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RESEARCH ARTICLE

Enhanced nitrate reductase activity offers *Arabidopsis* ecotype Landsberg *erecta* better salt stress resistance than Col-0

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Keywords

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ABSTRACT

- The nitrogen utilization efficiency of plants varies depending on the plant species. In modern agriculture, nitrogen fertilizer is used to increase crop production, with the amount of fertilizer addition increasing steadily worldwide.
- This study included the two most used ecotypes of *Arabidopsis thaliana*, Landsberg *erecta* (*Ler*) and Col-0, which were used to identify differences at the molecular level. We found that the efficiency of nitrogen utilization and salt stress resistance differed between these two ecotypes of the same species.
- We demonstrated distinct salt stress resistance between *Ler* and Col-0 depending on the differences in nitrate level, which was explained by different regulation of the *NIA2* gene expression in these two ecotypes.
- Our results demonstrate that the genes and promoters regulate expression of these genes and contribute to trait differences. Further studies are required on genes and promoter elements for an improved understanding of the salinity stress resistance mechanism in plants.

INTRODUCTION

Nitrogen is one of the most important macromolecules for plant growth and is absorbed by roots mainly as nitrate or ammonium from the soil (Frink *et al.* 1999). This nitrogen is used during the synthesis of nucleic acids, amino acids and various secondary metabolites (Hanrahan & Chan 2005). However, the concentration of nitrogen in the soil is limited and nitrogen compounds cannot be synthesized by plants; therefore, a large amount of nitrogen fertilizer is now applied to agricultural land each year to increase the productivity of crops. Although plants absorb more than 50% of the nitrogen applied to the soil, some nitrogen seeps into the environment, causing contamination (Garnett *et al.* 2009; Good & Beatty 2011). If the nitrogen utilization rate of crops can be increased by understanding nitrogen signalling, crop productivity can be maximized using lower nitrogen fertilizer applications (Good & Beatty 2011; Hirel *et al.* 2011). However, the signalling mechanism and nitrogen utilization are complex systems affected by various factors (Forde 2002; Lillo *et al.* 2004; Guan *et al.* 2017; Liu *et al.* 2017). Many studies have examined these factors and systems; however, more studies are required to gain further understanding.

Plants optimize their growth through various signalling mechanisms when nitrogen is scarce in the environment (Marchive *et al.* 2013; Yu *et al.* 2016; Liu *et al.* 2017). For example, the expression of various nitrogen transport proteins can be increased to more effectively absorb nitrogen from the soil (Liu *et al.* 1999). In addition, various nitrogen movement pathways toward shoots are suppressed to distribute more nitrogen to the roots to promote root development, which allows roots to extend into the soil area where nitrogen is more abundant (Remans *et al.* 2006). External nitrate levels can be sensed by the NRT1.1 transceptor protein, which also serves as a nitrate transporter (Ho *et al.* 2009). NRT1.1 regulates the expression of major nitrate assimilation genes and nitrate intake functions (Ho *et al.* 2009).

Salt stress is another issue that should be further studied, as salt stress is becoming very severe because of the irrigation strategies used in modern agricultural systems (Rengasamy 2006). Our understanding of the signal transduction cascade during salinity stress has improved considerably. One well-known mechanism is the salt stress resistance mechanism that employs salt overly sensitive (SOS) genes (Halfter *et al.* 2000; van Zelm *et al.* 2020). The SOS pathway is a calcineurin B-like protein (CBL)–CBL-interacting protein kinase (CIPK) pathway (Liu & Zhu 1998). Calcium increases inside the cell in response to salt stress and binds to SOS3/CBL4, enabling SOS3/CBL4 to bind to SOS2/CIPK24 and ultimately forming the SOS2-SOS3 complex. Sodium is released from the plant cell by the H^+/cation antiporter SOS1/NHX7, which is phosphorylated by the SOS2-SOS3 complex (Halfter *et al.* 2000). The correlation between nitrogen and salt stress is important, given that modern agricultural practices use both nitrogen fertilizers and irrigation facilities. To investigate the correlation between salt stress and nitrogen, we selected *Arabidopsis* Ler and Col-0 plants, two ecotypes commonly used in *Arabidopsis* research, and found that resistance to salt stress in these plants varied depending on the nitrate concentration. In the present study, we demonstrated that the differences in salt stress resistance between *Ler* and Col-0 depending on nitrate level was explained by differences in regulation of *NIA2* gene expression in these two ecotypes.

MATERIAL AND METHODS

Plant growth conditions

T-DNA was inserted into exon NIA2 knockout mutants, *nia2-1,2* (SALK 138297c; SALK 088070). *A. thaliana* (Col-0 and L*er*) was used for the experiment. The seeds of *A. thaliana* Col-0 and L*er* were stored under bactericidal conditions at 4°C for 3 days, then grown in half-strength Murashige and Skoog (MS) medium with 16-h light/8-h dark, 23 ± 1°C, 50–55 µmol of photon m^{-2} s^{-1}, and approximately 70% humidity in a growth chamber. Control plants were grown in 1/2 MS medium with 5 mM nitrate, 2% sucrose and 0.5% phytagel (pH 5.8). The seedlings were supplemented with various concentrations of salt (0, 100, 150 or 200 mM NaCl) in the MS medium. As a single nitrogen source, 5 mM KNO_3 was added to each treatment; K_2SO_4 was added to all media without KNO_3 to adjust the K^+ concentration to 5 mM.

Quantitative RT-PCR (qRT-PCR)

The RNA extracted from the 50 mg samples using TRIzol Reagent was converted into cDNA using the method described in the RevertAid First Strand cDNA Synthetic Kit (H1622; Thermo Fisher Scientific, www.thermofisher.com). qRT-PCR was performed at the Bio-Rad Laboratories. Actin2 was used as internal control (Kozera & Rapacz 2013). The primers used are given in Table 1. Three independent replications were performed for each sample.

Chlorophyll assay

Chlorophyll was isolated from 7-day-old seedlings using the spectroscopic light intensity measurement method (Porra *et al.*, 1989). First, a 100-mg leaf sample was pulverized into a fine powder in liquid nitrogen. The ground sample was placed in a 1.5-ml tube with 80% acetone solution at 4°C made up to a 700-µl volume. The solution was gently mixed in the dark for 30 min then centrifuged at 848x*g*, 4°C for 15 min. The absorbance was measured using the following equations:

chlorophyll *a* (mg g^{-1}) = [12.7 (A663) 2.69 (A645)] V/1000 W.
chlorophyll *b* (mg g^{-1}) = [22.9 (A645) 4.86 (A663)] V/1000 W.
total chlorophyll (*a* + *b*) (mg g^{-1}) = [8.02 (A663) + 20.20 (A645)] V/1000 W.

where: V: volume of extract, W: weight of fresh leaves.

Nitrate reductase (NR) assay

Seedlings were grown for 7 days in 5 mM KNO_3, after which the experimental plants received 200 mM NaCl. The treatments were: nitrate-free, 5 mM nitrate, nitrate-free + 200 mM NaCl, and 5 mM nitrate + 200 mM NaCl, for 0, 6, 24 or 48 h. Nitrate reductase (NR) was extracted and measured using an NR Assay Kit (Catalogue # CAK1014; Cohesion Biosciences). A 100-mg sample was extracted from 1 ml extract and centrifuged at 4000 × *g* for 10 min. The NR activity was calculated by measuring absorbance at 520 nm.

Nitrate content analysis

The seedlings of 100 mg Col-0 and L*er* were exposed to the above different treatments for 0, 6, 24 or 48 h after 7 days of growth in the control medium. The samples were crushed in liquid nitrogen, after which distilled water (1 ml) was added to the sample; then it was placed in boiled distilled water for 20 min. The mixture was centrifuged at 13,000 rpm at 4°C for 10 min, and 100 µl supernatant was added to a new e-tube and 400 µl salicylic acid added. The samples were then cultured at room temperature (25–30°C) for 30 min. Simultaneously, 9.5 ml 8% NaOH solution was placed in a 15-ml Falcon tube and, after 30 min, the culture fluid was treated and cooled to 4°C for 5 min. The absorption of nitrate was measured at 410 nm and the absorbance calculated (Zhao & Wang 2017).

Table 1. Sequence of primers used in this study.

Primer name	Primer sequences (5′-3′)
NRT1.1	F: TAAGGGATCAGGAAGCGGGA
qrt	R: AAGAGGATGCATGTTGCCCA
NRT1.2	F: AGGTCTCAAGATGGGAAGGC
qrt	R: GGCGAGCATGCCACCGTGA
NRT2.1	F: AAAGACAAATTCGGAAAGATTCTG
qRT	R: AAGTACTCGGCGATAACATTATCA
NLP7qRT	F: GCTGAAAGTTGATGCAGGAACG
	R: CAGGAGCTCCCTAGATTTGTCG
NIA1qRT	F: GAAACTAGCAATGCTCGCCG
	R: ATC CTC GGT TCT GT TGCGT
NIA2qRT	F: CAT TT CCT TTG CGC CAC CA
	R: AGC TCG AAG TAG CCA ACC AC
BG1qRT	F: TTACTATACTTCAGTGTTTGCAAAAG
	R: CTAGAGTTCTTCCCTCAGCTTG
RD29AqRT	GATATCGACAAGGATGTGCCG
	R: GTATCCAGGTCTTCCCTTCGC
ACTIN2	F: TGTGGATCTCCAAGGCCGAGTA
	R: CCCCAGCTTTTTAAGCCTTTGATC
NRT1.1promoeter-seq	F: TGCAGAACAACTGAATGGGC
	R:CTTGAAGATGGATTAATGATCTCTCTC
NIA2promoter-seq	F:CTTCCTATGTTAACACATTGATAATTCTTT
	R:ATCACACGTATGGGACCATTTGTGA
NIA1promoter-seq	F:TACAAATCCATACAAGAGTCTATCTTC
	R:TCGAGATTTTATTCACCATAGTTTC
NLP7promoter-seq	F:CGAGACCGTGGTATTGTATATACCAG
	R:GTTATTGCATGTCAGGACTCATGTCA
NRT1.5qRT	F:CGGACTTGTGATTGCTGTCATAGC
	R:GGAGCCTGCCAGAAGATGCTT
NRT1.8qRT	F:GGCTTCAGATTCTTGGATAGAGC
	R:AACCACAGAGTAGAGGATGGTGC
NIA2 promoter-chip	F:CGGCTTTGTGTCACGAATAAGAAAA
	R: CCTGTAATACAATATAAAAATGTGATCAAAA

The ^{15}N uptake assay

The ^{15}N intake activity was analysed using $^{15}NO_3$ following the methodology described by Lin *et al.* (2008). Plants were exposed to 200 mM NaCl and $K^{15}NO_3$. In the KNO_3 5 mM 1/2 MS medium, the seedlings grown for 7 days were exposed to treatments of nitrate-free + 200 mM NaCl and 5 mM nitrate + 200 mM NaCl for 24 h and 48 h. After transfer to 0.1 mM $CaSO_4$ for 1 min, 5 mM $K^{15}NO_3$ (99% atom) became the single N source and was reformed for 2 h; this sample was then transferred to MS nutrient solution. The roots were dried at 70°C, ground and weighed. The ^{15}N content was analysed

using a continuous isotope mass spectrometer (Thermo-MAT253) combined with a defect-free analyser (Flash 2000 HT; Thermo Fisher Scientific, Waltham, MA, USA).

Determination of proline content

The proline content was measured as described previously (Bates *et al.* 1973). Proline was extracted from 100 mg of seedlings using liquid nitrogen and 1 ml 3% sulfosalicylic acid. The ninhydrin reagent (80% ice acetic acid, 6.8% phosphoric acid, 70.17 mM ninhydrin) was reacted with 200 µl liquid extract for 60 min at 100°C. An ice bath was used to complete the reaction. The reaction mixture was the extracted with toluene (200 µl) and vortexed. The absorption of the toluene layer was measured at 520 nm using a UV/VIS spectrophotometer.

Anthocyanin measurement

We used 100 mg seedlings for each sample. We added 400µl MeOH (1% v/v HCl), wrapped seedlings in foil and incubated at 4°C overnight. We then added 400 µl distilled water and 400 µl chloroform and vortexed before centrifugation (848xg) at room temperature for 2 min. Anthocyanin level was determined using a spectrophotometer at A535 and A650. Anthocyanin = (A535–A650)/mg FW (Neff & Chory 1998).

Determination of abscisic acid (ABA) content

The ABA content and concentration were analysed using the meth of Liu *et al.* (2014). A total of 200 mg seedlings on day 7 were treated with nitrate-free, 5 mM nitrate, nitrate-free + 200 mM NaCl, or nitrate 5 mM nitrate + 200 mM NaCl for 0, 6, 24 or 48 h. The ABA concentration was measured according to the manufacturer's instructions (Phytodetek ELISA).

Chromatin immunoprecipitation (ChIP)

All processes of this experiment were conducted according to the methods described by Saleh *et al.* (2008). The 2-week-old seedlings were treated with salt stress as shown in the figure 1A. Then cross-linking buffer was added into the samples, vacuum-infiltrated and 2 M glycine solution added to stop the cross-linking reaction. Then water was removed and cold nucleation buffer added. After centrifugation, cold nucleation buffer was added and the mixture sonicated three times. Pre-equilibrated salmon sperm DNA/protein A agarose beads and acetyl K9 antibody were used for immunoprecipitation. After reverse cross-linking and protein digestion, the DNA precipitation process was conducted using the kit (cat. No.28104, Qiagen).

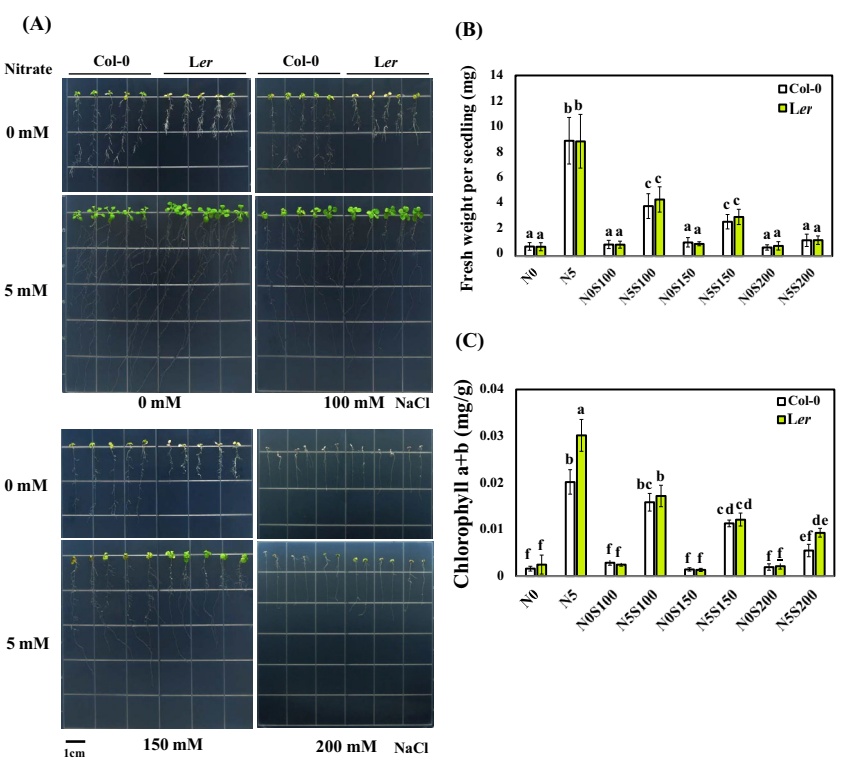

Fig. 1. Growth of L*er* and Col-0 under salt stress with or without nitrate. (A) Phenotypes of Col-0 and L*er* under different salt stress condition. In 5 mM nitrate MS agar containing 2% sucrose, Col-0 and L*er* seeds were germinated for 3 days and transferred to medium containing 0 mM nitrate (N0), grown for 4 days then transferred to 0 or 5 mM nitrate with 0, 100, 150 or 200 mM NaCl (N0: nitrate-free + 2.5 mM K_2SO_4, N5: nitrate 5 mM, N0S200: nitrate-free + 2.5 mM K_2SO_4 + 200 mM NaCl and N5S200: nitrate 5 mM + 200 mM NaCl). (B) FW of Col-0 and L*er* after exposure to the above conditions. Seeds of Col-0 and L*er* were germinated and grown for 3 days in N5, transferred N0 for 4 days, then to 0 or 5 mM nitrate with NaCl for 9 days. (**C**) Chlorophyll content of Col-0 and L*er* in response to the above (A) conditions. Values are mean ± SE of three independent experiments. Different letters indicate significant differences at $P < 0.05$ according to one-way ANOVA and Tukey's test.

Gene accession numbers and statistical analyses

The *A. thaliana* information resource accession numbers for the gene sequences used in this study are shown in (Table 1). Statistical analyses were performed using one-way ANOVA, followed by Tukey's test to compare means at the 95% confidence level.

RESULTS

Differentiation of salt stress resistance in L*er* and Col-0

Nitrate is the most abundant nitrogen source in agricultural land, and nitrate concentrations in agricultural soils typically range between 1 and 5 mM (Owen & Jones 2001). To determine the effect of nitrate on the salt stress resistance of plants, we examined the growth performance of two *Arabidopsis* ecotypes, L*er* and Col-0 under nitrate-free (0 mM nitrate: N0) or adequate conditions (5 mM nitrate: N5). The seedlings were grown for 3 days after germination in N5 medium then transferred to the test media containing nitrate and NaCl in combination. They were allowed to grow for a further 9 days before photographs were taken (nitrate-free + 200 mM NaCl N0S200; 5 mM nitrate + 200 mM NaCl: N5S200; Fig 1A, B). In the N0 condition, Col-0 grew better than L*er*, which was the same when salt was added. However, in the N5 condition, L*er* grew better, and the salt stress resistance was higher than that in Col-0. The chlorophyll content showed the same pattern as seen in the plant growth performance (Fig. 1C). To investigate differences in salinity tolerance of L*er* and Col-0 depending on nitrogen availability, we compared transcript levels of *RD29A*, a stress marker gene in L*er* and Col-0 seedlings incubated under N0, N5, N0S200 or N5S200 conditions for 0, 6, 24 or 48 h. When the roots and shoots were collected separately and qRT-PCR was performed, *RD29A* was highly expressed in the shoots of both L*er* and Col-0 incubated at N0S200 and N5S200 for 6 h, whereas *RD29A* was high in roots of L*er* only when incubated at N5S200 for 24 h (Fig. 2A, B). Thus, L*er* was more responsive to salt stress at N5S200, especially in the roots. To investigate whether a stress response other than changes in *RD29A* expression occurred in L*er* more than in Col-0, proline, anthocyanin and ABA content were measured. There was no difference in proline content between L*er* and Col-0 in the N5S200 condition (Fig. 2C), while anthocyanin and ABA content were low in L*er* at N5S200 after 48 h (Fig. 2D, E). β-glucosidase homologue 1 (*BG1*), which encodes the enzyme BG1 that plays an important role in releasing free biologically active (+)-ABA from the inactive ABA glucose ester (ABA-GE) (Lee *et al.* 2006), also decreased in L*er* at 24 and 48 h after stress treatment in roots (Fig. S1A, B). These conflicting results indicate that the different underlying salinity stress resistance mechanisms in L*er* and Col-0 are complicated.

Arabidopsis L*er* takes up more nitrate under salt stress than Col-0

Because the growth response to nitrate was different between L*er* and Col-0, we questioned whether nitrate contributed to this difference. The nitrate content was higher in L*er* than in Col-0 under normal conditions (Fig. 3A); however, as the incubation time increased, the difference gradually narrowed: after 48 h, the nitrate content in L*er* and Col-0 did not differ. To examine why nitrate concentrations was higher in L*er* than in Col-0 under normal conditions, we examined whether there was a difference in transcript levels of the nitrate transporter genes. The transcript levels of *NRT1.1*, *NRT2.1*, *NRT1.2*, *NRT1.5* and *NRT1.8* were slightly higher or similar in L*er* than in Col-0 under normal conditions, and the difference became larger at N5S200 after 48 h (Fig. 3B, Fig. S2). However, the high transcript levels of these transporters in L*er* at N5S200 after 48 h did not lead to a higher nitrate content in L*er*. L*er* absorbed less nitrate than Col-0 in the N0S200 condition; however, in N5S200 for 24 and 48 h, L*er* absorbed more nitrate than Col-0 (Fig. 4).

The NR activity of L*er* was higher than that of Col-0 under salt stress due to increased accumulation of *NIA2* transcripts

As shown in Fig. 5A, NR activity was much higher in L*er* at N5S200 after 48 h, which explains why L*er* and Col-0 did not differ in nitrate content despite the higher absorption of nitrate by L*er*. NR enzymes play a very important role in nitrogen assimilation and are regulated by various mechanisms. We confirmed that the transcripts of *NIA1* and *NIA2* genes accumulated more in L*er* than in Col-0 at N5S200 for 24 and 48 h (Fig 5B, C, Fig. S3A, B). Therefore, NR-encoding genes are transcriptionally regulated to increase enzyme activity under the N5S200 condition.

Difference in *NIA2* promoter between L*er* and Col-0, and loss of some of promoter sequences in L*er*

For the *NIA1* and *NIA2* gene transcripts to be more highly expressed in L*er* than in Col-0, the transcription regulators that bind to the gene promoters might be expressed more in L*er* than in Col-0. Alternatively, there might be differences in promoter elements of the *NIA1* and *NIA2* genes. NLP7, which binds to the *NIA2* gene promoter (Alvarez *et al.* 2020), was expressed more in L*er* than in Col-0 (Fig. S3C, D), leading us to determine the transcription regulator binding to the *NLP7* promote, which has not previously been reported. We sequenced approximately 1-kb upstream promoters of *NLP7*, *NRT1.1*, *IA1*, and *NIA2* genes and found that there was only a very small difference between L*er* and Col-0, except in the *NIA2* promoter (Fig. S4). There was a TA repeat sequence in the upstream −546-bp region of the *NIA2* gene promoter, where there was a difference between L*er* and Col-0 in sequencing (Fig. 6A). In this area, Col-0 had approximately 40 bp more than L*er*. Because the chromatin structure of the promoter has a significant effect on gene expression level, we examined whether the 40-bp loss in the *NIA2* gene promoter of L*er* led to a difference in the histone acetylation level. A ChIP assay was performed using an antibody raised against the H3K9 acetyl group, which confirmed that the acetylation level of H3K9 in the upstream −546-bp region of the *NIA2* gene promoter was much higher in Col-0 than in L*er* (Fig. 6B). In N5S200, more transcripts of *NIA2* accumulated and NR activity was higher in L*er* than in Col-0 (Fig. 5A–C). Therefore, L*er* may have a stronger salt stress resistance than Col-0 due to the difference in transcriptional regulation of *NIA2* based on loss of the TA region in this gene. To verify whether this was correct, we obtained two independent lines of *nia2 ko* seeds from The

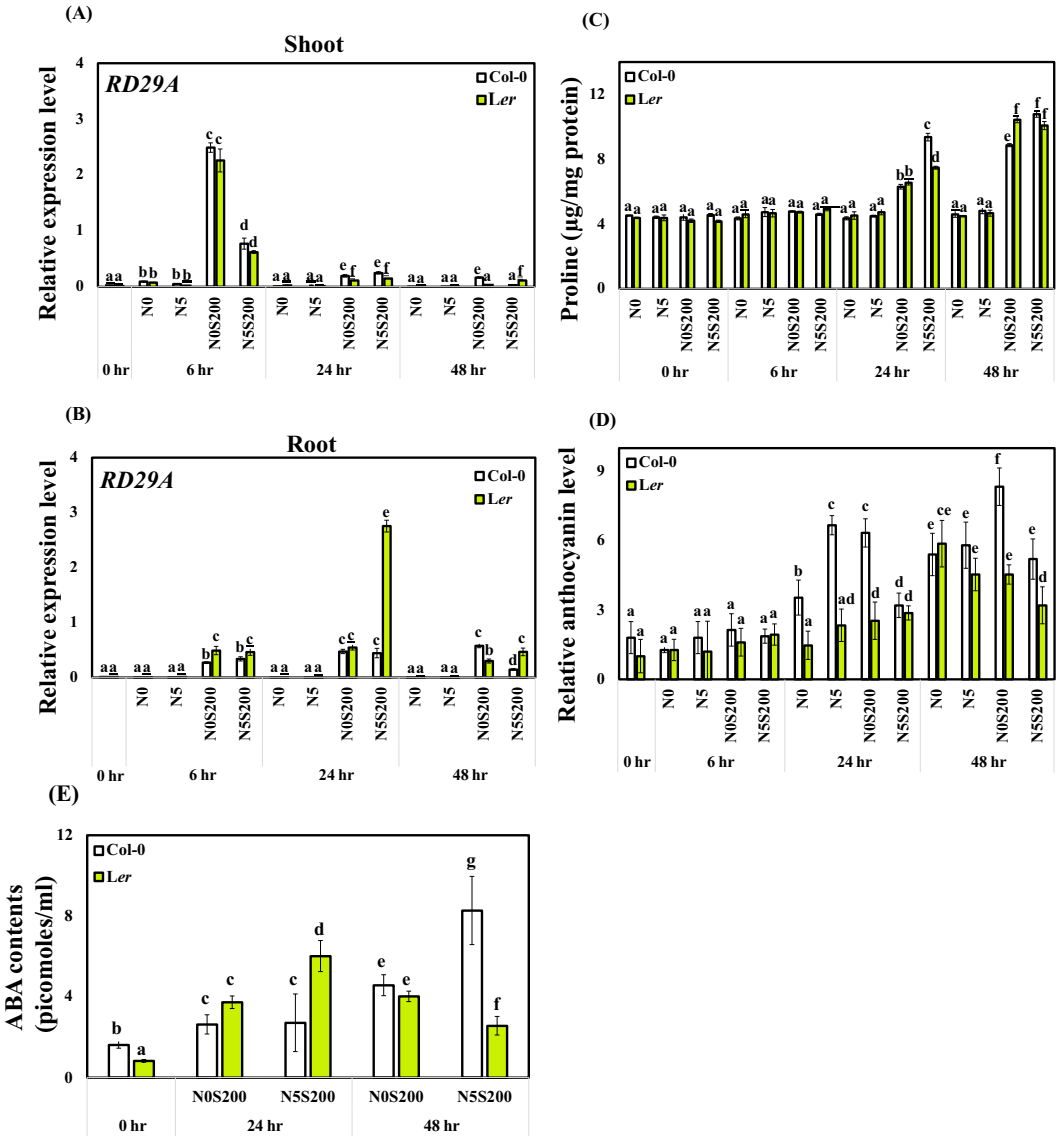

Fig. 2. Evaluation of salt stress markers in L*er* and Col-0. (A) and (B) Transcript level of *RD29A* in Col-0 and L*er* under indicated conditions from 50 mg of seedlings, separated into roots and shoots for tissue-specific quantification. (C) and (D), Proline (C) and anthocyanin (D) in Col-0 and L*er* seedlings. (E) ABA (ELISA) in Col-0 and L*er* at different times and salt conditions. Values are mean ± SE of three independent experiments. Different letters indicate significant differences at $P < 0.05$, according to one-way ANOVA and Tukey's test.

Arabidopsis Information Resource (TAIR) and investigated their salinity resistance under the N5S200 condition. As shown in Fig. 6C, 8 days after transfer to the saline media, root growth was significantly better in L*er* than in Col-0; however, the two lines of the *nia2 ko* plants had much weaker salinity stress than in L*er*. Root length after transfer to salinity demonstrated that *Ler* roots could still grow in salt stress (Fig. 6D). Therefore, NR activity is highly associated with salt stress resistance in *Arabidopsis*.

DISCUSSION

The abundance of nitrate in the soil is positively correlated with plant growth, although very high nitrate levels may prevent growth (Saiz-Fernández *et al.* 2020). Nitrate is a common nitrogen-containing molecule in soil (Haynes & Goh 1978; Crawford & Forde 2002). The soil nitrate level can be detected by nitrate transporter, NRT1.1, which is a dual functional protein that acts as both a sensor protein and a nitrate transporter (Ho *et al.* 2009; Hu *et al.* 2009). Sensing soil nitrate concentration is very important for plant growth and development. How would plants react if high salt stress is applied to the growth environment with varying nitrate concentrations? In the present study, we investigated whether there was a difference in salt stress resistance of plants under two different conditions, one that lacked nitrates and one with adequate nitrates in a growth environment. Additionally, we examined whether these responses differed in L*er* and Col-0, two of the most common ecotypes in *Arabidopsis* research.

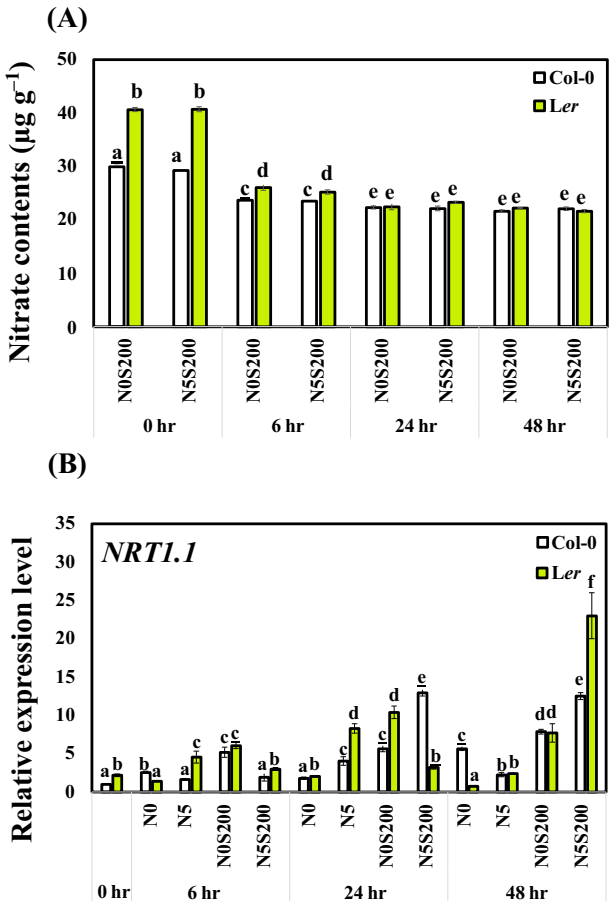

Fig. 3. Nitrate content and transcript level of *NRT* genes in Col-0 and L*er* under N0S200 and N5S200. (A) nitrate content of Col-0 and L*er* in response to various nitrate and salt stress. Col-0 and L*er* seeds were germinated and further grown in basic medium (5 mM nitrate) for 7 days, then transferred to N0S200 and N5S200 medium. (B), Transcript level of *NRT1.1* in Col-0 and L*er* in ± nitrate ± salt. A total of 50 mg seedlings were collected and ground for RNA extraction; for tissue-specific quantification, roots and shoots were separated. Transcripts of *NRT1.1* were obtained via quantitative RT-PCR. Values are mean ± SE of three independent experiments. Different letters indicate significant differences at $P < 0.05$ according to one-way ANOVA and Tukey's test.

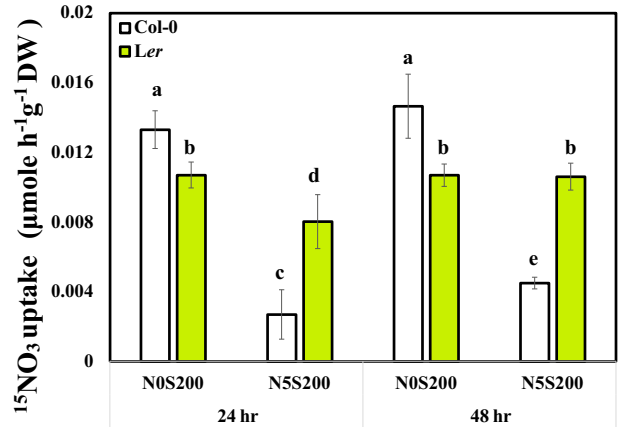

Fig. 4. Nitrogen uptake in Col-0 and Ler. Col-0 and L*er* were cultured in N5 medium for 7 days, then in N0S200 and N5S200 for 2 days and 1 day. Seedlings were then given N5 for 2 h and washed in $CaSO_4$. Values are mean ± SE of three independent experiments. Different letters indicate significant differences at $P < 0.05$, according to one-way ANOVA and Tukey's test.

When salt stress was included in nitrate-deficient conditions, growth of both ecotypes was inhibited, with the effect more severe in L*er* than in Col-0 (Fig. 1A). In contrast to the nitrate-deficient conditions, if there was adequate nitrate in the medium, L*er* could resist salt stress much better than Col-0 (Fig. 1A). Salinity stress triggers various signalling reactions in plants, helping them endure this stress (Liu & Zhu 1998; Halfter *et al.* 2000; Liu *et al.* 2000). In one stress marker gene, *RD29A*, transcript level did not clearly differ between Col-0 and L*er* in all growth conditions examined, whereas its transcript level was greatly enhanced in roots of L*er* in the N5S200 condition (Fig. 2A, B). Thus, salinity stress signalling was more efficient in L*er* than in Col-0, especially in the roots. The proline content (Hasegawa *et al.* 2000), anthocyanins (Kim *et al.* 2017) and ABA (He & Cramer 1996), which are indicators of salinity stress response, revealed that proline did not differ significantly between L*er* and Col-0 in the N5S200 condition after 48 h (Fig. 2C). Both ABA and anthocyanins help plants to cope with salt stress. Expression of various stress marker genes is enhanced by ABA and plays an important role in salt stress-signalling (see review of Vishwakarma *et al.* 2017). Therefore, the enhanced salt stress resistance of L*er* was not caused by increased ABA or anthocyanin content (Fig. 2D, E), which were lower in L*er* than in Col-0; therefore, L*er* might be less stressed than Col-0 under adequate nitrate and salt stress conditions.

In 5 mM nitrate medium, L*er* had better salinity stress resistance than Col-0; therefore, we examined whether there was any difference in nitrate content between these two ecotypes. Under normal conditions, nitrate was higher in L*er* than in Col-0 for the N0S200 and N5S200 conditions, whereas nitrate of both ecotypes gradually became similar as the salinity stress treatment period increased up to 48 h (Fig. 3A). The reason for this may be that the transcript level of nitrate transporter genes in L*er* increased more than in Col-0. Among *NRT1.1*, *NRT2.1* and *NRT2.1*, the transcript level of *NRT1.1* was twice as high in L*er* than in Col-0 in N5S200 at 48 h (Fig 3B, Fig. S2A, B). This difference was much larger between L*er* and Col-0 for transcript levels of *NRT1.5* and *NRT1.8*, which were examined because these transporters are closely related to salinity stress (Li *et al.* 2010; Chen *et al.* 2012). *NRT1.5* and *NRT1.8* levels in both roots and shoots of L*er* increased in the N5S200 after 48 h. Originally, it was assumed that the *NRT1.5* transcript level would be lower in L*er* to explain its increased salinity stress resistance; however, the results showed that the opposite was true (Fig. S2C–F). The *NRT1.8* transcript level explained the higher salt resistance of L*er* than of Col-0; however, it is difficult to conclude that the better salt stress tolerance of L*er* was due to higher nitrate distribution to the roots. Nitrate in the soil is a common nitrogen-containing molecule (Haynes & Goh 1978); therefore, if nitrate uptake from the soil is adequate and the absorbed nitrate is used appropriately for assimilation, the metabolic reactions required for plant stress resistance will function. In N0S200 for 48 h, Col-0 absorbed more nitrate than L*er*; however, in N5S200 for 48 h, L*er* absorbed more

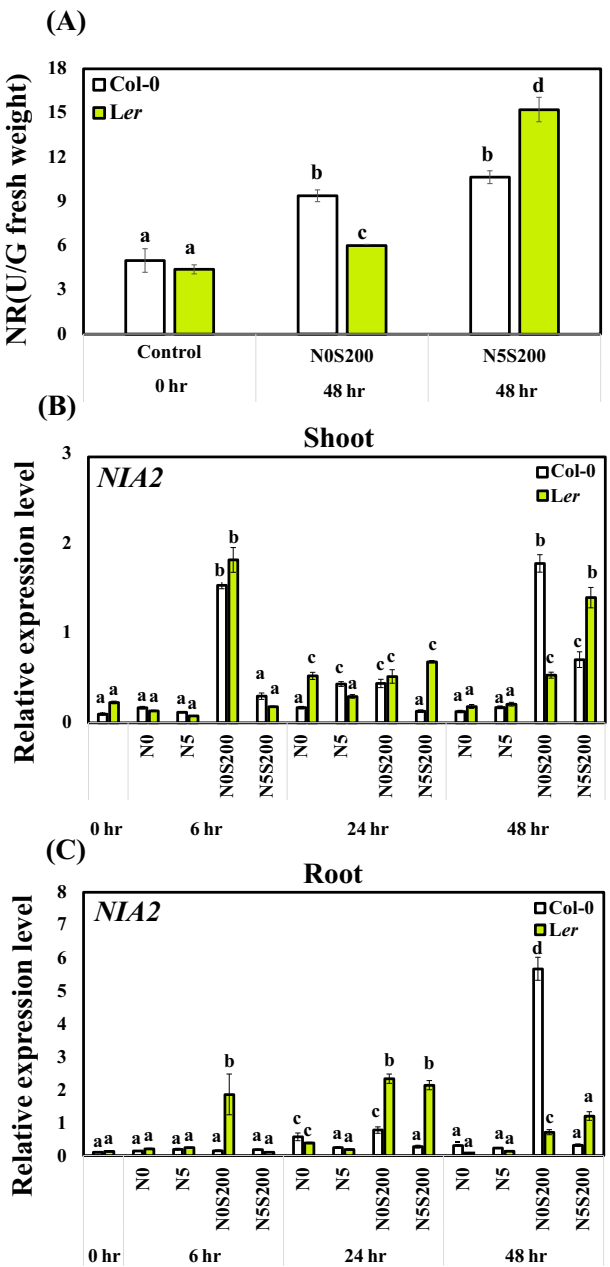

Fig. 5. Nitrate reductase activity of Col-0 and L*er*. (A) Nitrate reductase activity after salt treatment in Col-0 and L*er*. Col-0 and L*er* were grown in N5 for 7 days then transferred to N0S200 and N5S200 and further incubated for 48 h before nitrate reductase determination. (B), (C) Transcript level of *NIA2* in Col-0 and L*er* ± nitrate ± salt over 48 h from 50 mg seedlings (shoot and root separately) for total RNA quantification and quantitative RT-PCR. Values are mean ± SE obtained of three independent experiments. Different letters indicate significant differences at $P < 0.05$ according to one-way ANOVA and Tukey's test.

nitrate than Col-0 (Fig. 4). These reaction patterns were identical to those for NR activity (Fig. 5A). L*er* absorbs more nitrate than Col-0 because more nitrate transporter proteins, such as NRT1.1, are expressed in L*er* than in Col-0 (Fig 3, Fig. S2). Moreover, the transcript levels of NR-encoding genes, *NIA1* and *NIA2*, clearly differ between N0S200 and N5S200 conditions, with L*er* having higher transcripts of *NIA1* and *NIA2* in N5S200 than Col-0 (Fig 5B, C, Fig. S3A, B). Thus, the increased nitrate uptake and NR activity of L*er* in the N5S200 was partly due to transcriptional regulation of the corresponding genes.

Transcriptional control plays an important role in the improved salt stress tolerance of L*er*; therefore, we examined differences in the promoters of several nitrate signalling components. The 1-kb upstream promoter sequence of *NRT1.1*, *NLP7* and *NIA1* genes involved in nitrate signalling did not differ in the base sequence between L*er* and Col-0. However, there was a distinct difference between L*er* and Col-0 in the *NIA2* gene promoter. Approximately 40 bp in the upstream promoter −550-bp region of the *NIA2* gene were absent in the L*er* genome. As shown in Fig. 6A, this area consists of AT iteration sequences. We investigated whether this region had a different nucleosome modification pattern to determine whether it played an important role in expressing *NIA2*. The histone acetylation level was lower in the upstream promoter −550-bp region of *NIA2* in L*er* than in Col-0. Histone acetylation reduction generally leads to a reduction in binding of certain transcription factors (TFs) (Struhl, 1998). If this promoter region is essential for coupling of the negative regulator, L*er* would have difficulty combining the negative regulator because 36 bp are lost from this promoter region. This might explain, in part, why *NIA2* in L*er* was more highly expressed than in Col-0. Further studies are required to identify the exact mechanism to explain the difference in *NIA2* transcript levels in L*er* and Col-0. The promoter and upstream region of the *OsDREB1b* gene are known to change in nucleosome arrangement in response to low temperature stress (Roy *et al.* 2014). In particular, there were significant changes in the core promoter, TATA box, including hyperacetylation of histone H3K9. These results provide evidence to support nucleosome modification in the promoter region has an important role in regulation of gene expression. To investigate whether the decrease in NR activity might cause a decrease in salinity stress resistance, we investigated whether *nia2 ko* mutant plants were less resistant to salt stress than L*er* in the N5S200 condition, and found that our prediction is correct (Fig. 6C) and supports our hypothesis. The difference in salinity stress resistance based on nitrate concentrations of L*er* and Col-0 is not only due to the difference in *NIA2* gene expression. The strength of salinity resistance, which varies depending among species and ecotypes, is likely derived from differences in genes. Our results demonstrate that the genes themselves and the promoters regulating gene expression can contribute to trait differences. One biochemical process that is affected by salt stress is nitrate assimilation pathways, as reported in several crops as having negative effects on nitrogen metabolism (Baki *et al.* 2000; Flores *et al.* 2000; Carillo *et al.* 2005). Therefore, it is likely that the presence of NR with high activity under salt conditions will contribute to salt stress resistance. Controlling expression of this gene to maintain high levels of this enzyme may also be important. In the near future, it is assumed that expression regulation of genes might be possible when the promoter sequence of specific genes is edited using gene editing technology. To do so, future studies on genes and promoter elements are necessary to better understand the mechanism of salinity stress resistance in plants.

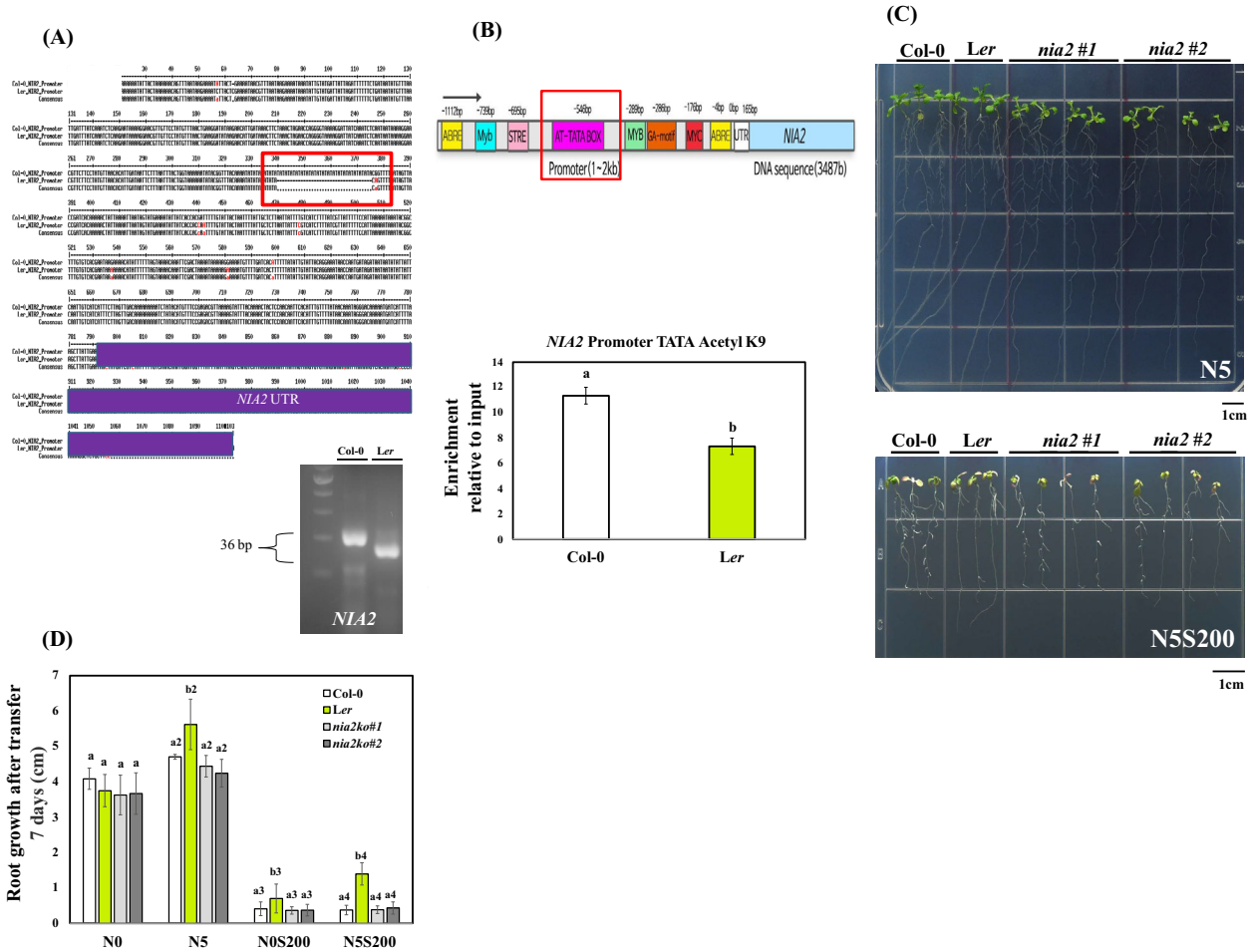

Fig. 6. Acetylation level of *NIA2* promoter in Col-0 and L*er*. (A) Transcription factor-binding sites in upstream 1200-bp sequences of *NIA2* promoter in Col-0 and L*er*. (B) Primers used for quantification of *NIA2* promoter sequence in a red box, showing differences in base sequence of Col-0 and L*er*; fold enrichment of acetyl k9 antibody of *NIA2* promoter regions shown. (C) Two *nia2* ko homo lines obtained from TAIR, Col-0 and L*er*, germinated on basic medium (N5) then after growth on N5S200 medium for 7 days. (D) Root growth showing quantitative differences between *nia2*ko and Col-0, L*er*. Values are mean ± SE of three independent experiments. Different letters indicate significant differences at $P < 0.05$ according to one-way ANOVA and Tukey's test.

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AUTHOR CONTRIBUTIONS

Seokjin Lee: salt stress phenotype assay, qRT-PCR, NR assay, ChIP assay; Jun Ho Choi: qRT-PCR, promoter sequencing; Hai An Truong: ABA content determination; Ye ji Lee: nitrate content measurement; Hojoung Lee: experimental design, manuscript writing.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Relative expression level of *BG1* in roots and shoots. S1 (A) and (B) as in Fig 2(A) seedlings collected for tissue-specific quantification. Expression of *BG1*, an ABA-related gene, was investigated in shoots and roots. Values are mean ± SE of three independent experiments. Different letters indicate significant differences at $P < 0.05$, according to one-way ANOVA and Tukey's test.

Figure S2. Transcript levels of *NRT* in Col-0 and L*er* under N0S200 and N5S200. (A)–(F) Col-0 and L*er* seeds germinated and grown for 7 days in 5 mM MS agar containing 2% sucrose then transferred to the indicated medium. A sample of 50 mg seedlings were collected (separating roots and shoots) and ground for total RNA extraction. Transcript levels of *NRT1.2*, *NRT2.1*, *NRT1.5*, *NRT1.8* obtained *via* quantitative RT-PCR. Values are mean ± SE of three independent experiments. Different letters indicate significant differences at $P < 0.05$ according to one-way ANOVA and Tukey's test.

Figure S3. Relative expression of *NIA1*, *NLP7* in Col-0 and L*er* under N0S200 and N5S200. (A)–(D) Col-0 and L*er* seeds were germinated and grown for 7 days in 5 mM MS agar containing 2% sucrose then transferred to the indicated medium for incubation for 0, 6, 24 or 48 h. A sample of 50 mg seedlings were collected (separating roots and shoots) and ground for

total RNA extraction. Transcript levels of (A), (B) *NIA1*, and (C), (D) *NLP7* obtained *via* quantitative RT-PCR. Values are mean ± SE of three independent experiments. Different letters indicate significant differences at $P < 0.05$ according to one-way ANOVA and Tukey's test.

Figure S4. Promoter sequencing of *NIA1* and *NLP7* genes in L*er*. (A)–(C) Sequence alignment between Col-0 and L*er NIA1*, *NLP7 and NRT1.1* promoters.

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