frontiers in Plant Science

ORIGINAL RESEARCH
published: 27 March 2019
doi: 10.3389/fpls.2019.00384

NRT1.1 Regulates Nitrate Allocation and Cadmium Tolerance in Arabidopsis

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Abiotic stress induces nitrate (NO_3^-) allocation to roots, which increases stress tolerance in plants. NRT1.1 is broadly involved in abiotic stress tolerance in plants, but the relationship between NRT1.1 and NO_3^- allocation under stress conditions is unclear. In this study, we found that Arabidopsis wild-type Col-0 was more cadmium (Cd^{2+})-tolerant than the *nrt1.1* mutant at 20 μM $CdCl_2$. Cd^{2+} exposure repressed *NRT1.5* but upregulated *NRT1.8* in roots of Col-0 plants, resulting in increased NO_3^- allocation to roots and higher [NO_3^-] root-to-shoot (R:S) ratios. Interestingly, *NITRATE REGULATORY GENE2* (*NRG2*) was upregulated by Cd^{2+} stress in Col-0 but not in *nrt1.1*. Under Cd^{2+} stress, *nrg2* and *nrg2-3chl1-13* mutants exhibited similar phenotypes and NO_3^- allocation patterns as observed in the *nrt1.1* mutant, but overexpression of *NRG2* in Col-0 and *nrt1.1* increased the [NO_3^-] R:S ratio and restored Cd^{2+} stress tolerance. Our results indicated that *NRT1.1* and *NRG2* regulated Cd^{2+} stress-induced NO_3^- allocation to roots and that *NRG2* functioned downstream of *NRT1.1*. Cd^{2+} uptake did not differ between Col-0 and *nrt1.1*, but Cd^{2+} allocation to roots was higher in Col-0 than in *nrt1.1*. Stressed Col-0 plants increased Cd^{2+} and NO_3^- allocation to root vacuoles, which reduced their cytosolic allocation and transport to the shoots. Our results suggest that NRT1.1 regulates NO_3^- allocation to roots by coordinating Cd^{2+} accumulation in root vacuoles, which facilitates Cd^{2+} detoxification.

Keywords: ***CHL1/NRT1.1/NPF6.3*, *NITRATE REGULATORY GENE 2*, nitrate allocation, cadmium stress, vacuole, Arabidopsis**

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *22 December 2018*
Accepted: *13 March 2019*
Published: *27 March 2019*

Citation:
Jian S, Luo J, Liao Q, Liu Q, Guan C and Zhang Z (2019) NRT1.1 Regulates Nitrate Allocation and Cadmium Tolerance in Arabidopsis. Front. Plant Sci. 10:384. doi: 10.3389/fpls.2019.00384

INTRODUCTION

Heavy metal pollution in soil is an important environmental issue worldwide, which gives rise to agricultural and public health concerns (Bertin and Averbeck, 2006; Mohammed et al., 2011; Åkesson et al., 2014). In China, for example, approximately 7% of the soil is cadmium (Cd) contaminated, 0.5% of which is severely polluted (Zhang et al., 2015). Cd can be released to the

Abbreviations: J_{max}, maximum electron transport rate; MDA, malondialdehyde; NR, nitrate reductase; *NRG2*, *NITRATE REGULATORY GENE2*; PPED, photosynthetic photon flux intensity; R:S, root-to-shoot; SINAR, stress-initiated nitrate allocation in roots; SOD, superoxide dismutase; TPU, triose phosphate utilization; $V_{c,max}$, maximum carboxylation rate of Rubisco.

soil by excessive use of chemical fertilizers and pesticides, utilization of industrial wastewater and sludge, and atmospheric deposition (Woodis et al., 1977; He and Singh, 1994; Wong et al., 2003; Ottosen et al., 2007; Roberts, 2014). Thus, people are being exposed to Cd-associated toxicity via the consumption of cereals and vegetables grown in Cd-contaminated soils (Chaney, 2015). Susceptibility to Cd^{2+} stress is species-specific in plants (Chen, 1996), which provides opportunities to select and breed Cd^{2+}-tolerant species/varieties. However, this requires a complete understanding of the underlying mechanisms of Cd^{2+} tolerance in plants.

Nitrate (NO_3^-) is one of the two forms of inorganic nitrogen nutrient taken up by terrestrial plants. It also acts as a signal molecule regulating a wide range of genes and biological processes involved in nitrogen utilization, general plant lateral root development, and response to environmental fluctuations (Gowri et al., 1992; Redinbaugh and Campbell, 1993; Gutiérrez et al., 2007; Hirel et al., 2007; Miller et al., 2007; Krouk et al., 2010; Wang et al., 2012; Ruffel et al., 2014; Vidal et al., 2014; Bouguyon et al., 2016). Under normal conditions, most of the absorbed NO_3^- is transported to the shoots for reduction by NR or as a temporary nitrogen pool stored in vacuoles, which is driven by the H^+ transport energized by the tonoplast H^+-ATPase and H^+-PPase (Martinoia et al., 1981, 2007; Han et al., 2016). NO_3^- allocation between the roots and the shoots in plants is important for nitrogen utilization and adaptation to abiotic stresses (Fan et al., 2007; Li et al., 2010; Han et al., 2016). The long-distance transport of NO_3^- is mediated by two nitrate transporters, NRT1.5 and NRT1.8. The former is found in pericycle cells where it is responsible for loading the NO_3^- to the xylem, whereas the latter is found in xylem parenchyma cells, where it contributes to NO_3^- unloading from xylem (Lin et al., 2008; Li et al., 2010; Chen et al., 2012). Under adverse environmental conditions, *NRT1.5* expression is downregulated and *NRT1.8* expression is upregulated in roots. As a result, more NO_3^- allocates to roots, which subsequently increases plant stress tolerance (Li et al., 2010; Chen et al., 2012). This phenomenon, known as SINAR, has been widely observed under several abiotic stresses, including the Cd^{2+} stress (Chen et al., 2012; Zhang et al., 2014). Ethylene and jasmonic acid are involved in the regulation of *NRT1.5* and *NRT1.8* under stress (Zhang et al., 2014).

NRT1.1, which was first cloned in 1993 (Tsay et al., 1993), is essential for NO_3^- uptake and signaling (Ho et al., 2009). In addition to its function as NO_3^- transporter, NRT1.1 also plays important roles in vegetative and reproductive growth (Guo et al., 2001), stomatal opening (Guo et al., 2003), root architecture (Remans et al., 2006; Mounier et al., 2014), and transport of chloride and phytohormones (IAA/ABA/jasmonic acid/GAs) (Tsay et al., 1993; Guo et al., 2002; Krouk, 2016; Corratgé-Faillie and Lacombe, 2017). Moreover, it induces tolerance in plants to abiotic stresses, such as proton stress, salt stress, Cd^{2+} stress, and iron deficiency (Mao et al., 2014; Liu et al., 2015; Abouelsaad et al., 2016; Fang et al., 2016). NRT1.1-mediated plant stress tolerance is closely associated with NO_3^- uptake, assimilation, and accumulation. It has been reported that *NRT1.1* mediates the expression of NO_3^- regulatory genes such as *NRG2* (Ho et al., 2009; Hu et al., 2009; Xu et al., 2016) and NO_3^- assimilation genes such as *NIAs* and *NiR*, as well as some other *NRTs* (Ho et al., 2009; Undurraga et al., 2017). However, the relationship between *NRT1.1* and SINAR, including the control mechanisms, is not well understood.

In this study, we found that *NRT1.1* regulated the expression of *NRT1.5* and *NRT1.8* under Cd^{2+} stress, which increased NO_3^- allocation to roots as a mechanism to resist Cd^{2+} stress. Furthermore, we demonstrated that *NRG2* functioned downstream of *NRT1.1* in regulating NO_3^- allocation. NO_3^- was required to facilitate Cd^{2+} allocation to the roots, where it was mainly stored in the vacuoles for detoxification. Our results provide insights into the effects of the nitrate regulatory gene network on the regulation of plant stress tolerance.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana wild-type (Col-0), *nrt1.1*, *nrg2* single and double mutants (*chl1-1*, *chl1-5*, *chl1-13*, *nrg2-1*, *nrt2-2*, *nrg2-3chl1-13*), and *NRG2* overexpression lines (*35S::NRG2*/*Col-0*, *35S::NRG2*/*chl1-5*) were used in this study. Seeds were sown in nutrition soil and placed in a growth chamber (300 μmol photons·m^{-2}·s^{-1}, 16-h photoperiod, 22°C) to germinate and grow. Ten days after sowing, seedlings with two true leaves were transplanted to 600-ml pots and cultivated hydroponically in nutrient medium. The growth medium contained 1.25 mM KNO_3, 0.625 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.5 mM $Ca(NO_3)_2$, 1.25 μM Fe-EDTA, 17.5 μM H_3BO_3, 3.5 μM $MnCl_2$, 0.25 μM $ZnSO_4$, 0.05 μM $NaMoO_4$, and 0.125 μM $CuSO_4$. The MES buffer (2.5 mM) was used to maintain the pH of the growth medium at pH 5.8. The growth medium was refreshed every 4 days, and the position of the pots was interchanged when refreshing the solution to eliminate any edge effects. Four weeks after sowing, plants were exposed to Cd stress by adding 20 μM $CdCl_2$ to the growth medium for 3 days. Control plants were grown without $CdCl_2$.

^{15}N Tracer Assay

Plants of Col-0, *chl1-1*, *chl1-5* were grown in the normal nutrient medium for 4 weeks, followed by a 12-h treatment with 200 μM $CdCl_2$. Then, roots were washed with 0.1 mM $CaSO_4$ for 1 min and labeled with 20% atom abundance of $Ca(^{15}NO_3)_2$ (pH = 5.8) for 40 min. The roots were washed with 0.1 mM $CaSO_4$ and deionized water. Shoots and roots were sampled separately and oven-dried at 70°C for 48 h. Then the samples were pulverized using a TissuLyser (Tissuelyer-48, Jingxin Co. Ltd., China), and ^{15}N abundance in samples was measured using a continuous-flow isotope ratio mass spectrometer coupled with a carbon-nitrogen elemental analyzer (ANCA-MS; PDZ Europa).

Determination of Photosynthetic Parameters

Photosynthesis of fully expanded rosette leaves was measured between 09:00 and 15:00 with a LI-6400 portable photosynthesis

system (Li-Cor Inc., Lincoln, NE, United States). The air temperature in the cuvette was 22°C, the PPED was 200 μmol m^{-2} s^{-1}, the CO_2 concentration (C_a) was 500 μmol mol^{-1} (controlled with a CO_2 mixer), and the VPD was between 1.0 and 1.5 kPa. Before measurement, leaves were placed in the cuvette to adjust for 10 min. Data were recorded after equilibration to a steady state.

Photosynthetic CO_2-response curves (A-C_i curves) were measured with a PPFD of 200 μmol m^{-2} s^{-1} at nine points of C_a (800, 600, 500, 400, 300, 200, 150, 100, 50 μmol mol^{-1}). Prior to the measurement, leaves were placed in the cuvette to equilibrate for 30 min under a PPFD of 200 μmol m^{-2} s^{-1} and C_a of 400 μmol mol^{-1}. Temperature and VPD in the cuvette during measurement were maintained as described above. Data were recorded after equilibration to a steady state. $V_{c,max}$, J_{max}, and TPU were calculated according to Sharkey et al. (2007). The parameters K_c, K_o, R_d, and Γ^*, which were used to calculate $V_{c,max}$, J_{max}, and TPU, were estimated as 27.24 Pa, 16.58 Pa, 1 μmol·m^{-2}·s^{-1}, and 3.74 Pa, respectively.

Determination of Proline, Malondialdehyde (MDA) Concentration, and Superoxide Dismutase (SOD) Activity

Plants were grown in the normal nutrient medium for 4 weeks and then treated with 20 μM $CdCl_2$ for 3 days. Fresh leaves were collected to measure proline and MDA concentrations and SOD activity. Proline concentration was measured using the ninhydrin colorimetry method (Bates et al., 1973; Sharma and Dubey, 2005). Briefly, fresh samples (0.5 g) were homogenized in 5 ml of 3% aqueous sulfosalicylic acid using a mortar and a pestle. Homogenates were centrifuged at 10,000 × g for 10 min at 4°C, and the supernatants were collected and used for proline analysis. In a test tube, 2 ml of supernatant was added to 2 ml of acidic ninhydrin and 2 ml of glacial acetic acid; then, the mixture was placed in a boiling water bath for 15 min. The processed mixture was extracted with 4 ml toluene by thoroughly vigorous stirring. After keeping the tube at room temperature for 30 min, the absorption of the toluene solution was measured spectrophotometrically at 520 nm. A standard curve was created using L-proline.

Malondialdehyde concentration was measured using the thiobarbituric acid method (Kramer et al., 1991). Fresh samples (0.5 g) were homogenized in 5 ml 5% (w/v) trichloroacetic acid (TCA) with a mortar and a pestle and centrifuged at 10,000 × g for 10 min at 4°C. Then, 2 ml supernatant were combined with 2.5 ml TBA reagent [0.6% (w/v) TBA in 10% (w/v) TCA], heated at 100°C for 10 min, cooled, and centrifuged at 4000 × g for 10 min. The concentration of MDA was calculated from the absorbance at 600, 532, and 450 nm.

Superoxide dismutase activity was determined according to the method described by Giannopolitis and Ries (1977). The shoot tissues were thoroughly ground with a mortar and a pestle in liquid nitrogen. Then, the samples were homogenized in 0.1 M phosphate buffer containing 0.1 mM EDTA (pH 7.8) and centrifuged at 13,000 × g and 4°C for 10 min. The supernatants were used for determining the SOD activity. The SOD reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitro blue tetrazolium (NBT), 10 μM EDTA-Na_2, 2.0 μM riboflavin, and modest volume of extract. Ultrapure water was added to a final volume of 3 ml. The mixtures in the glass test tubes were illuminated for 20 min and the absorbance at 560 nm was measured spectrophotometrically. Identical mixtures that were not illuminated served as blank controls (background).

Determination of Chlorophyll Concentration

Chlorophyll concentration in rosette leaves was determined by extraction with 80% acetone for 24 h at room temperature in the dark (Wellburn and Lichtenthaler, 1984). The absorbance of the extract was measured at 663 and 645 nm to calculate chlorophyll *a*, *b*, and total chlorophyll concentrations.

Measurements of Biomass, Nitrate, and Cd^{2+} Concentration

Four-week-old plants treated with $CdCl_2$ for 3 days were sampled and separated into shoots and roots. The samples were oven-dried at 70°C until the weight remained constant (dry weight).

NO_3^- was extracted from the samples using deionized water in a boiling water bath for 15 min and determined spectrophotometrically at 410 nm by nitration of salicylic acid (Cataldo et al., 1975).

Four-week-old plants exposed to $CdCl_2$ for 3 days were harvested and washed with 0.1 mM $CaCl_2$ for 1 min, followed by rinsing with deionized water for four times. Shoots and roots were separately collected and oven-dried at 70°C until the weight remained constant (dry weight). Samples were digested thoroughly with HNO_3 at 180°C and the Cd^{2+} concentration was determined with an ICP Mass Spectrometer (NexION 350X, PerkinElmer).

Determination of V-ATPase and V-PPase Activities

Root tissues (0.5 g) of four-week-old plants were used for V-ATPase and V-PPase activities determination according to Krebs et al. (2010) with some modifications. V-ATPase and V-PPase activities in 100 μl microsomal membranes were determined calorimetrically by measuring the release of inorganic phosphate (Pi) after an incubation of 30 min at 37°C. The V-ATPase assay medium contained 25 mM Tris-Hepes (pH 7.6), 3 mM $MgSO_4$·7 H_2O, 50 mM KCl, 0.5 mM NaN_3, 0.1 mM $NaVO_4$·12 H_2O, and 3 mM ATP-Tris. V-PPase activity was assayed in a reaction medium containing 25 mM Tris-Hepes (pH 7.6), 3 mM $MgSO_4$·7 H_2O, 50 mM KCl, 0.5 mM NaN_3, 0.1 mM $NaVO_4$·12 H_2O, and 3 mM $Na_4P_2O_7$.

Isolation of Intact Protoplasts and Vacuoles for Determination of Cd^{2+} and NO_3^- Levels

Root tissues (0.3 g) of four-week-old plants were used to isolate intact protoplasts and vacuoles according to Robert et al. (2007).

The purified protoplasts were divided into two fractions, one of which was used for the releases of vacuoles according to the method described in Dürr et al. (1975). The purified protoplasts and vacuoles were used for the determination of NO_3^- and Cd^{2+} concentrations (Vögeli-Lange and Wagner, 1990; Han et al., 2016). NO_3^- concentrations in protoplasts and vacuoles were measured by a continuous-flow auto-analyzer (Auto Analyzer 3, Bran and Luebbe) as described previously by Han et al. (2016). Cd^{2+} concentrations in the protoplasts and vacuole were determined with an ICP Mass Spectrometer (NexION 350X, PerkinElmer) as described in Huang et al. (2012).

RNA Extraction and Transcript Analysis

Four-week-old plants were treated with 200 μM $CdCl_2$ for 6 h and the roots were harvested for total RNA analysis. Total RNA was extracted with TRIzol (Invitrogen, United States), precipitated with an equal volume of isopropanol, washed with 75% ethanol, and dissolved with RNase-free water. The cDNA templates were synthesized using the PrimeScript™ RT Kit with gDNA Eraser (Perfect Real Time) (TAKARA, Japan) following the manufacturer's protocol. The relative expression of genes in roots was determined by quantitative RT-PCR performed in an Applied Biosystems StepOne™ Real-Time PCR System using SYBR Premix Ex-Taq (TAKARA) according to the manufacturer's protocol. Primers used in the assays are listed in **Supplementary Table S1**. The expression data were normalized to *Actin2* or *sand*.

Statistical Analysis

A completely randomized design was applied in the experiments by having four biological replicates in each treatment. The comparisons of the means between Cd^{2+} stress treatments and controls were performed using the two-tailed Student's *t*-test. The effects of Cd^{2+} stress treatment, along with the genotypes and their interactions, were evaluated using two-way analysis of variance (ANOVA). Multiple comparisons were performed using the least significant difference (*LSD*) multiple range test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Nrt1.1 Improves Cd^{2+} Stress Tolerance in Plants

Cd^{2+} stress degraded chlorophyll *a*, *b*, and total chlorophyll in *nrt1.1* mutants (*chl1-1* and *chl1-5*) by 16.35–24.87, 4.26–12.10, and 11.07–20.75%, respectively, as compared with those in the controls, resulting in more severe chlorosis in *nrt1.1* than in Col-0 (**Figures 1A,B** and **Supplementary Figures S1A–C**). Cd^{2+} stress significantly reduced shoot biomass of *nrt1.1*, but had no effect on Col-0; Cd^{2+} exposure did not affect the root biomass in any genotype (**Figure 1C**). P_n, $V_{c,max}$, J_{max}, and TPU in *nrt1.1* were strongly decreased under Cd^{2+} stress, with significantly lower values than those in Col-0. Photosynthesis in Col-0 was

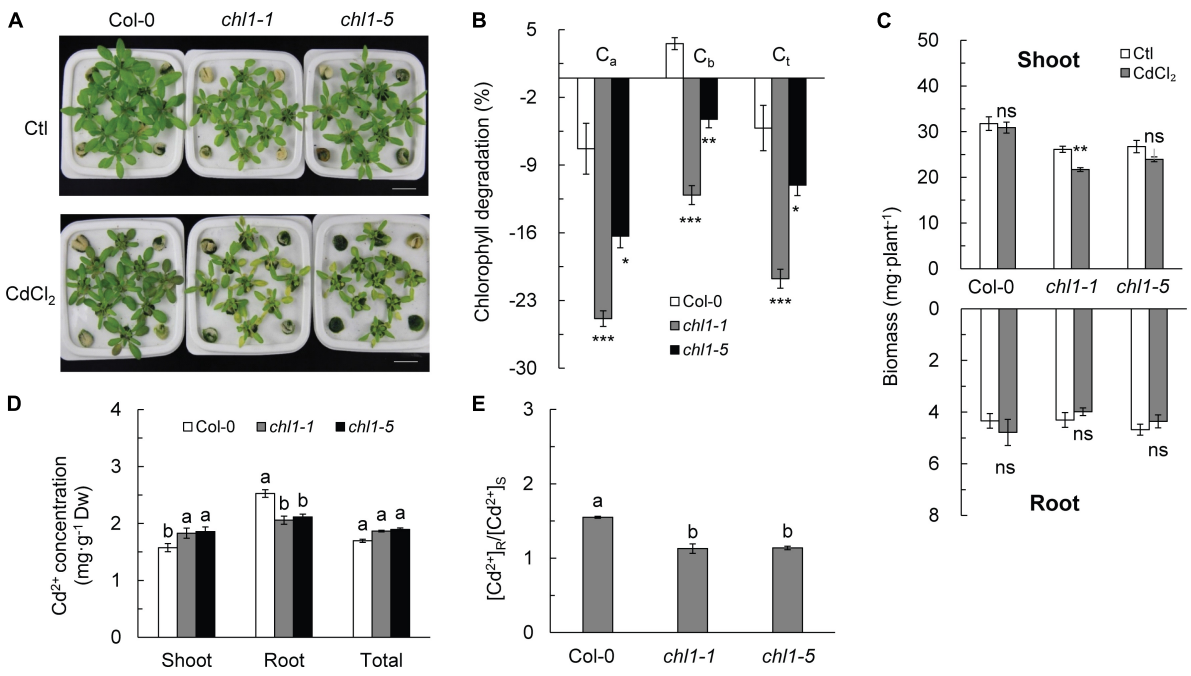

FIGURE 1 | Effects of Cd^{2+} stress on growth and Cd^{2+} distribution in Col-0 and *nrt1.1* plants. **(A)** Images of four-week-old Col-0 and the *nrt1.1* plants, which were maintained in the absence (Ctl, control) or presence of 20 μM $CdCl_2$ for 3 days. **(B)** Chlorophyll degradation rate (%) in $CdCl_2$ treated plants relative to untreated control plants. **(C)** Shoot and root biomasses in Col-0 and *nrt1.1*. **(D)** Cd^{2+} concentration in Col-0 and *nrt1.1* plants maintained without or with 20 μM $CdCl_2$ for 3 days. **(E)** $[Cd^{2+}]$ R:S ratio in Col-0 and *nrt1.1* plants. Data represent means ± SE ($n = 4$). Columns with the same letter indicate no significant difference at $P < 0.05$ using the *LSD* method. Scale bars = 1 cm. Bars with one (*), two (**), and three (***) asterisks indicate significant differences from the control at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, using the two-tailed Student's *t*-test.

not affected by Cd^{2+} stress (**Supplementary Figures S2A–D**). The *chl1-9* mutant is defective in nitrate uptake but shows a normal primary nitrate response (Ho et al., 2009). In the presence of Cd^{2+}, *chl1-9* had chlorosis similar to *chl1-1* and *chl1-5* (**Supplementary Figure S3**), indicating that the nitrate signaling function of *NRT1.1* is independent of the underlying mechanism of Cd^{2+} stress tolerance in Arabidopsis.

Cd^{2+} stress strongly increased the MDA concentration in *nrt1.1*, especially in the roots, where it increased by 310.4% in *chl1-1* and 481.4% in *chl1-5* as compared to that in the corresponding controls. However, the MDA concentration in Col-0 was not affected in the shoots and only increased by 41.8% in the roots (**Supplementary Figure S4A**). Cd^{2+} stress significantly increased the proline concentration in all genotypes, with increases of 84.5 and 59.8%, 54.7 and 55.1%, and 35.4 and 43.3% observed in the shoots and roots of Col-0, *chl1-1*, and *chl1-5*, respectively, as compared to the proline concentration in the corresponding controls (**Supplementary Figure S4B**). Cd^{2+} stress significantly increased the SOD activity in the shoots of Col-0 but reduced it in the *nrt1.1* mutant, which had a significantly lower SOD activity than Col-0 (**Supplementary Figure S4C**).

We compared the concentration and distribution of Cd^{2+} in plants exposed to Cd^{2+} stress and found that the Cd^{2+} concentration was significantly lower in the shoots of Col-0 plants than in those of the *nrt1.1* plants. In contrast, the Cd^{2+} concentration in the roots of Col-0 plants was markedly higher than that of the *nrt1.1* plants (**Figure 1D**). However, the whole-plant Cd^{2+} concentration did not vary significantly between Col-0 and *nrt1.1* (**Figure 1D**). Therefore, the [Cd^{2+}] R:S ratio was significantly higher in Col-0 than in *nrt1.1* (**Figure 1E**). These results indicated that Col-0 plants allocated more Cd^{2+} to the roots during Cd^{2+} stress, whereas *nrt1.1* plants distributed more Cd^{2+}

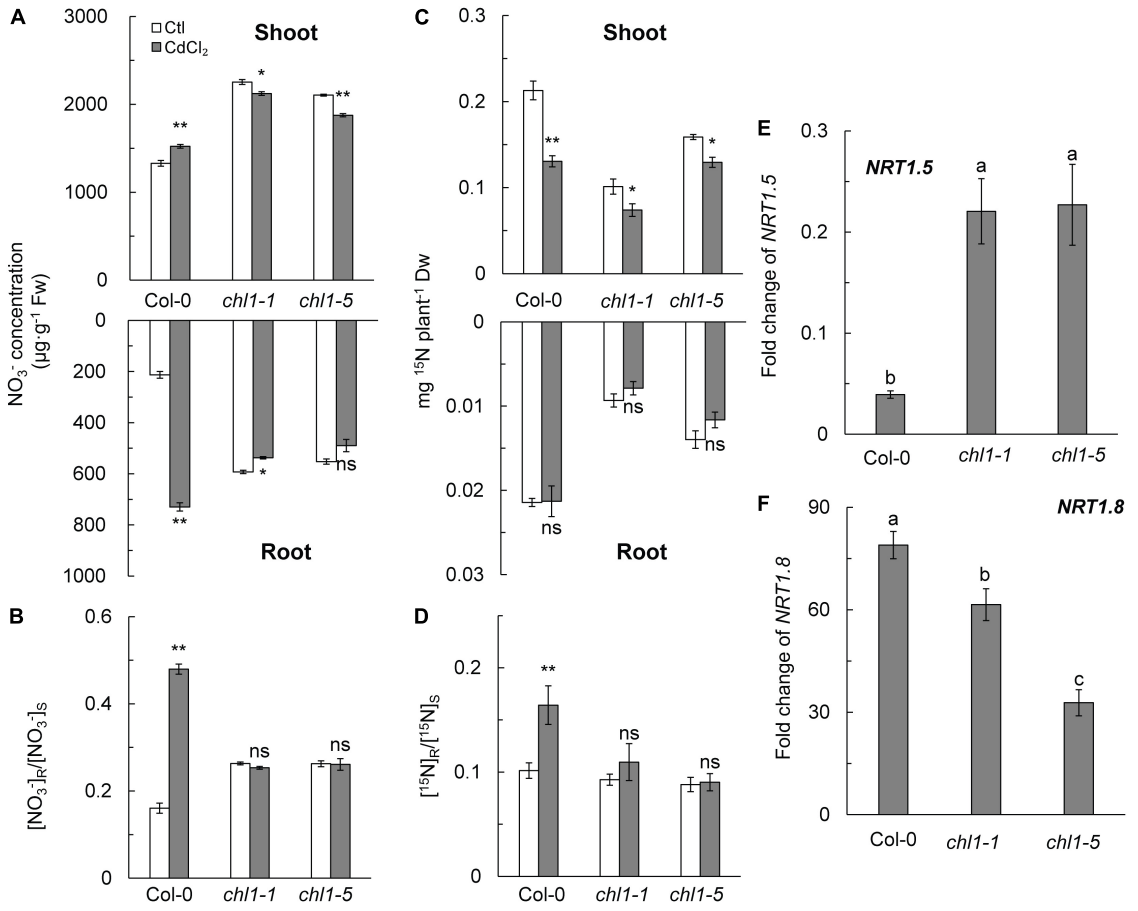

FIGURE 2 | Effects of Cd^{2+} stress on nitrate allocation and the fold change of *NRT1.5* and *NRT1.8*. **(A)** NO_3^- concentration in the shoots and roots of Col-0 and *nrt1.1*. **(B)** [NO_3^-] R:S ratio. **(C)** ^{15}N levels in the shoots and roots. Four-week-old plants maintained without or with 200 μM $CdCl_2$ for 12 h were labeled with 20% atom abundance of $Ca(^{15}NO_3)_2$ for 40 min. **(D)** [^{15}N] R:S ratio. **(E)** Fold change of *NRT1.5* expression calculated by dividing the expression of *NRT1.5* under $CdCl_2$ treatment with the expression of *NRT1.5* in the control. **(F)** Fold change of *NRT1.8* expression calculated by dividing the expression of *NRT1.8* under $CdCl_2$ treatment with the expression of *NRT1.5* in untreated controls. For the gene expression assay, plants were maintained without or with 200 μM $CdCl_2$ for 6 h; roots were harvested for mRNA isolation. Data represent means ± SE (n = 4). Columns with the same letter indicate no significant difference at $P < 0.05$ using the LSD method. Scale bars = 1 cm. Bars with one (*) and two (**) asterisks indicate significant differences from the control at $P < 0.05$, and $P < 0.01$, respectively, using the two-tailed Student's t-test.

to shoots, which was consistent with the observed Cd^{2+} toxicity in the shoots.

Since the iron (Fe) status of plants is critical for Cd^{2+} uptake and tolerance (He et al., 2017), we measured the Fe concentration but did not detect any genotype-dependent differences, neither under control nor Cd^{2+} stress conditions (**Supplementary Figure S5**). This observation suggested that the chlorosis in *nrt1.1* leaves under Cd^{2+} stress did not affect the Fe status.

NRT1.1 Mediates Nitrate Allocation in Roots Under Cd^{2+} Stress

NRT1.1 is a nitrate transporter. To determine whether the phenotype of Cd^{2+} stress-induced chlorosis is nitrate-dependent, we used ammonium succinate as the sole nitrogen source during Cd^{2+} treatment and found that the phenotype of Cd^{2+} toxicity disappeared (**Supplementary Figure S6**). The results suggested that NRT1.1-mediated Cd^{2+} tolerance is nitrate-dependent.

A previous study reported that nitrate allocation in plants is correlated with Cd^{2+} stress tolerance (Li et al., 2010). Thus, we measured the NO_3^- concentration and allocation in roots and shoots. The results showed that Cd^{2+} stress significantly reduced the NO_3^- concentration in roots and shoots of *nrt1.1*, whereas the NO_3^- concentration in roots and shoots of Col-0 was significantly increased by 2.43-fold and 0.13-fold as compared with those in control plants, respectively (**Figure 2A**). As a result, the $[NO_3^-]$ R:S ratio in Col-0 was significantly increased by Cd^{2+} treatment, whereas this ratio remained constant in *nrt1.1* at approximately 0.26 (**Figure 2B**). The short-term ^{15}N trace experiment further confirmed that allocation of absorbed nitrate to the roots was higher in Col-0 (14.00%) than in *chl1-1* (9.66%) or *chl1-5* (8.25%) under Cd^{2+} stress (**Figures 2C,D**).

The expression of nitrate long-distance transport genes *NRT1.5* and *NRT1.8* were also assayed in this study. As shown in **Figure 2E** and **Supplementary Figure S7A**, *NRT1.5* expression was strongly inhibited in Col-0 roots during Cd^{2+} exposure, whereas its expression was only slightly downregulated in *nrt1.1* mutants exposed to the same stress. In contrast, Cd^{2+} stress strongly upregulated the expression of *NRT1.8* in roots of all genotypes, with the highest expression levels measured in Col-0 (**Figure 2F** and **Supplementary Figure S7B**). The expression of *NRT1.5* and *NRT1.8* resulted in a higher $[NO_3^-]$ R:S ratio under Cd^{2+} stress.

NRG2 Participates in *NRT1.1*-Mediated Nitrate Allocation

Surprisingly, *NRG2* expression in Col-0 roots was also significantly upregulated by Cd^{2+} stress, whereas the expression of *NRG2* in *chl1-1* and *chl1-5* was not significantly affected

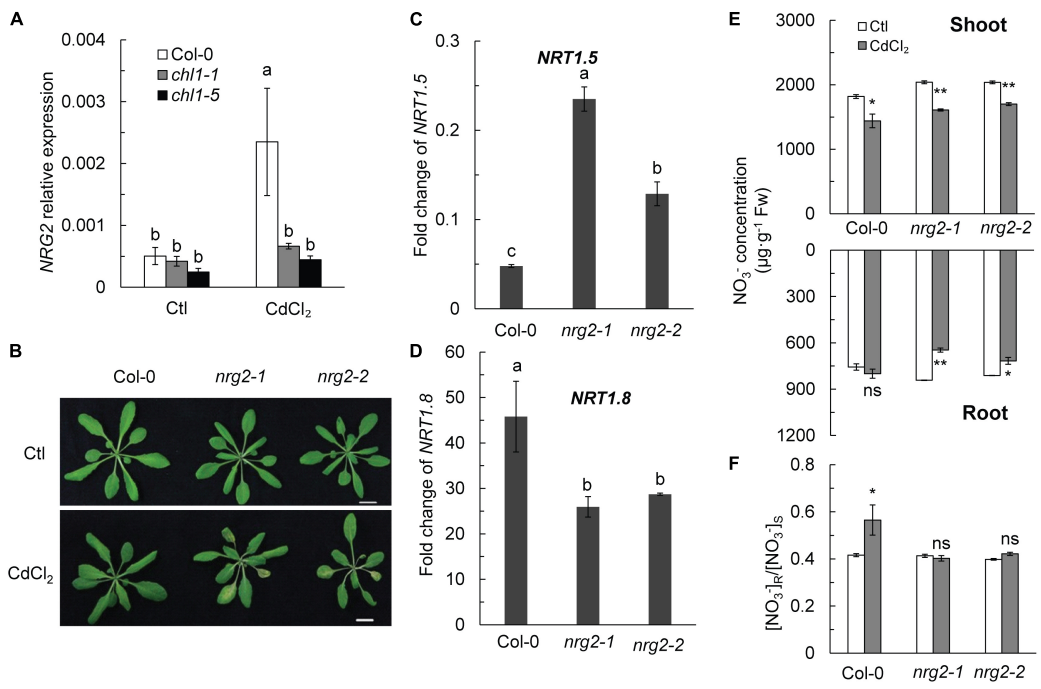

FIGURE 3 | Phenotypes, NO_3^- concentration, $[NO_3^-]$ R:S ratio, and gene expression change in Col-0 and *nrg2*. **(A)** *NRG2* relative expression in Col-0 and *nrt1.1* in the absence or presence of Cd^{2+}. **(B)** Images of four-week-old Col-0 and *nrg2* plants grown without or with 20 μM $CdCl_2$ for 3 days. **(C)** Fold change of *NRT1.5* expression calculated as the expression of *NRT1.5* under $CdCl_2$ treatment divided by the expression of *NRT1.5* in the untreated control. **(D)** Fold change of *NRT1.8* expression calculated as the expression of *NRT1.8* under $CdCl_2$ treatment divided by the expression of *NRT1.5* in the untreated control. For the gene expression assay, plants were maintained without or with 200 μM $CdCl_2$ for 6 h, roots were harvested for mRNA determination. **(E)** NO_3^- concentration in shoot and roots of Col-0 and *nrg2* mutants. **(F)** $[NO_3^-]$ R:S ratio. Data represent means ± SE (n = 4). Columns with the same letter indicate no significant difference at $P < 0.05$ using the LSD method. Scale bars = 1 cm. Bars with one (*) and two (**) asterisks indicate significant differences from the control at $P < 0.05$, and $P < 0.01$ respectively, using the two-tailed Student's t-test.

(**Figure 3A**). Therefore, we hypothesized that *NRG2* is involved in *NRT1.1*-mediated Cd^{2+} tolerance in Arabidopsis. Interestingly, we observed the Cd^{2+} toxicity phenotype in two *NRG2* single mutants, *nrg2-1* and *nrg2-2*, after a 3-day treatment with 20 μM $CdCl_2$ (**Figure 3B**).

The expression profile of *NRT1.5* and *NRT1.8* in *nrg2* mutants was similar to that in *chl1-1* and *chl1-5*. *NRT1.5* was more significantly downregulated in Col-0 than in *nrg2*, and *NRT1.8* was also more markedly upregulated in Col-0 than in the mutant (**Figures 3C,D** and **Supplementary Figures S7C,D**). Accordingly, Col-0 allocated more NO_3^- to the roots and had an increased [NO_3^-] R:S ratio under Cd^{2+} stress (**Figures 3E,F**). In contrast, the NO_3^- concentration was lower in *ngr2* shoots and roots than in control plant shoots and roots; thus, the *ngr2* mutant maintained a constant [NO_3^-] R:S ratio during the Cd^{2+} exposure (**Figures 3E,F**). These results indicated that the Cd^{2+} stress response in plants with depleted *NRG2* gene was similar to that in the *nrt1.1* mutant.

An *nrg2-3chl1-13* double mutant line was also used in this study. By exposing the double mutant plants to 20 μM Cd^{2+} for 3 days, we observed a Cd^{2+} toxicity phenotype that was similar to that of the *chl1-13* single mutant (**Figure 4A**). In the presence of Cd^{2+}, the *nrg2-3chl1-13* mutant had an increased NO_3^- concentration in the shoots and a decreased NO_3^- concentration in the roots, which lowered the [NO_3^-] R:S ratio under Cd^{2+} stress (**Figures 4B,C**). The results indicated that *NRG2* and *NRT1.1* are involved in the same pathway regulating the expression of *NRT1.5* and *NRT1.8*, and, consequently, controlling NO_3^- allocation and Cd^{2+} stress tolerance.

To further elucidate the relationship between *NRG2* and *NRT1.1* in regulating NO_3^- allocation as a major response to Cd^{2+} stress, we constitutively overexpressed *NRG2* in Col-0 and *chl1-5* under the control of the 35S promoter. We found that Cd^{2+} stress did not induce the Cd^{2+} toxicity phenotype in *35S::NRG2*/Col-0 or *35S::NRG2*/*chl1-5*, both of which with an *NRG2* expression that was significantly higher than that in Col-0 (**Figures 5A,B**). Overexpression of *NRG2* in Col-0 and *chl1-5* reduced the NO_3^- concentration in shoots but increased (or maintained) the NO_3^- concentration in roots under Cd^{2+} stress (**Figure 5C**). Hence, the [NO_3^-] R:S ratio under Cd^{2+} stress in *35S::NRG2*/Col-0 and *35S::NRG2*/*chl1-5* was significantly increased as compared to that in Col-0 (**Figure 5D**). These data indicated that *NRG2* acted as a downstream element of *NRT1.1* to regulate NO_3^- allocation under Cd^{2+} stress.

Coordination of Cd^{2+} and NO_3^- Allocation in Root Tissues

Cd^{2+} stress triggered both Cd^{2+} and NO_3^- allocation in roots of Col-0 plants (**Figures 1D,E**, **2A,B**, **3E,F**). Previous studies showed that the cellular vacuoles function in nitrogen storage and heavy metal sequestration (Andreev, 2001; Sharma et al., 2016). In our study, we measured the distribution of Cd^{2+} and NO_3^- in the vacuole and protoplast, along with the

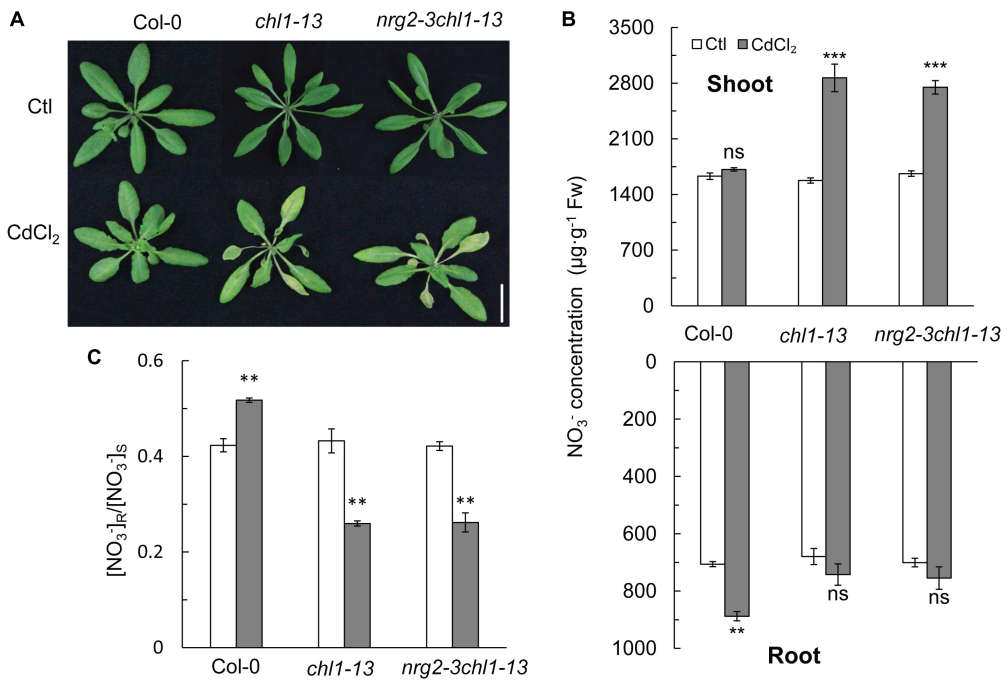

FIGURE 4 | Phenotypes, NO_3^- concentration, and [NO_3^-] R:S ratio in Col-0, *chl1-13*, and *nrg2chl1-13* mutants. **(A)** Images of phenotypes of Col-0, *chl1-13*, and *nrg2-3chl1-13* grown without or with 20 μM $CdCl_2$ for 3 days. **(B)** NO_3^-concentration in Col-0, *chl1-13*, and *nrg2chl1-13* in shoots and roots. **(C)** [NO_3^-] R:S ratio of Col-0, *chl1-13*, and *nrg2chl1-13*. Data represent means ± SE (n = 4). Scale bars = 1 cm. Bars with two (**) and three (***) asterisks indicate significant differences from the control at $P < 0.01$, and $P < 0.001$, respectively, using the two-tailed Student's t-test.

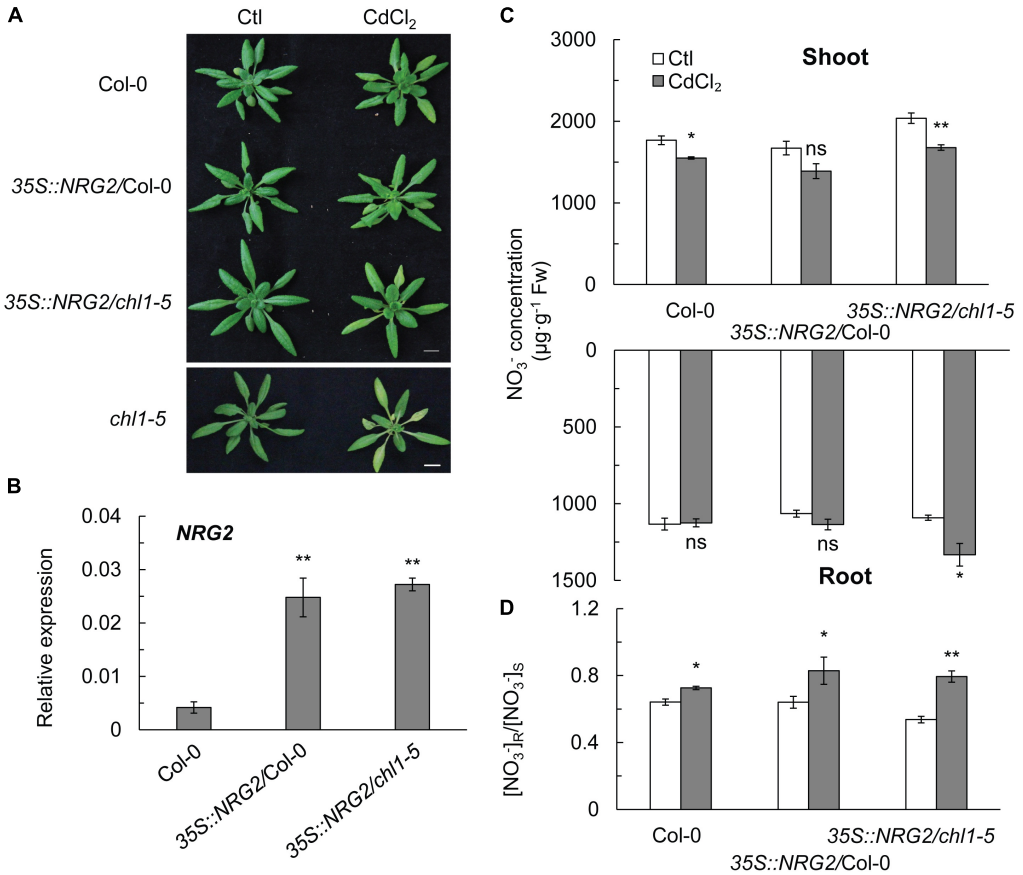

FIGURE 5 | Phenotypes, NO_3^- concentration, and [NO_3^-] R:S ratio in Col-0, *chl1-5*, and *NRG2* overexpression lines. Four-week-old plants were grown without or with 20 μM $CdCl_2$ for 3 days, and shoots and roots were harvested for nitrate determination. **(A)** Phenotypes of Col-0, *35S::NRG2/*Col-0, *35S::NRG2/chl1-5*, and *chl1-5* in maintained in the absence or presence of 20 μM $CdCl_2$. **(B)** *NRG2* relative expression in Col-0 and *NRG2* overexpression plants. **(C)** NO_3^- concentration of Col-0, *35S::NRG2/*Col-0, and *35S::NRG2/chl1-5* in shoots and roots. **(D)** [NO_3^-] R:S ratio in Col-0 and *NRG2* overexpression plants. Scale bars = 1 cm. Data represent means ± SE (n = 4). Bars with one (*) and two (**) asterisks indicate significant differences from the control at $P < 0.05$, and $P < 0.01$, respectively, using the two-tailed Student's t-test.

proton pump activity of the tonoplast, which promotes ion accumulation in the vacuole.

As shown in **Figure 6**, the activities of the V-ATPase and V-PPase in roots tissues of Col-0 were increased under Cd^{2+} stress, whereas the activities of those two enzymes were significantly decreased in *chl1-1* and *chl1-5* roots (**Figures 6A,B**). Cd^{2+} stress reduced the NO_3^- content in both protoplasts and vacuoles, with a greater reduction in the vacuole NO_3^- content in *chl1-1* and *chl1-5* mutant plants, resulting in a lower proportion of vacuole NO_3^- as compared to the total NO_3^- in *nrt1.1* protoplasts (**Figure 6C**). NO_3^- accumulation in the cytosol (P-V) was significantly increased in *nrt1.1* mutant plants and was markedly higher than that in Col-0 plants (**Figure 6D**).

Under Cd^{2+} stress, the proportion of vacuole Cd^{2+} relative to the total Cd^{2+} in root protoplasts was lower in *nrt1.1* than in Col-0 (**Figure 6E**), indicating that substantial amounts of Cd^{2+} accumulated in the cytosol of those *nrt1.1* root protoplasts (**Figure 6F**). Thus, we concluded that the increased allocation of NO_3^- and Cd^{2+} to the root vacuoles is an effective strategy for Col-0 to increase the tolerance to Cd^{2+} stress.

DISCUSSION

NRT1.1 Improves Cd^{2+} Stress Tolerance by Nitrate Allocation in Roots

We found that Col-0 plants were more tolerant to Cd^{2+} stress than *nrt1-1* and *nrt1-5* plants. Interestingly, Col-0 also allocated more nitrate to roots, which was not observed in the *nrt1.1* mutant. It is well known that nitrate is reallocated to the roots under abiotic stress, including heavy metal stress (Hernandez et al., 1997). Nitrate reallocation to plant roots has been characterized as a typical mechanism that increases the stress tolerance in plants (Li et al., 2010; Chen et al., 2012). Our results suggested that the difference in nitrate allocation between Col-0 and *nrt1.1* is linked to their difference in Cd^{2+} tolerance. In this study, the data indicated that the enhanced nitrate allocation to the roots is correlated with transcript level changes of *NRT1.5*

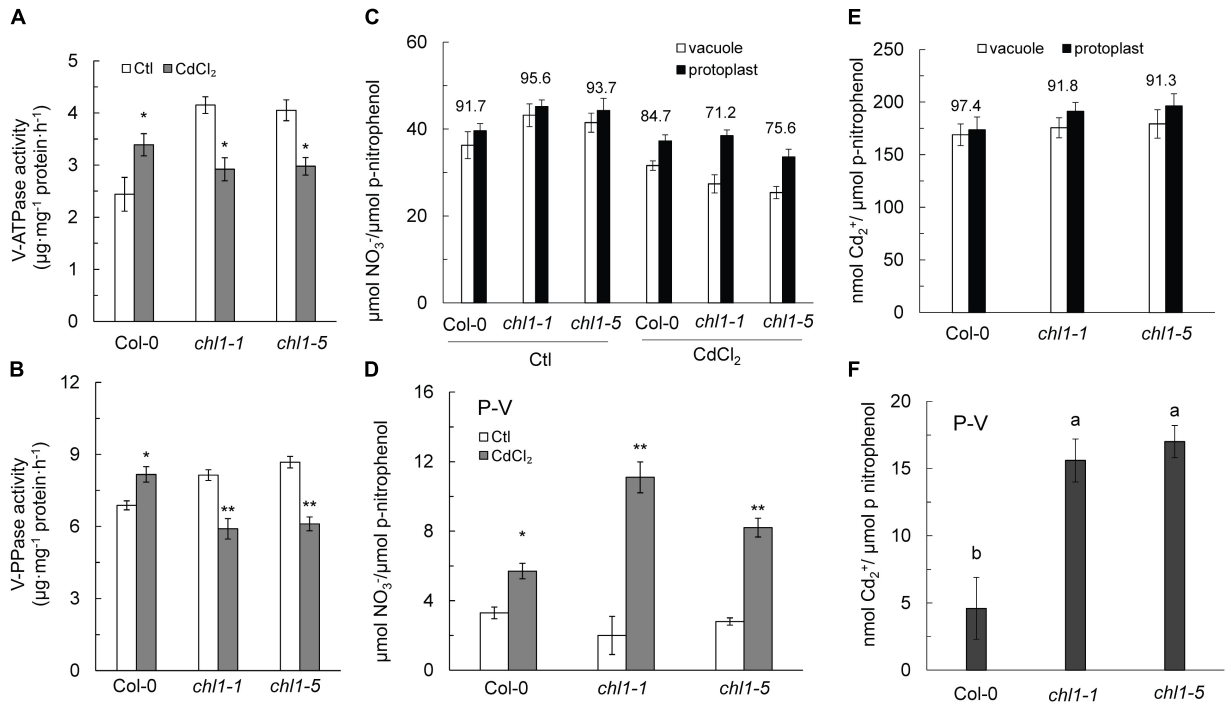

FIGURE 6 | Reduced VSC of NO_3^- and Cd^{2+} in the roots of *nrt1.1* mutant plants maintained without or with 20 μM $CdCl_2$. **(A)** V-ATPase and **(B)** V-PPase (tonoplast proton-pump) activities in root tissue of Col-0 and *nrt1.1* mutant. **(C)** NO_3^- distribution between the vacuole and protoplast in Col-0 and *nrt1.1* root tissue maintained without or with 20 μM $CdCl_2$. **(D)** Total NO_3^- in the cytosol under 20 μM $CdCl_2$ treatment, calculated by total NO_3^- in the protoplast minus total NO_3^- in vacuoles. **(E)** Cd^{2+} distribution between the vacuole and protoplast in Col-0 and *nrt1.1* root tissue maintained without or with 20 μM $CdCl_2$ treatment. **(F)** Total Cd^{2+} in the cytosol maintained without or with 20 μM $CdCl_2$ treatment, calculated as the difference between total Cd^{2+} in the protoplast and total Cd^{2+} in vacuoles. Data represent means ± SE (n = 4). Values above the bars represent the percentage of total vacuolar NO_3^- or Cd^{2+} in root tissue divided by total NO_3^- or Cd^{2+} in total protoplasts in root tissue. Bars with one (*) and two (**) asterisks indicate significant differences from the control at $P < 0.01$, and $P < 0.001$, respectively, using the two-tailed Student's t-test. Bars with the same letter indicate no significant difference at $P < 0.05$ using the *LSD* method.

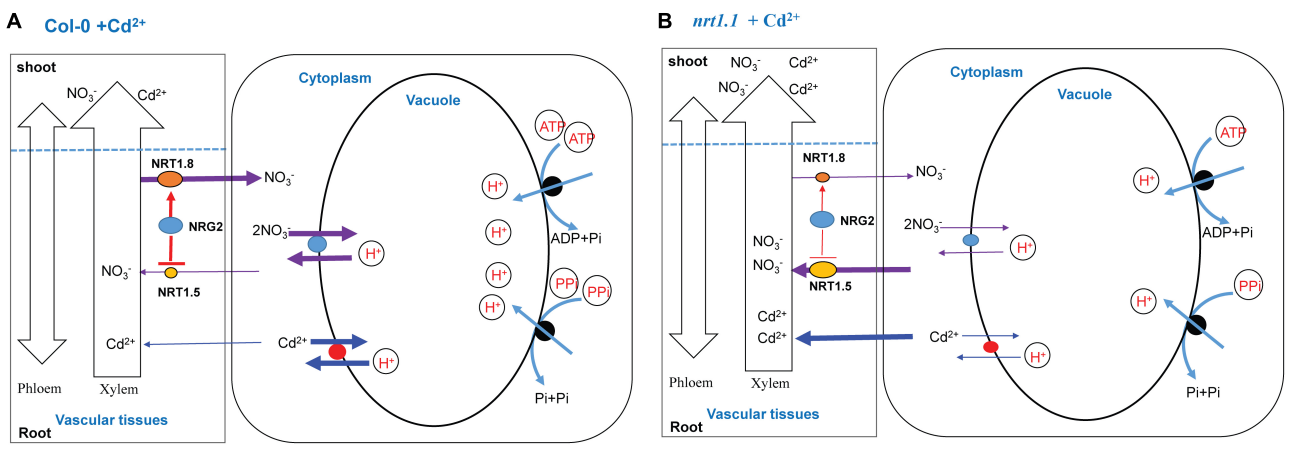

FIGURE 7 | A schematic model of the coordinated regulation by *NRT1.1* and *NRG2* in plants exposed to Cd^{2+} stress. The red lines indicate the route of the regulation pathway. The size of arrows represents the flow rate. **(A)** When Col-0 plants are exposed to Cd^{2+}, *NRT1.1* induces *NRG2* expression in roots and further downregulates *NRT1.5* expression and upregulates *NRT1.8* expression. As a result, more nitrate allocates to the roots. In addition, Cd^{2+} absorbed by the root system remains in the roots, and Cd^{2+} and NO_3^- are coordinatively allocated between vacuole and cytosol by enhancing tonoplast proton-pump activities (V-ATPase and V-PPase). Thus, the Cd^{2+} and NO_3^- contents are higher in the vacuole than in the cytoplasm, which facilitates Cd^{2+} detoxification and promotes growth. **(B)** In the *nrt1.1* mutant, *NRG2* expression is not changed by Cd^{2+} stress. The expression profiles of *NRT1.5* and *NRT1.8* are not affected as significantly as in Col-0 under Cd^{2+} stress. Hence, a greater proportion of nitrate absorbed by the root system is transported to the aboveground plant parts. Moreover, as compared with the control, the tonoplast proton-pump activities (V-ATPase and V-PPase) in *nrt1.1* mutants are lower under Cd^{2+} stress, resulting in enhanced Cd^{2+} and NO_3^- allocation to the cytosol and transport to aboveground plant parts. Ultimately, *nrt1.1* plants are damaged under Cd^{2+} stress.

and *NRT1.8*, which corroborated earlier reports by Li et al. (2010) and Chen et al. (2012) using *nrt1.5* and *nrt1.8* mutants. Our results, along with the earlier reports, indicated that nitrate plays an essential role in the tolerance to Cd^{2+} stress via the regulatory function of NRT1.1.

Although nitrate allocation to roots improves stress tolerance in plants, the relevant physiological and molecular mechanisms are not well characterized. Cd^{2+} induces oxidative stress in plants (Mendoza-Cózatl et al., 2005; Hayat et al., 2007; Cuypers et al., 2010). Our results revealed that nitrate accumulation in roots is correlated with increases in antioxidant capacity, as indicated by less membrane lipid peroxidation, higher proline level, and stronger SOD activity (**Supplementary Figures S2A–C**), suggesting that the anti-oxidative system protected the plants from Cd^{2+} damage. As predicted, Col-0 maintained a higher photosynthetic rate (**Supplementary Figure S1A**). Nitrate reduction is an energy-intensive process (Sunil et al., 2013). Assimilation of nitrate in the leaves directly involves reducing compounds such as NADH, NADPH, reduced ferredoxin, and ATP derived from photosynthesis (Gonzalez-Dugo et al., 2010). Storage of nitrate in the roots can reduce the energy competition between nitrate reduction and photosynthesis, thus, reducing the adverse effect of Cd^{2+} on photosynthesis.

NRG2 Works Together With NRT1.1 in the Regulation of Nitrate Allocation

NRG2 is an important nitrate regulatory gene that has been reported to regulate nitrate signaling in Arabidopsis in part by modulating *NRT1.1* expression (Xu et al., 2016). The *nrg2* mutant showed lower nitrate accumulation in the roots than the wild-type due to reduced expression of *NRT1.1* and upregulation of *NRT1.8* (Xu et al., 2016). However, the relationship between these genes in regulating nitrate allocation remained unknown. Our results showed distinct expression patterns of *NRT1.5* and *NRT1.8* in Col-0 and the *nrt1.1* mutant, indicating that *NRT1.1* regulates the expression of *NRT1.5* and *NRT1.8*. Interestingly, we observed a significant upregulation of *NRG2* in Col-0 plants when grown under Cd^{2+} stress (**Figure 3A**), indicating that *NRG2* is involved in the response of nitrate-dependent Cd^{2+} stress.

By testing the *nrg2* single mutant, we observed a Cd^{2+} stress phenotype, and the expression patterns of *NRT1.5* and *NRT1.8* were similar to that in the *nrt1.1* mutant (**Figures 3B–D**). The *nrg2-3chl1-13* double mutant also had chlorosis similar to the *nrt1.1* plants and a reduced [NO_3^-] R:S ratio under Cd^{2+} stress (**Figures 4A–C**). The results indicated that *NRG2* and *NRT1.1* are involved in the same regulatory pathway for nitrate allocation under Cd^{2+} stress. Xu et al. (2016) reported that *NRG2* functions upstream of *NRT1.1* in nitrate signaling. By overexpressing the *NRG2* gene in *chl1-5* plants, we found that both Cd^{2+} stress induced chlorosis and nitrate allocation to the roots were restored in *35S::NRG2/chl1-5* plants (**Figures 5A–D**). The results demonstrated that *NRG2* functions downstream of *NRT1.1* to regulate Cd^{2+} stress-induced nitrate allocation. Our results also suggested that *NRG2* can cooperate with *NRT1.1* in nitrate signaling, depending on the growth conditions.

Cd^{2+} Coordinates NO_3^- Allocation to Root Vacuoles to Improve Cd^{2+} Stress Tolerance in Plant

Previous studies have demonstrated that stress-induced alterations of the expression of *NRT1.5* and *NRT1.8* depend on the presence of nitrate in the growth medium (Chen et al., 2012). Interestingly, the phenomenon of Cd^{2+} toxicity disappeared, as was also observed in previous studies, when the plants were supplied with ammonium as the sole nitrogen nutrient (**Supplementary Figure S5**). It indicated that Cd^{2+} toxicity is closely correlated with NO_3^- allocation in plants. This observation was corroborated by studies performed in Arabidopsis, which showed that NO_3^- and Cd^{2+} uptake increase simultaneously (Mao et al., 2014). Studies in rice showed that an excess of NO_3^- increases the uptake and accumulation of Cd^{2+} (Yang et al., 2016). In this study, however, the uptake of Cd^{2+} in Col-0 was not different from that in *nrt1.1* mutants, but Col-0 plants allocated more Cd^{2+} to the roots (**Figures 1D,E**), which was consistent with the NO_3^- allocation pattern (**Figures 2A,B**). These results suggest that the presence of NO_3^- facilitates the allocation of Cd^{2+} to the roots, which increases the tolerance to Cd^{2+} stress.

The cellular vacuole plays important roles in maintaining the ion homeostasis in the plant cell and in regulating the responses to several abiotic and biotic stresses (Andreev, 2001). It acts as a storage pool of NO_3^-, in which the NO_3^- concentration is an order of magnitude higher than that in the cytoplasm (Martinoia et al., 1981, 2000). Sequestration into the vacuole is one of the key mechanisms of heavy metal detoxification in plants (Sharma et al., 2016). The transfer of NO_3^- and Cd^{2+} into the vacuole depends on a set of different transporters (De Angeli et al., 2006; Korenkov et al., 2006, 2007, 2009; Morel et al., 2009; Ueno et al., 2011). Nevertheless, both processes are mediated by V-ATPase and V-PPase energy pumps located on the membrane of tonoplast (Krebs et al., 2010; Sharma et al., 2016). We found that the activities of V-ATPase and V-PPase in Col-0 plants exposed to Cd^{2+} stress were significantly higher than those in the *nrt1.1* mutants (**Figures 6A,B**), leading to higher Cd^{2+} and NO_3^- levels in root vacuoles (**Figures 6C,E**) and a stronger reduction of cytosolic Cd^{2+} and NO_3^- levels in Col-0 plants as compared with those in *nrt1.1* plants (**Figures 6D,F**). Thus, there was less Cd^{2+} transport from the roots to the shoots, diminishing Cd^{2+} stress-induced injuries in the leaves. However, further studies are required to elucidate how the distribution of Cd^{2+} and NO_3^- between root vacuoles and cytosol is regulated.

CONCLUSION

Our results indicate that Cd^{2+} stress-initiated nitrate allocation to roots (SINAR) is associated with the antioxidant system to diminish stress-induced chlorosis. Furthermore, we found that root vacuoles are involved in the coordinated accumulation of Cd^{2+} and NO_3^- under Cd^{2+} stress.

In Col-0 plants, *NRG2* acted as a downstream element of *NRT1.1* in the regulation of NO_3^- allocation to the

roots by downregulating *NRT1.5* and upregulating *NRT1.8* under Cd^{2+} stress. Moreover, a larger proportion of absorbed Cd^{2+} remained in the roots, where Cd^{2+} and NO_3^- were allocated from the cytosol to the vacuole by increasing the tonoplast proton-pump activities (V-ATPase and V-PPase). Thus, intracellular sequestration reduced the transport of Cd^{2+} from roots to shoot, which is beneficial for Cd^{2+} detoxification and growth (**Figure 7A**).

In the *nrt1.1* mutant, *NRG2* expression was not changed by Cd^{2+} stress. Furthermore, the expression patterns of *NRT1.5* and *NRT1.8* in *nrt1.1* were not affected as significantly as those in Col-0 under Cd^{2+} stress. Hence, a larger proportion of NO_3^- absorbed by the root system was transported to the aboveground parts. Moreover, as compared to those in the controls, the tonoplast proton-pump activities (V-ATPase and V-PPase) in the *nrt1.1* mutant were lower under Cd^{2+} stress, resulting in enhanced Cd^{2+} and NO_3^- allocation to the cytosol and transport to aboveground parts. Ultimately, the *nrt1.1* mutant was more seriously injured under Cd^{2+} stress (**Figure 7B**). Our findings provide an insight into the underlying mechanism of the network of *NRT1.1* and *NRG2* in regulating Cd^{2+} stress tolerance in plants.

AUTHOR CONTRIBUTIONS

SJ and ZZ conceived the original screening and research plans and supervised the experiments. SJ performed most of the experiments and agreed to serve as the author responsible for contact and ensured communication. JL and QLiao provided technical assistance to SJ. SJ and ZZ designed the experiments and analyzed the data. QLiu and CG interpreted the result. ZZ conceived the project and wrote the article with contributions of all the authors and supervised and completed the writing.

FUNDING

This study was financially supported in part by the National Key R&D Program of China (2017YFD0200100), National Natural Science Foundation of China (Grant No. 31101596, 31372130), Hunan Provincial Recruitment Program of Foreign Experts, the National Oilseed Rape Production Technology System of China; "2011 Plan" supported by The Chinese Ministry of Education, and Research and Innovation Project of Postgraduates in Hunan Province (CX2015B242).

ACKNOWLEDGMENTS

We thank Dr. Ji-Ming Gong, Shanghai Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, for providing *A. thaliana* mutant materials (*chl1-1, chl1-5*), Dr. Yi-Fang Tsay, Institute of Molecular Biology, Academia Sinica, Tai Wan District, for providing the *A. thaliana* mutant lines *chl1-9*, and Dr. Yong Wang, Shan Dong Agricultural University, for providing *NRG2* mutant lines.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00384/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.