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RESEARCH PAPER

Iron uptake system mediates nitrate-facilitated cadmium accumulation in tomato (*Solanum lycopersicum*) plants

Bing Fang Luo[1,*], Shao Ting Du[2,*], Kai Xing Lu[3], Wen Jing Liu[1], Xian Yong Lin[1] and Chong Wei Jin[1,†]

[1] Ministry of Education Key Laboratory of Environmental Remediation and Ecosystem Health, College of Natural Resources and Environmental Science, Zhejiang University, Hangzhou, 310058, China
[2] College of Environmental Science and Engineering, Zhejiang Gongshang University, Hangzhou, 310035, China
[3] Laboratory of Plant Molecular Biology, College of Science and Technology Ningbo University, Ningbo, 315211, China

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: jincw@zju.edu.cn

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Abstract

Nitrogen (N) management is a promising agronomic strategy to minimize cadmium (Cd) contamination in crops. However, it is unclear how N affects Cd uptake by plants. Wild-type and iron uptake-inefficient tomato (*Solanum lycopersicum*) mutant (T3238*fer*) plants were grown in pH-buffered hydroponic culture to investigate the direct effect of N-form on Cd uptake. Wild-type plants fed NO_3^- accumulated more Cd than plants fed NH_4^+. Iron uptake and *LeIRT1* expression in roots were also greater in plants fed NO_3^-. However, in mutant T3238*fer* which loses FER function, *LeIRT1* expression in roots was almost completely terminated, and the difference between NO_3^- and NH_4^+ treatments vanished. As a result, the N-form had no effect on Cd uptake in this mutant. Furthermore, suppression of *LeIRT1* expression by NO synthesis inhibition with either tungstate or L-NAME, also substantially inhibited Cd uptake in roots, and the difference between N-form treatments was diminished. Considering all of these findings, it was concluded that the up-regulation of the Fe uptake system was responsible for NO_3^- -facilitated Cd accumulation in plants.

Key words: Ammonium, cadmium, iron uptake, nitrate.

Introduction

Cadmium (Cd) is recognized as a significant pollutant due to its high toxicity (Ronald, 2000; Pan and Wang, 2011). In most instances, dietary uptake through eating crops grown in Cd-contaminated soil is the most prevalent source of environmental Cd exposure for humans. Therefore, scientists have made great efforts to identify strategies for reducing/avoiding Cd accumulation by crops grown in Cd-contaminated soils. It is known that several plant nutrients have many direct as well as indirect effects on the availability of Cd in the soil and the uptake of Cd into plants (Sarwar *et al.*, 2010). For example, phosphate (Pi) favours the precipitation of Cd^{2+} (Hong *et al.*, 2010), while ferrous iron (Fe^{2+}) competes with Cd^{2+} for the same membrane transporters in plant cells (Vert *et al.*, 2002; Kovacs *et al.*, 2010). Growers are already applying nutrients to obtain a good crop yield. To alleviate Cd accumulation, the proper management of plant nutrients may be the only change needed due to the pre-existing interactions between Cd and plant nutrients. The use of nutrient management could be a relatively inexpensive, time-saving, and effective agronomic strategy to minimize Cd contamination in crops.

Nitrogen (N) is the main nutrient plants require as well as one of the most frequent factors limiting crop production (Daniel-Vedele *et al.*, 2010). Therefore, management of N has become an important agronomic practice. Physiologically, when nitrate (NO_3^-) is taken up by plants, there is a simultaneous uptake of protons (H^+), resulting in an increase in rhizosphere pH. Conversely, when ammonium (NH_4^+) is taken up, the H^+ are released into the rhizosphere, resulting in a decrease in rhizosphere pH (Marschner, 1995). The soil pH strongly affects the availability of Cd in the soil (Grant *et al.*, 1999). Because of this, it has often been

suggested that NH_4^+ fertilizers could result in enhanced Cd uptake due to a decrease in soil pH, compared with the NO_3^- fertilizers (Sarwar *et al.*, 2010). Numerous studies have provided evidence in support of this hypothesis. For example, a pot experiment (carried out on soils with weak buffer capacity), showed that NH_4^+ application clearly lowered rhizosphere pH and significantly increased Cd accumulation in sunflower plants, compared with NO_3^- application (Zaccheo *et al.*, 2006). However, contrary evidence has been obtained in several other studies. In a hydroponics experiment, Xie *et al.* (2009) found that *Thlaspi caerulesscens* plants fed NO_3^- accumulated much more Cd than the plants supplied with NH_4^+, even though the solution pH was lower in plants treated with NH_4^+. In a soil cultivation experiment, Jalloh *et al.* (2009) also observed that the rice plants fed NO_3^- had a higher Cd concentration than the plants fed NH_4^+. These conflicting findings indicate that the N-form may have another effect on Cd uptake in plants besides the indirect effect, which is changing the pH of the rhizosphere.

In addition to being an essential nutrient, NO_3^- also serves as a signalling molecule. It is known to regulate root architecture, stimulate shoot growth, delay flowering, regulate abscisic acid-independent stomata opening, and relieve seed dormancy (Walch-Liu *et al.*, 2005; Ho *et al.*, 2009; Tian *et al.*, 2009). In addition, NO_3^- has also been implicated in regulating the uptake of many nutrients. For instance, resupplying NO_3^- to tomato plants rapidly up-regulated expression of the NH_4^+ transporter *LeAMT2*, the Pi transporter *LePT2*, and *Kdc1* (a homologue of a carrot K^+ channel) (Wang *et al.*, 2001). In addition, the *Arabidopsis chl1-5* mutant, which is deficient for the NRT1.1 NO_3^- transporter, displays low NO_3^- uptake and has suppressed expression of *AtIRT1* (Muños *et al.*, 2004). IRT1 is a divalent plasma membrane cation transporter essential to the uptake of ferrous iron from the soil in non-graminaceous monocots and dicots (Vert *et al.*, 2002; Curie and Briat, 2003; Jeong and Guerinot, 2009). Interestingly, several studies provide strong evidence that the iron transporter IRT1 is also primarily responsible for Cd^{2+} influx into root cells (Vert *et al.*, 2002; Clemens, 2006; Verbruggen *et al.*, 2009; Lux *et al.*, 2011). This fact combined with the implication of NO_3^- in regulating *IRT1* led us to hypothesize that NO_3^- may affect Cd accumulation in plants through the regulation of root cell Fe uptake system.

In this study, tomato (*Solanum lycopersicum*) plants were used to investigate the above hypothesis. Evidence is provided that NO_3^- application directly enhances Cd uptake of plants, compared with NH_4^+ application. This enhancement is attributed to the up-regulation of root Fe uptake systems, which require the FER protein to function.

Materials and methods

Chemicals

The chemicals used in this study were purchased as: DAF-FM DA (diaminofluorescein-FM diacetate) from Beyotime Institute of Biotechnology (http://www.beyotime.com/), L-NAME (N^{ω}-nitro-L-arginine methyl ester hydrochloride) from the Rego Institute of Biotechnology (http://regobio.testmart.cn/), Trizol reagent from Invitrogen (http://www.invitrogen.com/), and tungstate and MES (4-morpholineethanesulfonic acid) from Sangon (http://www.sangon.com/).

Plant culture

Uniform size tomato (*Solanum lycopersicum* cv. Micro-Tom) seedlings were transferred to 1.0 l pots filled with aerated, full-strength complete nutrient solution. The nutrient solution had the following composition (in μM): NaH_2PO_4, 750; $MgSO_4$, 500; K_2SO_4, 375; KNO_3, 750; $(NH_4)_2SO_4$, 375; $CaCl_2$, 1000; H_3BO_3, 10; $MnSO_4$, 0.5; $ZnSO_4$, 0.5; $CuSO_4$, 0.1; $(NH_4)_6Mo_7O_{24}$, 0.1; and Fe-EDTA, 25. The solution pH was adjusted to 5.5 using 1 M NaOH. All the plants were grown in the controlled-environment growth chamber at 70% relative humidity with a daily cycle of 14 h day at 28 °C, and 10 h night at 22 °C. The daytime light intensity was 300–350 μmol photons m^{-2} s^{-1}. After 12 d of growth in the nutrient solution, plants were subjected to different N-form treatments. For the treatment of NO_3^- as the sole nitrogen source, 1.5 mM KNO_3 was applied to the solution. For the treatment of NH_4^+ as the sole N source, 0.75 mM $(NH_4)_2SO_4$ and 0.75 mM K_2SO_4 were added. For both N-form treatments, nutrient solutions were buffered with 2 mM MES at pH 5.5. Other nutrients were the same as above. Both N-form treatments were split into two sub-treatments, 0 and 2 μM Cd, added as $CdCl_2$. For the experiments illustrated in Fig. 5, the Fe uptake-inefficient mutant, T3238*fer*, and its wild type, T3238 (Brown *et al.*, 1971), were used, and the treatment methods were the same as the Cd-added treatments described above. For the experiments illustrated in Figs 6 and 7, either 0.4 mM L-NAME or 0.15 mM tungstate, were added into Cd-contained NO_3^- /NH_4^+ solutions at the start of N-form treatments. The solutions in all of the treatment containers were renewed daily. The shoots and roots of plants after 8 d of treatments were harvested for further analysis.

Real-time reverse transcription-PCR analyses

Root samples were frozen in liquid nitrogen immediately after collection and stored at –80 °C. About 100 mg of tissue were ground in liquid nitrogen and total RNA was extracted with TRIzol. The first-strand cDNA was synthesized with the total RNA by PrimeScript reverse transcription (RT) reagent kit (TaKaRa). All RNA samples were checked for DNA contamination before cDNA synthesis. The mRNA levels of *FER*, *LeFRO1*, and *LeIRT1* were detected by the SYBR Green RT-PCR kit (TaKaRa) with the following pairs of gene-specific primers: *FER* fw, 5′-TGAATCTTCTGGCACAACG-3′; rev, 5′-CCAATGATGGAGGCTTTATC-3′, *LeFRO1* fw, 5′-GCAAGACACCAGAAATCCTAC-3′, rev: 5′-ATCAGATGGGTTGGGCTT-3′; *LeIRT1* fw, 5′-AGCACTTGGGATAGCATTG-3′; rev, 5′-ACTGACATTC CACCAGCAC-3′. The RT-PCR analysis was performed with ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following cycling conditions: 10 s at 95 °C, 35 cycles of 95 °C for 5 s, 60 °C for 30 s. A pair of α-*tubulin* housekeeping gene primers were used for a control in the PCR: fw: 5′-CCTGAACAACTCATAAGTGGC-3′; rev, 5′-AGATTGGTGTAGGTAGGGCG-3′. Each cDNA sample was run in triplicates. Amplification of PCR products was monitored via intercalation of SYBR-Green. Relative expression units (REU) were calculated according to the equation as described previously (Jin *et al.*, 2009).

In situ *measurement of NO in the roots*

Nitric oxide was imaged using DAF-FM DA (diaminofluorescein-FM diacetate). The DAF-FM DA has been successfully used to detect NO production in both plants and animals. Roots were

loaded with 10 μM DAF-FM DA in 20 mM HEPES/NaOH buffer (pH 7.4) for 30 min, washed three times in fresh buffer and observed under a Nikon Eclipse E600 epifluorescence microscope equipped with a Nikon B-2A filter block (450–490 nm excitation filter, 505 nm dichroic mirror, 520 nm barrier filter). A 100 W high-pressure mercury-vapour lamp was used as a light source (HB-10103AF-Hg, Nikon). Exposure settings were constantly maintained during the fluorescence microscopy. Signal intensities of green fluorescence in the images were quantified according to the method of Guo and Crawford (2005) by using Photoshop software (Adobe Systems). Data are presented as the mean of fluorescence intensity relative to the root tips of Cd-free plants fed NH_4^+.

Analysis of elements' content

The dried root and shoot samples were wet digested in the concentrated HNO_3/HCl at 120 °C until there was no brown nitrogen oxide gas emitting, then further digested with $HClO_4$ at 180 °C until the solution became transparent. Digestates were diluted by ultrapure water, and the concentrations of Cd and Fe in the digestates were analysed by ICP-OES (iCAP 6300). The concentrations of P in the digestates were evaluated by the vanadate–molybdate colorimetric method (Hesse, 1971).

Statistics

All statistical analyses were conducted with SAS software (SAS Institute, Cary, NC). Means were compared by *t* test or Fisher's least significant difference test at $P < 0.05$ in all cases.

Results

Effect of N-form on plant growth and uptake of Cd

As discussed above, N-form may have a direct effect on Cd uptake in plant roots besides the indirect effect of altering rhizosphere pH. Distinguishing the 'N-form effect' from the 'pH effect' is important for understanding the mechanism of how the N-form affects Cd accumulation in plants. In this study, a pH-buffered culture solution was used to separate the two variables, so as to investigate whether N-form had a direct effect on Cd accumulation in tomato plants. In Cd-free growth solutions, after 8 d of treatment, the plants fed NO_3^- had a 16% greater root biomass and 17% greater shoot biomass than the plants fed NH_4^+. In Cd-added growth solutions, N-form had similar effects on the plant biomass (Fig. 1a, b).

The Cd accumulation in plants was also affected by the N-form. In Cd-added growth solutions, the roots and shoots from NO_3^- treatment contained 83% and 85% higher Cd concentrations, respectively, than those from NH_4^+ treatment (Fig. 2a, b). The amount of Cd absorbed per weight of roots (CAPR) was calculated. As shown in Fig. 2c, the plants grown with NO_3^- had about 2-fold higher CAPR than the plants grown with NH_4^+, indicating that NO_3^- nutrition facilitates the Cd uptake of roots.

Effect of N-form on Fe uptake

Cd uptake in plants has been linked to the Fe uptake system and, therefore, the Fe concentration in plants was checked. In Cd-free growth solutions, the Fe concentration in roots from the NO_3^- treatment was increased by 68% compared with those from the NH_4^+ treatment (Fig. 3a) while, in Cd-added growth solutions, it was increased by up to 163%. By contrast, in both Cd-free and Cd-added growth solutions, the Fe concentrations of shoots from NO_3^- treatments were slightly lower than those from NH_4^+ treatments (Fig. 3b). The amount of Fe absorbed per weight of roots (FAPR) was also calculated. As shown in Fig. 3c, in Cd-free growth solutions, FAPR in the NO_3^- treatment was 31% higher than that in the NH_4^+ treatment. Interestingly, in Cd-added growth solutions, this NO_3^- -enhanced FAPR was further strengthened, in some cases by up to 90%, compared with

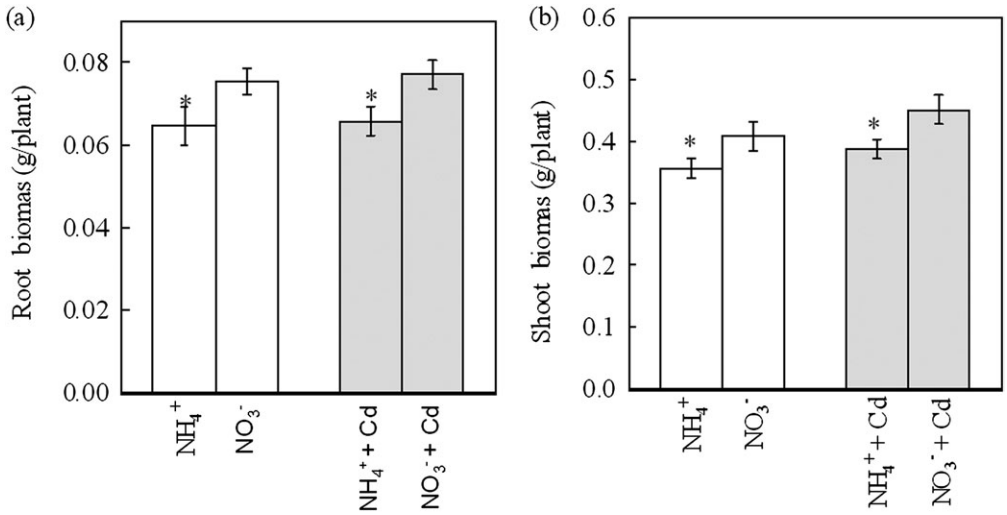

Fig. 1. Effect of N-form on growth of Micro-Tom tomato plants under Cd-free or Cd-exposed condition. (a) The root biomass. (b) The shoot biomass. The plants were pre-cultured in the growth solution contained both NO_3^- and NH_4^+ for 12 d and were then transferred to Cd-free or 2 μM Cd-added growth solutions with either NO_3^- or NH_4^+ as the sole nitrogen source. The pH in the all treatments was buffered at 5.5 using MES. The shoots and roots of plants after 8 d of treatments were harvested for biomass analysis. Data are means ±SD (n=4). * Significant differences ($P < 0.05$) between NO_3^- and NH_4^+ treatments.

the NH_4^+ treatment. These results suggest that NO_3^- also facilitates Fe uptake in roots, particularly with Cd exposure.

Fe (III) reduction and the transport of Fe (II) across the plasma membrane with ferric chelate reductase (FCR) and IRT1 are pivotal steps involved in Fe uptake by dicots (Curie and Briat, 2003; Jeong and Guerinot, 2009). *LeFRO1* which codes for FCR and *LeIRT1,* which codes for IRT1 in tomato plants, both display tightly regulated expression levels by the FER protein (Ling *et al.*, 2002; Bereczky *et al.*, 2003; Li *et al.*, 2004). It was found here that the expressions of *FER* and *LeFRO1* in roots was not affected or only slightly affected by N-form (Fig. 4a, b). Interestingly, expressions of *LeIRT1* were strongly affected by the N-form. In Cd-free growth solutions, the NO_3^- treatment had a 4.5-fold higher *LeIRT1* expression than the NH_4^+ treatment, while in Cd-added growth solutions the NO_3^-

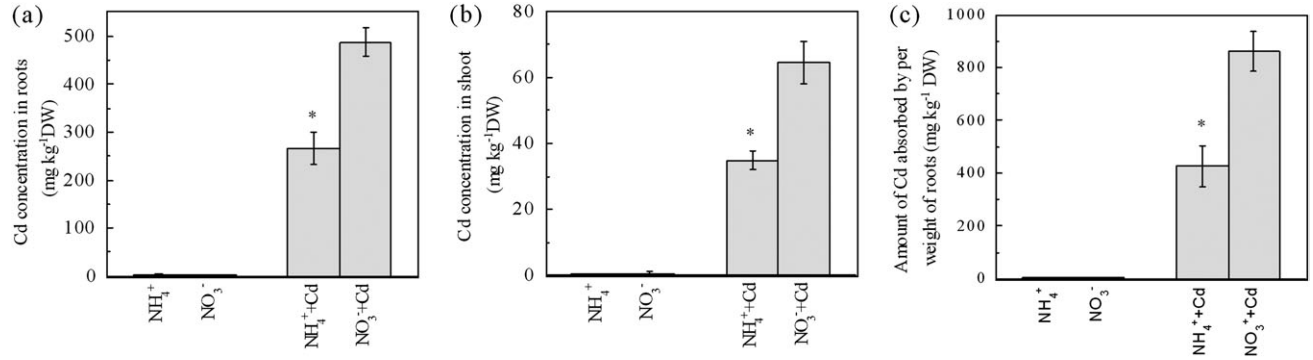

Fig. 2. Effects of N-form on Cd concentration and Cd uptake of Micro-Tom tomato plants. (a) The root Cd concentrations. (b) The shoot Cd concentrations. (c) The amount of Cd absorbed by per weight of roots. Treatments are the same as in Fig. 1. Data are means ±SD (n=4). * Significant differences ($P < 0.05$) between NO_3^- and NH_4^+ treatments.

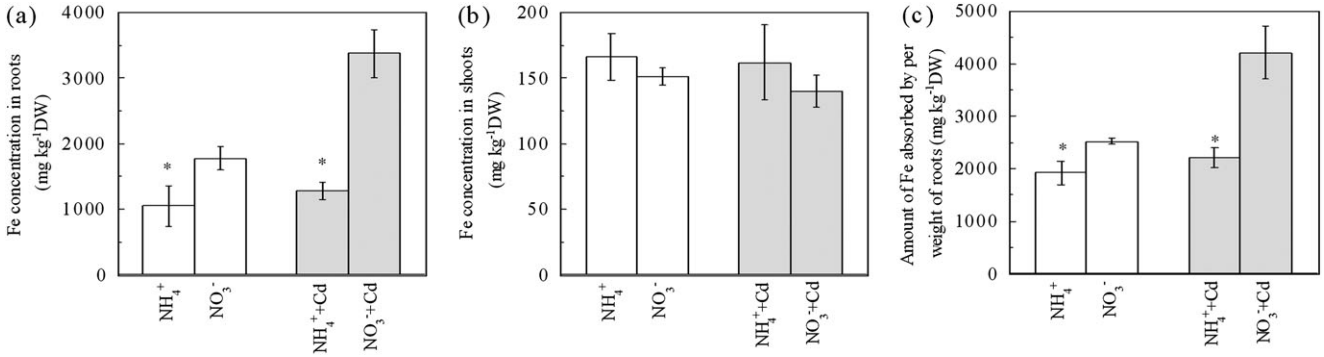

Fig. 3. Effects of N-form on Fe uptake of Micro-Tom tomato plants under Cd-free or Cd-exposed condition. (a) The root Fe concentrations. (b) The shoot Fe concentrations. (c) The amount of Fe absorbed by per weight of roots. Treatments are the same as in Fig. 1. Data are means ±SD (n=4). * Significant differences ($P < 0.05$) between NO_3^- and NH_4^+ treatments.

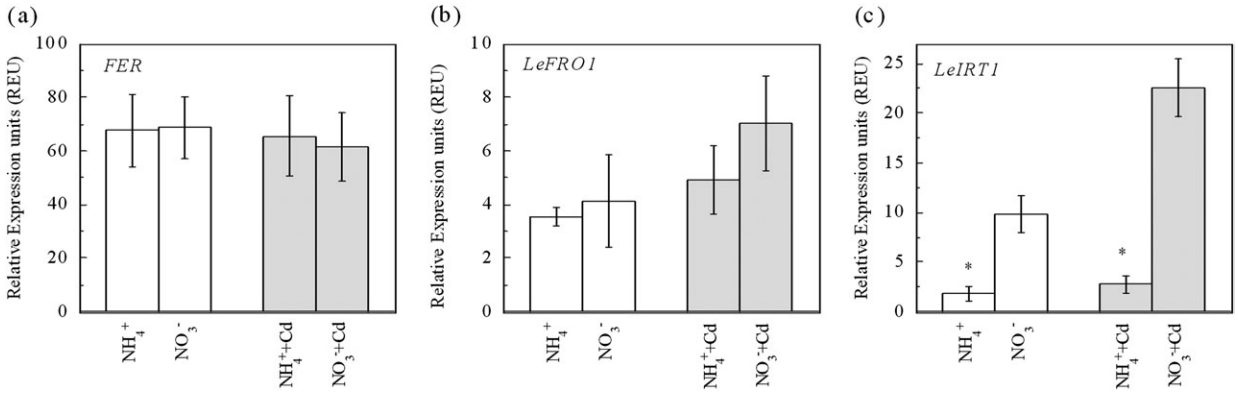

Fig. 4. Effects of N-form on expression levels of *FER* (a), *LeFRO1* (b), and *LeIRT1*(c) in Micro-Tom tomato roots under Cd-free or Cd-exposed condition. Treatments are the same as in Fig. 1. Data are means ±SD (n=7). * Significant differences ($P < 0.05$) between NO_3^- and NH_4^+ treatments.

treatment had a 7.2-fold increase in expression level (Fig. 4c). The results indicate that enhancement of *LeIRT1* expression may be responsible for the elevation of Fe uptake under NO_3^- conditions.

Effect of FER mutation on NO_3^- -enhanced Cd uptake

Loss of FER function in T3238*fer* tomato mutants leads to failure of Fe deficiency responses, including the expression of *LeIRT1* (Ling *et al.*, 2002). Therefore, the mutant, T3238*fer,* and its wild type, T3238, were used to investigate the role of Fe uptake systems in NO_3^- -facilitated Cd uptake. In Cd-added growth solutions, the expression of *LeIRT1* in roots of T3238 was significantly higher in NO_3^- treatments than in NH_4^+ treatments (Fig. 5a). This result is similar to the Micro-Tom wild-type plants described above. However, in T3238*fer* the expressions of *LeIRT1* in both N-form treatments were almost completely terminated compared with those in T3238. Furthermore, in this mutant strain there was not a statistically significant difference in *LeIRT1* expression between the two N-form treatments (Fig. 5a).

In accordance with the findings in Micro-Tom, the Cd concentrations of both roots and shoots in T3238 were also significantly higher in the NO_3^- treatment than in the NH_4^+ treatment (Fig. 5b). In T3238*fer*, however, the root Cd concentration was not affected by N-form (Fig. 5c). Interestingly, the shoot Cd concentration in this mutant was still unexpectedly higher in the NO_3^- treatment than in the NH_4^+ treatment, but the difference between them was far less than that in T3238. For T3238*fer*, shoot Cd concentration after NO_3^- treatment increased by 37% compared with the NH_4^+ treatment, whereas for T3238, concentration was increased 128% (Fig. 5b, c). The CAPR in roots of T3238 was significantly higher in the NO_3^- treatment than in the NH_4^+ treatment (Fig. 5b), but in T3238*fer* there was no difference between the two N-form treatments (Fig. 5c). These results, along with the finding that the N-form fails to affect *LeIRT1* expression in T3238*fer* mutants, indicate that the Fe uptake system is required for NO_3^- facilitation of Cd uptake in wild-type plants.

Effect of NO synthesis inhibition on NO_3^- -enhanced Cd uptake

Inhibition of nitric oxide (NO) synthesis has also been demonstrated to suppress the expression of *LeIRT1* (Graziano and Lamattina, 2007; Jin *et al.*, 2009). The nitrate reductase (NR) and the NO-synthase (NOS) enzymes have been recognized as major sources of NO generation in plants (Shapiro, 2005). Therefore, the NR inhibitor tungstate or the NOS inhibitor L-NAME was used to investigate the effect of NO synthesis inhibition on NO_3^- -enhanced Cd uptake. Interestingly, NO_3^- treatment resulted in a higher NO-associated green fluorescence in roots than did the NH_4^+ treatment (Fig. 6a). By quantifying the signal intensities of fluorescence, the NO contents in roots of the plants fed NO_3^- were increased by more than 2-fold compared with those of plants fed NH_4^+ in both Cd-free and Cd-added growth solutions (Fig. 6b). The presence of either tungstate or L-NAME in Cd-added growth solution substantially suppressed NO production in both N-form treatments, and eliminated any difference in NO levels between the two treatments. The NO_3^- -enhanced expression of *LeIRT1* in roots was also completely inhibited by either inhibitor, and there was no resulting difference between the two N-form treatments (Fig. 7a). Consequently, the application of either inhibitor greatly reduced the Cd concentration in NO_3^- -treated roots, which was even lower than in the NH_4^+ -treated roots (Fig. 7b). For shoot Cd concentrations, although they were significantly reduced by either inhibitor in both N-form treatments, the NO_3^- treatment still had a higher value (Fig. 7c). The CAPR was then calculated. As shown in Fig. 7d, when either L-NAME or tungstate were included in the growth solutions, the NO_3^- treatment had only 41% or 33% higher CAPR, respectively, than the NH_4^+ treatment, whereas in the growth solutions containing

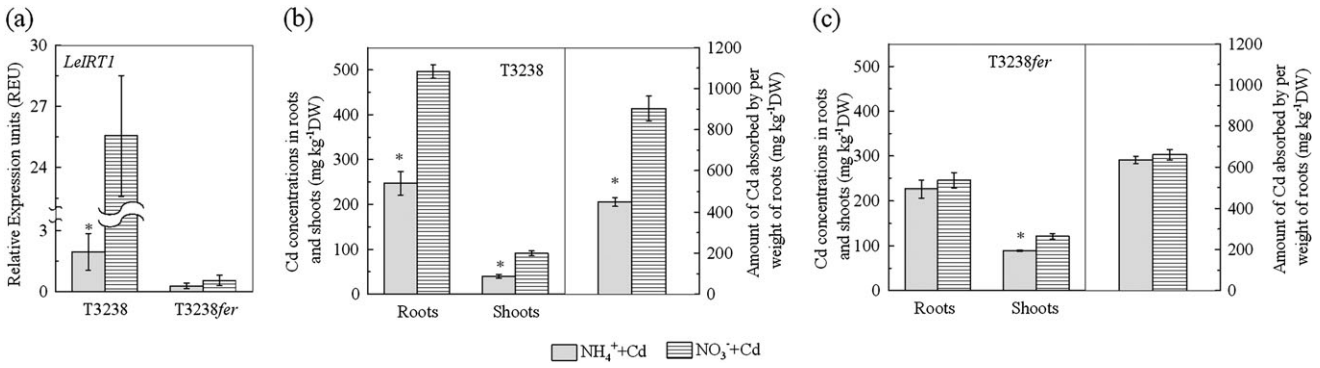

Fig. 5. Effects of N-form on *LeIRT1* expressions, Cd concentrations and Cd uptake capacities in T3238 wild-type plants and T3238*fer* mutants under Cd exposure condition. (a) The expression levels of *LeIRT1* in roots of T3238 and T3238*fer*. (b) The Cd concentrations (left figure) and the amount of Cd absorbed by per weight of roots (right figure) in T3238. (c) The Cd concentrations (left figure) and the amount of Cd absorbed by per weight of roots (right figure) in T3238 *fer*. The T3238 wild-type plants and the T3238*fer* mutants were transferred to 2 μM Cd-added growth solutions with either NO_3^- or NH_4^+ as the sole nitrogen source. The pH in the all treatments was buffered at 5.5 using MES. The shoots and roots of plants after 8 d of treatments were harvested for analysis. Data are means ±SD (n=4). * Significant differences ($P < 0.05$) between NO_3^- and NH_4^+ treatments.

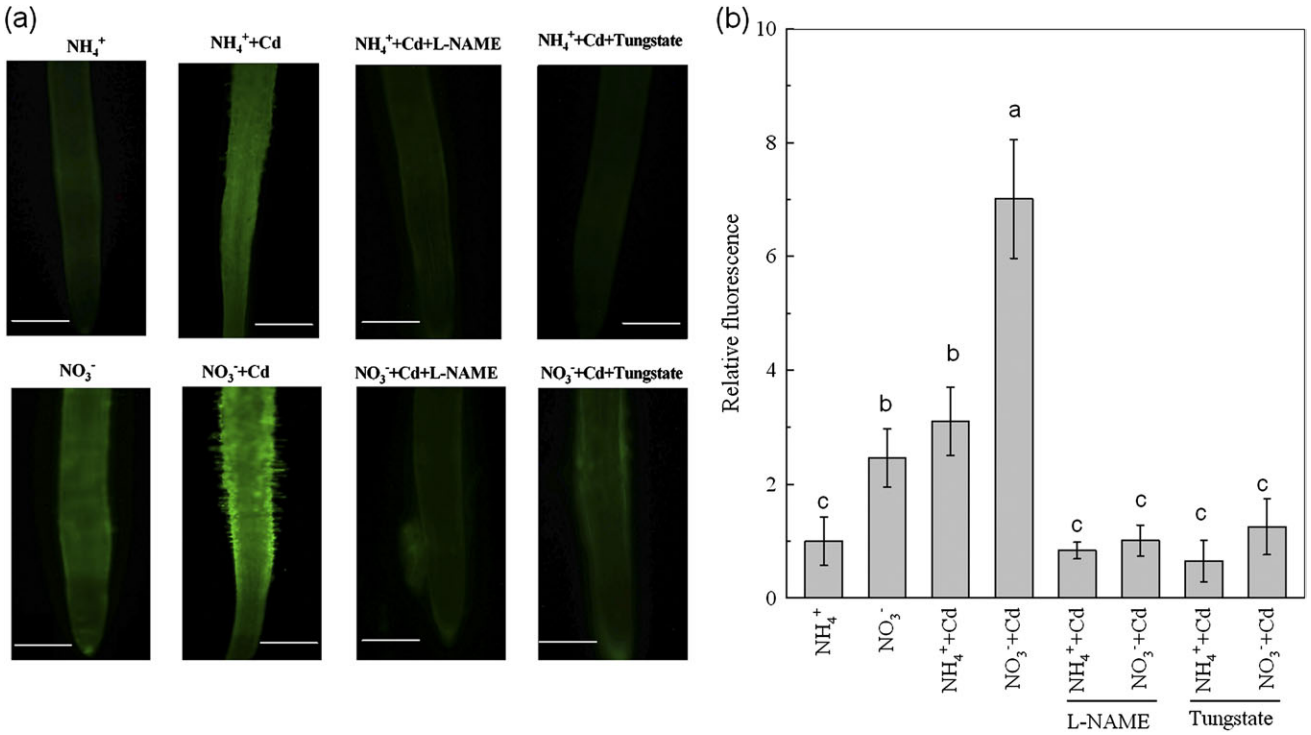

Fig. 6. Effects of N-form on NO production in roots of Micro-Tom tomato plants under Cd-free or Cd-exposed conditions. (a) Photographs of NO production shown as green fluorescence in representative roots (bar=1 mm). (b) NO production expressed as relative fluorescence. The plants were transferred to Cd-free and 2 μM Cd-added growth solutions with either NO_3^- or NH_4^+ as the sole nitrogen source. Meanwhile, either 0.4 mM L-NAME or 0.15 mM tungstate were added to the Cd-treated solutions when the N-form treatments were started. The pH in the all treatments was buffered at 5.5 using MES. The roots of plants after 8 d of treatments were harvested for NO analysis. Data are means ±SD (n=15). Different letters indicate significant differences ($P < 0.05$) among the treatments.

neither L-NAME nor tungstate, the NO_3^- treatment had about 100% higher CAPR than the NH_4^+ treatment. These results suggest that inhibition of NO synthesis could diminish the difference in Cd uptake between the two N-form treatments.

Discussion

Nitrate has a direct effect on enhancing Cd uptake

In the pH-buffered growth solutions, it was observed that NO_3^- nutrition facilitates Cd uptake in roots compared with NH_4^+ nutrition (Fig. 2). The Cd availability in nutrient solutions may be unintentionally altered due to N-form treatments. However, the computer modelling by GEOCHEM-PC (Parker *et al.*, 1995) showed that the composition of Cd species in nutrient solutions were similar between NO_3^- and NH_4^+ treatments, and all were present in soluble forms (see Supplementary Table S1 at *JXB* online). Furthermore, during plant growth, the pH in the pH-buffered growth solutions was kept constant, thus the variation of Cd availability in the rhizosphere due to N uptake-induced alteration of pH can be discounted. Therefore, the actions of NO_3^- -facilitated Cd uptake in plants should be directly related to cellular processes rather than the rhizospheric process. Nevertheless, one matter to clarify here is that NH_4^+ may have deleterious effects on plants when used as the sole N source for plant growth. Acidification of the rhizosphere due to NH_4^+ uptake is often considered to be a fundamental cause of NH_4^+ toxicity, particularly since relief from toxicity symptoms has often been observed when growth solutions are pH-buffered (Gigon and Rorison, 1972; Vollbrecht and Kasemir, 1992; Herbert *et al.*, 2001). In this study, pH-buffered growth solutions were used, and therefore no visual toxic symptoms on plants were observed throughout NH_4^+ treatment. The biomass for the NH_4^+ treatment was only slightly less than the NO_3^- treatment (Fig. 1). Furthermore, it was observed that the concentrations of P in both shoots and roots were higher in the plants fed NH_4^+ than in the plants fed NO_3^- (see Supplementary Fig. S1 at *JXB* online). These results indicate that the NH_4^+ treatment in pH-buffered solutions did not impair the nutrient uptake systems. Therefore, it is reasonable to conclude that NO_3^- nutrition facilitates Cd uptake in roots and that the lower Cd uptake in NH_4^+ treatment is not due to deleterious effects induced by NH_4^+ uptake.

In contrast to our results, it has been observed that NH_4^+ nutrition facilitates Cd accumulation in soil-grown winter rape (*Brassica napus* L.) and tobacco (*Nicotiana tabacum* L.) plants more so than NO_3^- nutrition (Eriksson, 1990; Tsadilasa *et al.*, 2005). The reason for these conflicting results may be because NH_4^+ has an indirect effect on

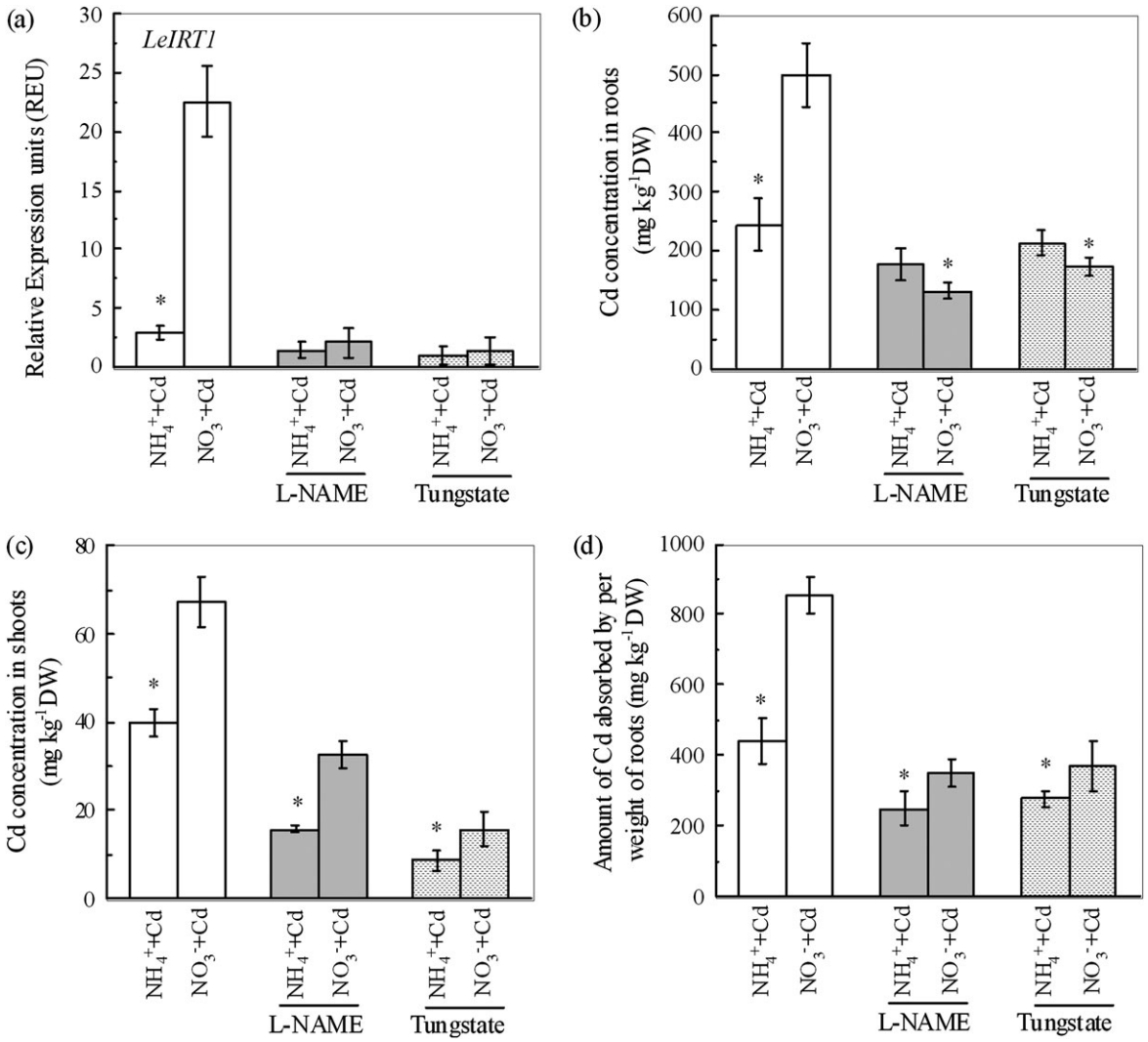

Fig. 7. The role of NO in regulating *LeIRT1* expression, Cd concentration, and Cd uptake capacity in roots of Micro-Tom tomato plants from different N-form treatment. (a) The expression levels of *LeIRT1* in roots. (b) The Cd concentrations in roots. (c) The Cd concentrations in shoots. (d) The Cd uptake capacities in roots. Treatments are the same as in Fig. 6. Data are means ±SD (n=4). * Significant differences ($P < 0.05$) between NO_3^- and NH_4^+ treatments.

increasing root Cd uptake due to a decrease of rhizosphere pH (De Roton *et al.*, 1996; Sarwar *et al.*, 2010). In soils with a weak buffering capacity, the effect of pH on Cd uptake due to NH_4^+ may be more predominant than the direct effect of NO_3^- facilitating Cd uptake as discussed above, whereas the opposite is probably true in soils with a strong buffer capacity. Therefore, distinguishing the indirect effects of pH from the direct effects of N-form and comprehensively considering each is a critically important step in determining whether pH amendments or N-forms should be prioritized when proposing a strategy for reducing Cd accumulation in crops grown in Cd-contaminated soils.

The system involved in Fe uptake is required for NO_3^- -enhanced Cd uptake

In most instances, the greater uptake of one ion can either depress the uptake of another ion with similar charge (antagonism) or stimulate the uptake of an ion with opposite charge (synergism). Therefore, the ion synergism may explain why the NO_3^- nutrition results in higher accumulation of Cd in the plants. However, the mechanism behind the above ion synergism remains unknown. As discussed above, reduction of Fe (III) to ferrous Fe by FCR and subsequent transport across the plasma membrane by IRT1 are pivotal steps involved in the Fe uptake of dicots (Robinson *et al.*, 1999; Jeong and Guerinot, 2009), while IRT1 is of particular interest in this study because it is also a plasma membrane transporter of Cd^{2+} (Vert *et al.*, 2002; Verbruggen *et al.*, 2009; Lux *et al.*, 2011). The linkage between Fe uptake and NO_3^- -enhanced Cd uptake was therefore analysed. It was observed here that NO_3^- treatment could also facilitate NO_3^- Fe uptake in the roots compared with the NH_4^+ treatment (Fig. 3). Furthermore, although the expression of *LeFRO1* in roots undergoing NO_3^- treatment was only increased slightly, the expression of *LeIRT1* NO_3^- treatment was greatly increased compared with the NH_4^+ treatment (Fig. 4b, c). Although FCR and IRT1 work

together to enhance Fe uptake under Fe-deficient conditions, IRT1 seems to be more important than FCR in Fe uptake under Fe-sufficient conditions. When the plants were grown in soil, the *Arabidopsis* FCR-null mutant *frd1-1* and the wild type had similar Fe concentrations, but the IRT1-null mutant *irt1-1* contained considerably lower Fe concentrations than the wild type (Yi and Guerinot, 1996; Vert *et al.*, 2002). Therefore, although *LeFRO1* expression is not increased with the up-regulation of *LeIRT1* expression, it is still reasonable to suggest that increasing Fe (II) transporter IRT1 may be responsible for increasing Fe uptake in the NO_3^- treatment.

The expression of *LeIRT1* is tightly regulated by the FER protein (Ling *et al.*, 2002). T3238*fer* tomato mutants with loss of FER function exhibit severe chlorosis and die early on unless supplied with ferrous iron or grafted onto a wild-type rootstock (Brown *et al.*, 1971; Ling and Ganal, 2000). It was found here that the expressions of *LeIRT1* in the Fe uptake-inefficient mutant T3238*fer* were similar between the NO_3^- and NH_4^+ treatments, and were almost completely non-existent compared with those in the wild type T3238 (Fig. 5a). Accordingly, in T3238*fer*, the Cd uptake in roots was not affected by the N-form, but in T3238 it was significantly higher in the NO_3^- treatment than in the NH_4^+ treatment (Fig. 5b, c). These results combined with the finding that both Fe uptake and *LeIRT1* expression were increased by NO_3^- (Figs 3, 4b), indicate that the system involved in Fe uptake is required for the enhancement of Cd uptake by NO_3^- in tomato plants. Although loss of FER function resulted in the inhibition of the NO_3^- -induced enhancement of *LeIRT1* expression and Cd uptake in the T3238*fer* mutant, the expression of *fer* in the wild-type plants was not affected by the N-form (Fig. 4a). It is speculated that FER is essential, but is not the limiting factor for the regulation of NO_3^- -induced enhancement of Cd uptake in tomato plants.

Several studies have demonstrated that NO is a signal controlling the Fe uptake system in roots (Graziano and Lamattina, 2007; Besson-Bard *et al.*, 2009; Chen *et al.*, 2010; Ramirez *et al.*, 2010; García *et al.*, 2010). Accordingly, in the present study, it was observed that suppression of *LeIRT1* expression in roots was by the inhibition of NO synthesis. Significant decreases in the Cd concentration in plants fed NO_3^- were observed, which diminished the difference in Cd uptake between NO_3^- and NH_4^+ treatments (Fig. 7). The results provide more evidence for our above conclusion that the Fe uptake system is required for NO_3^- induction of Cd uptake. Interestingly, it was also observed here that NO_3^- treatment resulted in a higher NO level in roots than did the NH_4^+ treatment in both Cd-free and Cd-supplemented growth solutions (Fig. 6). Theoretically, the NR-dependent NO production depends on the NR activity. The increase in nitrate availability enhances NR activity (Shaner and Boyer, 1976), whereas NH_4^+ is an inhibitor of NR (Jin *et al.*, 2011). Accordingly, a higher NO level in roots of NO_3^- treatment is probably due to activation of NR activity by NO_3^-. This viewpoint, combined with the fact that NO is a signal controlling the Fe uptake system in roots, allowed us to propose that NO_3^- -induction of NO production in roots may be the original signal causing the induction of the Fe uptake system, resulting in enhanced Cd uptake. This hypothesis will be the focus of our future research. It is interesting to note that the NOS inhibitor L-NAME could also inhibit the NO production in Cd-added NO_3^- treatment (Fig. 6). This may be due to the fact that accumulation of Cd in plants could also induce NO production by NOS (Besson-Bard *et al.*, 2009).

It is worth noting that NO availability in plants also affects the expression of *NRT2.1*, the gene encoding a high-affinity NO_3^- transporter. Elevation of NO levels in roots by Cd exposure induces the expression of *NRT2.1*, while the opposite is true for roots treated with L-NAME (Besson-Bard *et al.*, 2009). Therefore, it is reasonable to propose that NO_3^- -induced NO production may, in turn, facilitate NO_3^- uptake in roots, forming a positive feedback loop. In addition, because Cd in plants also induces NO production (Besson-Bard *et al.*, 2009), the induction of *IRT1* expression by NO not only may increase Cd uptake in roots, but may also enhance the production of NO. Taken together, the NO-mediated cross-talking between NO_3^- - and Fe-sensing pathways may take place in roots, which may aid the plants' Cd uptake.

Overall, although previous reports have provided other evidence concerning NO_3^- nutrition facilitating Cd uptake in roots compared with NH_4^+ nutrition in different plant species, the mechanism behind this process has not previously been examined. Here, using wild-type tomato plants, Fe uptake-inefficient mutants, and NO synthesis inhibitors, it has been demonstrated that the effects of NO_3^- on root Cd uptake are attributed to an up-regulation of the system involved in Fe uptake. The increase of NO production may be a signalling pathway controlling the above process. To our knowledge, this is the first report to uncover why NO_3^- -based fertilizers result in more Cd accumulation in plants than NH_4^+ -based fertilizers in many cases, even though NO_3^- -based fertilizers are expected to decrease the Cd availability in the rhizosphere. Furthermore, this study also helped determine whether pH amendments or N-forms should be prioritized when proposing a strategy for safe crop production in contaminated soil.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Effects of N-form on P concentrations in tomato plants during Cd exposure.

Supplementary Table S1. Comparison of Cd and Fe forms between NO_3^- and NH_4^+ media.

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References

Bereczky Z, Wang HY, Schubert V, Ganal M, Bauer P. 2003. Differential regulation of nramp and irt metal transporter genes in wild type and iron-uptake mutants of tomato. *Journal of Biological Chemistry* **278,** 24697–24704.

Besson-Bard A, Gravot A, Richaud P. 2009. Nitric oxide contributes to cadmium toxicity in Arabidopsis by promoting cadmium accumulation in roots and by up-regulating genes related to iron uptake. *Plant Physiology* **149,** 1302–1315.

Brown JC, Chaney RL, Ambler JE. 1971. A new tomato mutant inefficient in the transport of iron. *Physiologia Plantarum* **24,** 48–53.

Chen WW, Yang JL, Qin C, Jin CW, Mo JH, Ye T, Zheng SJ. 2010. Nitric oxide acts downstream of auxin to trigger root ferric-chelate reductase activity in response to iron deficiency in *Arabidopsis thaliana*. *Plant Physiology* **154,** 1885–1892.

Clemens S. 2006. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie* **88,** 1707–1719.

Curie C, Briat JF. 2003. Iron transport and signaling in plants. *Annual Review of Plant Biology* **54,** 183–206.

Daniel-Vedele F, Krapp A, Kaiser WM. 2010. Cellular biology of nitrogen metabolism and signaling. *Plant Cell Monographs* **17,** 145–172.

De Roton C, Tancogne J, Cadilhac JL. 1996. Effects of the soil pH and form of nitrogen fertilization on the uptake of trace-metals in Burley tobacco. CORESTA congress, Yokohama, Japan.

Eriksson JE. 1990. Effects of nitrogen-containing fertilizers on solubility and plant uptake of cadmium. *Water, Air, and Soil Pollution* **49,** 355–368.

García MJ, Lucena C, Romera FJ, Alcántara E, Pérez-Vicente R. 2010. Ethylene and nitric oxide involvement in the up-regulation of key genes related to iron acquisition and homeostasis in *Arabidopsis*. *Journal of Experimental Botany* **61,** 3885–3899.

Gigon A, Rorison IH. 1972. The response of some ecologically distinct plant species to nitrate- and to ammonium-nitrogen. *Journal of Ecology* **60,** 93–102.

Grant CA, Bailey LD, McLaughlin MJ, Singh BR. 1999. Management factors which influence cadmium concentration in crops. In: McLaughlin MJ, Singh BR, eds. *Cadmium in soils and plants*. Dordrecht: Kluwer, 151–198.

Graziano M, Lamattina L. 2007. Nitric oxide accumulation is required for molecular and physiological responses to iron deficiency in tomato roots. *The Plant Journal* **52,** 949–960.

Guo FQ, Crawford NM. 2005. Arabidopsis nitric oxide synthase1 is targeted to mitochondria and protects against oxidative damage and dark-induced senescence. *The Plant Cell* **17,** 3436–3450.

Herbert J, Kronzucker Dev T, Britto Romola J, Davenport Mark Tester. 2001. Ammonium toxicity and the real cost of transport. *Trends in Plant Science* **6,** 335–337.

Hesse PR. 1971. Soil phosphorus: its measurements and its uptake by plants. *Australian Journal of Soil Research* **35,** 227–239.

Ho CH, Lin SH, Hu HC, Tsay YF. 2009. CHL1 functions as a nitrate sensor in plants. *Cell* **138,** 1184–1194.

Hong CO, Chung DY, Lee do K, Kim PJ. 2010. Comparison of phosphate materials for immobilizing cadmium in soil. *Archives of Environmental Contamination Toxicology* **58,** 268–274.

Jalloh MA, Chen JH, Zhen FR, Zhang GP. 2009. Effect of different N fertilizer forms on antioxidant capacity and grain yield of rice growing under Cd stress. *Journal of Hazardous Materials* **162,** 1081–1085.

Jeong J, Guerinot ML. 2009. Homing in on iron homeostasis in plants. *Trends in Plant Science* **14,** 280–285.

Jin CW, Du ST, Chen WW, Li GX, Zhang YS, Zheng SJ. 2009. Elevated carbon dioxide improves plant Fe nutrition through enhancing the Fe-deficiency-induced responses under Fe-limited conditions in tomato. *Plant Physiology* **150,** 272–280.

Jin CW, Du ST, Shamsi IR, Luo BF, Lin XY. 2011. NO synthase-generated NO acts downstream of auxin in regulating Fe-deficiency-induced root branching that enhances Fe-deficiency tolerance in tomato plants. *Journal of Experimental Botany* **62,** 3875–3884.

Kovacs K, Kuzmann E, Vertes A, Levai L, Cseh E, Fodor F. 2010. Effect of cadmium on iron uptake in cucumber roots: a Mössbauer-spectroscopic study. *Plant and Soil* **327,** 49–56.

Li L, Cheng X, Ling HQ. 2004. Isolation and characterization of Fe(III)-chelate reductase gene *LeFRO1* in tomato. *Plant Molecular Biology* **54,** 125–136.

Ling HQ, Bauer P, Bereczky Z, Keller B, Ganal M. 2002. The tomato *fer* gene encoding a bHLH protein controls iron uptake responses in roots. *Proceedings of the National Academy of Sciences, USA* **99,** 13938–13943.

Ling HQ, Ganal MW. 2000. Towards map-based cloning of two genes involved in iron uptake of tomato. *Journal of Plant Nutrition* **23,** 1953–1967.

Lux A, Martinka M, Vaculík M, White PJ. 2011. Root responses to cadmium in the rhizosphere: a review. *Journal of Experimental Botany* **62,** 21–37.

Marschner H. 1995. *Mineral nutrition of higher plants*, 2nd edn. London: Academic Press.

Muños S, Cazettes C, Fizames C, Gaymard F, Tillard P, Lepetit M, Lejay L, Gojon A. 2004. Transcript profiling in the *chl1-5* mutant of Arabidopsis reveals a role of the nitrate transporter NRT1.1 in the regulation of another nitrate transporter, NRT2.1. *The Plant Cell* **16,** 2433–2447.

Pan K, Wang WX. 2011. Trace metal contamination in estuarine and coastal environments in China. *Science of Total Environment* doi:10.1016/j.scitotenv. 2011.03.013

Parker DR, Norvell WA, Chaney RL. 1995. GEOCHEM-PC: a chemical speciation program for IBM and compatible computers. In: Loeppert RH, Schwab AP, Goldberg S, eds. *Chemical equilibrium and reaction models*. Special Publication 42. Madison, WI: Soil Science Society of America, 253–269.

Ramirez L, Zabaleta EJ, Lamattina L. 2010. Nitric oxide and frataxin: two players contributing to maintain cellular iron homeostasis. *Annals of Botany* **105,** 801–810.

Robinson NJ, Procter CM, Connolly EL, Guerinot ML. 1999. A ferric-chelate reductase for iron uptake from soils. *Nature* **397,** 694–697.

Ronald E. 2000. *Handbook of chemical risk assessment: health hazards to humans, plants, and animals*. Boca Raton, FL: Lewis Publishers, 1–43.

Sarwar N, Saifullah, Malhi SS, Zia MH, Naeem A, Bibi S, Farid G. 2010. Role of mineral nutrition in minimizing cadmium accumulation by plants. *Journal of the Science of Food and Agriculture* **90,** 925–937.

Shaner DL, Boyer JS. 1976. Nitrate reductase activity in maize (*Zea mays* L.) leaves. I. Regulation by nitrate flux. *Plant Physiology* **58,** 499–504.

Shapiro AD. 2005. Nitric oxide signaling in plants. *Vitamins and Hormones* **72,** 339–398.

Tian QY, Sun P, Zhang WH. 2005. Ethylene is involved in nitrate-dependent root growth and branching in *Arabidopsis thaliana*. *New Phytologist* **184,** 918–931.

Tsadilasa CD, Karaivazogloub NA, Tsotsolisb NC, Stamatiadisc S, Samarasa V. 2005. Cadmium uptake by tobacco as affected by liming, N form, and year of cultivation. *Environmental Pollution* **134,** 239–246.

Verbruggen N, Christian H, Schat H. 2009. Moleclar mechanisms of metal hyperaccumulation in plants. *New Phytologist* **181,** 759–776.

Vert G, Grotz N, Dédaldéchamp F, Gaymard F, Guerinot ML, Briat JF, Curie C. 2002. IRT1, an Arabidopsis transporter essential for iron uptake from the soil and plant growth. *The Plant Cell* **14,** 1223–1233.

Vollbrecht P, Kasemir HI. 1992. Effects of exogenously supplied ammonium on root development of Scots Pine (*Pinus sylvestris* L.) seedlings. *Botanica Acta* **105,** 306–312.

Walch-Liu P, Filleur S, Gan Y, Forde BG. 2005. Signaling mechanisms integrating root and shoot responses to changes in the nitrogen supply. *Photosynthesis Research* **83,** 239–250.

Wang HY, Garvin DF, Kochian LV. 2001. Nitrate-induced genes in tomato roots. Array analysis reveals novel genes that may play a role in nitrogen nutrition. *Plant Physiology* **127,** 345–359.

Xie HL, Jiang RF, Zhang FS, McGrath SP, Zhao FJ. 2009. Effect of nitrogen form on the rhizosphere dynamics and uptake of cadmium and zinc by the hyperaccumulator. *Thlaspi caerulescens. Plant and Soil* **318,** 205–221.

Yi Y, Guerinot ML. 1996. Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. *The Plant Journal* **10,** 835–844.

Zaccheo P, Crippa L, Pasta VD. 2006. Ammonium nutrition as a strategy for cadmium mobilisation in the rhizosphere of sunflower. *Plant and Soil* **283,** 43–56.