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# Ammonium aggravates salt stress in plants by entrapping them in a chloride over-accumulation state in an NRT1.1-dependent manner

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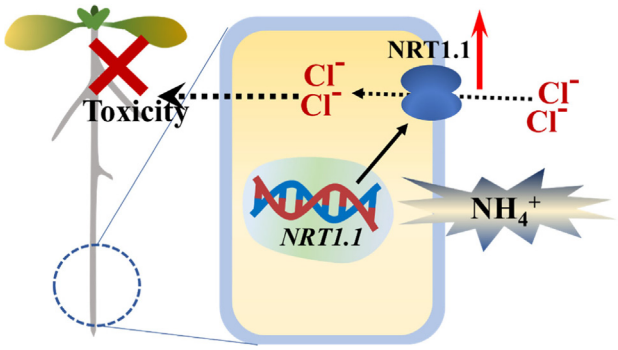
## HIGHLIGHTS

- The plants are hypersensitive to salt stress in $NH_4^+$ medium due to $Cl^-$ uptake.
- Excess of $Cl^-$ in plants grown in $NH_4^+$ medium is associated with the Cl- uptake by NRT1.1.
- The impact of $Cl^-$ toxicity in flooded-coastal areas requires serious consideration.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Global climate change has exacerbated flooding in coastal areas affected by soil salinization. Ammonium ($NH_4^+$) is the predominant form of nitrogen in flooded soils, but the role played by $NH_4^+$ in the plant response to salt stress has not been fully clarified. We investigated the responses of *Arabidopsis thaliana*, *Oryza sativa*, and *Nicotiana benthamiana* plants fed with $NH_4^+$. All species were hypersensitive to NaCl stress and accumulated more $Cl^-$ and less $Na^+$ than those fed with $NO_3^-$. Further investigation of *A. thaliana* indicated that salt hypersensitivity induced by the presence of $NH_4^+$ was abolished by removing the $Cl^-$ but was not affected by the removal of $Na^+$, suggesting that excess accumulation of $Cl^-$ rather than $Na^+$ is involved in $NH_4^+$-conferred salt hypersensitivity. The expression of nitrate transporter NRT1.1 protein was also up-regulated by $NH_4^+$ treatment, which increased root $Cl^-$ uptake due to the $Cl^-$ uptake activity of NRT1.1 and the absence of uptake competition from $NO_3^-$. Knockout of NRT1.1 in plants decreased their root $Cl^-$ uptake and retracted the $NH_4^+$-conferred salt hypersensitivity. Our findings revealed that $NH_4^+$-aggravated salt stress in plants is associated with $Cl^-$ over-accumulation through the up-regulation of NRT1.1-mediated $Cl^-$ uptake. These findings suggest the significant impact of $Cl^-$ toxicity in flooded coastal areas, an issue of ecological significance.



## 1. Introduction

Soil salinization negatively influences plant growth and crop productivity in coastal regions (Deinlein et al., 2014; Li et al., 2014). Grattan and Grieve (1992) proposed that salinity-driven root inhibition was associated with inhibition of nutrient uptake via ion competition (e.g., $Na^+/K^+$, $Na^+/Ca^{2+}$, $Na^+/Mg^{2+}$, and $Cl^-/NO_3^-$) during transportation across cell membranes. Following root uptake of $Na^+$ and $Cl^-$, the accumulation of these two ions in plant tissues may exacerbate nutritional imbalances via competition with other nutritional ions. Because of the negative effect of salt stress on plant nutrition, the nutritional conditions of the growth environment may exert a compounding effect on the severity of salt stress in plants (Bazihizina et al., 2019; Geilfus,

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2019). However, to date, the role of nutrients in the growth medium in the severity of salt stress for plants remains unclear.

Nitrogen is the essential nutrient required in the greatest quantity by plants and is one of the most limiting factors in crop production (Daniel-Vedele et al., 2010). Nitrogen is taken up by the roots primarily in the forms of ammonium ($NH_4^+$) and nitrate ($NO_3^-$). The presence of $NO_3^-$ in the growth medium has been shown to enhance both the root uptake and xylem loading of $Na^+$, and as a result, $NO_3^-$ is considered to be a detrimental factor for plant growth under saline conditions (Álvarez-Aragón and Rodríguez-Navarro, 2017). Flooding in coastal areas is increasing due to rising sea levels and the increased frequency of prolonged rainfall events and storm surges, all due to global climate change (Wahl et al., 2015). Flooded soil environments tend to be oxygen depleted, causing nitrogen to remain in the form of $NH_4^+$ due to intense microbial denitrification (Benckiser et al., 2016). In light of these issues, the response of plants to NaCl stress in growth media with $NH_4^+$ as the nitrogen source has been addressed in several studies. For example, pea (*Pisum sativum* L.) (Frechilla et al., 2001), poplar (*Populus simonii*) (Meng et al., 2016), and wheat (*Triticum aestivuni L.*) (Lewis et al., 1989) plants all exhibited increased sensitivity to salt stress when they were grown with $NH_4^+$ instead of $NO_3^-$. However, some species such as smooth cordgrass (*Spartina alterniflora*) (Hessini et al., 2013), nigella (*Nigella sativa* L.) (Bensalem et al., 2020) and Carrizo citrange (*Citrus sinensis* L.) (Fernández-Crespo et al., 2012) exhibit decreased sensitivity to salt stress when grown in $NH_4^+$ medium, as compared to those grown in $NO_3^-$ medium. These findings indicate that the effect of $NH_4^+$ on salt sensitivity varies between plant species, underscoring the need for further clarification of the mechanisms underlying $NH_4^+$-mediated salt sensitivity in plants. It should be noted that in flooded environments, the conversion of $NO_3^-$ to $NH_4^+$ due to microbial denitrification may minimize the uptake competition between $NO_3^-$ and $Cl^-$ or improve the synergistic uptake of $NH_4^+$ and $Cl^-$, both of which would likely increase $Cl^-$ acquisition by plants. Although $Cl^-$ is a micronutrient for plants, excess accumulation can be toxic (Geilfus, 2018). In plants such as grapes (*Vitis* spp. L.) (Fort et al., 2013; Tregeagle et al., 2010), Carrizo citrange (Brumós et al., 2009), avocado (*Persea americana* L.) (White and Broadley, 2001), soybean (*Glycine* max L.) (Cox et al., 2018), and faba bean (*Vicia faba* L.) (Franzisky et al., 2019), $Cl^-$ toxicity has been shown to inhibit transpiration and photosynthesis and to decrease biomass production. These findings strongly indicate that in an $NH_4^+$-enriched growth medium, plant sensitivity to salt stress could be associated with an excess accumulation of $Cl^-$.

We investigated the above assumption by monitoring the plant responses to different supplied forms of nitrogen. Our results showed that $NH_4^+$-enriched growth medium aggravated salt stress in *A. thaliana*, *N. benthamiana*, and *Oryza sativa* plants. Further investigation of *A. thaliana* revealed that the excess accumulation of $Cl^-$ (rather than $Na^+$) was responsible for $NH_4^+$-conferred salt hypersensitivity, which was found to be dependent on the up-regulation of root $Cl^-$ uptake by nitrate transporter AtNPF6.3/NRT1.1. Our findings demonstrate the importance of considering the negative impacts of $Cl^-$ toxicity on crop production in frequently flooded coastal regions, as well as in other soil environments where $NH_4^+$ is the predominant form of plant-available nitrogen.

## 2. Materials and methods

### 2.1. Plant materials

The *A. thaliana* ecotypes Columbia-0 (Col-0) and Landsberg erecta (Ler) were used in the present study. The single mutants *chl1-5*, *nrt1.1-1* (salk_097431), *nrt1.2* (cs859605), *nrt2.1* (salk_141712), *nrt2.2* (salk_043543), *nrt2.5* (GK 213H10), and *nlp7* (cs868891); the double-mutant *nrt2.1 nrt2.2* (cs859604); and the transgenic plants *pNRT1.1*::*NRT1.1-GUS* (cs6513) and *pNRT1.1*::*NRT1.1-GFP* all had a Col-0 background. The mutants *chl1-6* (cs6154) and *nrt2.4* (cs27332) had a Ler background. Seeds of *chl1-5* and *pNRT1.1*::*NRT1.1-GFP* were kindly provided by Dr. Philippe Nacry (Biochimie et Physiologie Moléculaire des Plantes, Montpellier, France). The insertions in the above lines were verified in our previous studies (Fang et al., 2016).

### 2.2. Plant culture

The plants were grown in agar medium in sterile Petri dishes. The seeds were surface-sterilized using 25% NaClO and sown on basal medium containing 1% (w/v) sucrose, 0.8% (w/v) agar, and 2.5 mM 2-(N-morpholino) ethanesulfonic acid (MES). Rice (*Oryza sativa* L. ssp. *japonica*) seedlings were grown in a hydroponic medium. The nutrient composition of the basal medium was as follows: 2 mM $KNO_3$, 1 mM $CaCl_2$, 500 μM $NaH_2PO_4$, 500 μM $MgSO_4$, 25 μM Fe-EDTA, 10 μM $H_3BO_3$, 0.5 μM $ZnSO_4$, 0.5 μM $MnSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, and 0.1 μM $CuSO_4$, with an overall pH of 6.0. After vernalization at 4 °C for 2 days, the Petri dishes were moved to a controlled-environment growth chamber with a daily cycle of 12 h daylight at 24 °C and 12 h darkness at 22 °C. The 4-day-old seedlings were treated according to experimental requirements. For the treatment with $NH_4^+$ as the sole nitrogen source, $KNO_3$ was replaced by 1 mM $(NH_4)_2SO_4$ and 1 mM $K_2SO_4$. To assay the equivalent stoichiometric $Na^+$ sources supplied in different forms (NaCl, $Na_2SO_4$, or $Na_2C_4H_6O_4$), the $CaCl_2$ in the medium was replaced with $CaSO_4$ to avoid interference with the $Cl^-$ from $CaCl_2$. In order to avoid acidification of the medium induced by the uptake of $NH_4^+$, all growth media were buffered at pH 6.0 by the addition of 2.5 mM MES.

### 2.3. Root measurement

Seedlings were grown in media with various nitrogen sources and various doses of NaCl for 4 days and then photographed using a digital camera. The lengths of the primary roots in the images were analyzed using the ImageJ software (http://imagej.nih.gov/ij/).

### 2.4. Measurement of $Na^+$ and $Cl^-$ concentrations

Following 4 days of treatment with or without 25 mM NaCl, the seedlings were harvested and separated into roots and shoots. The samples were subsequently rinsed with ultrapure water and oven-dried at 75 °C for 2 days; their dry weights were recorded, and they were then ground to a fine powder. For $Na^+$ measurement, the dried samples were wet-digested as described by He et al. (2017). The digests were diluted with ultrapure water, and the $Na^+$ concentrations were analyzed using MP-AES (Agilent Technologies, USA). For $Cl^-$ measurement, the ground samples were incubated overnight in an acidic solution (0.1 M $HNO_3$, 10% acetic acid). Following centrifugation at 15,000 ×*g* for 5 min, the supernatant was filtered (0.22 μm pore size), and the $Cl^-$ concentration was determined using a modified silver titration method as described by Zhang et al. (2015).

### 2.5. Analysis of green fluorescent protein (GFP) and histochemical staining

After 2 days of treatment with the various nitrogen sources (with or without 25 mM NaCl), we observed the expression of GFP in the roots of *pNRT1.1*::*NRT1.1-GFP* plants using an epifluorescence microscope (Ni-U, Nikon, excitation 488 nm, emission 525–550 nm) and imaged using a cooled charge-coupled device camera attached to the microscope. The histochemical β-glucuronidase (GUS) staining in the roots of *pNRT1.1*::*NRT1.1-GUS* plants was performed as described by Fang et al. (2016). The plasma membrane integrity of the root tissues was detected after 4 days of treatment by staining with Evans blue solution (0.25%, w/v) (Guan et al., 2018). The products of GUS staining and Evans blue staining of the roots were observed using a microscope.

### 2.6. RNA extraction and qPCR analysis

The root tissue samples of *A. thaliana* plants were ground in liquid nitrogen, and the total RNA was isolated using TRIzol reagent according to the manufacturer's instructions. Complementary DNA was synthesized using the reverse transcription kit HiScript II Q RT SuperMix for qPCR (Vazyme, Biotech) according to the manufacturer's instructions. The mRNA levels of *NRT1.1* were detected using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Biotech) in a 25 μL reaction system with pairs of *NRT1.1*-specific primers: 5′-GCACATTGGCATTAGGCTTT-3′ (forward) and 5′-CTCAATCCCCACCTCAGCTA-3′ (reverse). The qPCR analysis was performed using the ABI StepOnePlus Real-Time PCR system. Two pairs of housekeeping gene primers were used as the control in the qPCR analysis: 5′-ACCCTAACGGGAAAGACGA-3′ (*UBQ10*-forward) and 5′-GGAGCCTGAGAACAAGATGAA-3′ (*UBQ10*-reverse), and GCTG TCCTTATCATTGACTCCACC (*EF1α*-forward) and TCATACCAGTCTCAAC ACGTCC (*EF1α*-reverse). For each sample, relative transcript abundances, calculated by the comparative Ct (threshold cycle) method and normalized to the geometric mean of the expression of the two housekeeping genes and relative to a control ($NO_3^-$ treatment), is given by $2^{\wedge\text{-}\Delta\Delta Ct}$ (Vandesompele et al., 2002).

### 2.7. Measurement of $Cl^-$ uptake

The net $Cl^-$ fluxes of the roots were measured in the maturation, elongation, and meristematic zones using a non-invasive microelectrode ion flux measurement system (SIET IPA-2, Applicable Electronics, Inc., Forestdale, MA, USA). The $Cl^-$ flux in each section of the roots was measured for 20 min using a microelectrode filled with chloride ionophore I-cocktail A (Sigma-Aldrich, code 99408) (Lee et al., 2013), which exhibited high sensitivity for $Cl^-$ over $NO_3^-$, $SO_4^{2-}$, $HPO_4^{2-}$, and $HCO_3^-$ (Kondo et al., 1989). The presence of other anions, therefore, did not substantially interfere with the Nernstian response of the electrode to $Cl^-$ in our study. The microelectrodes were calibrated in 1, 5, and 10 mM KCl prior to the $Cl^-$ flux measurement. Only electrodes with a Nernstian slope above −56 mV per decade and a correlation of 0.999 or higher were used. To compare the effects of different nitrogen sources on the net $Cl^-$ fluxes, the 4-day-old seedlings were pre-treated with either $NO_3^-$ or $NH_4^+$ media with 25 mM NaCl for 2 days, after which the net $Cl^-$ fluxes of each seedling were measured in a medium identical to that used in for pre-treatment. To assay the induction of NRT1.1-mediated $Cl^-$ uptake by $NH_4^+$, the seedlings were pre-treated in either $NO_3^-$ or $NH_4^+$ medium with or without NaCl for 2 days. The net $Cl^-$ fluxes of each seedling were then measured in $NH_4^+$ media with 25 mM NaCl.

### 2.8. Statistics analyses

Data were analyzed by one-way and two-way ANOVA with post-hoc Tukey HSD test. A $P$-value < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism, version 8 (GraphPad Software, La Jolla, CA).

## 3. Results

### 3.1. A. thaliana plants are hypersensitive to NaCl stress in $NH_4^+$ medium

To quantify the sensitivity of plants to NaCl stress in response to different forms of nitrogen, we monitored the elongation of the primary roots of *A. thaliana* Col-0 plants grown in salty medium supplied with either $NO_3^-$ or $NH_4^+$. After 4 days of 25 mM NaCl stress, the root elongation of plants grown with $NO_3^-$ as the nitrogen source was unaffected (Fig. 1a, b), but the plants supplied with $NH_4^+$ as their sole nitrogen source exhibited mild NaCl stress and dramatically restrained root elongation (reduction of approximately 40%) as compared to the NaCl-free treatment (Fig. 1a–c). Two-way ANOVA revealed a significant interaction between NaCl stress and nitrogen form ($P = 0.0011$). Similar results were observed in *Oryza sativa* and *N. benthamiana* seedlings treated with 50 mM and 25 mM NaCl, respectively (Fig. S1). However, when the Col-0 WT *A. thaliana* plants were exposed to 50 mM NaCl for 4 days, the NaCl-induced inhibition of root elongation in the $NH_4^+$ treatment was similar to that observed in the $NO_3^-$ treatment (two-way ANOVA, $P = 0.6483$, Fig. S2). To control for this effect, we used the 25 mM dose of NaCl in the remaining evaluations.

In the $NH_4^+$ treatments, the arrest in root elongation owing to mild NaCl stress was correlated with a clear decrease in the size of the meristem, as well as with the number of cortical cells in the meristem zone (Figs. 1d, S3). Furthermore, treatment with mild levels of NaCl and with $NH_4^+$ medium resulted in the destruction of the cell structure in the elongation zone (Fig. 1d). The loss of membrane integrity and the resulting cell death in the primary roots were also monitored by staining with Evans blue. As shown in Fig. 1e, the roots showed minimal staining with Evans blue in the $NO_3^-$ growth medium, with or without the 25 mM NaCl addition. However, in the $NH_4^+$ growth medium, mild NaCl treatment resulted in heavy staining of the cells in the elongation zone. This result was inconsistent with the finding of the destructed cell structure in the elongation zone.

### 3.2. Excess accumulation of $Cl^-$ rather than $Na^+$ induces salt hypersensitivity for plants in $NH_4^+$ conditions

Following NaCl treatment, the $Na^+$ levels in the roots and shoots of the plants grown with $NH_4^+$ were lower than those of the plants grown with $NO_3^-$ (Fig. 2a, b). However, the $Cl^-$ levels in both the roots and shoots of the plants in the $NH_4^+$ treatment were significantly higher than those of the plants in the $NO_3^-$ treatment. This was particularly evident in the roots, where $Cl^-$ levels were increased by more than 100% in the $NH_4^+$ treatment as compared with that in the $NO_3^-$ treatment (Fig. 2c, d). We then used 12.5 mM $Na_2SO_4$ or 12.5 mM $(Na)_2C_4H_6O_4$ (sodium succinate) instead of 25 mM NaCl to remove the $Cl^-$ from NaCl. As shown in Fig. 2e and f, the $Na_2SO_4$ and $(Na)_2C_4H_6O_4$ treatments had similar effects on the root elongation of seedlings to that of the NaCl and $NO_3^-$ treatment but had clearly superior effects on the root elongation as compared to the NaCl and $NH_4^+$ treatment. These results indicate that the NaCl hypersensitivity of the plants grown with $NH_4^+$ could be abolished by removing $Cl^-$, supporting the hypothesis that the excess accumulation of $Cl^-$ rather than $Na^+$ induces NaCl hypersensitivity in plants grown under $NH_4^+$ conditions. We next removed $Na^+$ from NaCl in the growth medium by replacing 25 mM NaCl with 25 mM KCl. This treatment had little effect on the root elongation in the $NO_3^-$ treatment, but it significantly restrained root elongation in the $NH_4^+$ treatment (Fig. 2g, h). Regardless, the KCl-induced effect could be mitigated by replacing KCl with $K_2SO_4$ (Fig. S4), suggesting that the reduced root elongation observed in the 25 mM KCl and $NH_4^+$ treatment was a result of $Cl^-$ toxicity rather than an excess of $K^+$ ions.

### 3.3. NaCl hypersensitivity of plants in $NH_4^+$ medium is dependent on NRT1.1

The inhibition of root growth by NaCl stress in $NO_3^-$ treatment was less than that observed in the $NH_4^+$ treatment, indicating that the nitrate transporters (NRTs) involved in root $NO_3^-$ acquisition may improve plant tolerance against mild NaCl stress in $NH_4^+$-enriched environments. Six NRTs (AtNPF6.3/NRT1.1, AtNPF4.6/NRT1.2, NRT2.1, NRT2.2, NRT2.4, and NRT2.5) have been shown to be involved in root $NO_3^-$ uptake in *A. thaliana* plants (Wang et al., 2018). In the $NO_3^-$ condition, all of the tested mutants showed root growth similar to that in their corresponding WT plants, regardless of the 25 mM NaCl addition (Fig. 3a–c). Unexpectedly, the NRT1.1-knockout mutants *nrt1.1-1* and *chl1-5* in the $NH_4^+$ medium showed ~60% and ~69% greater elongation of the primary root, respectively, than Col-0 plants when grown in NaCl medium (Fig. 3a, c). The NaCl-induced inhibition of primary root growth in the third NRT1.1-null mutant (*chl1-6*) was also more severe than that in the corresponding ecotype (Ler) in the $NH_4^+$ medium (Fig. 3b, c). These results

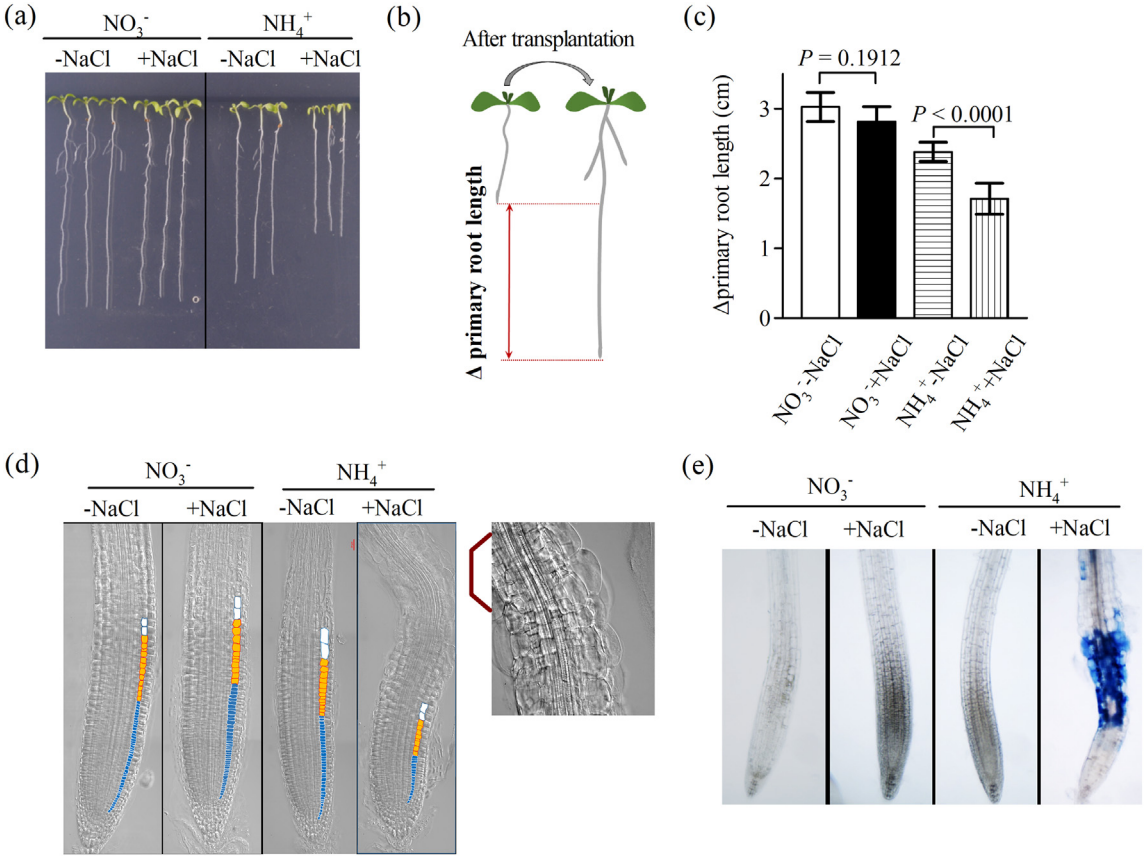


**Fig. 1.** Phenotype of Col-0 seedlings in response to 25 mM NaCl stress in treatments with different forms of nitrogen. (a) Representative images of the effects of different nitrogen sources on NaCl stress in Col-0 seedlings. (b) Scheme depicting the Δ primary root length of seedlings. (c) The Δ primary root length of the seedlings transferred to the treatment medium with different combinations of NaCl and forms of nitrogen. Bars and error bars indicate means ± standard deviations ($n$ = 6– 15 biological replicates). (d) Differential interference contrast images of the root tips. The cortical cell files within the meristem are marked in blue, and the same files within the transition zone are marked in orange. The first two cortical cells in the elongation zone are marked in white. The image on the right depicts the destroyed cell structure in the elongation zone of the roots subjected to the $NH_4^+$ treatment with NaCl. (e) Detection of cell death by histochemical staining with Evans blue. The 4-day-old Col-0 seedlings were transplanted to basal agar medium with either 2 mM $NO_3^-$ or 2 mM $NH_4^+$ as the sole nitrogen source and with (+NaCl) or without (–NaCl) 25 mM NaCl. The measurements were taken after 4 days of treatment. Statistical analysis: one-way ANOVA with post-hoc Tukey HSD test; $P$-values < 0.05 indicate significant differences between the –NaCl and +NaCl treatments within the same nitrogen condition.

suggested that NRT1.1 negatively regulated the plants' tolerance to NaCl stress in the $NH_4^+$ treatment. However, in contrast to the phenotypes observed in the *nrt1.1* mutants, none of the other five NRT-null single mutants (i.e., the *nrt1.2*, *nrt2.1*, *nrt2.2*, *nrt2.4*, and *nrt2.5* knockout mutants) showed improved tolerance to mild NaCl stress in the $NH_4^+$ medium as compared with their WT plants (Fig. 3c). We also examined NaCl sensitivity in an *nrt2.1 nrt2.2* double-mutant and found that this double-mutant also had a tolerance to mild NaCl stress similar to that of the Col-0 WT plants in the $NH_4^+$ medium (Fig. S5). These results indicate that NRT1.1 negatively mediates $NH_4^+$-induced NaCl hypersensitivity in a relatively specific manner.

We then examined the root growth of the *pNRT1.1*::*NRT1.1-GFP* transgenic plants in the *chl1-5* background. The results indicated that the improved elongation of the primary roots observed in the *chl1-5* mutants grown in the $NH_4^+$ medium (with NaCl) could be restrained by complementation with *pNRT1.1*::*NRT1.1-GFP* (Fig. 3d). Furthermore, Evans blue staining was absent in the elongation zone of *chl1–5* roots, but the roots were heavily re-stained with the complementation of *pNRT1.1*::*NRT1.1-GFP* (Fig. 3e).

### *3.4. Excess accumulation of $Cl^-$ in plants grown in $NH_4^+$ medium is associated with the $Cl^-$ uptake activity of NRT1.1*

In the $NH_4^+$ treatment with NaCl, the $Cl^-$ levels in the roots and shoots of *nrt1.1* mutants were demonstrably lower than those of Col-0 plants (Fig. 4a, b). In contrast to the results observed in Col-0, the $NH_4^+$ condition did not increase $Cl^-$ levels in the roots and shoots of nrt1.1 mutants compared with the NO3- treatment. Furthermore, we observed that the difference in the root growth of Col-0 and *nrt1.1* mutants under NaCl stress in the $NH_4^+$ medium was abolished by replacing $Cl^-$ with $SO_4^{2-}$ or $C_4H_6O_4^{2-}$ (Figs. 4c, S6). Additionally, in the $NH_4^+$ medium containing NaCl, the root growth restrained by the complementation of *pNRT1.1*::*NRT1.1-GFP* in the *chl1-5* background could be restored by removing $Cl^-$ (replacing the NaCl with $Na_2SO_4$) (Fig. S7).

A recent study revealed that NRT1.1 has $Cl^-$ permeability in the *Xenopus* oocyte system (Wen et al., 2017). In light of this, we compared the $Cl^-$ uptake between Col-0 and *chl1-5* using a non-invasive technique. The transmembrane $Cl^-$ fluxes of the roots were measured in the meristematic, elongation, and maturation zones of the roots grown in a medium with 25 mM NaCl (Fig. 5a, b). In Col-0 plants, the net $Cl^-$ influx in the elongation zone was greater than that of the other two root zones in both the $NO_3^-$ and $NH_4^+$ treatments, particularly so in the latter (Fig. 5b). This finding is consistent with the previous findings indicating that the elongation zone was the most sensitive site to mild NaCl stress in the $NH_4^+$ treatment (Fig. 1e). As expected, in the $NH_4^+$ treatment, the loss of the NRT1.1 function in the *chl1-5* mutant led to a significant decrease in net $Cl^-$ influx in the elongation and maturation zones but only a slight decrease in the meristematic zone, as compared to that in Col-0 plants (Fig. 5a, b). These results further demonstrated the $Cl^-$ uptake activity of AtNPF6.3/NRT1.1 in plants.

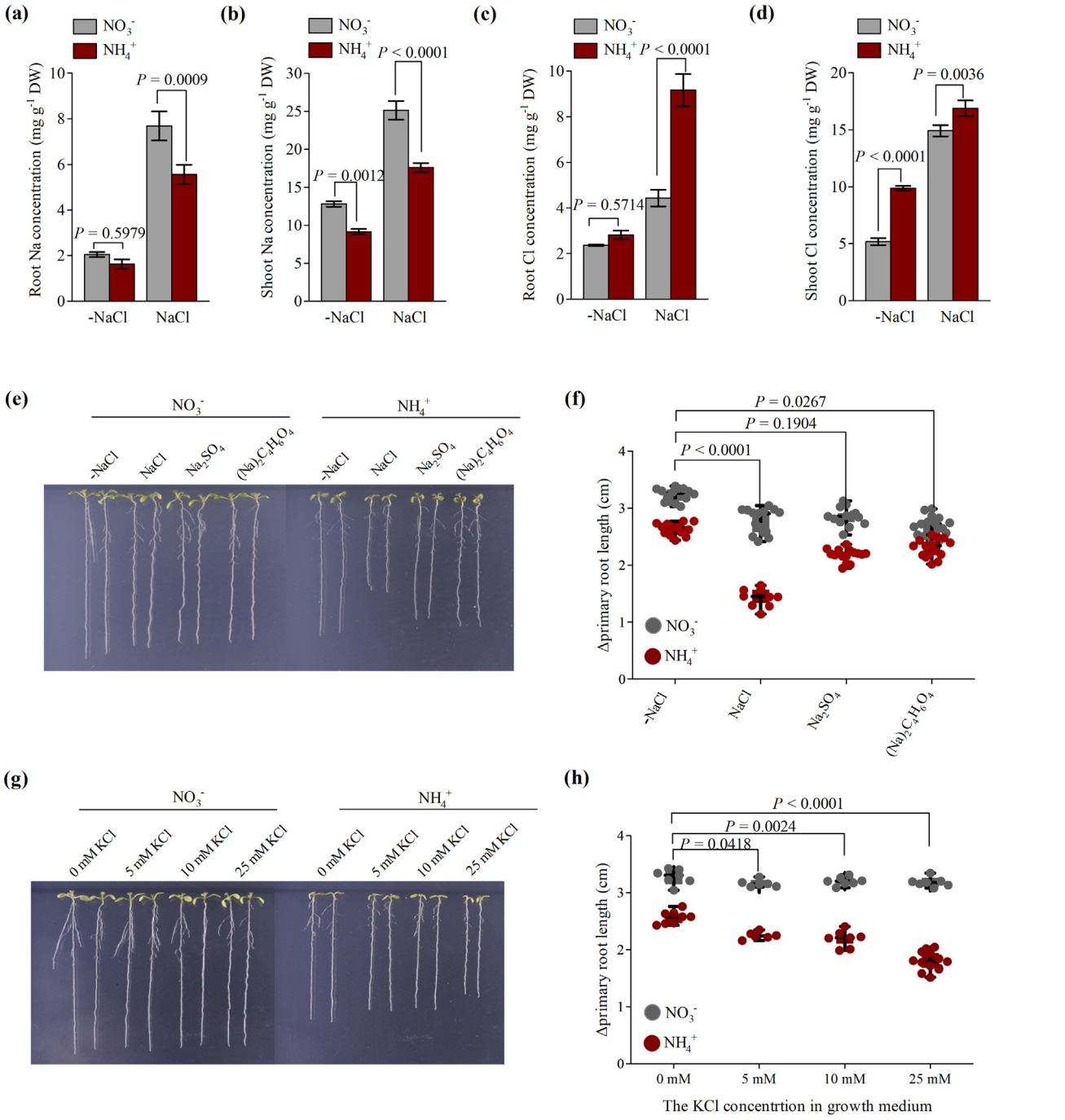


**Fig. 2.** Role of $Cl^-$ in $NH_4^+$-conferred salt hypersensitivity in Col-0 seedlings. (a–d) The Na and Cl concentrations in the roots and shoots of seedlings harvested after 4 days of treatment. The treatments were the same as those described in Fig. 1. Statistical analysis: one-way ANOVA with post-hoc Tukey HSD test; *P*-values < 0.05 indicate significant differences between the $NO_3^-$ and $NH_4^+$ treatments with the same NaCl condition. Bars and error bars indicate means ± standard deviations ($n = 5$ biological replicates). DW, dry weight. (e) Representative images of the effects of different $Na^+$ sources on Col-0 seedlings. (f) The Δ primary root length in response to different $Na^+$ sources. (g) Representative images of KCl dose-dependent responses of Col-0 seedlings. (h) The KCl dose responses of Δ primary root length. The 4-day-old Col-0 seedlings were transplanted to basal agar medium with either 2 mM $NO_3^-$ or 2 mM $NH_4^+$ as the sole nitrogen source and containing either 0 mM NaCl (–NaCl), 25 mM NaCl (+NaCl), 12.5 mM $Na_2SO_4$, or 12.5 mM $Na_2C_4H_6O_4$ (e, f), or various doses of KCl as indicated in the Figures (g, h). In (e, f), the $CaCl_2$ in the basal agar medium was replaced with $CaSO_4$ to avoid interference with the $Cl^-$ ions from $CaCl_2$. Seedlings were analyzed after 4 days of treatment. Statistical analysis: two-way ANOVA with post-hoc Tukey HSD test; *P*-values < 0.05 indicate significant interactions between the nitrogen form and $Na^+$ source or KCl dose. For each box plot, the dots represent each specific datum, the interior line indicates the mean, and the edges represent the estimates of the first and third quartiles ($n = 6$– 15 biological replicates).

No statistically significant differences were observed between Col-0 and *chl1-5* in terms of the net $Cl^-$ influx for all three root zones in the $NO_3^-$ treatments. In addition, Col-0 seedlings showed lower net $Cl^-$ influxes in the elongation and maturation zones (particularly in the former zone) in the $NO_3^-$ treatment, as compared to those in the $NH_4^+$ treatment (Fig. 5a). These results may be due to competition between $Cl^-$ and $NO_3^-$ during their uptake by NRT1.1. Accordingly, we examined the effect of additional $NO_3^-$ on the net $Cl^-$ influx of the elongation zone in an $NH_4^+$ medium containing 25 mM NaCl. As anticipated, the addition of 10 mM $NO_3^-$ rapidly decreased the net $Cl^-$ influx in Col-0 plants but had little effect on the net $Cl^-$ influx in the *chl1-5* mutant (Fig. 5c). This swift inhibitory effect indicates competition between $Cl^-$ and $NO_3^-$ during their uptake by NRT1.1.

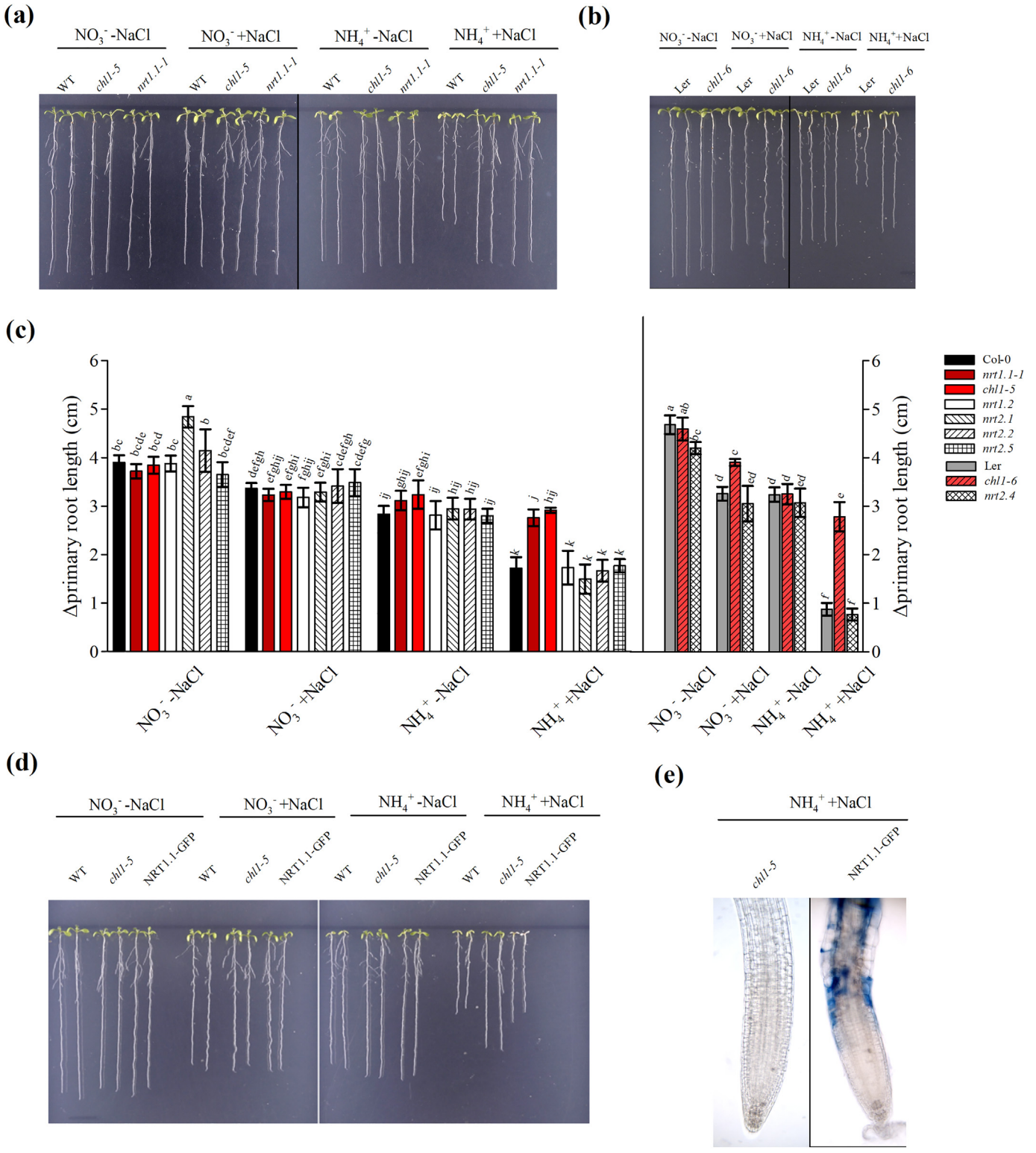


**Fig. 3.** Roles of nitrate uptake transporters (NRTs) in $NH_4^+$-conferred salt hypersensitivity. (a, b) Phenotypic comparison of *nrt1.1* mutants and their corresponding wild-type seedlings in response to mild NaCl stress in different nitrogen conditions. (c) The Δ primary root length of the wild-type and NRT-null mutant seedlings grown in media with different combinations of NaCl and forms of nitrogen. (d) Appearance of Col-0, *chl1-5* mutant, and *NRT1.1-GFP* transgenic seedlings grown in media with different combinations of NaCl and nitrogen forms. (e) Cell death in the *chl1-5* and *NRT1.1-GFP* transgenic seedling roots, as evaluated by Evans blue staining. The 4-day-old seedlings were treated as described in Fig. 1. The measurements were performed 4 days after seedling transfer. Statistical analysis: one-way ANOVA analysis with post-hoc Tukey HSD test. Different letters indicate significant differences within the same treatment (*P*-values < 0.05). Bars and error bars indicate means ± standard deviations ($n$ = 8– 15 biological replicates).

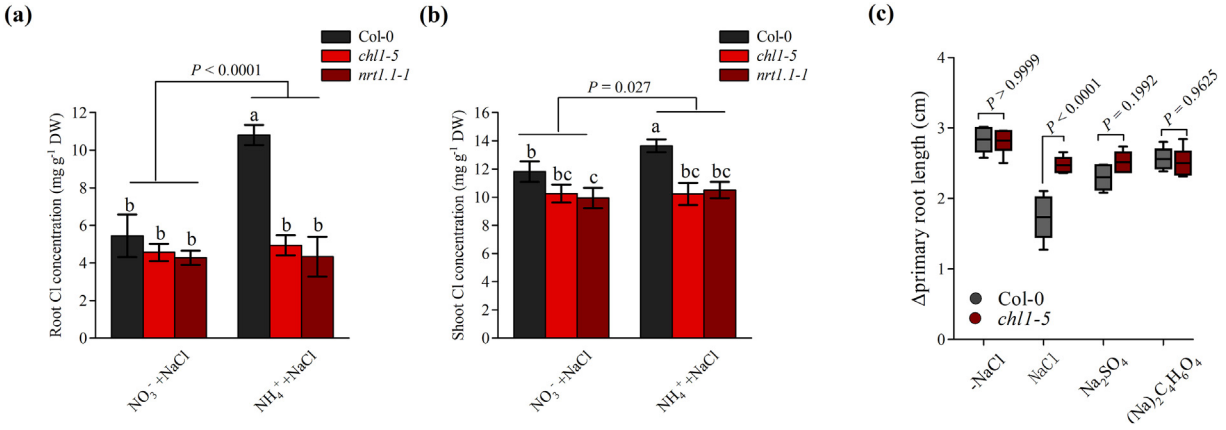


**Fig. 4.** Alleviation of NaCl toxicity in $NH_4^+$-fed *nrt1.1* mutants, associated with a low accumulation of $Cl^-$. (a, b) The $Cl^-$ concentrations in the roots and shoots of Col-0, *chl1-5*, and *nrt1.1-1* seedlings harvested after 4 days of treatment. The treatments were the same as those described in Fig. 1. Statistical analysis: one-way ANOVA analysis with post-hoc Tukey HSD; *P*-values < 0.05 indicate significant differences between Col-0 and *chl1-5* or *nrt1.1-1* mutants within the same treatment. Bars and error bars indicate means ± standard deviations (n = 5 biological replicates). (c) The Δ primary root length of Col-0 and *chl1-5* mutants treated with 2 mM $NH_4^+$ and the equivalent stoichiometric $Na^+$ source. The $CaCl_2$ in the basal agar medium was replaced with $CaSO_4$ to avoid interference with the $Cl^-$ ions from $CaCl_2$. Statistical analysis: one-way ANOVA with post-hoc Tukey HSD test; *P*-values < 0.05 indicate significant differences between Col-0 and *chl1-5* or *nrt1.1-1* mutants within the same treatment. For each box plot, the interior line indicates the mean, and the edges represent the estimates of the first and third quartiles (n = 6– 15 biological replicates).

### *3.5. NRT1.1 protein expression is increased in the $NH_4^+$ medium*

Real-time quantitative PCR analysis showed that the form of nitrogen and the mild NaCl treatment had little effect on the expression of *NRT1.1* in the roots (Fig. 6a). We then analyzed the expression of NRT1.1 protein in the roots. As shown in Fig. 6b, the NRT1.1-GFP protein expression (as indicated by GFP-associated fluorescence in the roots of *pNRT1.1*::*NRT1.1-GFP* transgenic plants) was minimally affected by mild NaCl stress in the $NO_3^-$ medium. However, when the plants were grown in $NH_4^+$ medium, the expression of NRT1.1-GFP protein was demonstrably increased, and this increase occurred regardless of the addition of NaCl (Fig. 6b). We tested the rate of net $Cl^-$ fluxes at the meristematic, elongation, and maturation zones of the roots following pre-culture with different nitrogen sources, with or without NaCl, for 2 days. The $Cl^-$ flux test was conducted in the $NH_4^+$ medium with NaCl to avoid any resulting interference from uptake competition between $Cl^-$ and $NO_3^-$ (Fig. 6c). In the Col-0 plants, pre-culture with $NH_4^+$ increased the net $Cl^-$ influx in the elongation and maturation zones in the same testing medium as compared to the pre-culture treatments with $NO_3^-$, although the net $Cl^-$ influx in the meristematic zone did not differ significantly between the two pre-culture treatments with different nitrogen forms (Fig. 6d–f). Furthermore, the increase in the net $Cl^-$ influx in Col-0 plants pre-cultured in $NH_4^+$ was not affected by the presence of NaCl in the pre-culture medium, confirming the above finding regarding the expression pattern of NRT1.1 protein in response to different nitrogen sources (Fig. 6d–f). In the NRT1.1-null mutant *chl1-5*, however, no significant differences were observed in the net $Cl^-$ influxes in all three measured zones between the two nitrogen-source pre-treatments, with or without the NaCl addition (Fig. 6d–f). We, therefore, posit that the increased $Cl^-$ uptake by the roots in the $NH_4^+$ growth medium is associated with the improved expression of NRT1.1 protein in the roots.

## 4. Discussion

Studies of salt stress in plants have largely focused on the effects of $Na^+$, neglecting the effect of $Cl^-$ (Li et al., 2016). In this study, we demonstrated that the excess accumulation of $Cl^-$, rather than $Na^+$, was the key factor responsible for salt hypersensitivity in plants grown in $NH_4^+$ conditions. $Cl^-$ toxicity may be a serious concern and should, therefore, be taken into account in frequently flooded coastal areas where $NH_4^+$ is the predominant form of soil nitrogen.

Although a transporter specifically responsible for root $Cl^-$ uptake from the growth medium has not yet been identified, several transport proteins have been shown to be permeable to $Cl^-$ and involved $Cl^-$ homeostasis in plants over the past 10 years (Li et al., 2016; Li et al., 2017b). CLCc compartmentalizes $Cl^-$ in the vacuoles (Jossier et al., 2010), while CCC (Colmenero-Flores et al., 2007), NPF2.4 (Li et al., 2016), SLAH1, and SLAH3 (Cubero-Font et al., 2016) facilitate $Cl^-$ loading into the root xylem; NPF2.5 extrudes $Cl^-$ from the root (Li et al., 2017a). Among these, NPF2.4 and NPF2.5 belong to the NITRATE EXCRETION TRANSPORTER (NAXT) subfamily (Segonzac et al., 2007; Tsay et al., 2007). The nitrate transporter NRT1.1 was also shown to have $Cl^-$ permeability in the *Xenopus* oocyte system (Wen et al., 2017). However, the permeability of such $NO_3^-$ transporters to $Cl^-$ in plant systems requires verification. In this study, we showed that this nitrate transporter exhibited $Cl^-$ permeability in *A. thaliana* plants (Fig. 5a, b) and that this function was enhanced in the $NH_4^+$ conditions as compared to the $NO_3^-$ conditions (Fig. 5a, b). This was likely the result of uptake competition between $Cl^-$ and $NO_3^-$. Furthermore, increased uptake of the cation $NH_4^+$ should be accompanied by increased anion uptake to maintain the charge balance in the roots. In this context, the uptake synergy between $Cl^-$ and $NH_4^+$ may be a driving force for $NH_4^+$-improved $Cl^-$ uptake by NRT1.1. Thus, enhanced $Cl^-$ uptake by NRT1.1 could be a mechanism for $NH_4^+$-conferred salt hypersensitivity in plants. In this study, *nrt1.1* mutants showed greater tolerances to $Cl^-$ exposure in the $NH_4^+$ medium, confirming this finding (Figs. 3a–b, 4c).

Intriguingly, loss of the NRT1.1 function also decreased the plant's $Na^+$ levels (Álvarez-Aragón and Rodríguez-Navarro, 2017), which should have improved the plant's tolerance to salt stress. This finding disagrees with our conclusion that excess $Cl^-$, rather than $Na^+$, was responsible for the NRT1.1-conferred salt hypersensitivity of plants grown in $NH_4^+$ medium (Figs. 4a–b, S8). This discrepancy may be due to the variance in growth conditions between the different experiments because only the presence of $NO_3^-$ ensured a lower $Na^+$ level in *nrt1.1* mutants than in the WT plants; this difference was abolished when $NO_3^-$ was removed from the growth medium (Álvarez-Aragón and Rodríguez-Navarro, 2017). Therefore, the mechanism of NRT1.1-intensified salt stress in plants is likely to be associated with the form of nitrogen present in the growth medium; in short, NRT1.1 aggravates $Na^+$ accumulation in plants grown in $NO_3^-$ conditions but entraps plants in a $Cl^-$-excess state in $NH_4^+$ conditions. In light of this finding and the disparate stress effects of $Na^+$ and $Cl^-$, the effect of different forms of nitrogen on the plant response to salt stress may be associated with the level

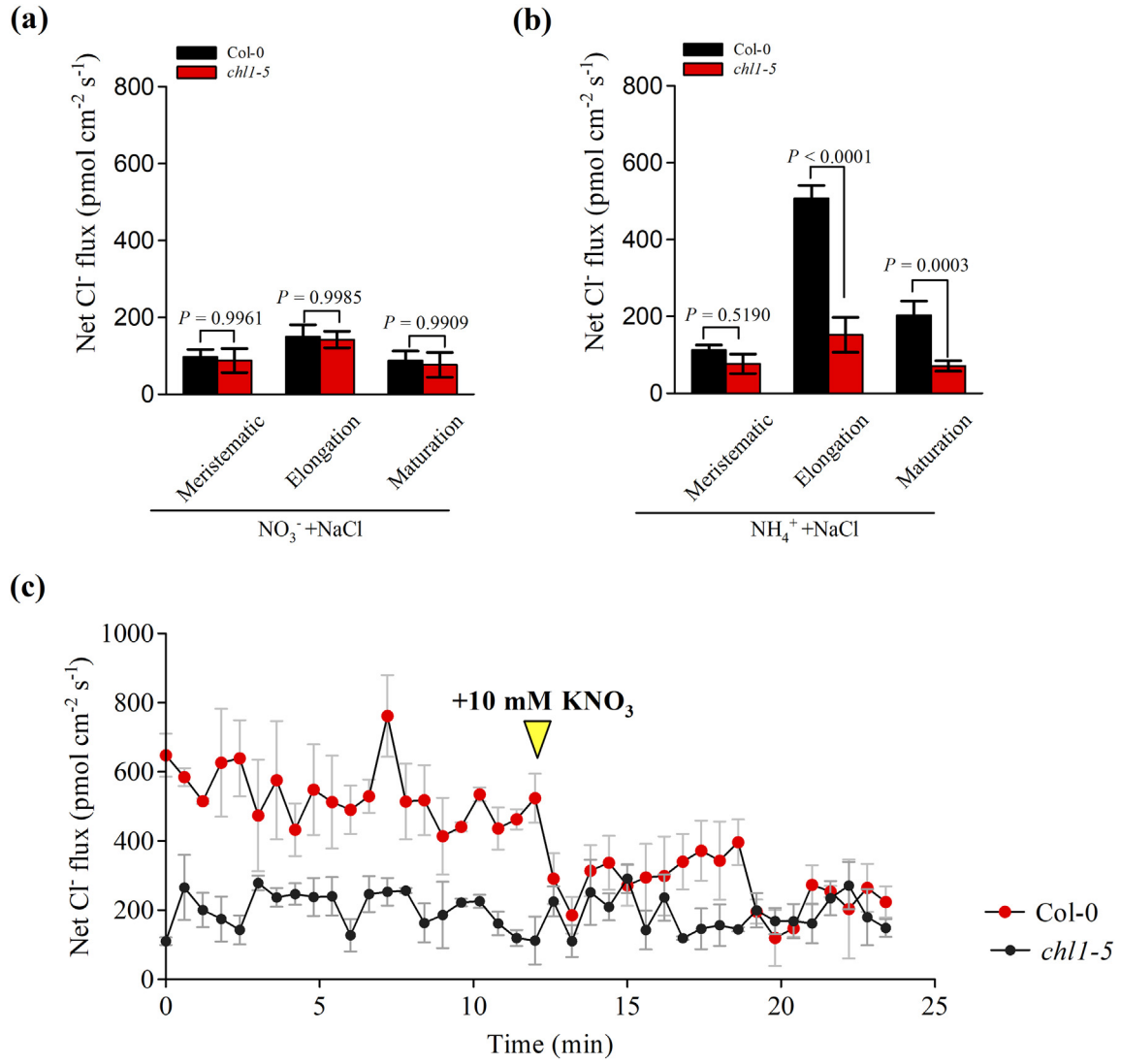


**Fig. 5.** Comparison of the net $Cl^-$ influx between Col-0 and *chl1-5* seedlings. (a, b) Net $Cl^-$ flux in roots of Col-0 and *chl1-5* mutants. The Col-0 and *chl1-5* seedlings were pre-cultured for 2 days in basal agar medium with either 2 mM $NO_3^-$ or 2 mM $NH_4^+$ as the sole nitrogen source and 25 mM NaCl. The net $Cl^-$ fluxes in the different root zones were measured in a testing solution that had an identical ion composition to that of the medium used for the corresponding seedling treatments. Statistical analysis: one-way ANOVA with post-hoc Tukey HSD test; *P*-values < 0.05 indicate significant differences in the net $Cl^-$ fluxes between Col-0 and *chl1-5* mutants within the same treatment. Bars and error bars indicate means ± standard deviations ($n = 4$ biological replicates). (c) The effect of additional $NO_3^-$ on the net $Cl^-$ flux in the elongation zone. The Col-0 and *chl1-5* seedlings were pre-cultured in basal agar medium with 2 mM $NH_4^+$ for 2 days. Subsequently, the net $Cl^-$ flux in the elongation zone of the roots was measured in the testing solution with a medium containing 2 mM $NH_4^+$ and 25 mM NaCl for the first 12 min and then further measured after the addition of 10 mM $NO_3^-$ (added in the form of $KNO_3$).

of salt stress. This is supported by the observation that no significant differences in root growth were observed between the $NH_4^+$ and $NO_3^-$ treatments when the NaCl stress was raised to 50 mM; Miranda et al. (2017) showed that 75 mM NaCl resulted in a higher $Na^+$ accumulation in plants grown with $NO_3^-$ and exhibited higher sensitivity to the salt stress than the plants grown with $NH_4^+$. It is important to note, as mentioned previously, that the effect of the form of nitrogen on salt sensitivity also varies between plant species (Ullrich, 2002). For example, smooth cordgrass (*Spartina alterniflora*) plants prefer $NH_4^+$ as a nitrogen source and exhibit better growth in response to salt stress when they were grown with $NH_4^+$ instead of $NO_3^-$ (Hessini et al., 2013; Hessini et al., 2009). This indicates that the salt sensitivity of a plant may also be affected by the nitrogen preference of the plant.

It should be noted that the expression of NRT1.1 protein in the roots was increased in the $NH_4^+$ treatment, which improved the $Cl^-$ uptake (Fig. 6b). Up-regulation of NRT1.1 should be detrimental for plants coping with salt stress in an $NH_4^+$-dominated soil environment; therefore, this may represent another mechanism for the $NH_4^+$-conferred salt hypersensitivity in plants. It is also noteworthy that NRT1.1 has been shown to negatively regulate plant tolerance to $NH_4^+$ stress (Hachiya et al., 2011; Jian et al., 2018). This finding was further confirmed in the present study, wherein we demonstrated that the NRT1.1-null mutants *chl1-5* and *nrt1.1-1* had better root growth than the Col-0 plants in the $NH_4^+$ treatments without NaCl (Fig. 3a). Because 2 mM $Cl^-$ (added in form of 1 mM $CaCl_2$) was also present in the $NH_4^+$ growth medium in our study, we examined whether NRT1.1-mediated $Cl^-$ uptake playing a role in $NH_4^+$ toxicity to plants. The replacement of $CaCl_2$ with $CaSO_4$ in the $NH_4^+$ growth medium did not improve the growth of WT plants over that of NRT1.1-null mutants, indicating that $Cl^-$ uptake by NRT1.1 should be not a cause of the growth restraint of plants grown in the $NH_4^+$ medium and containing low levels of $Cl^-$.In the present study, the two-way ANOVA indicated that NRT1.1 conferred sensitivity to NaCl stress more significantly in Ler than in Col-0 (Fig. S10), indicating that the genetic background of a genotype or species may affect the degree of Cl toxicity conferred by NRT1.1. Studies have shown that sensitivity to high $Cl^-$ concentrations varies greatly between plant species;

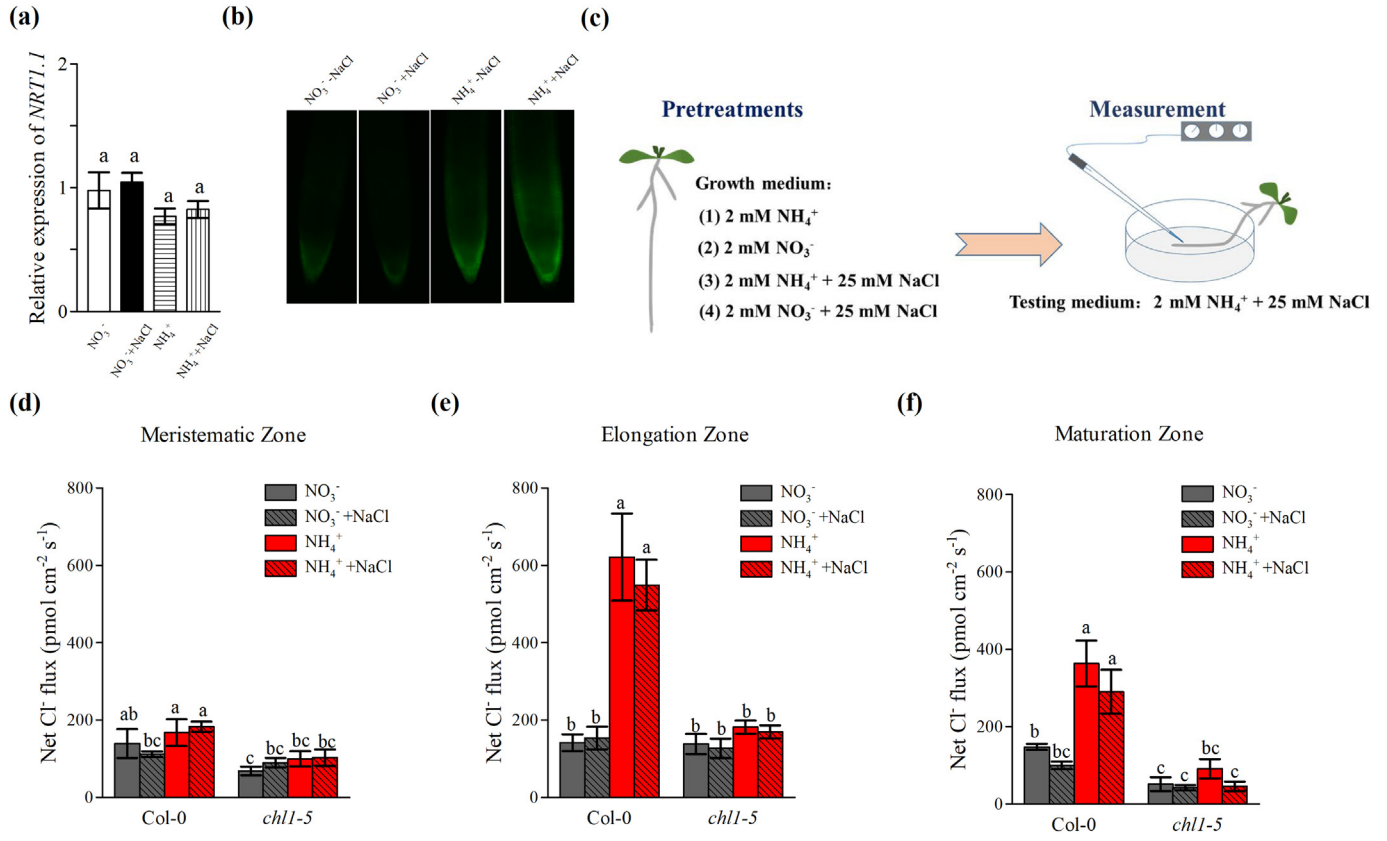


**Fig. 6.** Effect of $NH_4^+$ on the induction of NRT1.1-mediated $Cl^-$ uptake. (a) Expression of the *NRT1.1* gene in the roots of the Col-0 seedlings. (b) The expression of NRT1.1-GFP in *pNRT1.1*::*NRT1.1-GFP* plants. The 4-day-old seedlings were cultured in basal agar medium with either 2 mM $NO_3^-$ or 2 mM $NH_4^+$ as the sole nitrogen source, with or without 25 mM NaCl. The measurements were taken after 2 days of treatment. Bars and error bars indicate means ± standard deviations (n = 4 biological replicates). (c) Scheme depicting the pre-treatment of seedlings and the measurement of net $Cl^-$ flux. (d–f). Net $Cl^-$ flux in the meristematic, elongation, and maturation zones of roots. The 4-day-old Col-0 and *chl1-5* seedlings were pre-treated in the basal agar medium with either 2 mM $NO_3^-$ or 2 mM $NH_4^+$ as the sole nitrogen sources and with or without 25 mM NaCl, as depicted in Figure (c). The net $Cl^-$ fluxes in the different root zones were measured 2 days after treatment with 2 mM $NH_4^+$ and 25 mM NaCl. Statistical analysis: two-way ANOVA with post-hoc Tukey HSD test. Different letters indicate significant differences (*P*-values < 0.05). Bars and error bars indicate means ± standard deviations (n = 4 biological replicates).

for example, the toxicity threshold of $Cl^-$ concentrations accumulated in plant tissues is 4–7 mg $g^{-1}$ for citrus (*Citrus sinensis*) (Bell et al., 1997), 7–8 mg $g^{-1}$ for rice (*Oryza sativa*) (Yin et al., 1989), about 12 mg $g^{-1}$ for potato (*Solanum tuberosum*) (Corbett and Gausman, 1960), 25 mg $g^{-1}$ for cotton (*Gossypium raimondii*) (Tan and Shen, 1993), and 33 mg $g^{-1}$ for corn (*Zea mays*) (Parker et al., 1985). In this study, under the 25 mM NaCl treatment, the *A. thaliana* plants grown with $NH_4^+$ had about 9 mg $g^{-1}$ $Cl^-$ in their roots and showed evidence of toxicity (indicated by the inhibition of root growth) (Fig. 2c). *A. thaliana* likely has a lower toxic threshold for $Cl^-$ concentration than that of potato, cotton, and corn. The above findings suggested that screening crop cultivars with low efficacy of NRT1.1-conferred $Cl^-$ toxicity or a high toxic threshold of $Cl^-$ concentration may help to reduce the risk of decreasing crop yields in both coastal and non-coastal areas with frequent flooding and saline soils.

## 5. Conclusion

The $NH_4^+$-enrichment of the growth environment aggravates salt stress in *A. thaliana* plants as a result of excess accumulation of $Cl^-$, attributed to the increased $Cl^-$ acquisition by NRT1.1 and lack of competition from $NO_3^-$.

## CRediT authorship contribution statement

**Xing Xing Liu:** Investigation, Formal analysis, Writing - original draft. **Ya Xin Zhu:** Methodology. **Xian Zhi Fang:** Methodology. **Jia Yuan Ye:** Methodology. **Wen Xin Du:** Resources. **Qing Yang Zhu:** Resources. **Xian Yong Lin:** Writing - review & editing. **Chong Wei Jin:** Conceptualization, Writing - original draft.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.141244.

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