Journal of Experimental Botany, Vol. 70, No. 21 pp. 6363–6374, 2019
doi:10.1093/jxb/erz374 Advance Access Publication August 15, 2019
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RESEARCH PAPER

Nitrate transporter 1.1 alleviates lead toxicity in Arabidopsis by preventing rhizosphere acidification

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Received 16 April 2019; Editorial decision 4 August 2019; Accepted 5 August 2019

Editor: Hideki Takahashi, Michigan State University, USA

Abstract

Identification of the mechanisms that control lead (Pb) concentration in plants is a prerequisite for minimizing dietary uptake of Pb from contaminated crops. This study examines how nitrate uptake by roots affects Pb uptake and reveals a new resistance strategy for plants to cope with Pb contamination. We investigated the interaction between nitrate transporter (NRT)-mediated NO_3^- uptake and exposure to Pb in Arabidopsis using NRT-related mutants. Exposure to Pb specifically stimulated NRT1.1-mediated nitrate uptake. Loss of function of *NRT1.1* in *nrt1.1*-knockout mutants resulted in greater Pb toxicity and higher Pb accumulation in nitrate-sufficient growth medium, whereas no difference was seen between wild-type plants and null-mutants for *NRT1.2*, *NRT2.1*, *NRT2.2*, *NRT2.4*, and *NRT2.5*. These results indicate that only NRT1.1-mediated NO_3^- uptake alleviated Pb toxicity in the plants. Further examination indicated that rhizosphere acidification, which favors Pb entry to roots by increasing its availability, is prevented when *NRT1.1* is functional and both NO_3^- and NH_4^+ are present in the medium.

Keywords: Lead, nitrate, nitrate transporters, Pb resistance, pH, phytoremediation.

Introduction

Consumption of lead (Pb) can result in a variety of health-related ailments ranging from learning disabilities to cardiovascular disease (Chiodo *et al.*, 2007; Navas-Acien *et al.*, 2008). Crops can take up Pb from the soil and, under certain conditions, high levels can accumulate in the edible parts of crops (Jin *et al.*, 2005a, 2005b). The contamination of soil by Pb has continuously increased over recent decades as a result of various anthropogenic activities, such as waste disposal and the use of sewage water for irrigation (Clemens, 2006; Pourrut *et al.*, 2011). Consequently, minimizing Pb in soil, particularly in an agricultural context, is a subject of increasing concern for researchers across a number of different scientific disciplines. Using plant-based technology to remove Pb from contaminated soils (phytoremediation) has gained a great deal of attention because of its low costs and environmental friendliness (Tangahu *et al.*, 2011; Selamat *et al.*, 2014). However, there are few natural hyperaccumulator plants for Pb and most of those that have been reported grow slowly and have small biomass, which limits their efficiency in cleaning contaminated soil (Ernst, 2000; van der Ent *et al.*, 2013). Using a biological engineering approach to increase Pb accumulation in non-food crops with rapid growth and large biomass may be an alternative strategy to hyperaccumulating plants. A clear understanding of the factors that affect Pb uptake by plants is a prerequisite for ensuring that such a strategy is successful.

The amount of Pb taken up by plant roots is closely associated with its bioavailability in the growth medium (Kopittke *et al.*, 2008; Uzu *et al.*, 2009). The bioavailability of Pb in soil is affected by numerous factors including the cation exchange capacity (CEC), texture, redox potential, clay mineralogy, organic content, and pH of the soil, and also by the levels of other elements that are present (i.e. phosphorus and sulfur) (Jin *et al.*, 2005b). Among these factors, soil acidity has been recognized as one of the most important (Fischer *et al.*, 2014), because as pH decreases, the solubility of Pb increases in the form of free or solvated ions and ion pairs. Nutrient management, particularly nitrogen fertilization, significantly affects the pH of soil (Thomson *et al.*, 1993). Nitrogen is taken up by plants mainly in the form of ammonium (NH_4^+) and nitrates (NO_3^-). Physiologically, when NO_3^- is taken up by plants, there is a simultaneous uptake of protons (H^+) resulting in an increase in the pH of the rhizosphere. Conversely, when NH_4^+ is taken up, H^+ is released into the rhizosphere, resulting in a decrease in the pH (Marschner, 1995). In this context, the uptake of NO_3^- by roots may decrease the amount of Pb^{2+} acquired by plants by consumption of rhizospheric H^+, which thus lowers the level of soluble Pb^{2+}, whereas the opposite may be true for uptake of NH_4^+.

Owing to the fact that Pb is a non-essential element for plants, its entry into root cells may rely on transporters/channels for various bivalent nutrient cations (Pourrut *et al.*, 2011). In addition to H^+, our previous studies have shown that decreased uptake of the anion NO_3^- by roots is accompanied by decreased uptake of several cations, including Ca^{2+}, K^+, Fe^{2+}, and Cd^{2+} (Luo *et al.*, 2012; Mao *et al.*, 2014), indicating that the uptake of NO_3^- may be non-specifically coupled with the uptake of cations. Hence, the uptake of nitrate may favor the uptake of Pb^{2+}. However, this is in contrast with the effect noted above that nitrate uptake may decrease Pb bioavailability in the rhizosphere. Thus, it is not clear how NO_3^- uptake by roots affects Pb^{2+} uptake. The pathways of NO_3^- uptake by plant roots are complex. It has been reported that six NO_3^- transporters (NRTs) are involved in uptake in Arabidopsis (Wang *et al.*, 2012, 2018; Léran *et al.*, 2014). NRT1.1 is one of the most common NRTs with dual-affinity, being involved in both high- and low-affinity uptake (Liu *et al.*, 1999; Ye *et al.*, 2019), NRT1.2 is a low-affinity transporter (Huang *et al.*, 1999), whilst NRT2.1, NRT2.2, NRT2.4, and NRT2.5 only take part in high-affinity uptake (Cerezo *et al.*, 2001; Li *et al.*, 2007; Kiba *et al.*, 2012; Lezhneva *et al.*, 2014). These NRTs often show different responses to various stressors (Krapp *et al.*, 2011; Mao *et al.*, 2014; Fang *et al.*, 2016). Given the presumed association between NO_3^- and Pb^{2+} uptake, it is clearly important to determine whether the activities of the different NRTs affect the uptake of Pb.

In the present study, we investigated the interaction between NRT-mediated NO_3^- uptake and Pb^{2+} exposure in Arabidopsis. Our results revealed a new mechanism by which the plants responded to Pb^{2+} stress: exposure to Pb specifically induced NRT1.1-mediated NO_3^- uptake, which consequently prevented acidification of the rhizosphere and thus decreased entry of Pb into the plants by decreasing its bioavailability. Our findings may provide a new strategy for manipulating Pb levels in plants, such as improving the efficiency of removal of Pb from contaminated soils and minimizing the accumulation of Pb in food crops.

Materials and methods

Plant material

The following *Arabidopsis thaliana* plants were used in this study: the mutants *chl1-5* (Huang *et al.*, 1996), *nrt1.1-1* (salk_097431), *nrt1.2* (cs859605), *nrt2.1* (salk_141712), *nrt2.2* (salk_043543), *nrt2.5* (GK 213H10), *nlp7-2* (cs868891), and *chl1-9*, the double-mutant *nrt2.1 nrt2.2* (cs859604), the triple-mutants *abi1 hab1 abi2* and *abi1 hab1 pp2ca*, together with *pNRT1.1::NRT1.1-GFP* transgenic plants with a Columbia (Col-0) background, and also the mutants *chl1-6* (cs6154) and *nrt2.4* (cs27332) with a Landsberg *erecta* (Ler) background. Seeds of *chl1-5* and the *pNRT1.1::NRT1.1-GFP* transgenic plants were obtained from Dr Philippe Nacry (Biochimie et Physiologie Moléculaire des Plantes, Montpellier, France), seeds of *chl1-9* were obtained from Dr Yi-Fang Tsay (Institute of Molecular Biology, Academia Sinica, Taiwan), and seeds of the *abi1 hab1 abi2* and *abi1 hab1 pp2ca* triple-mutants were obtained from Dr Jian-Kang Zhu (Purdue University, USA). The insertions in these lines were verified using the primers listed in Supplementary Table S1 at *JXB* online.

Cultivation conditions

The experiments were performed on plants grown on agar medium in sterile square Petri dishes (10 × 10 cm). Briefly, the seeds were surface-sterilized and sown on basal agar medium containing 1% sucrose (w/v). The nutrient composition of the basal agar medium had a NO_3^-/NH_4^+ ratio of 3:1 and was as follows: 750 μM NaH_2PO_4, 500 μM $MgSO_4$, 375 μM K_2SO_4, 2.25 mM KNO_3, 375 μM $(NH_4)_2SO_4$, 1 mM $CaCl_2$, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.1 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, and 25 μM Fe-EDTA. Because *nrt1.1* mutants are hypersensitive to low pH (Fang *et al.*, 2016), the agar medium was adjusted to pH 6.5. For treatment with Pb, 4-d-old seedlings were transferred to Petri dishes with fresh basal medium with or without 300 μM lead acetate, $Pb(CH_3COO)_2$. Each dish contained 15 seedlings. To examine the effects of N source on the response to Pb^{2+}, we used basal medium adjusted to contain different NO_3^-:NH_4^+ ratios. The total N concentration fixed at 3.0 mM and the ratio was adjusted using KNO_3 and $(NH_4)_2SO_4$. The resulting differences in K concentrations were balanced by adjusting the K_2SO_4 concentration.

Measurement of Pb^{2+} concentrations

After 7 d of Pb^{2+} treatment, the seedlings were washed with pure water, divided into shoots and roots, and dried at 70 °C for 48 h. The dried samples were then wet-digested as previously described (Guan *et al.*, 2018), and diluted with ultrapure water. The concentrations of lead were analysed using a Microwave Plasma-Atomic Emission Spectrometer (4210 MP-AES, Agilent Technologies). Four biological replicates per treatment were analysed, each of which consisted of root or shoot tissues from 90 seedlings.

GFP expression analysis

Seedlings of the *pNRT1.1::NRT1.1-GFP* (green fluorescent protein) transgenic plants were cultured on complete nutrient agar medium with or without 300 μM $Pb(CH_3COO)_2$ for 3 d. The distribution and intensity of the green fluorescence in their roots were then observed under a microscope (Eclipse NI; Nikon) and imaged using a camera attached to the microscope. Four replicate seedlings per treatment were analysed.

Measurement of NO_3^- uptake

The net fluxes of NO_3^- were measured along the root axis in the meristematic, elongation, and maturation zones using a non-invasive

microelectrode ion flux measurement system (SIET IPA-2, Applicable Electronics, Inc., Forestdale, MA, USA) , which can directly record transmembrane ion influx and efflux in a non-contact manner by detecting the diffusion potentials outside of the membrane (Shabala and Newman, 1997). Seedlings were pre-cultured in complete nutrient medium with or without 300 μM $Pb(CH_3COO)_2$ for 3 d, and were then transferred to 5-cm diameter plates with a nutrient solution containing 750 μM NaH_2PO_4, 500 μM $MgSO_4$, 375 μM K_2SO_4, 2.25 mM KNO_3, 375 μM $(NH_4)_2SO_4$, 1000 μM $CaCl_2$, and 0.05% MES buffer (w/v), with a pH of 5.8. using NMT The NO_3^- fluxes of the roots were measured The flux of NO_3^- in each section of the roots was then measured for 30 min using an electrode as described by Hawkins *et al.* (2008). Six replicate seedlings per treatment were analysed.

Measurement of relative expression of NRT genes

Root samples of ~100 mg (FW) were collected and frozen in liquid nitrogen. Total RNA was extracted using RNAisoPlus (Takara) and was then used to synthesize first-strand cDNA using a PrimeScript RT reagent kit (Takara). The cDNA was mixed with an SYBR Green RT-PCR kit (Takara) and the corresponding primers in a 25-μl reaction system; the primers are listed in Supplementary Table S1. All quantitative reverse-transcription (qRT-)PCR analyses were performed in an Opticon 2 Real-Time PCR System (MJ Research, MA, USA). Relative transcript levels were determined and corrected with efficiency calculations as described in Fang *et al.* (2016), and normalizeding to the geometric mean of expression of *UBQ10* and *EF1α* for each sample (Vandesompele *et al.*, 2002). Four biological replicates per treatment were analysed, each of which consisted of 75–90 seedlings.

Determination of lipid peroxidation

We measured malondialdehyde (MDA), a product of lipid peroxidation, according to the method described by Zhang *et al.* (2016). Samples of ~0.1 g of root or shoot tissue were frozen in liquid nitrogen and homogenized in 1.5 ml of 10% trichloroacetic acid (TCA) at 4 °C. After centrifugation at 15 000 *g* for 10 min, the supernatant was collected and incubated with 1 ml of 0.6% thiobarbituric acid (TBA) (dissolved in 10% TCA) at 100 °C for 20 min. The absorbance was measured at 440, 532, and 600 nm using an ELISA (SpectraMax 13X, Melceemlar Devices, USA). A total of 4–5 biological replicates per treatment were analysed, each of which consisted of 45–90 seedlings.

Determination of pH in the agar rooting medium

The pH of the agar rooting medium was determined based on the method described by Hachiya *et al.* (2012). The rooting medium was collected into a 50-ml centrifuge tube, which was frozen at –20 °C overnight and thawed at room temperature to free the aqueous phase from the agar. The mixture was then filtered at room temperature and the pH of the supernatant was determined using a desktop pH electrode (Sartorius). Measurements were taken on agar from five replicate plates per treatment, each of which contained 15 seedlings.

Results

Pb^{2+} stress stimulates NO_3^- uptake by roots

Abiotic stress often negatively affects NO_3^- uptake by the roots (Mao *et al.*, 2014; Bai *et al.*, 2017). In the present study, we first evaluated the effect of Pb^{2+} exposure on NO_3^- uptake in the roots of Arabidopsis Columbia-0 (Col-0) using a non-invasive technique. The transmembrane NO_3^- fluxes of the roots were measured along the root axis in the meristematic, elongation, and maturation zones. Interestingly, although Pb^{2+} is a toxic metal for plants, 3 d of exposure to 300 μM Pb^{2+} in the agar medium increased the rate of net NO_3^- influx in all zones (Fig. 1). The net NO_3^- influx in the mature root zone was greater than that in the other two zones, and exposure to Pb^{2+} resulted in about twice the uptake of NO_3^- compared to the controls.

To investigate the molecular basis underlying the stimulation of root NO_3^- uptake in response to Pb^{2+} exposure, we examined the expression of six NRT genes that are involved in NO_3^- uptake by roots. We found that only the expression of *NRT1.1* was significantly increased after 3 d of 300 μM Pb^{2+} exposure (Fig. 2A) whilst the expression of the other five genes, *NRT1.2*, *NRT2.1*, *NRT2.2*, *NRT2.4*, and *NRT2.5* were not significantly affected (Fig. 2B–E). We then examined the time-course of *NRT1.1* expression in response to exposure to Pb^{2+} and found that there was limited induction during the first 12 h of exposure, but after that it increased significantly (Supplementary Fig. S1). These results indicated that the stimulation of NO_3^- uptake by Pb^{2+} may have been mediated by NRT1.1. Consistent with this, GFP fluorescence was observed in the roots of *pNRT1.1::NRT1.1-GFP* transgenic plants (which are in the *chl1-5* background, an *NRT1.1*-null mutant) and indicated that exposure to Pb^{2+} caused a clear increase in *NRT1.1-GFP* protein levels (Fig. 2F). We then compared the rate of net NO_3^- influx by the roots of wild-type Col-0 plants and two *NRT1.1*-null mutants, *chl1-5* and *nrt1.1-1*. Exposure to Pb^{2+} had no effect on influx in the *nrt1.1* mutant in any of the three root zones that we examined (Fig. 3), which was in contrast with the results for the Col-0 plants. These results demonstrated that induction of NRT1.1 activity was responsible for the increase in root NO_3^- uptake in the presence of exposure to Pb^{2+}.

Pb^{2+} resistance in Arabidopsis is specifically associated with NRT1.1

We investigated the association between NRTs and Pb resistance in Arabidopsis. Because NRT1.1 appeared likely to be responsible for the stimulation of NO_3^- uptake by Pb^{2+}, we first examined the effects of its loss of function on resistance to Pb stress. After 7 d of exposure to 300 μM Pb^{2+}, the inhibition of root growth in the *NRT1.1*-null mutants *chl1-5*, *nrt1.1-1*, and *chl1-6*, was greater than that in their corresponding wild-type plants (Fig. 4A, B, Supplementary Fig. S2). In addition, reductions in root elongation over time in response to exposure to Pb^{2+} were greater in the *NRT1.1*-null mutants compared to the wild-types (Supplementary Fig. S3) and there was a significant interaction between *NRT1.1* function and exposure to Pb^{2+} ($P<0.05$). These results indicated that NRT1.1 is required for Pb^{2+} resistance, and further evidence for this was provided by Measurements of root growth in *pNRT1.1::NRT1.1-GFP* transgenic plants, which showed that the inhibition of root elongation in the *NRT1.1*-null mutants could be rectified by complementation with *NRT1.1* (Supplementary Fig. S4). Lipid peroxidation is often used as an indicator of stress effects and therefore we measured the amounts of MDA (a product of this process; Hodges *et al.*, 1999) in the roots and shoots. After 7 d of exposure to 300 μM Pb^{2+}, the MDA levels in both the roots and shoots of the *chl1-5* and *nrt1.1-1* mutants were greatly increased, whereas there was no significant effect in

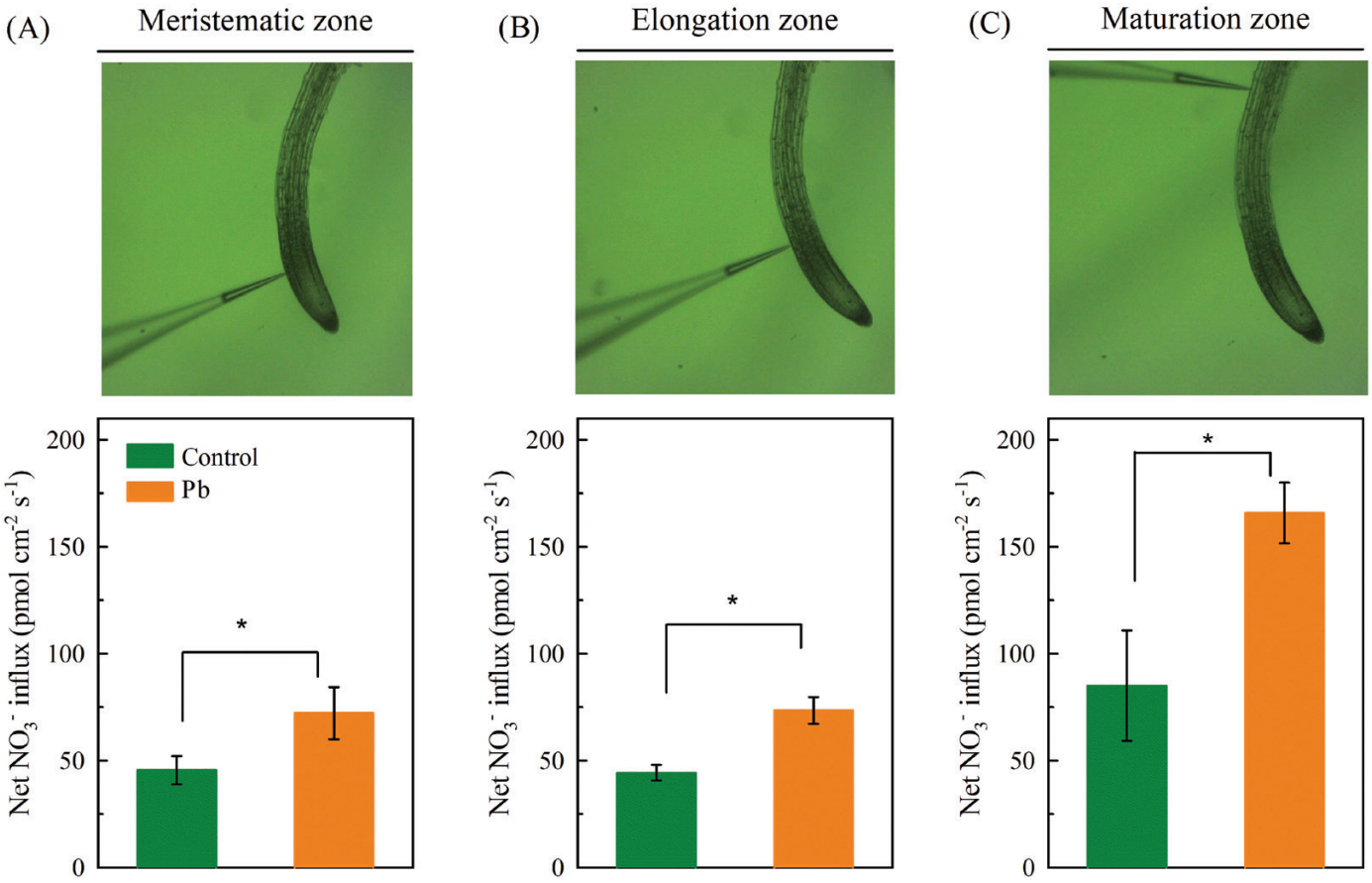

Fig. 1. Effects of Pb^{2+} stress on net NO_3^- influx in the roots of Arabidopsis Col-0 plants. Seedlings at 4 d old were transferred to medium with or without 300 μM $Pb(CH_3COO)_2$ and net NO_3^- influx was measured after 3 d using a non-invasive microelectrode ion flux measurement system. Measurements were taken in the meristematic (A), elongation (B), and maturation zones (C) of the roots. Data are means (±SE) of six biological replicates. Significant differences were determined using two-tailed Student's *t*-tests: **P*<0.05. (This figure is available in colour at *JXB* online.)

the Col-0 plants (Fig. 4C). This provided further support for NRT1.1 being required for Pb^{2+} resistance in Arabidopsis.

NRT1.1 also functions as a NO_3^- sensor, and this is independent of its uptake activity (Ho *et al.*, 2009; Bouguyon *et al.*, 2015). We therefore examined Pb^{2+} resistance in the *chl1-9* mutant, which is defective in terms of NO_3^- uptake but has a normal NO_3^- sensing function (Ho *et al.*, 2009). We found that *chl1-9* had a similar Pb^{2+} resistance phenotype in terms of root growth to the other *nrt1.1*-knockout mutants (Supplementary Fig. S5), indicating that NO_3^- uptake rather than sensing was the likely mechanism behind NRT1.1-conferred Pb^{2+} resistance. We also checked the Pb^{2+} resistance of the *nlp7*-knockout mutant, which shares common features with the *nrt1.1*-knockout mutant in terms of loss of many NO_3^- sensing functions, but it retains normal root NO_3^- uptake activity (Castaings *et al.*, 2009; Marchive *et al.*, 2013). As expected, the Pb^{2+} resistance of *nlp7* was similar to that of the wild-type plants (Supplementary Fig. S5). These results provided further support for NO_3^--uptake activity rather than NO_3^--sensing activity being involved in NRT1.1-mediated Pb^{2+} resistance.

We then examined associations between Pb toxicity and other NRTs and found that the *nrt1.2*, *nrt2.1*, *nrt2.2*, *nrt2.4*, and *nrt2.5* mutants all had similar root elongation to their corresponding wild-types (Supplementary Fig. S6). Owing to the fact that NRT2.1, NRT2.2, NRT2.4, and NRT2.5 are high-affinity NO_3^- uptake transporters, we also evaluated the effects of Pb^{2+} exposure on root growth in the corresponding *NRT*-null mutants under growth conditions with 0.2 mM NO_3^-. We found that root elongation in *nrt2.1*, *nrt2.2*, *nrt2.4*, and *nrt2.5* was similar to that of the corresponding wild-type plants (Supplementary Fig. S7). We also examined Pb sensitivity in the *nrt2.1 nrt2.2* double-mutant, which has only 20–40% of the high-affinity NO_3^- uptake of the wild-type (Li *et al.*, 2007; Lezhneva *et al.*, 2014). Again, we found that this double-mutant also had similar root elongation compared to the wild-type in growth medium containing 0.2 mM NO_3^- (Supplementary Fig. S8). These results suggested that NRT1.2, NRT2.1, NRT2.2, NRT2.4, and NRT2.5 are not involved in Pb^{2+} resistance in Arabidopsis, or at least they do not act like NRT1.1 in conferring resistance.

The level of Pb accumulated in Arabidopsis is specifically controlled by NRT1.1

We investigated how NRT1.1 affected the levels of Pb accumulated in plants. After 7 d of exposure to 300 μM Pb^{2+}, the concentrations in the roots of the *chl1-5* and *nrt1.1-1* mutants were ~3-fold higher than that in the Col-0 plants (Fig. 5). In addition, the mutants also had ~80% higher concentrations in their shoots. Similar differences was also found between the

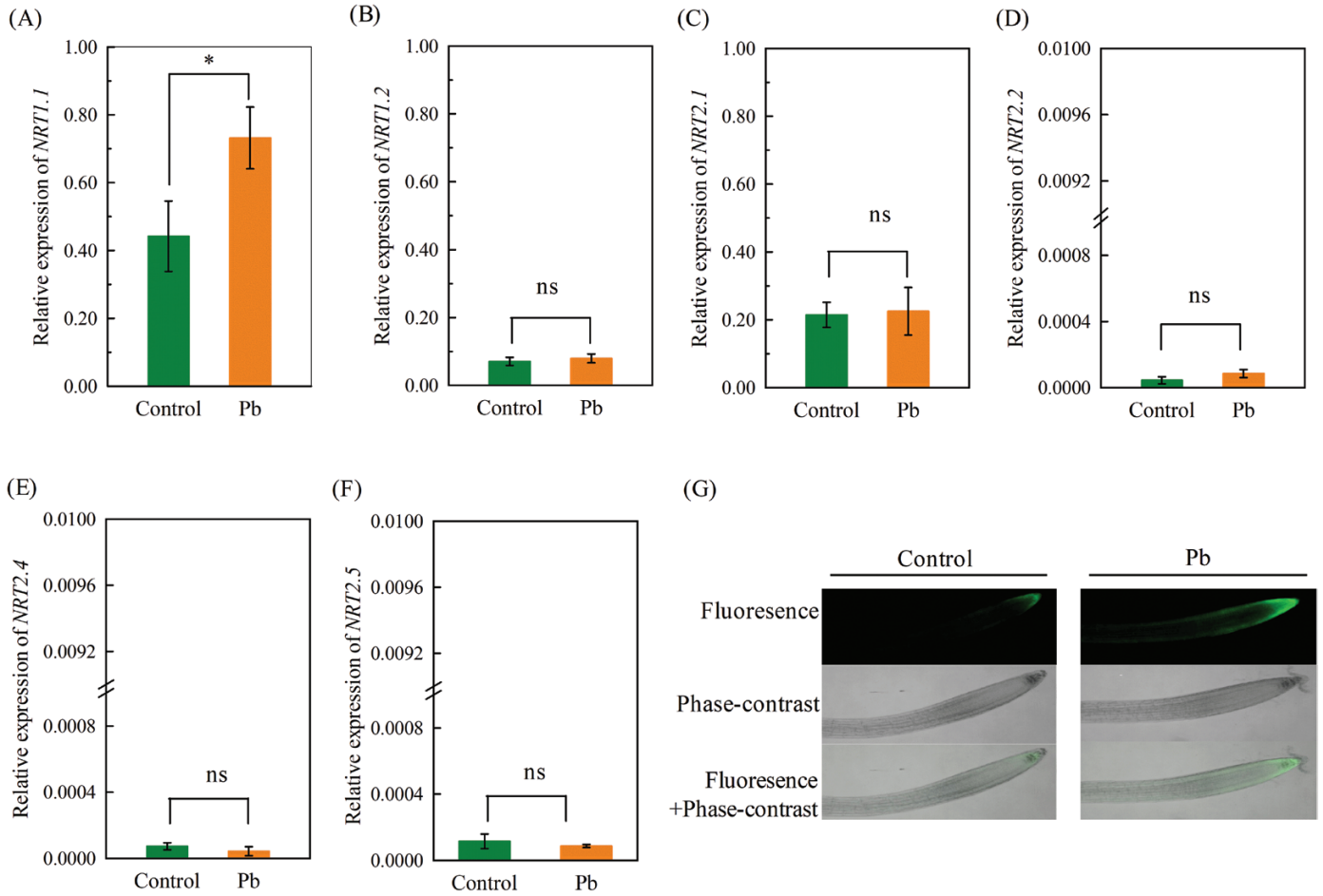

Fig. 2. Effects of Pb^{2+} stress on the expression of (A) *NRT1.1*, (B) *NRT1.2*, (C) *NRT2.1*, (D) *NRT2.2*, (E) *NRT2.4*, and (F) *NRT2.5* in the roots of Arabidopsis Col-0 plants, and (G) the localization of expression of NRT1.1-GFP in *pNRT1.1::NRT1.1-GFP* transgenic plants. Seedlings at 4 d old were transferred to medium with or without 300 μM $Pb(CH_3COO)_2$ and measurements were taken after 3 d. Relative expression levels were normalized to the geometric mean expression of *UBQ10* and *EF1α*. Data are means (±SE) of four biological replicates. Significant differences were determined using two-tailed Student's *t*-tests: **P*<0.05; ns, non-significant. (This figure is available in colour at *JXB* online.)

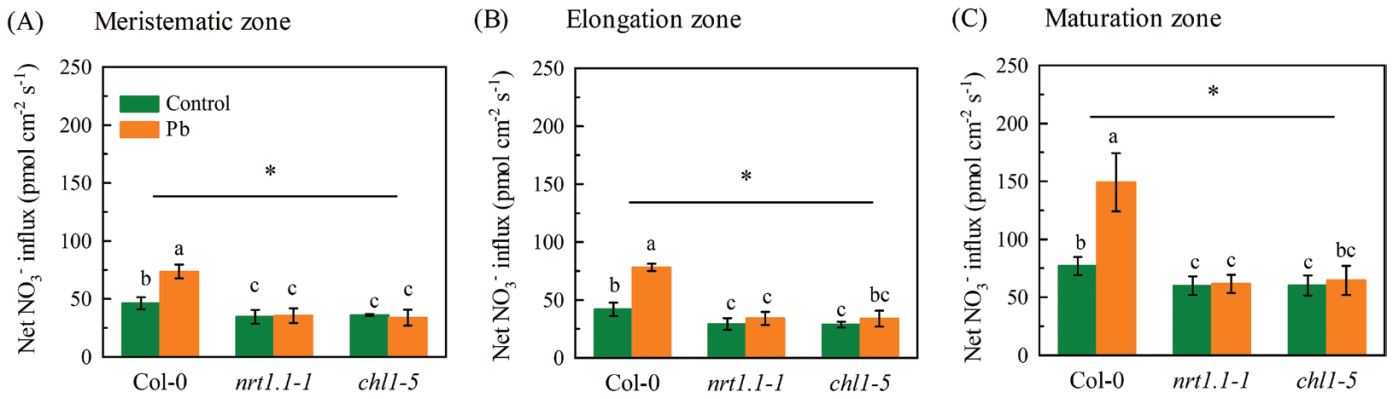

Fig. 3. Effects of Pb^{2+} stress on net NO_3^- influx in different root sections of Arabidopsis wild-type Col-0, and the *nrt1.1-1* and *chl1-5* mutants. Seedlings at 4 d old were transferred to medium with or without 300 μM $Pb(CH_3COO)_2$ and net NO_3^- flux was measured after 3 d using a non-invasive microelectrode ion flux measurement system (Fig. 1). Measurements were taken in the meristematic (A), elongation (B), and maturation zones. Data are means (±SE) of four biological replicates. Different letters indicate significant differences between means as determined using two-way ANOVA followed by Tukey's multiple comparisons test (*P*<0.05). Significant interactions between Pb treatment and genotype are indicated by an asterisk (**P*<0.05). (This figure is available in colour at *JXB* online.)

chl1-6 mutant and the L*er* wild-type (Supplementary Fig. S9). The concentrations in the *pNRT1.1::NRT1.1-GFP* transgenic plants demonstrated that the increase in Pb levels in the *NRT1.1*-null mutants could be rectified by complementation with *NRT1.1* (Supplementary Fig. S10). These results indicated that NRT1.1 negatively regulated the Pb concentrations in the plant tissues, providing further evidence that NRT1.1 is required for resistance to Pb^{2+} exposure in Arabidopsis. It is

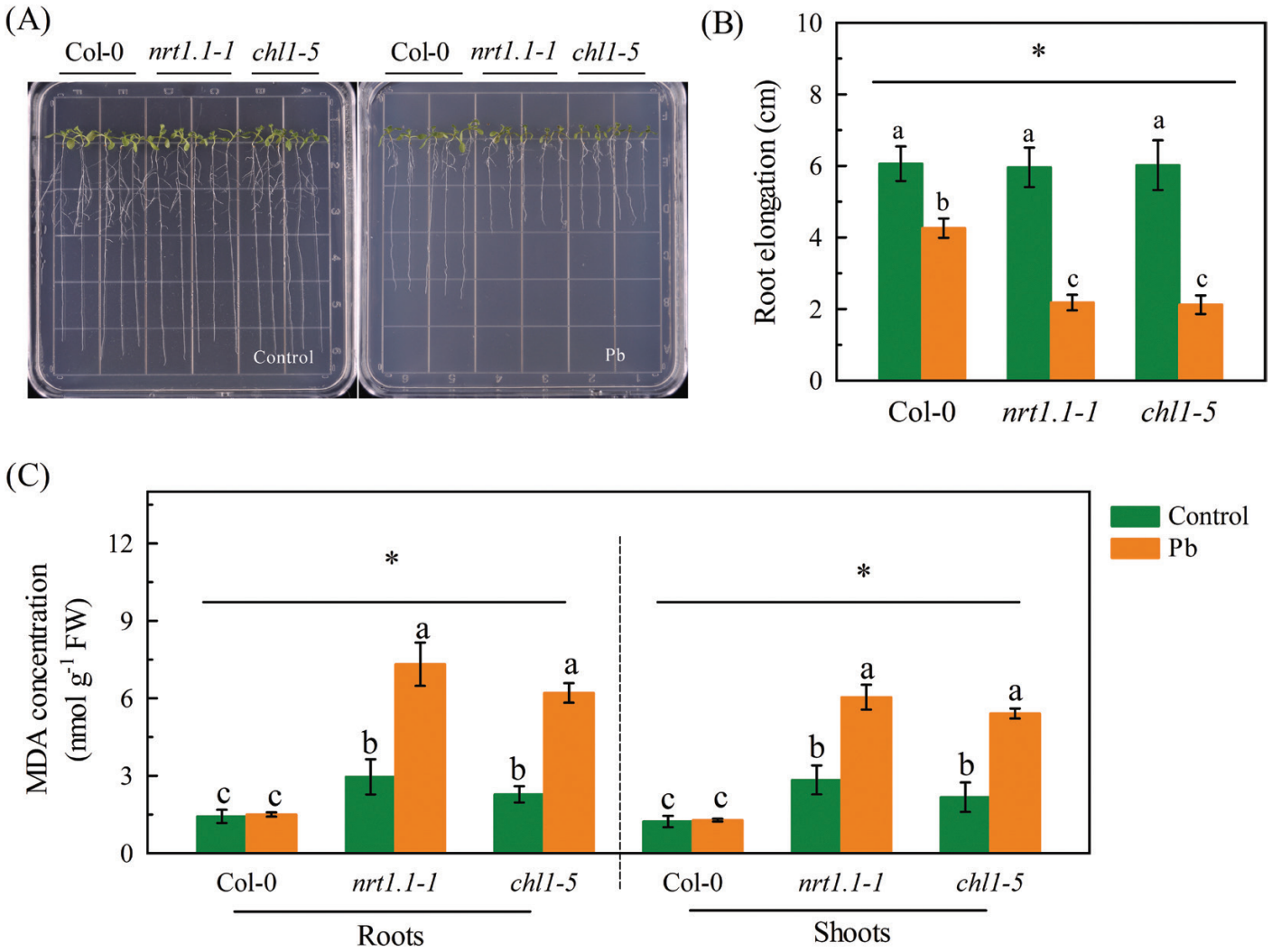

Fig. 4. Effects of Pb^{2+} stress on Arabidopsis wild-type Col-0, and the *nrt1.1-1* and *chl1-5* mutants. Seedlings at 4 d old were transferred to medium with or without 300 μM $Pb(CH_3COO)_2$ and measurements were taken after 7 d. (A) Representative images of plants, (B) root elongation, and (C) concentrations of malondialdehyde (MDA, a product of lipid peroxidation) in the shoots and roots. Data are means (±SE) of 4–5 biological replicates. Different letters indicate significant differences between means as determined using two-way ANOVA followed by Tukey's multiple comparisons test ($P<0.05$). Significant interactions between Pb treatment and genotype are indicated by an asterisk (*$P<0.05$). (This figure is available in colour at *JXB* online.)

worth noting that in all the plant lines examined, the Pb concentrations in the shoots were much lower than that in the roots. This was probably because Pb has extremely low mobility in plants (Pourrut *et al.*, 2011), and hence most will remain in the root tissues after being absorbed from the growth medium.

We also examined whether the other NRTs had a role in affecting the accumulation of Pb, and found that the *nrt1.2*, *nrt2.1*, *nrt2.2*, *nrt2.4*, and *nrt2.5* mutants had similar Pb concentrations in their roots and shoots as their corresponding wild-types when grown in both sufficient (2.25 mM) or low-NO_3^- (0.2 mM) conditions (Supplementary Fig. S11). in addition, the *nrt2.1 nrt2.2* double-mutant also had similar Pb concentrations to the Col-0 plants when grown with 0.2 mM NO_3^- (Supplementary Fig. S8). Thus, the NRT1.2, NRT2.1, NRT2.2, NRT2.4, and NRT2.5 transports do not play a role in affecting the Pb in Arabidopsis, leading us to conclude that NRT1.1 negatively regulates the Pb in a relatively specific manner.

NRT1.1-mediated reduction of Pb^{2+} uptake is associated with increased pH in the rhizosphere

As noted in the Introduction, uptake of NO_3^- may non-specifically couple with the uptake of cations and thus favor uptake of Pb^{2+}, whilst the simultaneous uptake of H^+ will increase the pH of the rhizosphere and thus reduce the bioavailability of Pb^{2+}. Given that we observed a decrease in uptake of Pb^{2+}, we measured the pH in the agar growth medium, which was initially set to 6.5. After 7 d of treatment with 300 μM Pb^{2+}, the pH in the media of the *NRT1.1*-knockout mutants decreased to ~4.0, whilst the pH in the Col-0 medium was ~5.0 (Fig. 6A).Computer modelling using GEOCHEM-PC (Parker *et al.*, 1995) indicated that the activity of Pb^{2+} in our growth medium was sharply reduced when the pH increased (Supplementary Fig. S12). It is therefore likely that the higher concentrations of Pb accumulated in the *nrt1.1*-knockout mutants probably resulted from the increased Pb^{2+} availability that occurred because of the lower pH in the rhizosphere.

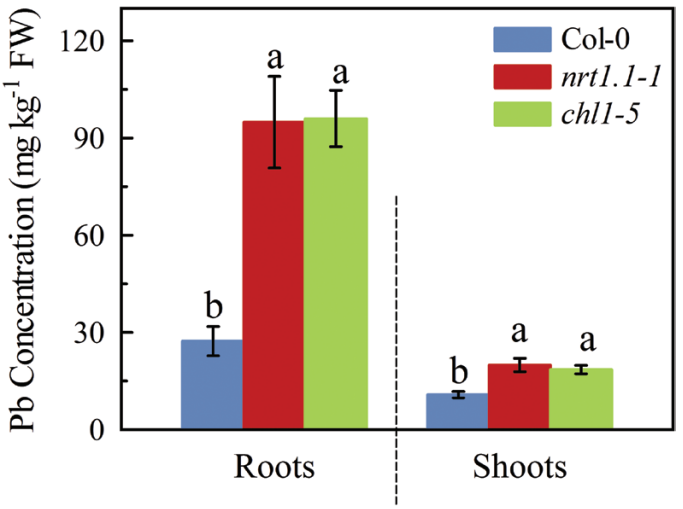

Fig. 5. Pb concentrations in Arabidopsis wild-type Col-0, and the *nrt1.1-1* and *chl1-5* mutants exposed to Pb^{2+} stress. Seedlings at 4 d old were transferred to medium with or without 300 μM $Pb(CH_3COO)_2$ and measurements were taken after 7 d. Data are means (±SE) of four biological replicates. Different letters indicate significant differences between means as determined using two-way ANOVA followed by Tukey's multiple comparisons test ($P<0.05$). (This figure is available in colour at *JXB* online.)

We then used MES to buffer the pH in the growth media. After 7 d of Pb^{2+} treatment, the presence of 0.05% MES (w/v) substantially reduced the differences in pH between the Col-0 rooting medium and those of the two *nrt1.1* mutants (Supplementary Fig. S13). This was associated with significantly reduced concentrations of Pb in both the roots and shoots of the mutants (Fig. 6C), suggesting that there was an interaction between NRT1.1 and the pH of the rhizosphere in the uptake of Pb. Thus, the results provided direct evidence that the negative effect of NRT1.1 on Pb uptake was associated with pH-determined Pb availability in the rhizosphere. Given that the toxicity of Pb is closely associated with the concentration that is accumulated in plant tissues (Pourrut *et al.*, 2011), we evaluated the effect of NRT1.1 on plant resistance to exposure in the presence of MES. As expected, the MES treatment almost completely removed the Pb^{2+}-related inhibition of root growth in the *nrt1.1* mutants (Fig. 6B). Taken together, the results indicated that upon exposure to Pb^{2+}, up-regulation of NRT1.1 in the roots reduced acidification of the rhizosphere and hence reduced Pb^{2+} uptake, thus creating a mechanism whereby the plants increased their resistance to Pb^{2+} toxicity.

An efficient contribution of NRT1.1 to Pb^{2+} resistance requires the co-supply of NO_3^- and NH_4^+

As the resistance to entry of Pb^{2+} into the plants associated with NRT1.1 depended on alterations in the pH of the rhizosphere, we wanted to assess how this was affected by the form in which nitrogen was supplied. We therefore fed Col-0, *nrt1.1-1*, and *chl1-5* plants with different ratios of NO_3^- to NH_4^+ (N:A ratio). When NH_4^+ was supplied as the sole N source, the root growth of Col-0 plants was greatly inhibited by exposure to Pb^{2+}, and they had similar root elongation to the *nrt1.1* mutants (Fig. 7A). This was associated with equally strong acidification of the rhizosphere (Fig. 7B), which would have resulted in high Pb^{2+} availability (Supplementary Fig. S12). When NO_3^- was supplied together with NH_4^+, the root growth of the Col-0 plants improved progressively as the N:A ratio increased, whilst in the *nrt1.1* mutants there was only an initial small increase in growth at an N:A ratio of 1:3. The variations in root growth were associated with corresponding changes in the pH of the growth media (Fig. 7B). When NO_3^- was supplied as the sole N source, exposure to Pb^{2+} had little effect on the root growth of any of any of the plants and this was associated with high pH in the growth media. These results indicated that an efficient contribution of NRT1.1 to Pb^{2+} resistance in Arabidopsis required the co-supply of NO_3^- and NH_4^+. We then measured the Pb concentrations in the plants and found that in growth media with either NH_4^+ or NO_3^- supplied as the sole N source there were no differences between Col-0 and the mutants in either the roots or shoots (Fig. 7C, D). However, when NH_4^+ and NO_3^- were co-supplied, both the mutants had higher Pb concentrations in their roots and shoots compared with the Col-0 plants, and this correlated with the differences in pH in the growth media and with the root growth phenotypes. These results further indicated that the presence of both NO_3^- and NH_4^+ in the growth medium enhances the contribution of NRT1.1 to Pb^{2+} resistance in Arabidopsis.

We also examined the effects of Pb^{2+} exposure on the expression of *NRT1.1* and on the influx of NO_3^- into roots in response to different N:A ratios. In Col-0, both the expression of *NRT1.1* and the influx of NO_3^- were similar between control and Pb^{2+}-treated plants when either NH_4^+ or NO_3^- was supplied as the sole N source, but both were up-regulated by exposure to Pb^{2+} when NH_4^+ and NO_3^- were co-supplied (Supplementary Figs S14, S15). Furthermore, two-way ANOVA of the root NO_3^- influx data confirmed that the NRT1.1 transporter was required for the up-regulation of NO_3^- influx that resulted from exposure to Pb^{2+} in the growth media containing both NH_4^+ and NO_3^-. These results suggested that co-supply of NH_4^+ and NO_3^- ensured the induction of NRT1.1-mediated NO_3^- uptake in response to exposure to Pb^{2+}.

Discussion

The removal of heavy metals from contaminated soils and minimizing their accumulation in food crops are two important challenges that span scientific disciplines (Alexander *et al.*, 2006; Tangahu *et al.*, 2011). Identification of the mechanisms that control the entry of these pollutants into plant tissues is essential if we wish to address these challenges by using biotechnology to engineer modifications of relevant metabolic pathways. In this study, we have demonstrated the existence of a mechanism by which Arabidopsis prevents uptake of Pb^{2+} by its roots, namely that exposure to Pb^{2+} stimulates NRT1.1-mediated uptake of NO_3^- and this results in removal of H^+ from the rhizosphere, which in turn lowers the bioavailability of Pb^{2+}, thus reducing its uptake into the plant.

Pb^{2+} can cause damage to plants at both the physiological and molecular levels (Pourrut *et al.*, 2011). The presence of high levels of Pb^{2+} can lead to inhibition in photosynthesis,

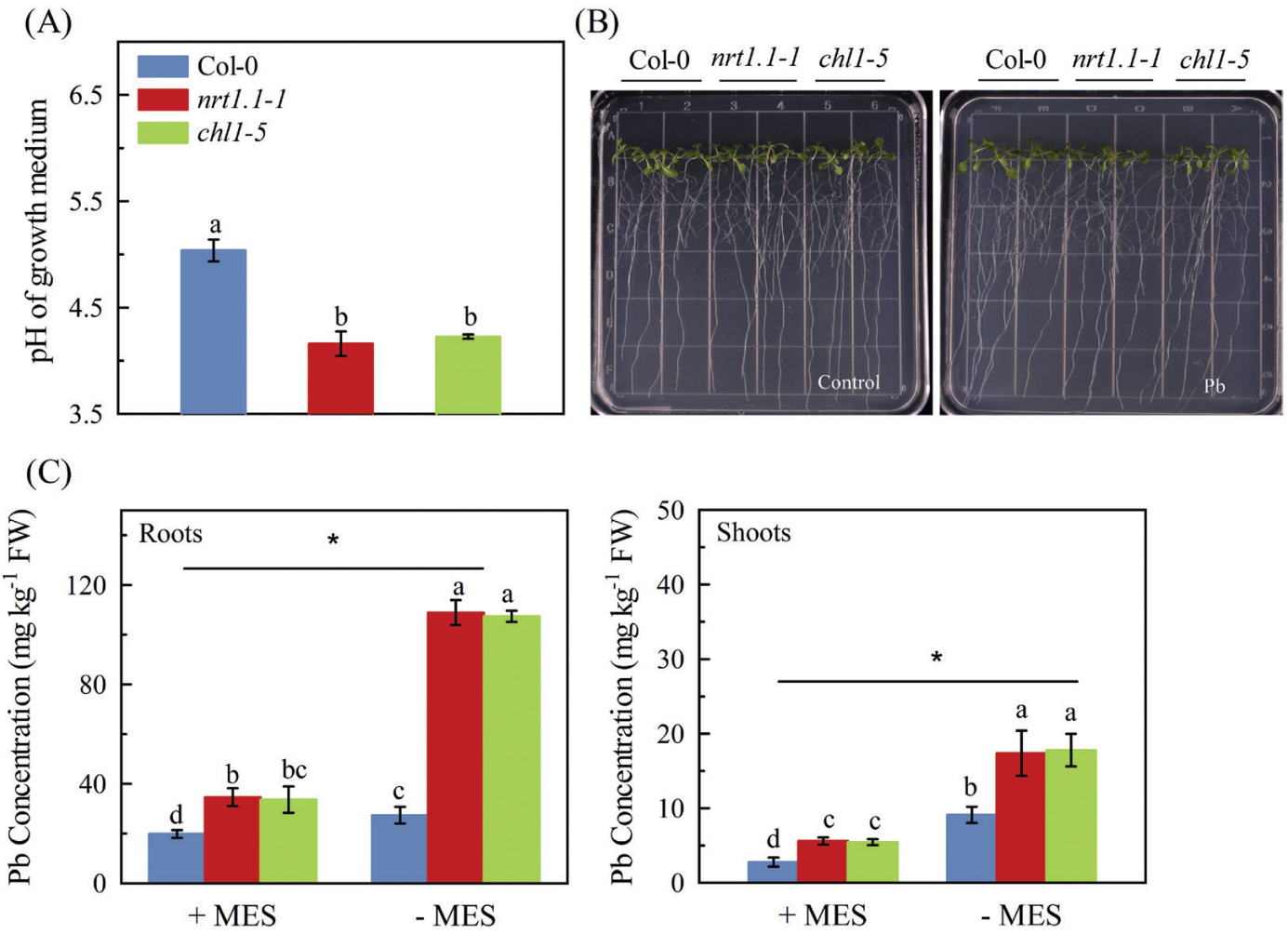

Fig. 6. Effects of pH on Pb^{2+} toxicity in Arabidopsis wild-type Col-0, and the *nrt1.1-1* and *chl1-5* mutants. Seedlings at 4 d old were transferred to medium with or without 300 μM $Pb(CH_3COO)_2$ and with or without the pH buffer MES, and measurements were taken after 7 d. The initial pH of the growth medium was 6.5. (A) The pH of the growth medium with Pb^{2+} and without pH-buffer. (B) Representative images of plants grown with pH buffer and with or without Pb^{2+}. (C) Pb concentrations in roots and shoots of plants grown with Pb^{2+} and with or without pH buffer. Data are means (±SE) of 4–5 biological replicates. Different letters indicate significant differences between means as determined using either one- or two-way ANOVA (depending on whether one or two different variables were considered) followed by Tukey's multiple comparisons test ($P<0.05$). Significant interactions between Pb treatment and genotype are indicated by an asterisk (*$P<0.05$). (This figure is available in colour at *JXB* online.)

an increase in the ATP/ADP ratio, detrimental changes in the cell cycle, breaks in single- and double-strands of DNA, and the accumulation of reactive oxygen species (ROS) (Verma and Dubey, 2003; Patra *et al.*, 2004; Romanowska *et al.*, 2005, 2006; Gichner *et al.*, 2008). Thus, Pb^{2+} stress might be expected to have an adverse effect on NO_3^- uptake by roots; however, we found that exposure to Pb^{2+} stimulated NO_3^- uptake in Arabidopsis roots under our experimental growth conditions (Fig. 1). Although several nitrate transporters (NRTs) are involved in NO_3^- uptake in Arabidopsis, we found that Pb^{2+} only stimulated NRT1.1, as evidenced by its increased expression in seedlings and increased levels of transcripts in the roots (Fig. 2). Furthermore, the loss of function of *NRT1.1* in *nrt1.1* mutants abolished the effect of Pb^{2+} on root NO_3^- uptake (Fig. 3). These results raised the question of how NRT1.1-controlled NO_3^- uptake was induced by exposure to Pb^{2+}. Time-course analysis showed that the induction of *NRT1.1* was initially slow and significant increases were not detected until after12 h of exposure (Supplementary Fig. S1), suggesting that induction was probably the result of the progression of Pb toxicity. Several studies have shown that Pb^{2+} stress results in a significant increases in the level of endogenous abscisic acid (ABA) in plants (Parys *et al.*, 1998; Atici *et al.*, 2005; Cenkci *et al.*, 2010), and ABA has been shown to significantly up-regulate the expression of *NRT1.1* in roots (Kiba *et al.*, 2011). This may provide a mechanism by which Pb^{2+} stimulates NRT1.1-controlled uptake ofNO_3^- but further research is needed.

Comparison of growth responses to Pb^{2+} exposure in various NRT-related mutants also indicated that only the NRT1.1 transporter was required for plant resistance to Pb^{2+} (Fig. 4, Supplementary Figs S2, S3). NRT1.1 was initially identified as a NO_3^- transporter responsible for root NO_3^- uptake (Tsay *et al.*, 1993), but it has also been shown to function as a NO_3^- sensor and an auxin transporter, and these two functions are independent of its uptake activity (Ho *et al.*, 2009; Krouk *et al.*, 2010; Bouguyon *et al.*, 2015). Consequently, NRT1.1 has been shown to be involved in many physiological processes, including the regulation of root growth (Guo *et al.*, 2001;

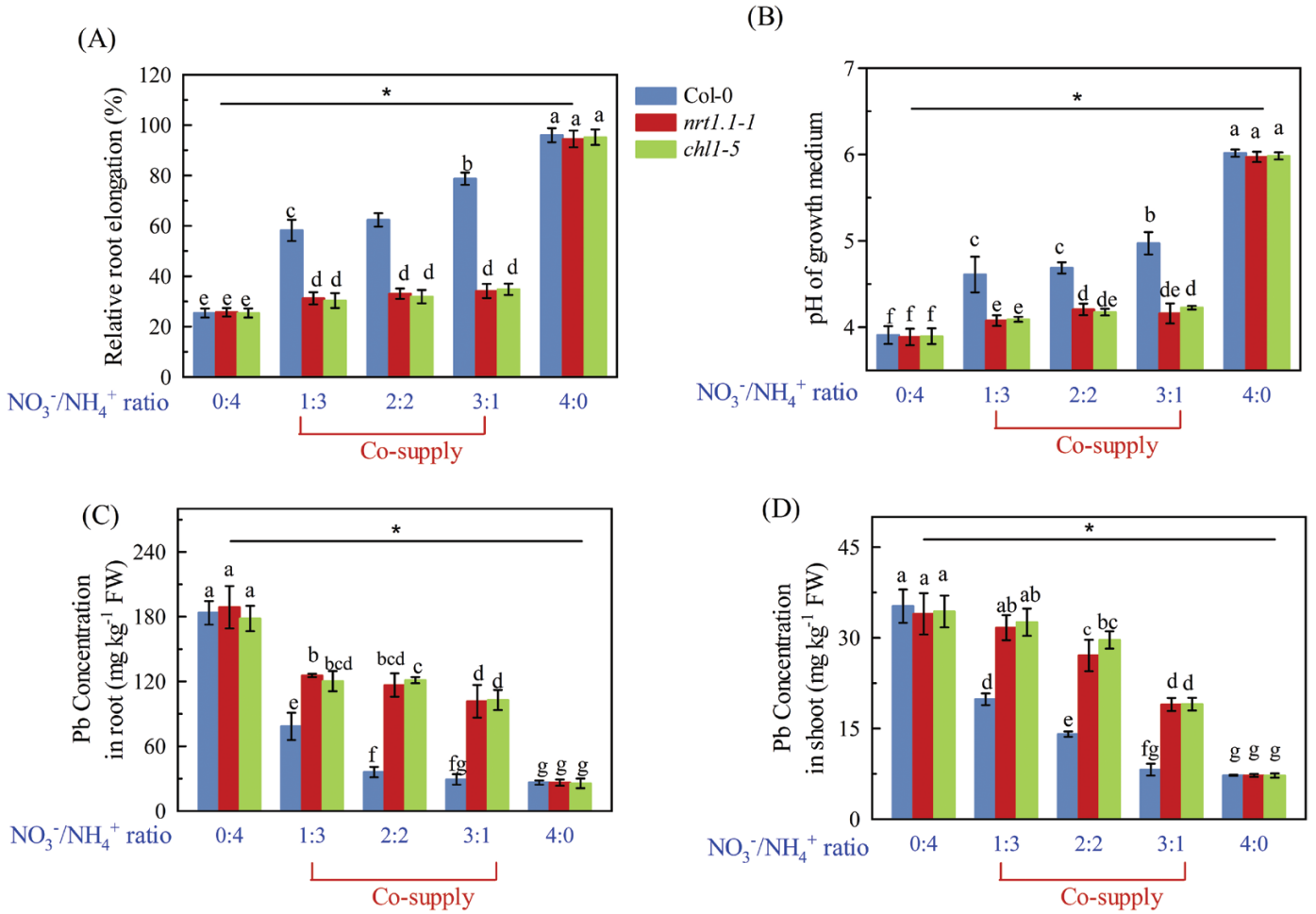

Fig. 7. Interactions between the effects of Pb^{2+} stress and the ratio of NO_3^- to NH_4^+ supplied to Arabidopsis wild-type Col-0, and the *nrt1.1-1* and *chl1-5* mutants. Seedlings at 4 d old were transferred to medium with or without 300 μM $Pb(CH_3COO)_2$ and containing different ratios of NO_3^- to NH_4^+, and measurements were taken after 7 d. (A) Root elongation, calculated as elongation with Pb relative to elongation of the same genotype without Pb under the same NO_3^-/NH_4^+ ratio. (B) pH of the growth medium, and Pb concentrations in (C) the roots and (D) the shoots. Data are means (±SE) of four biological replicates. Different letters indicate significant differences between means as determined using two-way ANOVA followed by Tukey's multiple comparisons test ($P<0.05$). Significant interactions between NO_3^-/NH_4^+ ratio and genotype are indicated by an asterisk (*$P<0.05$). (This figure is available in colour at *JXB* online.)

Remans *et al.*, 2006), control of the expression of the gene for another NO_3^- transporter, NRT2.1 (Muños *et al.*, 2004; Ho *et al.*, 2009; Bouguyon *et al.*, 2015), and regulation of resistance to NH_4^+, Cd^{2+}, and H^+ stress (Hachiya *et al.*, 2011; Mao *et al.*, 2014; Fang *et al.*, 2016). Most of these NRT1.1-associated processes are dependent on the presence of NO_3^- in the growth medium, as they act through either NO_3^- uptake activity or the NO_3^- sensing function (Wang *et al.*, 2012). In our current study, we found that the NRT1.1-conferred resistance to Pb^{2+} probably depends on the NO_3^--uptake activity (Supplementary Fig. S14). Thus, the induction of NRT1.1-mediated NO_3^--uptake as a result of exposure to Pb^{2+} (Fig. 1) may be part of the response mechanisms to enhance Pb resistance. As noted above, an increase in endogenous ABA in plants may be a factor leading to NRT1.1 induction under Pb^{2+} stress. In relation to this, found that the ABA-hypersensitive triple-mutants *abi1 hab1 abi2* and *abi1 hab1 pp2ca* (Fujii *et al.*, 2009) had a greater resistance to Pb^{2+} stress than the wild-type plants (Supplementary Fig. S16). Measurement of Pb concentrations showed that the effect of NRT1.1 resulted from a reduction in the entry of Pb^{2+} into the plants (Fig. 5). The amount of Pb^{2+} taken up by roots is highly associated with its availability in the rhizosphere, which is significantly affected by pH (Fischer *et al.*, 2014). Here, we found that the loss function of *NRT1.1* in *nrt1.1*-knockout mutants resulted in a lower pH in the growth medium (Fig. 6A), which favored an increase in Pb^{2+} activity, and thus increasing its uptake by the plants. This effect was greatly reduced when the pH-buffer MES was present in the growth medium (Fig. 6B, C), leading us to conclude that the reduction in uptake of Pb^{2+} associated with NRT1.1 occurred because of the consumption of H^+ by root cells during NO_3^- uptake, which increased the pH of the rhizosphere and thus decreased Pb^{2+} solubility. This was supported by the observation that the H^+ concentration of the growth medium was highly correlated with the Pb concentrations in the tissues of wild-type and mutant plants supplied with various N:A ratios (Supplementary Fig. S17).

NO_3^- uptake activity is a common function for NRTs (Wang *et al.*, 2012; Léran *et al.*, 2014), but we found that only NRT1.1-mediated uptake played a role in plant resistance to

Pb^{2+}, whilst the other NRTs that we examined had little effect. Our previous study had shown that efficient prevention of acidification in the rhizosphere requires a significant consumption of H^+, which can be achieved through adequate NO_3^- uptake by the roots (Fang *et al.*, 2016). Thus, insufficient uptake of NO_3^- may lead to insufficient consumption of H^+ that is inadequate to decrease Pb^{2+} solubility in the rhizosphere. In the current study, we found that knockout of *NRT1.1* resulted in a decrease in NO_3^- uptake rate of >50% in the presence of Pb^{2+} (Fig. 3), which suggested that the other five NRTs (i.e. *NRT1.2*, *NRT2.1*, *NRT2.2*, *NRT2.4*, and *NRT2.5*) are responsible for <50% of the total root NO_3^- uptake. The AtNRT2 transporters have functional redundancy for high-affinity nitrate uptake (Li *et al.*, 2007; Lezhneva *et al.*, 2014); however, the *nrt2.1nrt2.2* double-mutant had similar root elongation and Pb concentrations as wild-type plants when grown in low-nitrate medium (Supplementary Fig. S8). Hence, relatively low levels of NO_3^- uptake by the other five NRTs may explain why they were not as effective as NRT1.1 in inhibiting Pb^{2+} uptake. It is worth noting that Pb^{2+} can be easily precipitated by phosphates, which may have affected the phosphate availability under our growth conditions. Lowering the pH would increase the solubility of Pb-phosphate precipitates in the medium (Sauvé *et al.*, 1998) and hence the lower pH in the rhizosphere of the *nrt1.1*-knockout mutants compared with that of the wild-type would favor greater solubilization of phosphate precipitates. This suggests that it is more likely that the inhibition of root growth in the mutants was the result of greater Pb^{2+} solubility rather than insufficient phosphate availability.

Interestingly, co-supply of NH_4^+ and NO_3^- in the growth medium was required to ensure the induction of NRT1.1-mediated NO_3^- uptake in response to Pb^{2+} exposure (Supplementary Figs S14, S15). Although the NRT1.1-mediated process of resistance depended on NO_3^- uptake, the presence of NH_4^+ was also required, as evidenced by the fact that there were no differences in root growth and the pH in the growth medium between the wild-type and mutants when NO_3^- was the sole N source (Fig. 7A, B). As previously noted, NO_3^- uptake by roots consumes H^+, whereas NH_4^+ uptake produces H^+ (Marschner, 1995), and thus if a plant only takes up NO_3^- and not NH_4^+ then the pH in the rooting medium would increase. In addition, if a plant is fed with NO_3^- as its sole N source, feedback inhibition would limit the increase in pH in the rooting medium to a level that the plant could tolerate (Imsande, 1986; Helali *et al.*, 2010), because beyond that the plant would be damaged. This may be the reason why the pH of the growth medium for both the wild-type and the *nrt1.1* mutants increased and reached a maximum of ~6.0 as the N:A ratio increased and NO_3^- became the sole N source (Fig. 7B). This led to a similar root growth between these plants in the presence of Pb^{2+} (Fig. 7A). When NO_3^- and NH_4^+ were both present in the growth medium, the loss of function of *NRT1.1* may have resulted in H^+ consumption through NO_3^- uptake mediated by the other NRTs, but this was not sufficient to counteract H^+ production by NH_4^+ uptake, hence leading to greater acidification in the rhizosphere. This may explain why only the co-supply of NO_3^- and NH_4^+ could result in higher Pb accumulation and lower Pb^{2+} resistance in the *nrt1.1* mutants compared to the wild-type. It may be possible to use biotechnological modifications of NRT1.1 activity to effectively manipulate Pb levels in plants because NO_3^- and NH_4^+ are often both present in agricultural soils.

In summary, in this study we found that NRT1.1 negatively regulates Pb levels in Arabidopsis by preventing acidification of the rhizosphere through consumption of H^+ in the process of NO_3^- uptake, which has the effect of reducing the bioavailability of Pb^{2+}. Induction of *NRT1.1* may therefore be considered as a regulatory mechanism used by Arabidopsis to cope with Pb^{2+} stress.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Primers used in this study

Fig. S1. Time-course of induction of *NRT1.1* as the result of exposure to Pb^{2+}.

Fig. S2. Root growth responses of wild-type L*er* and the *chl1-6* mutant to exposure to Pb^{2+}.

Fig. S3. Time-course of root growth responses of wild-type Col-0, and the *nrt1.1-1* and *chl1-5* mutants to exposure to Pb^{2+}.

Fig. S4. Root growth responses of wild-type Col-0, the *chl1-5* mutant, and *pNRT1.1::NRT1.1-GFP* transgenic plants to exposure to Pb^{2+}.

Fig. S5. Root growth responses of wild-type Col-0, and the *nrt1.1-1*, *chl1-5*, *chl1-9*, and *nrt7-2* mutants to exposure to Pb^{2+}.

Fig. S6. Root growth responses of wild-type Col-0, the *nrt1.2*, *nrt2.1*, and *nrt2.2* mutants, and wild-type L*er* and the *nrt2.4* mutant to exposure to Pb^{2+} in standard growth medium.

Fig. S7. Root growth responses of wild-type Col-0, and the *nrt2.1*, *nrt2.2*, and *nrt2.5* mutants, and wild-type L*er* and the *nrt2.4* mutant to exposure to Pb^{2+} low-nitrate growth medium.

Fig. S8. Root elongation and tissue Pb concentrations in wild-type Col-0 and the *nrt2.1 nrt2.2* double-mutant in low-nitrate growth conditions.

Fig. S9. Pb concentrations in tissues of wild-type L*er* and the *chl1-6* mutant exposed to Pb^{2+}.

Fig. S10. Pb concentrations in tissues of wild-type Col-0, the *chl1-5* mutant, and *pNRT1.1::NRT1.1-GFP* transgenic plants exposed to Pb^{2+}.

Fig. S11. Pb concentration in tissues of wild-type Col-0, the *nrt1.2*, *nrt2.1*, *nrt2.2*, and *nrt2.5* mutants, wild-type L*er* and the mutant *nrt2.4* exposed to Pb^{2+} in sufficient (2.25 mM) or low (0.2 mM) nitrate growth conditions.

Fig. S12. Activity of free Pb in growth media at different values of pH as calculated using GEOCHEM-PC.

Fig. S13. pH values in growth media of wild-type Col-0, and the *nrt1.1-1* and *chl1-5* mutants exposed to Pb^{2+} in the presence of the MES pH buffer.

Fig. S14. Expression of *NRT1.1* in the roots of wild-type Col-0 plants exposed to Pb^{2+} and supplied with different ratios of NO_3^- to NH_4^+.

Fig. S15. Net NO_3^- flux in roots of wild-type Col-0, and the *nrt1.1-1* and *chl1-5* mutants exposed to Pb^{2+} and supplied with different ratios of NO_3^- to NH_4^+.

Fig. S16. Root growth responses of wild-type Col-0, and the *abi1 hab1 abi2* and *abi1 hab1 pp2ca* triple-mutants to exposure to Pb^{2+}.

Fig. S17. The relationships between H^+ concentration in the growth media and Pb concentrations in the roots and shoots of wild-type Col-0, and the *nrt1.1-1* and *chl1-5* mutants exposed to Pb^{2+} and supplied with different ratios of NO_3^- to NH_4^+.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (31622051 and 31670258), and the Fundamental Research Funds for the Central Universities (2017XZZX002-06).

References

Alexander PD, Alloway BJ, Dourado AM. 2006. Genotypic variations in the accumulation of Cd, Cu, Pb and Zn exhibited by six commonly grown vegetables. Environmental Pollution **144**, 736–745.

Atici Ö, Ağar G, Battal P. 2005. Changes in phytohormone contents in chickpea seeds germinating under lead or zinc stress. Biologia Plantarum **49**, 215–222.

Bai JH, Jia J, Huang C, Wang QG, Wang W, Zhang GL, Cui BS, Liu XH. 2017. Selective uptake of nitrogen by *Suaeda salsa* under drought and salt stresses and nitrogen fertilization using ^{15}N. Ecological Engineering **102**, 542–545.

Bouguyon E, Brun F, Meynard D, *et al*. 2015. Multiple mechanisms of nitrate sensing by Arabidopsis nitrate transceptor NRT1.1. Nature Plants **1**, 15015.

Castaings L, Camargo A, Pocholle D, *et al*. 2009. The nodule inception-like protein 7 modulates nitrate sensing and metabolism in *Arabidopsis*. The Plant Journal **57**, 426–435.

Cenkci S, Cigerci IH, Yildiz M, Özay C, Bozdag A, Terzi H. 2010. Lead contamination reduces chlorophyll biosynthesis and genomic template stability in *Brassica rapa* L. Environmental and Experimental Botany **67**, 467–473.

Cerezo M, Tillard P, Filleur S, Muños S, Daniel-Vedele F, Gojon A. 2001. Major alterations of the regulation of root NO_3^-uptake are associated with the mutation of *Nrt2.1* and *Nrt2.2* genes in *Arabidopsis*. Plant Physiology **127**, 262–271.

Chiodo LM, Covington C, Sokol RJ, Hannigan JH, Jannise J, Ager J, Greenwald M, Delaney-Black V. 2007. Blood lead levels and specific attention effects in young children. Neurotoxicology and Teratology **29**, 538–546.

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

Ernst WHO. 2000. Evolution of metal hyperaccumulation and phytoremediation hype. New Phytologist **146**, 357–358.

Fang XZ, Tian WH, Liu XX, Lin XY, Jin CW, Zheng SJ. 2016. Alleviation of proton toxicity by nitrate uptake specifically depends on nitrate transporter 1.1 in *Arabidopsis*. New Phytologist **211**, 149–158.

Fischer S, Kühnlenz T, Thieme M, Schmidt H, Clemens S. 2014. Analysis of plant Pb tolerance at realistic submicromolar concentrations demonstrates the role of phytochelatin synthesis for Pb detoxification. Environmental Science & Technology **48**, 7552–7559.

Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park SY, Cutler SR, Sheen J, Rodriguez PL, Zhu JK. 2009. *In vitro* reconstitution of an abscisic acid signalling pathway. Nature **462**, 660–664.

Gichner T, Znidar I, Száková J. 2008. Evaluation of DNA damage and mutagenicity induced by lead in tobacco plants. Mutation Research **652**, 186–190.

Guan MY, Zhang HH, Pan W, Jin CW, Lin XY. 2018. Sulfide alleviates cadmium toxicity in Arabidopsis plants by altering the chemical form and the subcellular distribution of cadmium. The Science of the Total Environment **627**, 663–670.

Guo FQ, Wang R, Chen M, Crawford NM. 2001. The *Arabidopsis* dual-affinity nitrate transporter gene *AtNRT1.1* (*CHL1*) is activated and functions in nascent organ development during vegetative and reproductive growth. The Plant Cell **13**, 1761–1777.

Hachiya T, Mizokami Y, Miyata K, Tholen D, Watanabe CK, Noguchi K. 2011. Evidence for a nitrate-independent function of the nitrate sensor NRT1.1 in *Arabidopsis thaliana*. Journal of Plant Research **124**, 425–430.

Hachiya T, Watanabe CK, Fujimoto M, Ishikawa T, Takahara K, Kawai-Yamada M, Uchimiya H, Uesono Y, Terashima I, Noguchi K. 2012. Nitrate addition alleviates ammonium toxicity without lessening ammonium accumulation, organic acid depletion and inorganic cation depletion in *Arabidopsis thaliana* shoots. Plant & Cell Physiology **53**, 577–591.

Hawkins BJ, Boukcim H, Plassard C. 2008. A comparison of ammonium, nitrate and proton net fluxes along seedling roots of Douglas-fir and lodgepole pine grown and measured with different inorganic nitrogen sources. Plant, Cell & Environment **31**, 278–287.

Helali SM, Nebli H, Kaddour R, Mahmoudi H, Lachaâl M, Ouerghi Z. 2010. Influence of nitrate–ammonium ratio on growth and nutrition of *Arabidopsis thaliana*. Plant and Soil **336**, 65–74.

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

Hodges DM, DeLong JM, Forney CF, Prange RK. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta **207**, 604–611.

Huang NC, Chiang CS, Crawford NM, Tsay YF. 1996. *CHL1* encodes a component of the low-affinity nitrate uptake system in *Arabidopsis* and shows cell type-specific expression in roots. The Plant Cell **8**, 2183–2191.

Huang NC, Liu KH, Lo HJ, Tsay YF. 1999. Cloning and functional characterization of an *Arabidopsis* nitrate transporter gene that encodes a constitutive component of low-affinity uptake. The Plant Cell **11**, 1381–1392.

Imsande J. 1986. Nitrate–ammonium ratio required for pH homeostasis in hydroponically grown soybean. Journal of Experimental Botany **37**, 341–347.

Jin CW, He YF, Zhang K, Zhou GD, Shi JL, Zheng SJ. 2005a. Lead contamination in tea leaves and non-edaphic factors affecting it. Chemosphere **61**, 726–732.

Jin CW, Zheng SJ, He YF, Zhou GD, Zhou ZX. 2005b. Lead contamination in tea garden soils and factors affecting its bioavailability. Chemosphere **59**, 1151–1159.

Kiba T, Feria-Bourrellier AB, Lafouge F, *et al*. 2012. The *Arabidopsis* nitrate transporter NRT2.4 plays a double role in roots and shoots of nitrogen-starved plants. The Plant Cell **24**, 245–258.

Kiba T, Kudo T, Kojima M, Sakakibara H. 2011. Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. Journal of Experimental Botany **62**, 1399–1409.

Kopittke PM, Asher CJ, Kopittke RA, Menzies NW. 2008. Prediction of Pb speciation in concentrated and dilute nutrient solutions. Environmental Pollution **153**, 548–554.

Krapp A, Berthomé R, Orsel M, Mercey-Boutet S, Yu A, Castaings L, Elftieh S, Major H, Renou JP, Daniel-Vedele F. 2011. *Arabidopsis* roots and shoots show distinct temporal adaptation patterns toward nitrogen starvation. Plant Physiology **157**, 1255–1282.

Krouk G, Lacombe B, Bielach A, *et al*. 2010. Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. Developmental Cell **18**, 877–878.

Léran S, Varala K, Boyer JC, *et al*. 2014. A unified nomenclature of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family members in plants. Trends in Plant Science **19**, 5–9.

Lezhneva L, Kiba T, Feria-Bourrellier AB, Lafouge F, Boutet-Mercey S, Zoufan P, Sakakibara H, Daniel-Vedele F, Krapp A. 2014. The Arabidopsis nitrate transporter NRT 2.5 plays a role in nitrate acquisition and remobilization in nitrogen-starved plants. The Plant Journal **80**, 230–241.

Li W, Wang Y, Okamoto M, Crawford NM, Siddiqi MY, Glass AD. 2007. Dissection of the *AtNRT2.1:AtNRT2.2* inducible high-affinity nitrate transporter gene cluster. Plant Physiology **143**, 425–433.

Liu KH, Huang CY, Tsay YF. 1999. CHL1 is a dual-affinity nitrate transporter of *Arabidopsis* involved in multiple phases of nitrate uptake. The Plant Cell **11**, 865–874.

Luo BF, Du ST, Lu KX, Liu WJ, Lin XY, Jin CW. 2012. Iron uptake system mediates nitrate-facilitated cadmium accumulation in tomato (*Solanum lycopersicum*) plants. Journal of Experimental Botany **63**, 3127–3136.

Mao QQ, Guan MY, Lu KX, Du ST, Fan SK, Ye YQ, Lin XY, Jin CW. 2014. Inhibition of nitrate transporter 1.1-controlled nitrate uptake reduces cadmium uptake in *Arabidopsis*. Plant Physiology **166**, 934–944.

Marchive C, Roudier F, Castaings L, Bréhaut V, Blondet E, Colot V, Meyer C, Krapp A. 2013. Nuclear retention of the transcription factor NLP7 orchestrates the early response to nitrate in plants. Nature Communications **4**, 1713.

Marschner H. 1995. Mineral nutrition of higher plants, 2nd edn. London, UK: 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.

Navas-Acien A, Schwartz BS, Rothenberg SJ, Hu H, Silbergeld EK, Guallar E. 2008. Bone lead levels and blood pressure endpoints. Epidemiology **19**, 496–504.

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

Parys E, Romanowska E, Siedlecka M, Poskuta JW. 1998. The effect of lead on photosynthesis and respiration in detached leaves and in mesophyll protoplasts of *Pisum sativum*. Acta Physiologiae Plantarum **20**, 313–322.

Patra M, Bhowmik N, Bandopadhyay B, Sharma A. 2004. Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. Environmental and Experimental Botany **52**, 199–223.

Pourrut B, Shahid M, Dumat C, Winterton P, Pinelli E. 2011. Lead uptake, toxicity, and detoxification in plants. Reviews of Environmental Contamination and Toxicology **213**, 113–136.

Remans T, Nacry P, Pervent M, Filleur S, Diatloff E, Mounier E,Tillard P, Forde BG, Gojon A. 2006. The *Arabidopsis* NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. Proceedings of the National Academy of Sciences, USA **103**, 19206–19211.

Romanowska E, Pokorska B, Siedlecka M. 2005. The effects of oligomycin on content of adenylates in mesophyll protoplasts, chloroplasts and mitochondria from Pb^{2+} treated pea and barley leaves. Acta Physiologiae Plantarum **27**, 29–36.

Romanowska E, Wróblewska B, Drozak A, Siedlecka M. 2006. High light intensity protects photosynthetic apparatus of pea plants against exposure to lead. Plant Physiology and Biochemistry **44**, 387–394.

Sauvé S, McBride M, Hendershot W. 1998. Lead phosphate solubility in water and soil suspensions. Environmental Science & Technology **32**, 388–393.

Selamat SN, Abdullah SR, Idris M. 2014. Phytoremediation of lead (Pb) and arsenic (As) by *Melastoma malabathricum* L. from contaminated soil in separate exposure. International Journal of Phytoremediation **16**, 694–703.

Shabala SN, Newman IA. 1997. H^+ flux kinetics around plant roots after short-term exposure to low temperature: identifying critical temperatures for plant chilling tolerance. Plant, Cell and Environment **20**, 1401–1410.

Tangahu BV, Sheikh Abdullah SR, Basri H, Idris M, Anuar N, Mukhlisin M. 2011. A review on heavy metals (As, Pb, and Hg) uptake by plants through phytoremediation. International Journal of Chemical Engineering **2011**, 1–31.

Thomson CJ, Marschner H, Römheld V. 1993. Effect of nitrogen fertilizer form on pH of the bulk soil and rhizosphere, and on the growth, phosphorus, and micronutrient uptake of bean. Journal of Plant Nutrition **16**, 493–506.

Tsay YF, Schroeder JI, Feldmann KA, Crawford NM. 1993. The herbicide sensitivity gene *CHL1* of *Arabidopsis* encodes a nitrate-inducible nitrate transporter. Cell **72**, 705–713.

Uzu G, Sobanska S, Aliouane Y, Pradere P, Dumat C. 2009. Study of lead phytoavailability for atmospheric industrial micronic and sub-micronic particles in relation with lead speciation. Environmental Pollution **157**, 1178–1185.

van der Ent A, Baker AJM, Reeves RD, Pollard AJ, Schat H. 2013. Hyperaccumulators of metal and metalloid trace elements: facts and fiction. Plant and Soil **362**, 319–334.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology **3**, research0034.1.

Verma S, Dubey RS. 2003. Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Science **164**, 645–655.

Wang YY, Cheng YH, Chen KE, Tsay YF. 2018. Nitrate transport, signaling, and use efficiency. Annual Review of Plant Biology **69**, 85–122.

Wang YY, Hsu PK, Tsay YF. 2012. Uptake, allocation and signaling of nitrate. Trends in Plant Science **17**, 458–467.

Ye JY, Tian WH, Jin CW. 2019. A reevaluation of the contribution of NRT 1.1 to nitrate uptake in Arabidopsis under low-nitrate supply. FEBS letters **593**, 2051–2059.

Zhang P, Zhang R, Fang X, Song T, Cai X, Liu H, Du S. 2016. Toxic effects of graphene on the growth and nutritional levels of wheat (*Triticum aestivum* L.): short- and long-term exposure studies. Journal of Hazardous Materials **317**, 543–551.