

RESEARCH PAPER

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

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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 (T3238fer) plants were grown in pH-buffered hydroponic culture to investigate the direct effect of N-form on Cd uptake. Wild-type plants fed  $\text{NO}_3^-$  accumulated more Cd than plants fed  $\text{NH}_4^+$ . Iron uptake and *LeIRT1* expression in roots were also greater in plants fed  $\text{NO}_3^-$ . However, in mutant T3238fer which loses FER function, *LeIRT1* expression in roots was almost completely terminated, and the difference between  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$ -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  $\text{Cd}^{2+}$  (Hong *et al.*, 2010), while ferrous iron ( $\text{Fe}^{2+}$ ) competes with  $\text{Cd}^{2+}$  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 ( $\text{NO}_3^-$ ) is taken up by plants, there is a simultaneous uptake of protons ( $\text{H}^+$ ), resulting in an increase in rhizosphere pH. Conversely, when ammonium ( $\text{NH}_4^+$ ) is taken up, the  $\text{H}^+$  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  $\text{NH}_4^+$  fertilizers could result in enhanced Cd uptake due to a decrease in soil pH, compared with the  $\text{NO}_3^-$  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  $\text{NH}_4^+$  application clearly lowered rhizosphere pH and significantly increased Cd accumulation in sunflower plants, compared with  $\text{NO}_3^-$  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 caerulescens* plants fed  $\text{NO}_3^-$  accumulated much more Cd than the plants supplied with  $\text{NH}_4^+$ , even though the solution pH was lower in plants treated with  $\text{NH}_4^+$ . In a soil cultivation experiment, Jalloh *et al.* (2009) also observed that the rice plants fed  $\text{NO}_3^-$  had a higher Cd concentration than the plants fed  $\text{NH}_4^+$ . 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,  $\text{NO}_3^-$  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,  $\text{NO}_3^-$  has also been implicated in regulating the uptake of many nutrients. For instance, resupplying  $\text{NO}_3^-$  to tomato plants rapidly up-regulated expression of the  $\text{NH}_4^+$  transporter *LeAMT2*, the Pi transporter *LePT2*, and *Kdcl1* (a homologue of a carrot  $\text{K}^+$  channel) (Wang *et al.*, 2001). In addition, the *Arabidopsis chll-5* mutant, which is deficient for the *NRT1.1*  $\text{NO}_3^-$  transporter, displays low  $\text{NO}_3^-$  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 Gueriot, 2009). Interestingly, several studies provide strong evidence that the iron transporter *IRT1* is also primarily responsible for  $\text{Cd}^{2+}$  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  $\text{NO}_3^-$  in regulating *IRT1* led us to hypothesize that  $\text{NO}_3^-$  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  $\text{NO}_3^-$  application directly enhances Cd uptake of plants, compared with  $\text{NH}_4^+$  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 ( $\text{N}^{\text{G}}$ -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  $\mu\text{M}$ ):  $\text{NaH}_2\text{PO}_4$ , 750;  $\text{MgSO}_4$ , 500;  $\text{K}_2\text{SO}_4$ , 375;  $\text{KNO}_3$ , 750;  $(\text{NH}_4)_2\text{SO}_4$ , 375;  $\text{CaCl}_2$ , 1000;  $\text{H}_3\text{BO}_3$ , 10;  $\text{MnSO}_4$ , 0.5;  $\text{ZnSO}_4$ , 0.5;  $\text{CuSO}_4$ , 0.1;  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ , 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  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . After 12 d of growth in the nutrient solution, plants were subjected to different N-form treatments. For the treatment of  $\text{NO}_3^-$  as the sole nitrogen source, 1.5 mM  $\text{KNO}_3$  was applied to the solution. For the treatment of  $\text{NH}_4^+$  as the sole N source, 0.75 mM  $(\text{NH}_4)_2\text{SO}_4$  and 0.75 mM  $\text{K}_2\text{SO}_4$  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  $\mu\text{M}$  Cd, added as  $\text{CdCl}_2$ . For the experiments illustrated in Fig. 5, the Fe uptake-inefficient mutant, *T3238fer*, 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  $\text{NO}_3^-/\text{NH}_4^+$  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'-GCAAGACACCA-GAAATCCTAC-3'; rev: 5'-ATCAGATGGGTTGGGCTT-3'; *LeIRT1* fw, 5'-AGCACTTGGGATAGCATTG-3'; rev, 5'-ACTGACATC 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  $\alpha$ -tubulin housekeeping gene primers were used for a control in the PCR: fw: 5'-CCTGAACAACCTCATAAGTGCC-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  $\mu\text{M}$  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  $\text{NH}_4^+$ .

#### Analysis of elements' content

The dried root and shoot samples were wet digested in the concentrated  $\text{HNO}_3/\text{HCl}$  at 120  $^\circ\text{C}$  until there was no brown nitrogen oxide gas emitting, then further digested with  $\text{HClO}_4$  at 180  $^\circ\text{C}$  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

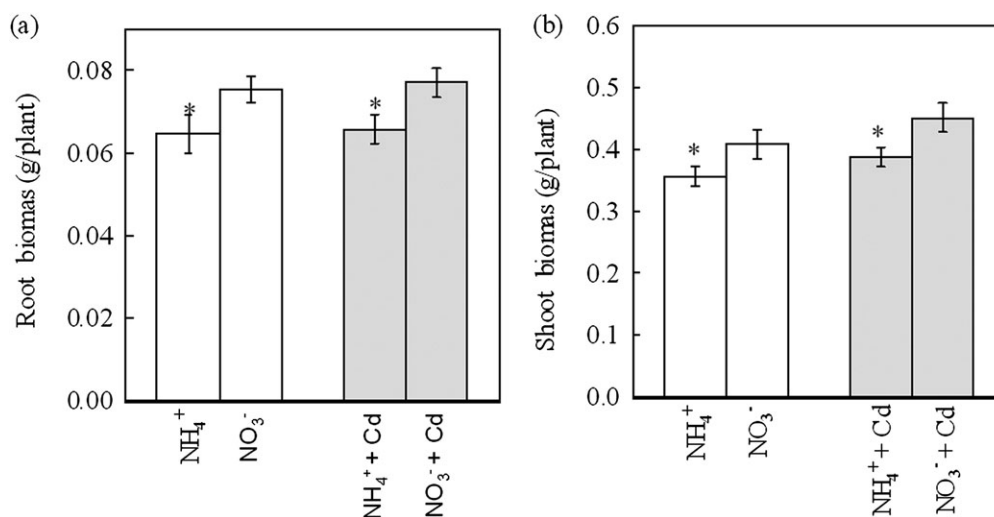
As discussed above, N-form may have a direct effect on Cd uptake in plant roots besides the indirect effect of altering rhizosphere pH. Distinguishing the 'N-form effect' from the 'pH effect' is important for understanding the mechanism of how the N-form affects Cd accumulation in plants. In this

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  $\text{NO}_3^-$  had a 16% greater root biomass and 17% greater shoot biomass than the plants fed  $\text{NH}_4^+$ . 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  $\text{NO}_3^-$  treatment contained 83% and 85% higher Cd concentrations, respectively, than those from  $\text{NH}_4^+$  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  $\text{NO}_3^-$  had about 2-fold higher CAPR than the plants grown with  $\text{NH}_4^+$ , indicating that  $\text{NO}_3^-$  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  $\text{NO}_3^-$  treatment was increased by 68% compared with those from the  $\text{NH}_4^+$  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  $\text{NO}_3^-$  treatments were slightly lower than those from  $\text{NH}_4^+$  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  $\text{NO}_3^-$  treatment was 31% higher than that in the  $\text{NH}_4^+$  treatment. Interestingly, in Cd-added growth solutions, this  $\text{NO}_3^-$ -enhanced FAPR was further strengthened, in some cases by up to 90%, compared with

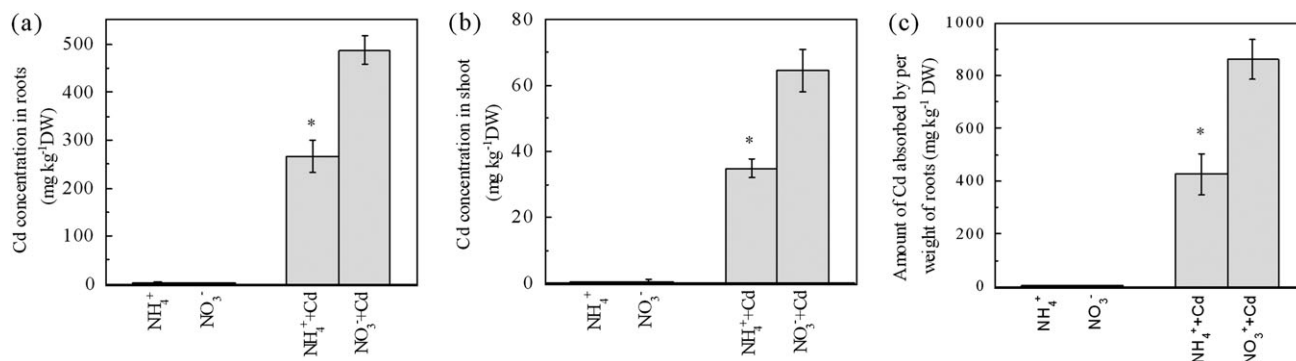


**Fig. 1.** Effect of N-form on growth of Micro-Tom tomato plants under Cd-free or Cd-exposed condition. (a) The root biomass. (b) The shoot biomass. The plants were pre-cultured in the growth solution contained both  $\text{NO}_3^-$  and  $\text{NH}_4^+$  for 12 d and were then transferred to Cd-free or 2  $\mu\text{M}$  Cd-added growth solutions with either  $\text{NO}_3^-$  or  $\text{NH}_4^+$  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  $\pm$  SD ( $n=4$ ). \* Significant differences ( $P < 0.05$ ) between  $\text{NO}_3^-$  and  $\text{NH}_4^+$  treatments.

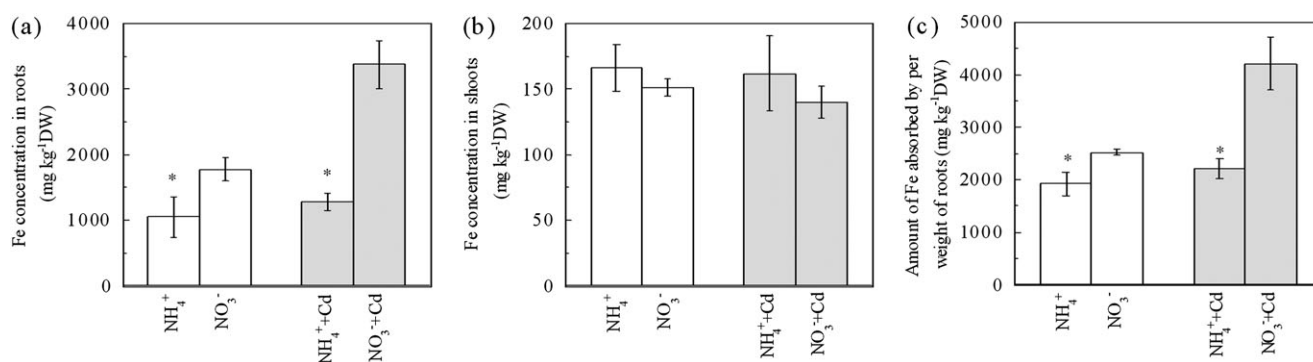
the  $\text{NH}_4^+$  treatment. These results suggest that  $\text{NO}_3^-$  also facilitates Fe uptake in roots, particularly with Cd exposure.

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

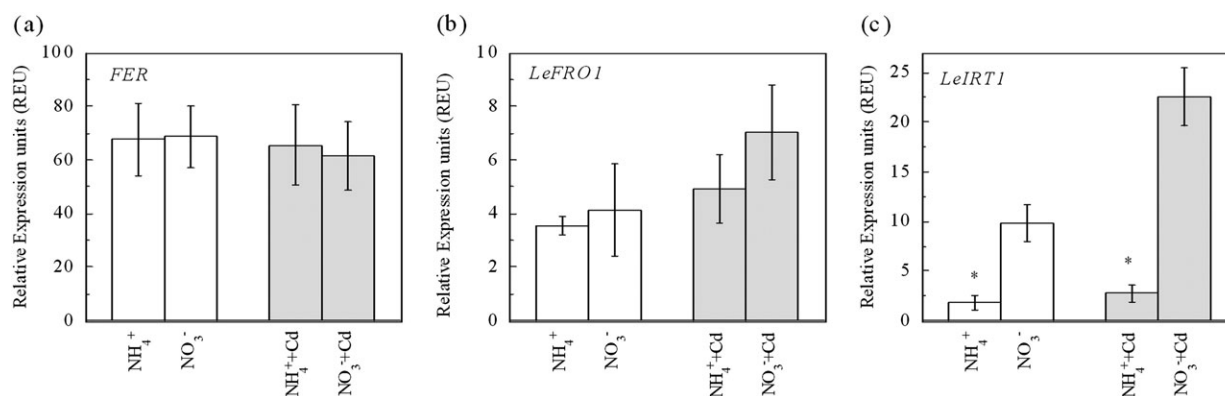
levels by the FER protein (Ling *et al.*, 2002; Berezcky *et al.*, 2003; Li *et al.*, 2004). It was found here that the expressions of *FER* and *LeFRO1* in roots was not affected or only slightly affected by N-form (Fig. 4a, b). Interestingly, expressions of *LeIRT1* were strongly affected by the N-form. In Cd-free growth solutions, the  $\text{NO}_3^-$  treatment had a 4.5-fold higher *LeIRT1* expression than the  $\text{NH}_4^+$  treatment, while in Cd-added growth solutions the  $\text{NO}_3^-$



**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  $\pm$ SD ( $n=4$ ). \* Significant differences ( $P < 0.05$ ) between  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\pm$ SD ( $n=4$ ). \* Significant differences ( $P < 0.05$ ) between  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\pm$ SD ( $n=7$ ). \* Significant differences ( $P < 0.05$ ) between  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$  conditions.

#### Effect of *FER* mutation on $\text{NO}_3^-$ -enhanced Cd uptake

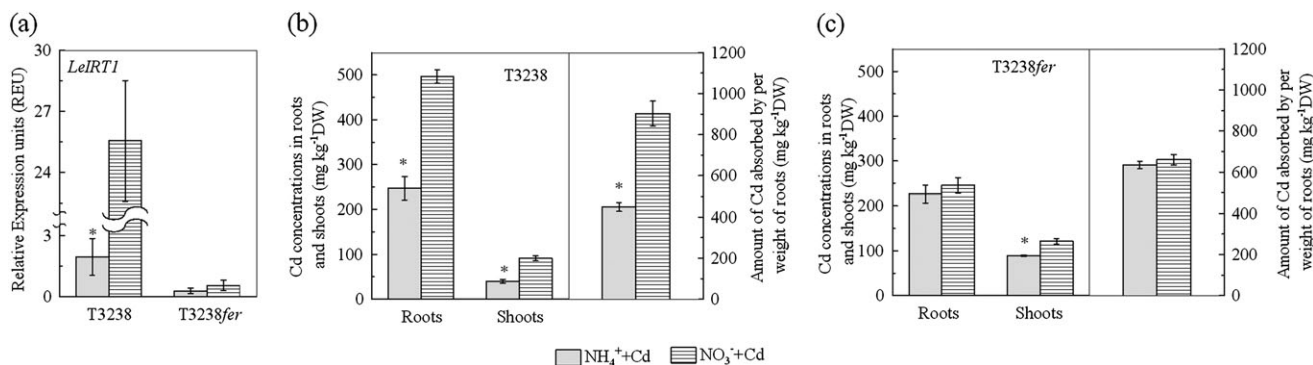
Loss of *FER* function in T3238*fer* tomato mutants leads to failure of Fe deficiency responses, including the expression of *LeIRT1* (Ling *et al.*, 2002). Therefore, the mutant, T3238*fer*, and its wild type, T3238, were used to investigate the role of Fe uptake systems in  $\text{NO}_3^-$ -facilitated Cd uptake. In Cd-added growth solutions, the expression of *LeIRT1* in roots of T3238 was significantly higher in  $\text{NO}_3^-$  treatments than in  $\text{NH}_4^+$  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  $\text{NO}_3^-$  treatment than in the  $\text{NH}_4^+$  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  $\text{NO}_3^-$  treatment than in the  $\text{NH}_4^+$  treatment, but the difference between them was far less than that in T3238. For T3238*fer*, shoot Cd concentration after  $\text{NO}_3^-$  treatment increased by 37% compared with the  $\text{NH}_4^+$  treatment, whereas for T3238, concentration was increased 128% (Fig. 5b, c). The CAPR in roots of T3238 was significantly higher in the  $\text{NO}_3^-$  treatment than in the  $\text{NH}_4^+$  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

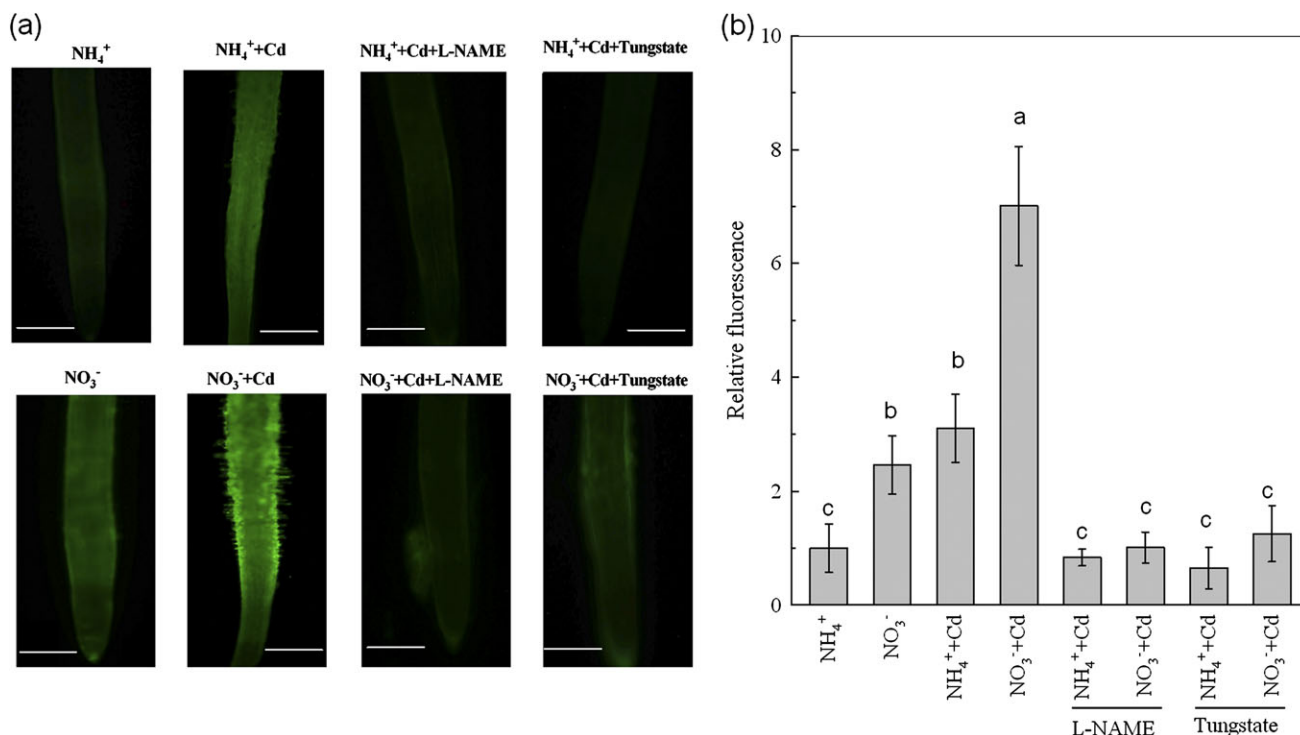
the Fe uptake system is required for  $\text{NO}_3^-$  facilitation of Cd uptake in wild-type plants.

#### Effect of NO synthesis inhibition on $\text{NO}_3^-$ -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  $\text{NO}_3^-$ -enhanced Cd uptake. Interestingly,  $\text{NO}_3^-$  treatment resulted in a higher NO-associated green fluorescence in roots than did the  $\text{NH}_4^+$  treatment (Fig. 6a). By quantifying the signal intensities of fluorescence, the NO contents in roots of the plants fed  $\text{NO}_3^-$  were increased by more than 2-fold compared with those of plants fed  $\text{NH}_4^+$  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  $\text{NO}_3^-$ -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  $\text{NO}_3^-$ -treated roots, which was even lower than in the  $\text{NH}_4^+$ -treated roots (Fig. 7b). For shoot Cd concentrations, although they were significantly reduced by either inhibitor in both N-form treatments, the  $\text{NO}_3^-$  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  $\text{NO}_3^-$  treatment had only 41% or 33% higher CAPR, respectively, than the  $\text{NH}_4^+$  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  $\mu\text{M}$  Cd-added growth solutions with either  $\text{NO}_3^-$  or  $\text{NH}_4^+$  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  $\pm$ SD ( $n=4$ ). \* Significant differences ( $P < 0.05$ ) between  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\mu\text{M}$  Cd-added growth solutions with either  $\text{NO}_3^-$  or  $\text{NH}_4^+$  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  $\pm$ SD ( $n=15$ ). Different letters indicate significant differences ( $P < 0.05$ ) among the treatments.

neither L-NAME nor tungstate, the  $\text{NO}_3^-$  treatment had about 100% higher CAPR than the  $\text{NH}_4^+$  treatment. These results suggest that inhibition of NO synthesis could diminish the difference in Cd uptake between the two N-form treatments.

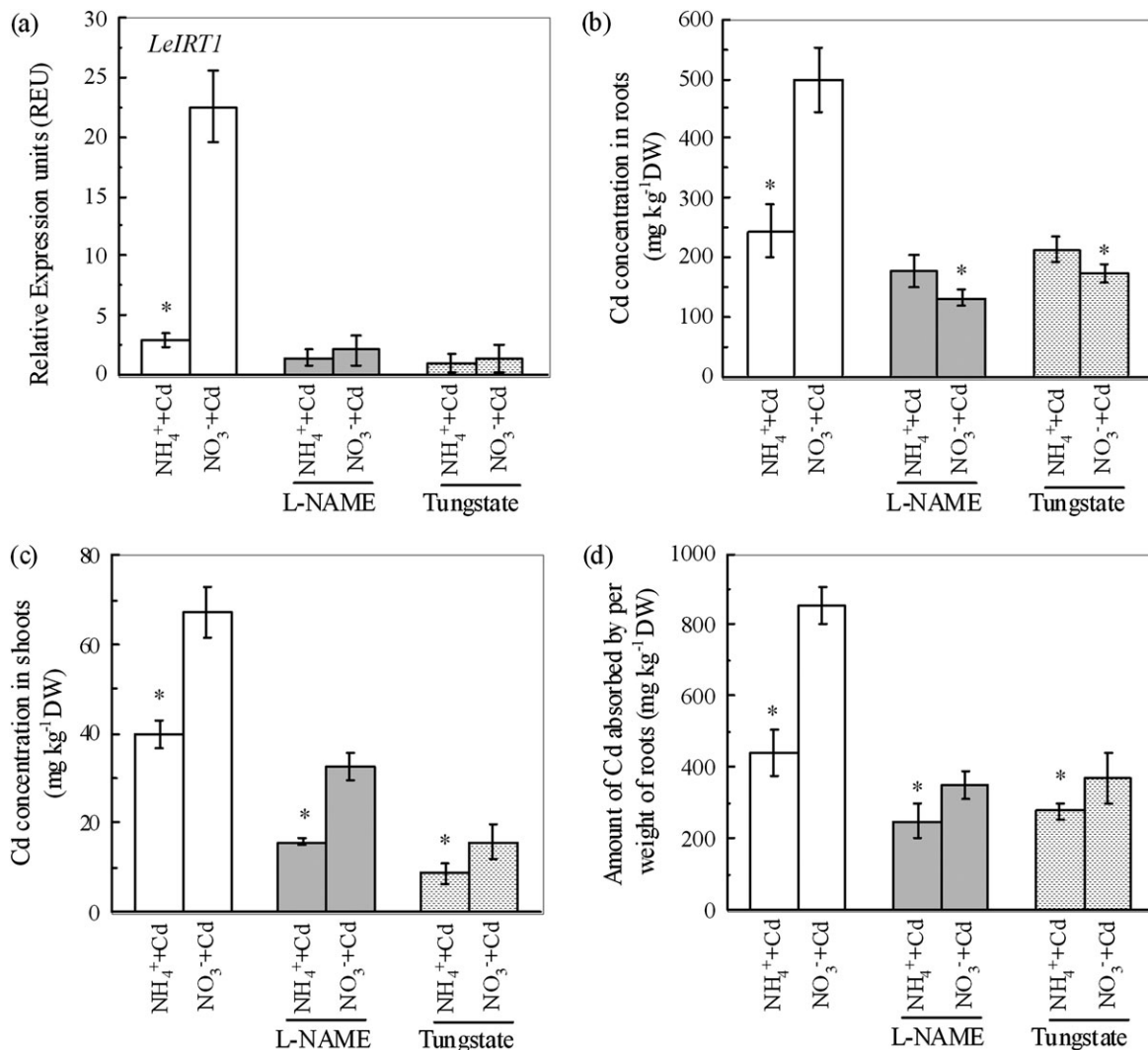
## Discussion

### *Nitrate has a direct effect on enhancing Cd uptake*

In the pH-buffered growth solutions, it was observed that  $\text{NO}_3^-$  nutrition facilitates Cd uptake in roots compared with  $\text{NH}_4^+$  nutrition (Fig. 2). The Cd availability in nutrient solutions may be unintentionally altered due to N-form treatments. However, the computer modelling by GEO-CHEM-PC (Parker *et al.*, 1995) showed that the composition of Cd species in nutrient solutions were similar between  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$ -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  $\text{NH}_4^+$  may have deleterious effects on plants when used as the sole N source for plant growth. Acidification of the rhizosphere due to  $\text{NH}_4^+$  uptake is often considered to be a fundamental cause of  $\text{NH}_4^+$  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  $\text{NH}_4^+$  treatment. The biomass for the  $\text{NH}_4^+$  treatment was only slightly less than the  $\text{NO}_3^-$  treatment (Fig. 1). Furthermore, it was observed that the concentrations of P in both shoots and roots were higher in the plants fed  $\text{NH}_4^+$  than in the plants fed  $\text{NO}_3^-$  (see Supplementary Fig. S1 at *JXB* online). These results indicate that the  $\text{NH}_4^+$  treatment in pH-buffered solutions did not impair the nutrient uptake systems. Therefore, it is reasonable to conclude that  $\text{NO}_3^-$  nutrition facilitates Cd uptake in roots and that the lower Cd uptake in  $\text{NH}_4^+$  treatment is not due to deleterious effects induced by  $\text{NH}_4^+$  uptake.

In contrast to our results, it has been observed that  $\text{NH}_4^+$  nutrition facilitates Cd accumulation in soil-grown winter rape (*Brassica napus* L.) and tobacco (*Nicotiana tabacum* L.) plants more so than  $\text{NO}_3^-$  nutrition (Eriksson, 1990; Tsadilasa *et al.*, 2005). The reason for these conflicting results may be because  $\text{NH}_4^+$  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  $\pm$ SD ( $n=4$ ). \* Significant differences ( $P < 0.05$ ) between  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NH}_4^+$  may be more predominant than the direct effect of  $\text{NO}_3^-$  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 $\text{NO}_3^-$ -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  $\text{NO}_3^-$  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  $\text{Cd}^{2+}$  (Vert *et al.*, 2002; Verbruggen *et al.*, 2009; Lux *et al.*, 2011). The linkage between Fe uptake and  $\text{NO}_3^-$ -enhanced Cd uptake was therefore analysed. It was observed here that  $\text{NO}_3^-$  treatment could also facilitate  $\text{NO}_3^-$  Fe uptake in the roots compared with the  $\text{NH}_4^+$  treatment (Fig. 3). Furthermore, although the expression of *LeFROI* in roots undergoing  $\text{NO}_3^-$  treatment was only increased slightly, the expression of *LeIRT1*  $\text{NO}_3^-$  treatment was greatly increased compared with the  $\text{NH}_4^+$  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 *LeFROI* 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  $\text{NO}_3^-$  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 Ganai, 2000). It was found here that the expressions of *LeIRT1* in the Fe uptake-inefficient mutant T3238*fer* were similar between the  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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  $\text{NO}_3^-$  treatment than in the  $\text{NH}_4^+$  treatment (Fig. 5b, c). These results combined with the finding that both Fe uptake and *LeIRT1* expression were increased by  $\text{NO}_3^-$  (Figs 3, 4b), indicate that the system involved in Fe uptake is required for the enhancement of Cd uptake by  $\text{NO}_3^-$  in tomato plants. Although loss of FER function resulted in the inhibition of the  $\text{NO}_3^-$ -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  $\text{NO}_3^-$ -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  $\text{NO}_3^-$  were observed, which diminished the difference in Cd uptake between  $\text{NO}_3^-$  and  $\text{NH}_4^+$  treatments (Fig. 7). The results provide more evidence for our above conclusion that the Fe uptake system is required for  $\text{NO}_3^-$  induction of Cd uptake. Interestingly, it was also observed here that  $\text{NO}_3^-$  treatment resulted in a higher NO level in roots than did the  $\text{NH}_4^+$  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  $\text{NH}_4^+$  is an inhibitor of NR (Jin *et al.*, 2011). Accordingly, a higher NO level in roots of  $\text{NO}_3^-$  treatment is probably due to activation of NR activity by  $\text{NO}_3^-$ . This viewpoint, combined with the fact that NO is a signal controlling the Fe

uptake system in roots, allowed us to propose that  $\text{NO}_3^-$ -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  $\text{NO}_3^-$  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  $\text{NO}_3^-$  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  $\text{NO}_3^-$ -induced NO production may, in turn, facilitate  $\text{NO}_3^-$  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  $\text{NO}_3^-$ - 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  $\text{NO}_3^-$  nutrition facilitating Cd uptake in roots compared with  $\text{NH}_4^+$  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  $\text{NO}_3^-$  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  $\text{NO}_3^-$ -based fertilizers result in more Cd accumulation in plants than  $\text{NH}_4^+$ -based fertilizers in many cases, even though  $\text{NO}_3^-$ -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  $\text{NO}_3^-$  and  $\text{NH}_4^+$  media.

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