 3 weeks using the Plant RNA extraction Kit (Intron, South Korea). After quantification of the RNA using a NanoDrop (BioSpec-nano, Shimadzu, Japan), first-strand cDNA was synthesized from 2 µg of RNA in RNase-free water using PrimeScript™ RT reagent kit (Takara, Otsu, Japan) according to the manufacturer’s instructions. Semi-quantitative PCR was employed for the analysis of NtHb1 expression by using gene-specific primers. Actin was used as an internal control. The expression levels of cadmium transporter genes in Arabidopsis were evaluated by using qRT-PCR. Isolation of total RNA was performed by using transgenic Arabidopsis seedlings (T3 homozygous) grown on 1/2 MS agar plates supplemented with 0 and 50 µM CdSO4 for 3 weeks, and cDNA was synthesized as described above. Quantitative RT-PCR was accomplished using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, United States). The reaction mixture consisted of 10 µl of SYBER Supermix (SsoAdvanced® Universal SYBR™ Green Supermix), 1 µl of cDNA, 7 µl of nuclease-free water, and 1 µl of each primer to a final volume of 20 µl. The following qRT-PCR reaction thermal conditions were used: 95°C for 30 s, followed by 40 cycles at 95°C for 15 s and 57°C for 20 s. The Arabidopsis ACTIN2 gene was used as an internal quantitative control, and the relative expression level of each gene was calculated based on the 2−ΔΔCt method (Pfaffl, 2001). Each qRT-PCR experiment was performed three times with different cDNA sets obtained from three independent biological replicates. The gene-specific primers are shown in Supplementary Table S1.

Western Blot Analysis

Arabidopsis wild-type plants (Col-0) were grown on 1/2 MS agar plates containing 0 and 50 µM CdSO4 for 3 weeks, and subsequently, the roots and shoots were separated and homogenized in extraction buffer (20 mM Tris–HCl, pH 8.0, 1 mM DTT (dithiothreitol), 0.3 mM EDTA and protease inhibitor cocktail). Protein samples (40 µg) were loaded and separated on SDS-PAGE gels and then transferred to nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences) and probed with AtHb1 primary antibodies (Abmart Co.,) and the binding of secondary antibody rabbit anti-mouse horseradish peroxidase-conjugated IgG (Amersham Biosciences) was detected by enhanced chemiluminescence (Amersham Biosciences). Rubisco Ponceau S-stained large subunit was used as a loading control. Three independent biological experiments were performed.

Analysis of Cadmium Tolerance

To measure cadmium tolerance, seeds of transgenic Arabidopsis lines (T3 homozygous) were surface-sterilized, germinated and incubated on 1/2 MS agar plates supplemented with 0 and 50 µM of cadmium sulfate for 3 weeks. This cadmium concentration reduced the fresh weight of wild-type seedlings by 50%. The Arabidopsis seedlings were photographed after 3 weeks. These experiments were performed in three independent biological replicates for each line. To calculate the cadmium tolerance, the fresh weight of each seedling (n = 90) on cadmium plates was divided by the fresh weight of seedlings grown on control plates (1/2 MS), and finally expressed as the relative fresh weight (%).

Measurement of Cadmium Concentration in Plants

For the Cd concentration measurement, control and transgenic Arabidopsis seeds (T3 homozygous) were surface-sterilized, germinated and incubated on 1/2 MS agar supplemented with 50 µM of cadmium sulfate for 3 weeks. The seedlings on each plate were harvested after 21 days, washed with ice-cold 5 mM CaCl2 (three times) and dried for 72 h at 60 °C. Then, the dried sample (0.5 g) was digested by using concentrated HNO3 and HClO4 in a Teflon Digestion Vessel (Savillex, United States). ICP Mass Spectroscopy (Varian 720-MS, United States) was employed to specify the concentration of Cd at a wavelength of 214.44 nm at the National Instrumentation
Localization of Cadmium in Plants

Leadmium™ Green AM (Invitrogen, United States) was used to monitor the localization of Cd in Arabidopsis roots following the manufacturer’s instructions. Briefly, control and transgenic Arabidopsis seeds were germinated and grown on 1/2 MS medium containing 0 and 50 μM of cadmium. After 21 days, the root samples were harvested and rinsed in distilled water. The working solution of Leadmium™ Green AM was obtained through dilution (1:10) of its stock solution [1 μg/μl in DMSO in 0.85% NaCl]. Next, the samples were immersed in staining solution [0.04% (v/v) of Leadmium™ Green AM in 0.85% NaCl] and kept in the dark. After 1 h, the samples were briefly washed with saline solution (0.85% NaCl) (Du et al., 2015) and visualized using a fluorescence microscope (Leica, MZ10F; Germany) with 490 nm excitation and 501–520 nm emission filters under proper magnification (12.5-25X). At least 10 plants were examined per line, and three independent biological experiments were performed.

Measurement of Anti-oxidative Enzyme Activity

Antioxidant enzymes assays were performed using the crude extract of the leaves as the enzyme source. Briefly, leaf samples (0.2 g) of 3-week-old transgenic Arabidopsis plants grown on 1/2 MS medium supplemented with or without 50 μM of cadmium sulfate were ground to a fine powder in liquid nitrogen and then homogenized in 1.2 ml of 0.2 M potassium phosphate buffer (pH 7.8 containing 0.1 mM EDTA) and centrifuged at 15,000 × g at 4°C for 20 min to obtain a crude enzyme extract.

To determine the SOD activity (SOD; EC 1.15.1.1), the modified NBT method of Bayer and Fridovich (Bayer and Fridovich, 1987) was employed. Briefly, the assay solution consisted of 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 9.9 mM L-methionine, 55 μM NBT, 0.025% Triton X-100 and 1 mM riboflavin. Following the addition of the leaf extract, the reaction was initiated after exposing the samples to a fluorescent lamp (15 W) for 10 min. The absorbance of the reaction mixture was measured at 560 nm against a similar mixture lacking the leaf extract as a control. The SOD standard curve was used to evaluate the enzyme activity (per gram fresh weight) of the samples.

Glutathione reductase activity (GR; EC 1.8.1.7) was measured as previously described (Smith et al., 1918). The assay solution (1 ml) was prepared in 100 mM potassium phosphate buffer (pH 7.5) and 0.75 mM DTNB, 0.1 mM NADPH, 1 mM GSSG (glutathione disulfide), and 10 μl of leaf extract. GSSG was added to start the reaction, and the absorbance increment at 412 nm was measured when DTNB was reduced to TNB by GSH in the reaction. The activity of GR was determined based on the extinction coefficient of TNB (14.15 M⁻¹ cm⁻¹ at 240 nm) and expressed in terms of millimoles of H₂O₂ per minute per gram fresh weight.

Measurement of Cd Efflux

Seeds of control (pBI121) and NtHb1-expressing Arabidopsis were germinated and grown on 1/2 MS media plate for...
**FIGURE 1** | Cd tolerance and accumulation in the control and NtHb1-expressing Arabidopsis. (A) qRT-PCR results showing higher levels of NtHb1 transcripts in transgenic Arabidopsis. Control plants contain the empty vector pBI121, and NtHb1-4 and NtHb1-5 refer to two different lines of NtHb1-expressing transgenic Arabidopsis. EtBr-stained qRT-PCR product of the actin gene (AtActin) was used as a loading control. (B) Cadmium tolerance of transgenic Arabidopsis (Continued)
3 weeks, and subsequently, all plants were transferred to 50 μM Cd (cadmium sulfate) agar plates, incubated for 24 h, washed with distilled water, and placed in a Cd-free liquid medium (DW). After 6 and 24 h, the Cd concentrations in the liquid media were determined as described in the above section. Three independent biological experiments were performed.

**GUS Staining Analysis**

Detection of AtHb1::GUS activity was performed as previously described (Prasinos et al., 2005). Briefly, 7-day-old seedlings grown vertically on 1/2 MS agar containing 0 and 50 μM Cd were incubated in GUS staining solution (50 mM NaPO4 buffer, pH 6.8, 0.5 M EDTA, pH 8.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 20% Triton X-100 and 2 mM X-gluc), for 2 h at 37°C. For short-term experiments, 7-day-old seedlings grown vertically on 1/2 MS agar were treated with 50 μM of cadmium for 2, 6, 12, and 24 h. To observe GUS expression in the leaves, the samples were washed with ethanol (70%) to remove green chlorophyll pigments. Images were captured with a microscope (Zeiss Axiosoplan2). This experiment was repeated 3 times, and 10 seedlings were observed each time.

**Statistical Analysis**

All data were subjected to analysis of variance (Two-way ANOVA) by using SAS software (version 9.1), and comparisons of the means were performed according to Tukey’s test at P ≤ 0.05 or P ≤ 0.01.

**RESULTS**

**Over-Expression of NtHb1 Enhances Cd Tolerance but Decreases Cd Accumulation in Transgenic Arabidopsis**

To examine whether NtHb1 increases Cd tolerance in Arabidopsis similar to that in tobacco (Lee and Hwang, 2015), NtHb1 was over-expressed in Arabidopsis using the binary pBI121 vector. As shown in Figure 1A, two lines (4 and 5) of transgenic Arabidopsis expressing NtHb1 exhibited higher levels of NtHb1 transcript compared with that of the vector-only-expressing control Arabidopsis, which had no NtHb1 transcript. As expected, NtHb1-expressing Arabidopsis displayed higher tolerance to Cd than the control plants (Figures 1B, C). In contrast, the accumulation of Cd in NtHb1-Arabidopsis was lower than that in control plants (Figure 1D–F). Additionally, Cd was highly accumulated in the meristematic zone and stele (likely in the xylem).

**NO and ROS Are Less Induced by Cd in NtHb1-Expressing Arabidopsis**

Because NO production is enhanced in response to Cd (Besson-Bard et al., 2009) and Hb scavenges NO in Arabidopsis (Dordas et al., 2003; Perazzolli et al., 2004; Hebelstrup et al., 2006), the NO level was examined in control and transgenic Arabidopsis germinated and grown with 50 μM CdSO4 for 21 days. As shown in Figures 2A, B, the NO level was highly enhanced (6.0-fold) by Cd in control Arabidopsis expressing the pBI121 vector only. In contrast, the NO level was less increased (2-fold) by Cd in NtHb1-expressing Arabidopsis, indicating that Cd-induced NO accumulation was decreased by increased Hb in NtHb1-expressing Arabidopsis, leading to increased Cd tolerance. In addition, the increased accumulation of NO in the meristem and stele of Cd-treated plants (Figure 2A) corresponded to the pattern of Cd accumulation (Figure 1E).

Because ROS are also generated by Cd in Arabidopsis (Bi et al., 2009), the ROS level was measured using a fluorescent probe (CM-H2DCFDA). As shown in Figure 3, ROS accumulation was greatly increased (14-fold) by Cd in control plants, while the ROS content was less enhanced (3–4-fold) in NtHb1-expressing Arabidopsis. Interestingly, ROS evenly accumulated in the roots in response to Cd (Figure 3), while the Cd and NO levels were higher in the meristem and stele (Figure 2). To examine the involvement of anti-oxidative enzymes in decreasing ROS levels, the activities of CAT, SOD, and GR were measured in control and transgenic plants. As shown in Figure 4, all three enzymes were activated by Cd in both control and NtHb1-expressing Arabidopsis plants, but were highly induced in transgenic plants compared with levels in control plants.

**Expression of CAXs and PDR8 Is Enhanced While That of IRT1 Is Decreased in Response to Cd, Leading to Higher Cd Export in NtHb1-Expressing Arabidopsis**

To elucidate the mechanism by which Cd accumulation is decreased in NtHb1-Arabidopsis, qualitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed to examine the expression levels of various Cd transporters, including AtCAXI (vacuolar transporter, AT2G38170),
FIGURE 2 | NO production in response to Cd in the control and NtHb1-expressing Arabidopsis. (A) NO production was visualized using DAF-2DA in the control (pBI121) and NtHb1-expressing Arabidopsis (NtHb1-4 and NtHb1-5), which were germinated and grown for 3 weeks on 1/2 MS agar media without (left) and with (right) 50 µM CdSO₄. (B) The intensity of NO fluorescence (five plants from each line) was measured using Image J software. Each value corresponds to the means ± SE (n = 5). Different letters over the bars indicate significant differences according to Tukey's multiple comparison test (p ≤ 0.05).
FIGURE 3 | ROS levels in response to Cd in the control and NtHb1-Arabidopsis. (A) ROS staining with a fluorescent probe (CM-H$_2$DCFDA). Plants were germinated and grown for 3 weeks on 1/2 MS agar media without and with 50 µM Cd. Experiments were performed 3 times, and 10 plants from each line were examined in each experiment. (B) Quantification of the ROS shown in (A). The fluorescence signal was quantified using Image J software. Data are presented as the means of three independent experiments, and the error bars indicate SE. Different letters over the bars indicate significant differences according to Tukey’s multiple comparison test ($p \leq 0.05$).
AtIRT1 (uptake transporter, AT4G19690), AtHMA2 (efflux transporter, AT4G30110), AtHMA4 (xylem loading transporter, AT2G19110), AtNRAMP3 (vacuolar transporter, AT2G23150), AtABC1 (vacuolar transporter, AT1G30400), AtACA10 (putative uptake transporter, OsACA6 homolog, AT4g29900.1), and AtPCR1 (efflux transporter, AT1G14880.1). As shown in Figure 5A, the expression of AtCAX3 and AtPDR8 was increased by Cd in both control and NtHb1-expressing Arabidopsis plants, but transgenic plants showed greater increases and thus higher levels than those in control plants. In contrast, the expression of AtIRT1 and ACA10 was induced by Cd in control plants but reduced in NtHb1-Arabidopsis, resulting in a lower level of IRT1 and ACA10 in transgenic plants than in control plants. The expression of CAX1 and CAX2 in response to Cd was also higher in transgenic plants because the expression of these genes was already increased in transgenic plants in the absence of Cd. The expression of AtHMA2, AtHMA4, AtNRAMP3, AtABC1, and AtPCR1 did not show significant differences between control and transgenic plants in the absence and presence of Cd. Moreover, phytochelatins have been shown to play a significant role in Cd tolerance by sequestrating Cd in vacuoles (Pomponi et al., 2006; Brunetti et al., 2011). To examine whether phytochelatins are involved in Cd tolerance of NtHb1-expressing plants, we measured the expression level of AtPCS1 (phytochelatin synthase, AT5G44070) in control and NtHb1-expressing plants. As shown in Figure 5A, the transcript level of AtPCS1 did not change significantly in control and transgenic plants in the absence and presence of Cd, indicating that phytochelatins may not participate in enhancing the Cd tolerance observed in NtHb1-expressing transgenic plants. Furthermore, the Cd export rates of plants were examined. As shown in Figure 5B, NtHb1-expressing Arabidopsis showed higher Cd efflux rates than those of control plants, suggesting the involvement of CAX1–3 and PDR8 in reducing Cd levels in transgenic plants.

Taken together, these results suggest that the increased expression of CAX1–3 and PDR8 and the decreased expression of IRT1 and ACA10 are involved in reducing Cd accumulation by enhancing the Cd efflux and decreasing the Cd influx in NtHb1-expressing Arabidopsis.

Expression of CAX3, PDR8, IRT1, and ACA10 Is Regulated by NO and ROS in Arabidopsis

Because NtHb1-Arabidopsis showed a lower level of NO, it is likely that the expression of Cd transporters was regulated by the decreased NO content. To examine this hypothesis, we treated Col-0 Arabidopsis with SNP (an NO donor) for 24 h and subsequently examined the expression of CAX3, PDR8, IRT1, and ACA10 using qRT-PCR. As shown in Figure 6, when plants were germinated and grown for 3 weeks and thereafter treated with SNP for 24 h, the transcript levels of CAX3 and PDR8 were decreased, while those of IRT1 and ACA10 were increased, and these expression patterns were reversed in NtHb1-expressing Arabidopsis (Figure 5). In addition, the transcript levels of CAX3 and PDR8 were decreased, while those of IRT1 and ACA10 were increased in plants germinated and grown for 3 weeks...
FIGURE 5 | Expression of Cd transporter genes in response to Cd and Cd efflux in the control and NtHb1-Arabidopsis. (A) qRT-PCR results showing the transcript levels of various Cd transporter genes in transgenic Arabidopsis. Notably, pBI121 refers to control plants containing the empty vector pBI121, and NtHb1-4 and NtHb1-5 refer to two different lines of NtHb1-expressing transgenic Arabidopsis. qRT-PCR was performed using total RNA isolated from plants that were

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generated and grown for 3 weeks on 1/2 MS plates without or with 50 µM Cd. The relative transcript levels were assessed by normalization to actin transcript abundance as an endogenous control. (B) Cd efflux in plants. Control and transgenic plants were germinated and grown for 3 weeks on 1/2 MS agar plates, transferred to agar plates with 50 µM Cd, incubated for 24 h, and then placed in Cd-free liquid media for 6 h and 24 h. Subsequently, the Cd concentrations in liquid media were determined. All samples were run in triplicate (three biological repeats) for cDNA and each primer set, and the error bars indicate SE. Different letters over the bars indicate significant differences according to Tukey’s multiple comparison test ($p \leq 0.05$).

**FIGURE 5** | Short-term effect of SNP (NO donor) on the phenotype and gene expression of Cd transporters in Col-0 Arabidopsis. qRT-PCR results showing the transcript levels of various Cd-transporter genes in Col-0 Arabidopsis after short-term (24-h) exposure to SNP. qRT-PCR was performed using total RNA isolated from Col-0 plants, which were germinated and grown for 3 weeks on 1/2 MS agar plate and subsequently treated with 1/2 MS liquid (labeled as MS) and 500 µM SNP (labeled as SNP) for 24 h. All experiments were performed independently in triplicate, and the error bars indicate SE.

AtHb1 Expression Is Enhanced Greatly in the Roots and Slightly in the Shoots in Response to Cd

To examine whether AtHb1 is naturally involved in decreasing the NO level, which is induced by Cd in plants, the transcript level of AtHb1 in response to Cd was measured (Figure 8). The expression of AtHb1 was increased by 78% in the shoots and 428% in the roots in Col-0 Arabidopsis germinated and grown for 3 weeks on Cd media (Figure 8A). The AtHb1 expression level in the roots was 32 and 317% higher, respectively, than that in the shoots in the absence and presence of Cd. The AtHb1 transcript level was elevated by up to 14-fold in the roots within 24 h after Cd treatment (Figure 8B). Furthermore, the AtHb1 protein level was increased by 83% in the shoots and 305% in the roots in response to Cd, while the AtHb1 level was 47 and 246% higher, respectively, in the roots than in the shoots in the absence and presence of Cd (Figures 8F,G). In addition, transgenic Arabidopsis expressing the pAtHb1-GUS construct was generated to study the tissue localization of AtHb1. As shown in Figures 8C,E, AtHb1 was highly expressed in the roots but slightly expressed in cauline leaves. In the absence of Cd, AtHb1 was highly expressed in the root tips (meristems) and slightly expressed around the stele in the upper part of the roots. In contrast, AtHb1 expression was highly induced by Cd in every part of the roots, with the highest level of expression in the meristem, which corresponds to the NO pattern.
(Figures 2A, 8D), but AtHb1 induction was not clear in the shoots (Figures 8C,E). Regarding the 78% induction of the AtHb1 transcript levels by Cd in the shoots, the non-induction of GUS by Cd in pAtHb1-GUS plants may be ascribed to the shorter Cd treatment (7 days) compared with the 3-week Cd treatment in the AtHb1 transcript measurement, while GUS expression in the roots was enhanced by the 7-day Cd treatment. The time-dependent slight increase in Hb1 expression without Cd challenge (Figure 8D) was likely due to a buffer-induced hypoxia condition. These data suggest that Hb1 is naturally involved in removing NO, which is induced by Cd in the roots. AtHb1 expression was observed in cauline leaf tips and rosette leaf hydathodes in Arabidopsis Col-0 (Hebelstrup et al., 2006). Furthermore, AtHb1 expression was also observed in the shoot apical meristem of 16-day-old Arabidopsis plants (Hebelstrup and Jensen, 2008). By contrast, AtHb1 was only expressed in the roots of the plant (C-24), and it was absent in the shoots (Hunt et al., 2001).

**DISCUSSION**

Transgenic Arabidopsis expressing the hemoglobin gene NtHb1 showed an enhancement in Cd tolerance and reductions in Cd, NO, and ROS levels. The increase in Cd tolerance could be ascribed to the decreased Cd accumulation, as well as the reduction in oxidative stress, including NO and ROS. The reduction in the Cd level might be due to the enhanced expression of CAX3 and PDR8 and reduced expression of IRT1 and ACA10, which was attributed to the diminished levels of NO and ROS.

**NO Accumulation Is Increased by Cd and Inhibited by Hemoglobin in Arabidopsis**

Nitric oxide levels were enhanced by Cd in both control and NtHb1-expressing Arabidopsis, but NO was less increased (103%) in transgenic plants than in control plants (679%), resulting in lower levels of NO in transgenic plants (Figure 2). This finding suggests that the enhanced Hb1 diminishes the NO level, likely by scavenging more NO in NtHb1-expressing Arabidopsis.

The NO level was enhanced by Cd treatment in diverse plant species, including wheat (Groppa et al., 2008), Arabidopsis (Besson-Bard et al., 2009; De Michele et al., 2009; Zhu et al., 2012; Ye et al., 2013), white poplar (Balestrazzi et al., 2011), barley (Valentovicova et al., 2010), tobacco (Ma et al., 2010; Kulik et al., 2012; Lee and Hwang, 2015),
FIGURE 8 | Continued
pea (Lehotai et al., 2011), yellow lupine (Arasimowicz-Jelonek et al., 2012), Indian mustard (Verma et al., 2013), and soybean (Perez-Chaca et al., 2014). In contrast, some studies reported that NO content was decreased by Cd (Rodriguez-Serrano et al., 2009; Xiong et al., 2009; Ortega-Galisteo et al., 2012; Gupta et al., 2017).

Decreased NO and Cd Levels Reduce the ROS Level, Contributing to Cd Tolerance

In addition to the lower accumulation of NO in response to Cd, NtHb1-expressing Arabidopsis also had a lower ROS level (Figure 3). The lower levels of NO and ROS contribute to Cd tolerance higher in transgenic plants than that in control plants. Cd toxicity is mediated by NO by increasing ROS (De Michele et al., 2009; Arasimowicz-Jelonek et al., 2012; Kulik et al., 2012), RNS (Arasimowicz-Jelonek et al., 2012), and lipid peroxidation (Groppa et al., 2008; Zhu et al., 2012) as well as by inhibiting the anti-oxidative enzymes CAT and ascorbate peroxidase (De Michele et al., 2009). These reports suggest that Cd toxicity may not be enhanced if NO is not elevated in response to Cd. Consistently, in transgenic Arabidopsis expressing NtHb1, the NO level was less elevated in response to Cd, while Cd tolerance was more promoted than it was in control plants (Figures 1B,C, 2). Therefore, we concluded that the enhancement of Cd tolerance in NtHb1-Arabidopsis is attributed to the reduced accumulation of NO, which is responsible for Cd toxicity by promoting oxidative stress.

Furthermore, the lower ROS content in transgenic plants suggests that the ROS level is also decreased by Hb and that NO functions as ROS or produces ROS in Arabidopsis. In support of this result, it was reported that Hb was involved in decreasing ROS in Arabidopsis (Yang et al., 2005), and NO increased the ROS level (De Michele et al., 2009; Arasimowicz-Jelonek et al., 2012; Kulik et al., 2012). In addition, the highly enhanced activities of the anti-oxidative enzymes CAT, SOD, and GR by Cd in transgenic plants are responsible for the smaller increase of ROS (Figure 4). The higher activities of anti-oxidative enzymes in transgenic plants may be ascribed to the lower levels of NO and Cd. Interestingly, ROS accumulated evenly in roots in response to Cd (Figure 3), whereas the NO level was higher in the meristem and stele, indicating that the pattern of ROS production/accumulation is different from that of NO synthesis/accumulation in response to Cd in Arabidopsis.

Decreased NO and ROS Levels Reduce Cd Accumulation by Enhancing Cd Efflux via Regulation of the Expression of Cd Transporters

Cd-tolerant NtHb1-expressing Arabidopsis displayed lower levels of Cd and NO than those of control plants (Figures 1D–F, 2). This result is supported by reports that NO mediates Cd toxicity by enhancing Cd levels (Ma et al., 2010; Arasimowicz-Jelonek et al., 2012; Chmielowska-Bak and Deckert, 2013; Han et al., 2014). Among the putative Cd transporters examined, the expression of AtCAX1–3 (vacuolar transporter) and AtPDR8 (efflux transporter) was higher in NtHb1-Arabidopsis than in control plants in the presence of Cd, while the AtIRT1 (uptake transporter) and AtACA10 (putative uptake transporter) transcript levels were lower. This finding suggests that the altered expressions of these transporters are involved in decreasing Cd accumulation in NtHb1-Arabidopsis. This is supported by the enhanced Cd efflux in transgenic plants (Figure 5B). IRT1 expression is induced by Cd exposure in tobacco (Yoshihara et al., 2006), and the over-expression of IRT1 reduces Cd tolerance in Arabidopsis and rice, which is accompanied by increased accumulation of Cd (Connolly et al., 2002; Lee and An, 2009). The proposed roles for PDR8 and IRT1 in NtHb1-expressing Arabidopsis are supported by previous reports that PDR8 functions in pumping out Cd from the cytoplasm (Kim et al., 2007) and IRT1 plays a role in Cd absorption (Korsunova et al., 1999; Connolly et al., 2002). Like IRT1, ACA10 expression was induced by Cd in control plants but was reduced in transgenic plants, leading to a lower level than that in control plants (Figure 5A). In support of our result, the transcript level of OsACA6 (homologous to Arabidopsis ACA10) was increased by Cd treatment in rice, and tobacco expressing OsACA6 exhibited higher Cd tolerance and accumulation in the roots (Shukla et al., 2014). It has been reported that Cd uptake can be mediated by the transporters involved in uptake and transport of other divalent cations, such as Ca$^{2+}$, Fe$^{3+}$, and Mn$^{2+}$ (Vert et al., 2002; Sasaki et al., 2012; Chen et al., 2018). However, it was not elucidated how OsACA6 expression enhanced Cd levels in roots.

In contrast, it is not clear how CAXs are involved in reducing the Cd levels in transgenic plants because CAXs have been reported as tonoplast transporters that sequester Ca, Cd,
FIGURE 9 | A proposed model for the function of Hb1 in response to cadmium. The arrows show positive regulation, whereas the blunt lines show negative regulation.

and Mg into vacuoles in Arabidopsis (Pittman and Hirschi, 2016). Therefore, enhanced CAXs may indirectly participate in decreasing Cd accumulation in transgenic Arabidopsis through modulation of the cytosol Ca concentration. Furthermore, other studies suggest that CAXs may not contribute to reducing Cd accumulation in plants. AtCAX1-expressing petunia showed higher Cd tolerance and accumulation (Wu et al., 2011), and the ectopic expression of Arabidopsis CAX2 and CAX4 improved Cd tolerance along with higher Cd accumulation (Korenkov et al., 2007). In addition, CAXs can promote Cd tolerance by regulating ROS signaling and accumulation in plants. Knockout Arabidopsis of CAX1 showed less tolerance to Cd at low concentration of Ca and a higher ROS level compared with the WT plant (Baliardini et al., 2015). Very recently, CAX1 has been shown to increase the Cd tolerance and diminish ROS accumulation under Cd stress in Arabidopsis halleri (Ahmadi et al., 2018). Therefore, it is very possible that the higher expression level of CAXs in NtHb1-Arabidopsis also lowered ROS accumulation compared with the control plants, resulting in a higher Cd tolerance.

Sodium nitroprusside (NO donor) treatment experiments (Figure 6) demonstrated that expression of CAX3 and PDR8 was decreased by NO, but IRT1 expression was enhanced, emphasizing the correctness of the reversed expression pattern in NtHb1-expressing Arabidopsis. Expression of IRT1 is enhanced by NO, which leads to an increase in Cd uptake (Besson-Bard et al., 2009; Luo et al., 2012; Zhu et al., 2012). However, the repression of CAX3 and PDR8 expression, as well as induction of ACA10 expression, by NO was first reported in this research. Regarding the increased expression of CAX1~3, PDR8, and ACA10 in NtHb1-Arabidopsis in the absence of Cd (Figure 5), the higher level of Hb1 in transgenic plants is likely involved in enhancing the expression of CAX1~3, PDR8, and ACA10 by decreasing NO, even without a Cd challenge. In summary, the reduced accumulation of Cd in NtHb1-Arabidopsis was attributed to the enhanced expression of CAX1~3 and PDR8, and to the reduced expression of IRT1 and ACA10, which was caused by the decreased level of NO.

It seems that ROS, in addition to NO, is able to regulate the expression of Cd transporters. In response to H2O2 treatment, the transcript levels of CAX3 and PDR8 were decreased, while those of IRT1 and ACA10 were increased (Figure 7). NtCAX3 expression was increased and NtIRT1 expression was reduced in NtUBC1-expressing tobacco, which accumulated a lower level of ROS under Cd stress conditions (Bahmani et al., 2017). Moreover, NRAMP3 and NRAMP4 were up-regulated by H2O2 treatment (Molins et al., 2013), and ABCC3, ABCC4, and ABCC6 expression was also enhanced in wheat plants exposed to H2O2 (Bhati et al., 2015). The next step is to elucidate how NO or ROS regulates gene expression of Cd transporters.

In summary, we propose a model (Figure 9) showing that the over-expression of NtHb1 reduces NO and ROS levels, by which Cd efflux is enhanced and Cd influx is decreased.

CONCLUSION

The expression of NtHb1 in Arabidopsis reduces Cd levels and increases Cd tolerance by inhibiting expression of IRT1 and ACA10 and enhancing expression of PDR8 and CAXs via decreased NO and ROS (H2O2) levels. In addition, AtHb1 expression is greatly enhanced in the roots and slightly enhanced in the shoots in response to Cd.
AUTHOR CONTRIBUTIONS

SH acquired a research fund, designed and supervised whole research, and wrote the article with contributions of all authors. RB performed most experiments. DK and JN performed the minor parts of experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00201/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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