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

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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 NO₃⁻ 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 NO₃⁻ 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 NO₃⁻ and NH₄⁺ 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 (Chiolo 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 (NH₄⁺) and nitrates (NO₃⁻). Physiologically, when NO₃⁻ 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 NH₄⁺ is taken up, H⁺ is released into the rhizosphere, resulting in a decrease in the pH (Marschner, 1995). In this context, the uptake of NO₃⁻ by roots may decrease the amount of Pb²⁺ acquired by plants by consumption of rhizospheric H⁺, which thus lowers the level of soluble Pb²⁺, whereas the opposite may be true for uptake of NH₄⁺.

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 NO₃⁻ by roots is accompanied by decreased uptake of several cations, including Ca²⁺, K⁺, Fe²⁺, and Cd²⁺ (Luo et al., 2012; Mao et al., 2014), indicating that the uptake of NO₃⁻ may be non-specifically coupled with the uptake of cations. Hence, the uptake of nitrate may favor the uptake of Pb²⁺. 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 NO₃⁻ uptake by roots affects Pb²⁺ uptake. The pathways of NO₃⁻ uptake by plant roots are complex. It has been reported that six NO₃⁻ 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 (Kropp et al., 2011; Mao et al., 2014; Fang et al., 2016). Given the presumed association between NO₃⁻ and Pb²⁺ 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 NO₃⁻ uptake and Pb²⁺ exposure in Arabidopsis. Our results revealed a new mechanism by which the plants responded to Pb²⁺ stress: exposure to Pb specifically induced NRT1.1-mediated NO₃⁻ 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 ab1 hab1 ab2 and ab1 hab1 pp2a, 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 ab1 hab1 ab2 and ab1 hab1 pp2a 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 NO₃⁻/NH₄⁺ ratio of 3:1 and was as follows: 750 μM NaH₂PO₄, 500 μM MgSO₄, 375 μM K₂SO₄, 2.25 mM KNO₃, 375 μM (NH₄)₂SO₄, 1 mM CaCl₂, 10 μM H₃BO₃, 0.5 μM MnSO₄, 0.5 μM ZnSO₄, 0.1 μM CuSO₄, 0.1 μM (NH₄)₂MoO₄, 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(CH₃COO)₂. Each dish contained 15 seedlings. To examine the effects of N source on the response to Pb²⁺, we used basal medium adjusted to contain different NO₃⁻/NH₄⁺ ratios. The total N concentration fixed at 3.0 mM and the ratio was adjusted using KNO₃ and (NH₄)₂SO₄. The resulting differences in K concentrations were balanced by adjusting the K₂SO₄ concentration.

Measurement of Pb²⁺ concentrations

After 7 d of Pb²⁺ 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

Seeds 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(CH₃COO)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 NO₃⁻ uptake

The net fluxes of NO₃⁻ 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$_3$COO)$_2$ for 3 d, and were then transferred to 5-cm diameter plates with a nutrient solution containing 750 μM Na$_2$HPO$_4$, 500 μM MgSO$_4$, 375 μM K$_2$SO$_4$, 2.25 mM KNO$_3$, 375 μM (NH$_4$)$_2$SO$_4$, 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 normalizing 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, Molecemlar 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 as described by Hawkins et al. (2008). The rooting 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

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.

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the Col-0 plants (Fig. 4C). This provided further support for NRT1.1 being required for Pb2+ resistance in Arabidopsis.

NRT1.1 also functions as a NO3– sensor, and this is independent of its uptake activity (Ho et al., 2009; Bouguyon et al., 2015). We therefore examined Pb2+ resistance in the chl1-9 mutant, which is defective in terms of NO3– uptake but has a normal NO3– sensing function (Ho et al., 2009). We found that chl1-9 had a similar Pb2+ resistance phenotype in terms of root growth to the other nrt1.1-knockout mutants (Supplementary Fig. S5), indicating that NO3– uptake rather than sensing was the likely mechanism behind NRT1.1-conferred Pb2+ resistance.

We also checked the Pb2+ resistance of the nlp7-knockout mutant, which shares common features with the nrt1.1-knockout mutant in terms of loss of many NO3– sensing functions, but it retains normal root NO3– uptake activity (Castaings et al., 2009; Marchive et al., 2013). As expected, the Pb2+ resistance of nlp7 was similar to that of the wild-type plants (Supplementary Fig. S5). These results provided further support for NO3–-uptake activity rather than NO3–-sensing activity being involved in NRT1.1-mediated Pb2+ 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 NO3– uptake transporters, we also evaluated the effects of Pb2+ exposure on root growth in the corresponding NRT-null mutants under growth conditions with 0.2 mM NO3−. 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 NO3– 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 NO3− (Supplementary Fig. S8). These results suggested that NRT1.2, NRT2.1, NRT2.2, NRT2.4, and NRT2.5 are not involved in Pb2+ 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 Pb2+, 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
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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 Pb2+ exposure in Arabidopsis. It is

chl1-6 mutant and the Ler 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 Pb2+ exposure in Arabidopsis. It is

Fig. 2. Effects of Pb2+ 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(CH3COO)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 Pb2+ stress on net NO3− 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(CH3COO)2, and net NO3− 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.)
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-NO₃⁻ (0.2 mM) conditions (Supplementary Fig. S11). In addition, the nrt2.1 nrt2.2 double-mutant also had similar Pb concentrations in their roots and shoots as their corresponding wild-types when grown in both conditions (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.

As noted in the Introduction, uptake of NO₃⁻ may nonspecifically couple with the uptake of cations and thus favor uptake of Pb²⁺, whilst the simultaneous uptake of H⁺ will increase the pH of the rhizosphere and thus reduce the bioavailability of Pb²⁺. Given that we observed a decrease in uptake of Pb²⁺, we measured the pH in the agar growth medium, which was initially set to 6.5. After 7 d of treatment with 300 μM Pb²⁺, 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 Pb²⁺ 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 Pb²⁺ availability that occurred because of the lower pH in the rhizosphere.
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We then used MES to buffer the pH in the growth media. After 7 d of Pb²⁺ 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 Pb²⁺-related inhibition of root growth in the nrt1.1 mutants (Fig. 6B). Taken together, the results indicated that upon exposure to Pb²⁺, up-regulation of NRT1.1 in the roots reduced acidification of the rhizosphere and hence reduced Pb²⁺ uptake, thus creating a mechanism whereby the plants increased their resistance to Pb²⁺ toxicity.

**An efficient contribution of NRT1.1 to Pb²⁺ resistance requires the co-supply of NO₃⁻ and NH₄⁺**

As the resistance to entry of Pb²⁺ 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 NO₃⁻ to NH₄⁺ (N:A ratio). When NH₄⁺ was supplied as the sole N source, the root growth of Col-0 plants was greatly inhibited by exposure to Pb²⁺, 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 Pb²⁺ availability (Supplementary Fig. S12). When NO₃⁻ was supplied together with NH₄⁺, 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 NO₃⁻ was supplied as the sole N source, exposure to Pb²⁺ had little effect on the root growth 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 Pb²⁺ resistance in Arabidopsis required the co-supply of NO₃⁻ and NH₄⁺. We then measured the Pb concentrations in the plants and found that in growth media with either NH₄⁺ or NO₃⁻ 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 NH₄⁺ and NO₃⁻ 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 NO₃⁻ and NH₄⁺ in the growth medium enhances the contribution of NRT1.1 to Pb²⁺ resistance in Arabidopsis.

We also examined the effects of Pb²⁺ exposure on the expression of NRT1.1 and on the influx of NO₃⁻ into roots in response to different N:A ratios. In Col-0, both the expression of NRT1.1 and the influx of NO₃⁻ were similar between control and Pb²⁺-treated plants when either NH₄⁺ or NO₃⁻ was supplied as the sole N source, but both were up-regulated by exposure to Pb²⁺ when NH₄⁺ and NO₃⁻ were co-supplied (Supplementary Figs S14, S15). Furthermore, two-way ANOVA of the root NO₃⁻ influx data confirmed that the NRT1.1 transporter was required for the up-regulation of NO₃⁻ influx that resulted from exposure to Pb²⁺ in the growth media containing both NH₄⁺ and NO₃⁻. These results suggested that co-supply of NH₄⁺ and NO₃⁻ ensured the induction of NRT1.1-mediated NO₃⁻ uptake in response to exposure to Pb²⁺.

**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 Pb²⁺ by its roots, namely that exposure to Pb²⁺ stimulates NRT1.1-mediated uptake of NO₃⁻ and this results in removal of H⁺ from the rhizosphere, which in turn lowers the bioavailability of Pb²⁺, thus reducing its uptake into the plant.

Pb²⁺ can cause damage to plants at both the physiological and molecular levels (Pourrut et al., 2011). The presence of high levels of Pb²⁺ can lead to inhibition in photosynthesis,
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, Pb2+ stress might be expected to have an adverse effect on NO3− uptake by roots; however, we found that exposure to Pb2+ stimulated NO3− uptake in Arabidopsis roots under our experimental growth conditions (Fig. 1). Although several nitrate transporters (NRTs) are involved in NO3− uptake in Arabidopsis, we found that Pb2+ 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 Pb2+ on root NO3− uptake (Fig. 3). These results raised the question of how NRT1.1-controlled NO3− uptake was induced by exposure to Pb2+. Time-course analysis showed that the induction of NRT1.1 was initially slow and significant increases were not detected until after 12 h of exposure (Supplementary Fig. S1), suggesting that induction was probably the result of the progression of Pb toxicity. Several studies have shown that Pb2+ stress results in a significant increase 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 Pb2+ stimulates NRT1.1-controlled uptake of NO3− but further research is needed.

Fig. 6. Effects of pH on Pb2+ 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(CH3COO)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 Pb2+ and without pH-buffer. (B) Representative images of plants grown with pH buffer and with or without Pb2+. (C) Pb concentrations in roots and shoots of plants grown with Pb2+ 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.)

Comparison of growth responses to Pb2+ exposure in various NRT-related mutants also indicated that only the NRT1.1 transporter was required for plant resistance to Pb2+ (Fig. 4, Supplementary Figs S2, S3). NRT1.1 was initially identified as a NO3− transporter responsible for root NO3− uptake (Tsai et al., 1993), but it has also been shown to function as a NO3− 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;
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Remans et al. (2006), control of the expression of the gene for another NO$_3$− transporter, NRT2.1 (Muñoz 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 nrt2.1 nrt2.2 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 Ler 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 nrt1-2 mutants to exposure to Pb$^{2+}$.

Fig. S6. Root growth responses of wild-type Col-0, the nrt1.2, nrt2.1, and nrt2.4 mutants, and wild-type Ler 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 nrt1.2, nrt2.2, and nrt2.5 mutants, and wild-type Ler 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 Ler 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 Ler 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 Pb²⁺ and supplied with different ratios of NO₃⁻ to NH₄⁺.

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