The nitrate transporter NRT2.1 functions in the ethylene response to nitrate deficiency in *Arabidopsis*

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Abstract

The ethylene signaling pathway is closely associated with complex environmental stresses. Previous studies have reported impact of high nitrate (HN) availability on ethylene biosynthesis and regulation of ethylene on *NITRATE TRANSPORTER 2.1* (*NRT2.1*) expression. However, molecular interaction between *NRT2.1* transcript levels and the ethylene signaling pathway under nitrate deficiency is still elusive. Here, we report a low nitrate (LN) treatment-induced rapid burst of ethylene production and regulated expression of ethylene signaling components *CTR1*, *EIN3* and *EIL1* in wild-type *Arabidopsis thaliana* (Col-0) seedlings, and enhanced ethylene response reporter *EBS:GUS* activity in both Col-0 and the ethylene mutants *ein3-1eil1-1* and *ctr1-1*. LN treatment also caused up-regulation of *NRT2.1* expression, which was responsible for an enhanced high-affinity nitrate uptake. Comparison of ethylene production and *EBS:GUS* activity between *nrt1.1*, *nrt2.1* mutants and Col-0 indicated that this up-regulation of *NRT2.1* expression caused a positive effect on ethylene biosynthesis and signaling under LN treatment. On the other hand, ethylene down-regulated *NRT2.1* expression and reduced the high-affinity nitrate uptake. Together, these findings uncover a negative feedback loop between *NRT2.1* expression and ethylene biosynthesis and signaling under nitrate deficiency, which may contribute to finely tuning of plant nitrate acquisition during exploring dynamic soil conditions.

Key words: nitrate deficiency; ethylene biosynthesis and signaling; nitrate transporter NRT2.1.

Introduction

Nitrogen availability is a major environmental factor that regulates plant growth, development, and metabolism. Nitrate (NO_3^-) and ammonium (NH_4^+) represent the most readily available forms of nitrogen for root absorption from the soil. NO_3^- is highly mobile in soil and is the preferred nitrogen source in many soil types. NO_3^- uptake, reduction, and assimilation are essential for plant growth as well as nitrogen input in many terrestrial trophic chains (Crawford & Glass 1998; Daniel-Vedele, Filleur & Caboche 1998; Williams & Miller 2001).

There are two distinct NO_3^- uptake systems in higher plants, namely the low-affinity transport system (LATS), which is responsible for uptake when NO_3^- is plentiful (> 1 mM), and the high-affinity transport system (HATS), which scavenges NO_3^- from the soil at concentrations between 1 μM and 1 mM. So far, two gene families, *NRT1* and *NRT2*, have been identified as being involved in the LATS and HATS, respectively (Crawford & Glass 1998; Forde 2000).

In *Arabidopsis*, seven *NRT2* genes are significantly expressed in the roots and up-regulated at the transcript level by nitrogen starvation, indicating they are required for the HATS under nitrogen-limiting conditions (Daniel-Vedele *et al.* 1998; Okamoto, Vidmar & Glass 2003; Orsel *et al.* 2004; Zhuo *et al.* 1999). Of their protein products, NRT2.1 appears to be the most critical one for high-affinity NO_3^- uptake. The NRT2.1/NRT2.2 deletion mutant *atnrt2.1-1* shows strongly reduced HATS activity (27% of the activity seen in wild type) under various experimental conditions (Cerezo *et al.* 2001; Filleur *et al.* 2001; Li *et al.* 2007). Among the seven *NRT2* genes, only *NRT2.1* exhibits a statistically significant correlation between the transcript level and HATS influx, and it is stimulated by low external NO_3^- (Okamoto *et al.* 2003) and inhibited in response to downstream products of NO_3^- assimilation (e.g., NH_4^+ and certain amino acids) (Vidmar *et al.*

2000; Zhuo *et al.* 1999).

Ethylene is an important gaseous hormone that regulates many physiological responses in plants, including seed germination, cell elongation, fruit ripening and abscission, leaf and flower senescence, and resistance to pathogens and insect attack (Bleecker & Kende 2000; Johnson & Ecker 1998). Ethylene biosynthesis and signaling have been well characterized at the molecular level, providing many genetic tools that can be used to determine how ethylene signaling plays a role in the physiological responses of plants. Formation of the ethylene synthetic precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is catalysed by ACC synthase (ACS) and ACC oxidase(Kieber *et al.* 1993), while 2-aminoethoxyvinyl-glycine (AVG) inhibits ACS and ethylene biosynthesis. CONSTITUTIVE ETHYLENE RESPONSE 1 (CTR1) is a Raf-like kinase that acts as a negative regulator of ethylene signaling (Kieber *et al.* 1993). The mutant *ctr1-1* shows constitutive ethylene responses (Clark *et al.* 1998; Gao *et al.* 2003). ETHYLENE INSENSITIVE 3 (EIN3) and its closest homolog EIN3-LIKE 1 (EIL1) are two primary transcription factors (An *et al.* 2010; Chao *et al.* 1997) that stimulate response wide range of target genes leading to diverse plant ethylene responses (Guo & Ecker 2004). The double mutant *ein3-1eil1-1* displays strong ethylene insensitivity in terms of the triple response and defense response (Alonso *et al.* 2003).

Many previous reports have demonstrated that ethylene is closely associated with physiological and morphological responses to nutritional deficiency, including phosphorus starvation (Lopez-Bucio, Cruz-Ramirez & Herrera-Estrella 2003; Zhang, Lynch & Brown 2003), iron deficiency (Romera, Alcantara & de la Guardia 1999; Schmidt 2001; Zaid *et al.* 2003) and potassium deficiency (Jung, Shin & Schachtman 2009; Shin & Schachtman 2004). There are several studies on the involvement of ethylene in NO_3^--dependent physiological processes. For

example, the sensitivity of maize to ethylene is increased under conditions of nitrogen deficiency (He, Morgan & Drew 1992; Schmelz *et al.* 2003), and expression of the NO_3^- transporters *NRT1.1* and *NRT2.1* is sensitive to ethylene (Leblanc *et al.* 2008; Tian, Sun & Zhang 2009). In addition, Tian *et al.* (2009) detected a rapid rise in ethylene production upon exposure to HN conditions. However, there has been no detailed study elaborated the molecular mechanism of the interaction between NO_3^- deficiency and ethylene biosynthesis and signaling.

In this study, we used wild-type *Arabidopsis thaliana* (Col-0), the ethylene-insensitive mutant *ein3-1eil1-1*, the constitutive ethylene response mutant *ctr1-1*, the low-affinity NO_3^- transporter mutant *nrt1.1*, the high-affinity NO_3^- transporter mutant *nrt2.1*, and ethylene reporter lines *EBS:GUS* in both Col-0 and these mutant backgrounds to examine the effect of NO_3^- deficiency on ethylene biosynthesis and signaling. Moreover, we examined the role of *NRT2.1* in the ethylene-mediated response to NO_3^- deficiency.

Materials and methods

Plant materials

All *Arabidopsis thaliana* mutants and transgenic lines used in this study were of the Col-0 background. Seeds of wild-type Col-0 and the NO_3^- transporter mutants *nrt1.1* (SALK_138710C) and *nrt2.1* (CS859604) were obtained from the ABRC (Ohio State University, Columbus, OH, USA) seed stock center. The ethylene-insensitive mutant *ein3-1eil1-1* (Alonso *et al.* 2003), constitutive ethylene response mutant *ctr1-1* (Kieber *et al.* 1993), and *EBS:GUS* (Stepanova *et al.* 2007) line were described previously.

Multiple genotype combinations (*nrt1.1/EBS:GUS* and *nrt2.1/EBS:GUS*) were generated by

genetic crosses and selected on hygromycin B for homozygous progenies. Experiments were performed with F3- or F4-derived homozygous plants for each crossed line.

Plant growth conditions

All seeds were surface-sterilized by incubation for 1 min in 75% ethanol followed by 10 min in 10% (v: v) sodium hypochlorite, and rinsed with sterile distilled water for more than four times. The sterilized seeds were germinated on glass plates (diameter, 9 cm) containing nitrogen-sufficient (HN, 10 mM NO_3^-) medium for 7 days, then transferred to plates containing HN or nitrogen-deficient (LN, 0.2 mM NO_3^-) medium in the absence or presence of 10 μM ACC (Sigma, St. Louis, MO, USA) or 10 μM AVG (Sigma) for 24 h. Basic medium containing 0.5 mM $CaSO_4$, 0.5 mM $MgCl_2$, 1 mM KH_2PO_4, 2.5 mM MES (Sigma), 50 μM NaFeEDTA, 50 μM H_3BO_3, 12 μM $MnCl_2$, 1 μM $CuCl_2$, 1 μM $ZnCl_2$, and 0.03 μM $NH_4Mo_7O_{24}$, pH 5.8 (adjusted with NaOH), with 1% sucrose and 0.8% (w: v) agar were used. This basic medium was complemented with 10 mM KNO_3 as the sole nitrogen source in the nitrogen-sufficient medium. The K^+ concentration was adjusted to 10 mM by adding K_2SO_4 to the nitrogen-deficient medium. After 2 days of storage at 4°C in the dark, the plates were incubated vertically in a controlled environment with a 16-h light/8-h dark regimen at 20°C/23°C, 80% relative humidity, and 150 μ mol·m^{-2}·s^{-1} irradiation.

Measurement of ethylene production

To measure ethylene production in the wild-type plants and NO_3^- transporter mutants, 7-day-old plants grown on nitrogen-sufficient medium were transferred to 30 mL-vials containing media with distinct NO_3^- concentrations (0.2 or 10 mM), and then incubated at room temperature. After incubation for 0, 0.5, 1, 3, 6 , or 24 h, 0.3 mL of headspace air was sampled from each vial and the

ethylene content was measured using a 6850 series gas chromatography (GC) system (Hewlett-Packard, Palo Alto, CA, USA) equipped with an HP Plot alumina-based capillary column (Agilent Technologies, Palo Alto, CA, USA). The fresh tissue weight of each sample was measured.

Measurement of the net NO_3^- flux using the scanning ion-selective electrode technique (SIET)

The net fluxes of NO_3^- were measured non-invasively using SIET (BIO-003A system; Younger USA Science and Technology Corp., Amherst, MA, USA; Applicable Electronics Inc., Forestdale, MA, USA; Science Wares Inc., Falmouth, MA, USA). The principle behind this method and the instrument were described previously (Sun *et al.* 2009). Measurements were performed at room temperature (24-26°C). After treatment for 24 h on medium containing 0.2 or 10 mM NO_3^-, the roots of the seedlings were immediately equilibrated in measuring solution containing 0.2 mM NO_3^- for 30 min, and then transferred to a small plastic dish (diameter, 3 cm) containing 4 mL of fresh measuring solution. The root was immobilized by a small piece of quartz at the bottom of the dish. The microelectrode was vibrated in the measuring solution between 5 μm and 35 μm from the root surface along an axis perpendicular to the root. The background was recorded by vibrating the electrode in measuring solution not containing roots. Glass microelectrodes with 2-4 mm apertures were made and silanized by Xuyue Science and Technology Co., Ltd. (Beijing, China). KNO_3 (100 mM) was added as a backfilling solution, followed by 20 μM of a commercially available ionophore cocktail to measure NO_3^- (NO_3^--selective liquid ion exchange cocktail #72549; Sigma) in front of the microelectrode. Prior to the flux measurements, the ion-selective electrodes were calibrated using NO_3^- at concentrations of 0.1 and 1.0 mM. The net fluxes of NO_3^- at the

mature zone were measured. Each plant was measured for at least 10 min. The final flux values for each treatment represent the means of five plants. The measuring solution was composed of 0.2 mM $CaCl_2$, 0.1 mM KCl, 0.2 mM KNO_3, and 0.5 g/L MES (pH 5.8, adjusted with 1 M NaOH). All measurements of the net NO_3^- fluxes were performed at Xuyue Science and Technology Co., Ltd.

GUS reporter assay

GUS assays were performed as described previously (Jefferson, Kavanagh & Bevan 1987; Stepanova *et al.* 2005). Briefly, 7-day-old seedlings were grown as described above on agar plates, which were treated with or without ACC/AVG at specific concentrations (0.2 or 10 mM) of NO_3^-, for 24 h. The seedlings were rinsed three times with staining buffer without X-Gluc and stained with GUS staining buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM Na_2EDTA, 0.5 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, 0.5 mM $K_3[Fe(CN)_6]$, 0.1% Triton X-100, and 1 mg/mL X-Gluc) for 12 h at 37°C in the dark. The stained seedlings were then rinsed for 15 min in 70% ethanol, and mounted for 2 h in Hoyer's solution (chloral hydrate:water:glycerol; 8:3:1; w/v/v). GUS expression in the leaf and root mature zone was observed using a Leica MZFLIII dissecting microscope equipped with an Olympus DP-50 digital camera.

Gene expression analysis

Quantitative real-time RT-PCR analysis was performed to study the expression patterns of *NRT2.1*, *CTR1*, *EIN3*, and *EIL1* in response to different treatments, including varying NO_3^- concentrations (0.2 or 10 mM), the ethylene precursor ACC, and the ethylene synthesis inhibitor AVG. Total RNA was extracted from *Arabidopsis* roots with Trizol reagent (Invitrogen) and treated with RNase -Free DNase I (Promega). Total RNAs were reverse-transcribed to first-strand cDNA in a

20-μL volume with M-MLV Reverse Transcriptase (Promega). Samples were diluted to 100 μL with water, and 5 μL of each sample (approximately 8 ng of RNA) was amplified using SYBR GreenER qPCR SuperMix Universal (Invitrogen) in a 25-μL reaction containing 5 μL of diluted cDNA, 12.5 μL of SYBR GreenER qPCR SuperMix Universal, 0.5 μL of Rox Reference Dye, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, and 5 μL of water. A Bio-Rad iCycler iQ System (Bio-Rad Laboratories) was used to run quantitative RT-PCR with the following primer pair combinations:

NRT2.1: 5'-CTGGAGGGAACTTTGGATCAGGG-3' and 5'-GTCACAGGTAACGTGCAAGCGACTA-3'.

EIN3: 5'-GCATGTCCACATCGAGACAGTCG-3' and GAGTTCACTGGCCTTGGCTGAG-3'.

EIL1: 5'-TCTCCATCTCTGAAGTTGTGGGGAT-3' and 5'-TCCACCACAATCAAGAACAGAGCCT-3'.

CTR1: 5'-CTACGCTTTCTGCGGCGGCT-3' and 5'- GTCTGCTGCGCCCAGCTCTT-3'.

In addition, a housekeeping gene, *AtActin11*, was employed as a control: 5'-CCACATGCTATTCTGCGTTTGGACC-3' and 5'-CATCCCTTACGATTTCACGCTCTGC-3'.

Primers were designed across exon-exon junctions in the cDNA to avoid potential problems caused by contaminating genomic DNA. The amplification efficiency for each primer pair was calculated using serial cDNA dilutions. The expression values of the four genes were normalized to the corresponding controls. At least three independent experiments were performed to confirm the results. In each experiment, three biological replicates were used to generate means and determine the statistical significance.

Statistical and graphical analyses

The data were statistically analysed using SPSS 13.0 (SPSSInc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) with a Duncan post hoc test was used to test for differences in ethylene production, mean NO_3^- fluxes and the transcript levels of genes. Graphs were produced using Sigma Plot 12.0. All graphs and images were generated using Adobe Photoshop 7.0.

Results

Ethylene biosynthesis and signaling is enhanced under NO_3^- deficiency

To determine the effect of NO_3^- deficiency on ethylene biosynthesis, we measured ethylene production in Col-0 seedlings that were grown under HN conditions for 7 days and then transferred to either HN or LN medium and incubated for 0, 0.5, 1, 3, 6, or 24 h by GC. There was a significant increase in ethylene production in Col-0 seedlings after the transfer to LN medium (Fig.1). Maximal ethylene production was observed 0.5-1 h after transferred seedlings from HN to LN medium; however, there was an approximately two-fold difference in ethylene between the HN- and LN-treated samples. Ethylene production then declined gradually over time. Nevertheless, ethylene production in seedlings exposed to LN medium for 24 h was significantly greater than that in HN medium (Fig. 1). These results suggest that ethylene biosynthesis is enhanced under conditions of NO_3^- deficiency.

To further explore the effect of NO_3^- deficiency on the ethylene signaling pathway in *Arabidopsis*, three transgenic ethylene reporter lines (Col-0/*EBS:GUS*, *ctr1-1/EBS:GUS*, and *ein3-1eil1-1/EBS:GUS*) were used. Enhanced *EBS:GUS* activity was observed in the leaf and root mature zone of Col-0/*EBS:GUS* in response to LN treatment compared with HN treatment (Fig. 2a

and b). Not surprisingly, *EBS:GUS* activity was enhanced in the HN concentration treated with ACC, and was reduced in the LN concentration treated with AVG (Fig. 2b). Thus, NO_3^- deficiency may cause an enhanced effect on ethylene signaling. Supporting this hypothesis, *EBS:GUS* activity in both constitutive ethylene response mutant *ctr1-1/EBS:GUS* and ethylene-insensitive mutant *ein3-1eil1-1/EBS:GUS* was enhanced by LN treatment (Fig. 2c).

To determine the alternation of ethylene signaling pathway in response to high and low NO_3^- concentrations at molecular level, the expression of three critical genes in the ethylene signaling pathway (*CTR1*, *EIN3*, and *EIL1*) was examined. *CTR1* expression was slightly down-regulated by LN treatment compared with HN treatment, while *EIN3* and *EIL1* expression was strongly up-regulated by LN treatment (Fig.3), suggesting that the activity of the ethylene signaling pathway is enhanced by NO_3^- deficiency. Therefore, we confirmed that NO_3^- deficiency may play a positive role in ethylene biosynthesis and signaling.

NRT2.1 plays a critical role in high-affinity NO_3^- uptake under conditions of NO_3^- deficiency

To verify the role of NRT2.1 in NO_3^- deficiency signaling under our experimental conditions, the expression of *NRT2.1* in Col-0 and *nrt2.1* seedlings in response to high and low NO_3^- concentrations was examined. *NRT2.1* expression in Col-0 seedlings was strongly up-regulated by LN treatment, while in *nrt2.1* seedlings there was only a slight increase under NO_3^- deficiency (Fig. 4).

To confirm that high-affinity NO_3^- uptake was enhanced under NO_3^- deficiency, we used high-resolution SIET to measure the net NO_3^- fluxes in the maturation zone of the primary root in Col-0 and *nrt2.1* seedlings treated with 0.2 mM NO_3^-. HATS NO_3^- fluxes alternation at the surface of the mature zone between HN and LN treatments were different in Col-0 and *nrt2.1* seedlings

(Fig. 5a and b). In Col-0 seedlings, the average NO_3^- efflux was stimulated to 50.89 pmol·cm^{-2}·s^{-1} in HN medium, whereas the net NO_3^- flux switched to influx with LN treatment, assuming a value of 33.6 pmol·cm^{-2}·s^{-1} (Fig. 5a). In *nrt2.1* seedlings, although the average flux values were similar between HN and LN treatments ($P < 0.05$, one-way ANOVA), the NO_3^- efflux increased to 175 and 157 pmol·cm^{-2}·s^{-1}, respectively (Fig. 5a). The NO_3^- efflux in *nrt2.1* seedlings was approximately three-fold of that in Col-0 seedlings under HN conditions, and contrasted even more strongly with the influx in Col-0 under LN treatment (Fig. 5a and b). These results suggest that the up-regulated transcript level of *NRT2.1* induced by NO_3^- deficiency causes a remarkable enhancement of HATS NO_3^- uptake, whereas the deletion of *NRT2.1* gene sharply reduces the HATS NO_3^- uptake.

NRT2.1 is involved in the ethylene signaling pathway response to NO_3^- deficiency

To examine the effect of NRT2.1 on ethylene biosynthesis, we measured ethylene production in Col-0, *nrt1.1*, and *nrt2.1* seedlings that were grown under HN conditions for 7 days and then transferred to either HN or LN medium for 24 h using GC. The ethylene production of *nrt1.1* seedlings was increased under LN treatment compared with HN treatment, although still slightly, but significantly, less than that of Col-0 seedlings in LN medium (Fig. 6). In contrast, the ethylene production of *nrt2.1* seedlings in LN medium was sharply reduced by 76.5% compared with HN treatment, and approximately declined to 11.5% of that in Col-0 seedlings under LN condition (Fig. 6). These results indicate that NRT2.1, rather than NRT1.1, plays an important positive role in ethylene biosynthesis under NO_3^- deficiency stress.

To explore the role of NRT2.1 in the ethylene signaling pathway in *Arabidopsis*, two ethylene reporter lines (*nrt1.1/EBS:GUS* and *nrt2.1/EBS:GUS*) were used to compare GUS activity with

Col-0/*EBS:GUS* under HN and LN treatment. Similar to Col-0/*EBS:GUS*, enhanced *EBS:GUS* activity was observed in *nrt1.1/EBS:GUS* with LN treatment (Fig. 7a and Fig. 2a.). In contrast, reduced *EBS:GUS* activity was observed in *nrt2.1 EBS:GUS* under LN treatment (Fig. 7b.), suggesting that the deletion of *NRT2.1* has a negative effect on the ethylene signaling pathway. Not surprisingly, *EBS:GUS* activity was enhanced in HN medium treated with ACC, and was reduced in LN medium treated with AVG in both mutant reporter lines, confirming the *EBS:GUS* specificity of the two mutant ethylene reporter lines. Therefore, these results indicate that NRT2.1, rather than NRT1.1, causes an enhanced effect on ethylene signaling pathway.

On the other hand, to determine the effect of ethylene signaling on *NRT2.1* expression under NO_3^-deficiency, the impacts of ethylene biosynthesis precursor ACC and inhibitor AVG, and mutations of ethylene signaling components on transcript levels of *NRT2.1* under LN conditions were examined. In Col-0 seedlings, *NRT2.1* expression was down-regulated by ACC while it was up-regulated by AVG under LN conditions (Fig. 8a). Correspondingly, *NRT2.1* expression was down-regulated in *ctr1-1* but up-regulated in *ein3-1eil1-1* seedlings under LN conditions (Fig. 8b). These results suggest that ethylene has a negative effect on *NRT2.1* expression under NO_3^- deficiency.

Furthermore, to determine the effect of ethylene on HATS NO_3^- uptake, we used SIET to measure the HATS NO_3^- fluxes at the primary root maturation zone in Col-0, *ein3-1eil1-1*, and *ctr1-1* seedlings that were grown in HN medium for 7 days and then transferred to LN medium with or without ACC/AVG (10 μM) for 24 h. Average HATS NO_3^- influx in Col-0 seedlings under LN conditions was sharply enhanced to more than two-fold with AVG treatment, whereas the net NO_3^- flux switched to efflux with ACC treatment, assuming a mean value of 61.7 $pmol \cdot cm^{-2} \cdot s^{-1}$

(Fig. 9a and b). Furthermore, the HATS NO_3^- influx under LN conditions in the *ein3-1eil1-1* mutant was increased to approximate two-fold of that in Col-0 seedlings, whereas the NO_3^- flux in *ctr1-1* seedlings switched to efflux, assuming a mean value of 8.4 $pmol \cdot cm^{-2} \cdot s^{-1}$ (Fig. 9c and d). These results suggest that ethylene also plays a negative role in high-affinity NO_3^- uptake under LN conditions.

Discussion

Previous reports have shown that ethylene production is closely associated with nutritional deficiency. Borch *et al.* (1999) found that phosphorus-deficient *bean* roots produced twice as much ethylene per unit dry weight as roots supplied with adequate phosphorus. Similarly, it was reported that potassium deprivation in *Arabidopsis* roots stimulates ethylene production and up-regulates genes that are involved in ethylene biosynthesis and signaling (Jung *et al.* 2009; Shin & Schachtman 2004). In addition, roots from Fe-deficient cucumber, *tomato* and *pea* plants produce more ethylene than those from Fe-sufficient plants (Romera *et al.* 1999). Ethylene production is alsoassociated with NO_3^- supply. It was reported that in *maize* seedlings, root sensitivity to ethylene and subsequent aerenchyma formation was increased by 100-fold during periods of nitrogen deficiency (He *et al.* 1992; Schmelz *et al.* 2003). Tian *et al.* (2009) reported a rapid burst of ethylene production upon the exposure of wild-type *Arabidopsis* seedlings grown on LN concentration (0.1 mM) to HN concentration (10 mM). However, there has been no detailed study to explore the relationship between NO_3^- deficiency and ethylene production. Based on the report of Tian *et al.* (2009), we transferred *Arabidopsis* seedlings from HN medium to LN medium to examine the effect of NO_3^- deficiency on ethylene production. We found that the

ethylene production of Col-0 seedlings significantly increased following LN treatment (Fig. 1), suggesting that ethylene biosynthesis is induced by NO_3^- deficiency. Furthermore, we explored the effect of NO_3^- deficiency on the ethylene signaling pathway by comparing ethylene reporter *EBS:GUS* activity, which was previously used to monitor the reaction level of the ethylene signaling pathway (Stepanova *et al.* 2007), in Col-0/*EBS:GUS*, *ctr1-1/EBS:GUS*, and *ein3-1eil1-1/EBS:GUS* seedlings. We found that GUS activity in the three transgenic ethylene reporter lines was enhanced in response to LN treatment (Fig. 2). Our results also provide a possible explanation for NO_3^- deficiency-induced ethylene signaling by regulating the expression of *CTR1*, *EIN3*, and *EIL1*, which modulates ethylene signal transduction and downstream responses (Fig. 3). Moreover, *EBS:GUS* activity was enhanced in *ctr1-1/EBS:GUS* and *ein3-1eil1-1/EBS:GUS* mutants with LN treatment (Fig. 2c), suggesting that *ctr1-1* and *ein3-1eil1-1* can still transduce low NO_3^- stress signals and that other genes in the ethylene signaling pathway may be involved in NO_3^- deficiency-induced plant responses.

The role of NRT2.1 in high-affinity NO_3^- transport system has been demonstrated in many studies (Cerezo *et al.* 2001; Filleur *et al.* 2001). We verified the expression of *NRT2.1* and HATS NO_3^- fluxes under NO_3^--deficient conditions using Col-0 and *nrt2.1* seedlings. As expected, the expression of *NRT2.1* in Col-0 was strongly up-regulated by LN treatment, and there was a reduction of *NRT2.1* expression in *nrt2.1* seedlings (Fig. 4). Moreover, we used high-resolution SIET to measure the HATS NO_3^- flux in the maturation zone of primary roots in *Arabidopsis* seedlings. The HATS NO_3^- uptake of Col-0 seedlings was largely enhanced by LN treatment, while the HATS NO_3^- uptake of the *nrt2.1* mutant was similar between HN and LN treatments but significantly decreased compared with Col-0 (Fig. 5a and b), indicating the critical role of NRT2.1

in the high-affinity NO_3^- transport system.

To explore the role of NRT2.1 in ethylene responses to NO_3^- deficiency, we compared ethylene biosynthesis between Col-0 and the NO_3^- transporter mutants *nrt1.1* and *nrt2.1*, and we monitored the *EBS:GUS* expression in *nrt1.1/EBS:GUS* and *nrt2.1/EBS:GUS* seedlings, which were generated by genetic crosses. Comparable enhanced ethylene production was detected in Col-0 and *nrt1.1*, but not in *nrt2.1*, under LN treatment compared with HN treatment (Fig. 6), suggesting that NRT2.1, rather than NRT1.1, plays a positive role in ethylene biosynthesis in response to NO_3^- deficiency. Correspondingly, *EBS:*GUS activity was increased in *nrt1.1/EBS:GUS* (Fig. 7a) and decreased in *nrt2.1/EBS:GUS* (Fig. 7b) in LN medium compared with HN medium, indicating that *NRT2.1* may enhance the ethylene signaling pathway. Moreover, *EBS:GUS* activity was enhanced in HN medium treated with ACC and reduced in LN medium treated with AVG in both mutant reporter lines (Fig. 7), verifying the *EBS:GUS* specificity of the two mutant ethylene reporter lines. These results suggest that *NRT2.1* has a positive effect on ethylene biosynthesis and signaling.

Expression of the NO_3^- transporter *NRT2.1* was sensitive to the ethylene synthetic precursor ACC and ethylene synthesis antagonist AVG (Leblanc *et al.* 2008; Tian *et al.* 2009). Our study showed similar effects for ACC and AVG on *NRT2.1* expression under NO_3^- deficiency (Fig. 8a). Furthermore, we compared the *NRT2.1* transcript level in the ethylene-insensitive mutant *ein3-1eil1-1* and constitutive ethylene response mutant *ctr1-1* with that in Col-0 (Fig. 8b) and found that ethylene signaling may negatively modulate *NRT2.1* transcription under LN conditions. More importantly, HATS NO_3^- uptake in Col-0 seedlings was significantly decreased by ACC treatment and enhanced by AVG (Fig. 9a and b) ; in comparison, it was decreased in *ctr1-1* and

enhanced in *ein3-1eil1-1* in LN medium (Fig. 9c and d). This suggests that ethylene signaling has a negative effect on the high-affinity NO_3^- uptake in response to NO_3^- deficiency.

Based on aforementioned results, we proposed a hypothetical model to describe the interrelationships among NO_3^- deficiency, *NRT2.1* transcription, and ethylene biosynthesis and signaling in *Arabidopsis* seedlings (Fig. 10). In this model, *NRT2.1* expression is up-regulated at low external NO_3^- concentration, which enhances HATS NO_3^- uptake and NO_3^- stress tolerance and intensifies external NO_3^- deficiency stress. Meanwhile, NO_3^- deficiency may induce ethylene biosynthesis and signaling in a NRT2.1-dependent manner. Ethylene, in turn, down-regulates *NRT2.1* expression, which reduces HATS NO_3^- uptake and NO_3^- stress tolerance in plants, thereby alleviating external NO_3^- deficiency stress. Overall, we propose a negative feedback loop between the transcription of *NRT2.1* and ethylene biosynthesis and signaling induced by NO_3^- deficiency. Finally, the HATS NO_3^- uptake of plants relies on an internal comparative balance mechanism to account for external NO_3^- deficiency stress.

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Figure legends

Figure 1. Effect of NO_3^- deficiency on ethylene production in wild-type *Arabidopsis* (Col-0) seedlings. Ethylene production in Col-0 seedlings that were grown under HN (10 mM NO_3^-) conditions for 7 days and then transferred to HN or LN (0.2 mM NO_3^-) medium for 24 h was measured by GC. The data represent the mean ± SD of six replicates.

Figure 2. Effect of NO_3^- deficiency on activity of the ethylene reporter *EBS:GUS* in *Arabidopsis*. (a) *EBS:GUS* activity in the leaf and root mature zone of Col-0*/EBS:GUS* seedlings that were grown under HN (10 mM NO_3^-) conditions for 7 days and then transferred to HN or LN (0.2 mM NO_3^-) medium for 24 h. (b) *EBS:GUS* activity in the root mature zone of Col-0*/EBS:GUS* seedlings placed in HN, LN, HN with ACC (10 μM) and LN with AVG (10 μM) media for 24 h. (c) *EBS:GUS* activity in the roots of *ctr1-1/EBS:GUS* and *ein3-1eil1-1/EBS:GUS* seedlings in HN or LN medium for 24 h. Bars: (a) 500 μm, (band c) 200 μm. The images are representative of at least three independent experiments, with > 6 seedlings examined for each experiment.

Figure 3. *CTR1*, *EIN3*, and *EIL1* expression in Col-0 seedlings in response to NO_3^- deficiency. Seedlings were grown under HN (10 mM NO_3^-) conditions for 7 days and then transferred to HN or LN (0.2 mM NO_3^-) medium for 24 h. The data represent the means ± SD of three replicates.

Figure 4. *NRT2.1* expression in Col-0 seedlings and the *nrt2.1* mutant. Seedlings were grown in HN (10 mM NO_3^-) medium for 7 days and then transferred to HN or LN (0.2 mM NO_3^-) medium for 24 h. The data represent the means ± SD of three replicates.

Figure 5. Influence of NO_3^- deficiency on net plasma membrane NO_3^- fluxes at the maturation zone in *Arabidopsis* primary roots. (a) HATS NO_3^- fluxes in Col-0 and *nrt2.1* seedlings that were grown under HN (10 mM NO_3^-) conditions for 7 days and then transferred to HN or LN (0.2 mM

NO_3^-) medium for 24 h was measured by SIET with 0.2 mM NO_3^- in the measuring solution. (b) Mean values of NO_3^- fluxes from (a). Each point represents the mean ±SD of more than five individual plants. Significant differences between treatments are indicated with different letters ($P < 0.05$, one-way ANOVA).

Figure 6. Ethylene prodution in Col-0, *nrt1.1*, and *nrt2.1 Arabidopsis* seedlings in response to NO_3^- deficiency. Ethylene production in Col-0, *nrt1.1*, and *nrt2.1* seedlings that were grown in HN (10 mM NO_3^-) medium for 7 days and then transferred to HN or LN (0.2 mM NO_3^-) medium for 24 h was measured by GC. The data represent the mean ± SD of six replicates. Bars with different letters indicate significant differences at $P < 0.05$ (ANOVA).

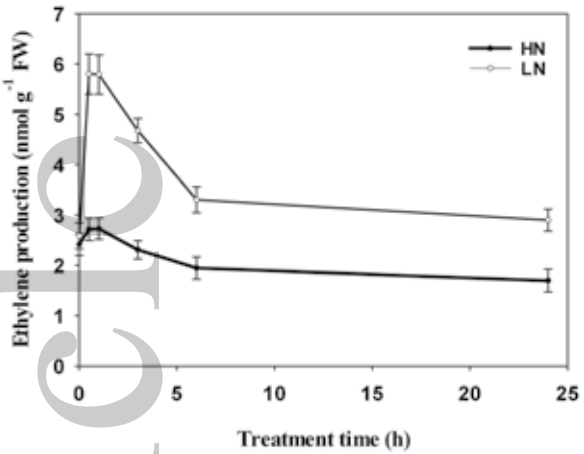
Figure 7. Effect of NRT2.1 on ethylene reporter *EBS:GUS* activity in response to NO_3^- deficiency. *EBS:GUS* activity in *nrt1.1/EBS:GUS* (a) and *nrt2.1/EBS:GUS* (b) seedlings that were grown in HN (10 mM NO_3^-) medium for 7 days and then transferred to HN or LN (0.2 mM NO_3^-) medium in the absence or presence of ACC or AVG (10 μM) for 24 h. Bars, 200 μm. The images are representative of at least three independent experiments, with > 10 seedlings examined for each experiment.

Figure 8. Effect of ethylene on *NRT2.1* expression. (a) *NRT2.1* transcript levels in Col-0 seedlings that were grown under HN (10 mM NO_3^-) conditions for 7 days and then transferred to LN (0.2 mM NO_3^-) medium upon the addition of ACC or AVG (10 μM) for 24 h. (b) *NRT2.1* transcript levels in *ein3-1eil1-1*and *ctr1-1* seedlings treated with LN. The data represent the means ± SD of three replicates.

Figure 9. Influence of ethylene on net plasma membrane NO_3^- fluxes at the maturation zone in *Arabidopsis* primary roots under NO_3^- deficiency. (a) HATS NO_3^- fluxes in Col-0 seedlings that

were grown under HN (10 mM NO_3^-) conditions for 7 days and then transferred to LN (0.2 mM NO_3^-) medium with/without the addition of ACC or AVG (10 μM) for 24 h. (c) HATS NO_3^- fluxes in Col-0, *ein3-1eil1-1*, and *ctr1-1* seedlings treated with LN for 24 h. (b) and (d) Mean values of NO_3^- fluxes from (a) and (c), respectively. Each point represents the mean ± SD of more than five individual plants. Significant differences between treatments are indicated with different letters ($P < 0.05$, one-way ANOVA).

Figure 10. Proposed model illustrating the interaction among NO_3^- deficiency, *NRT2.1* transcript level, and ethylene biosynthesis and signaling. Up arrow, increase.



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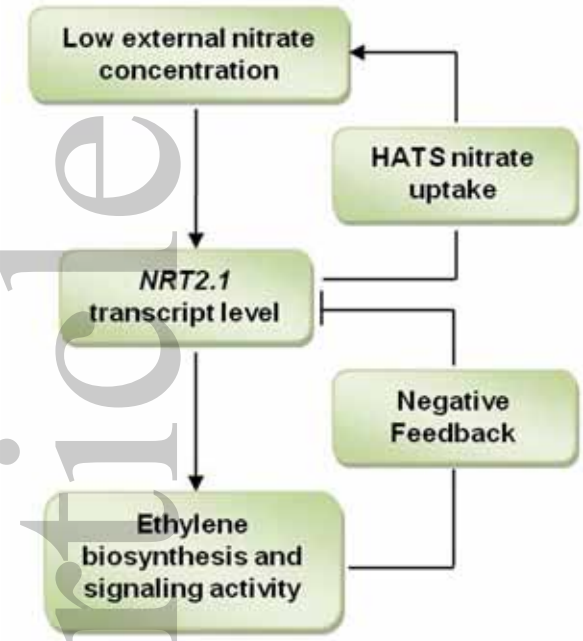

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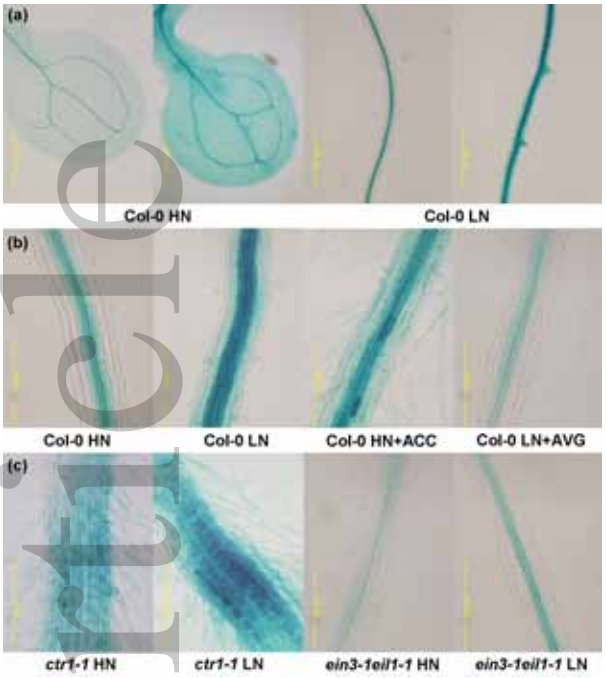

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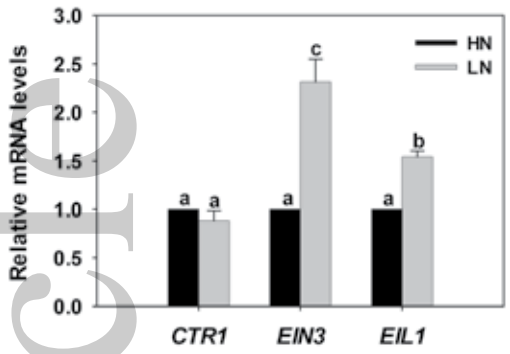

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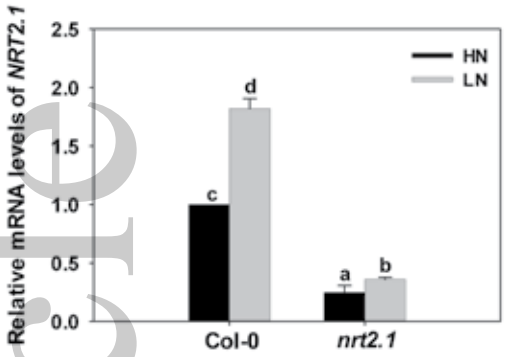

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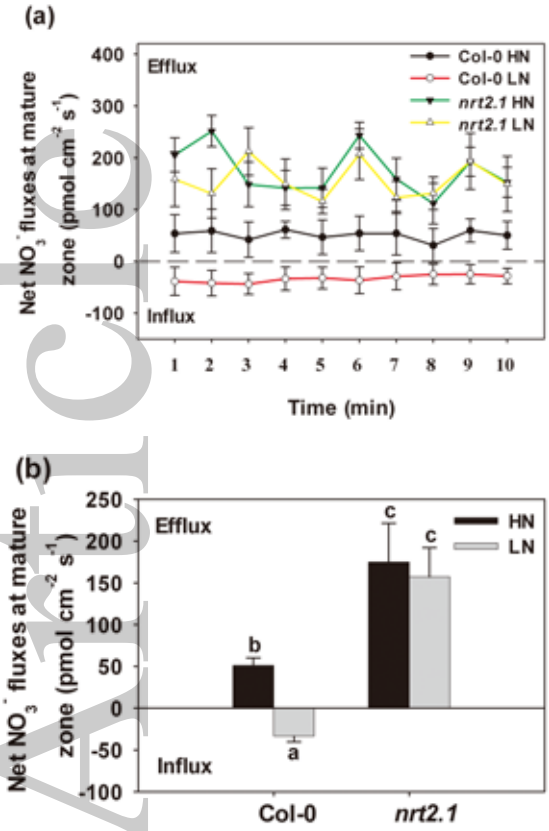

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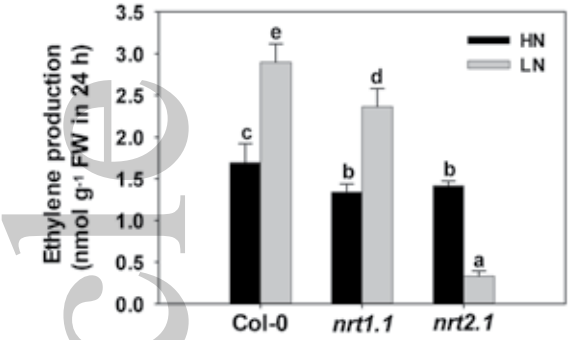

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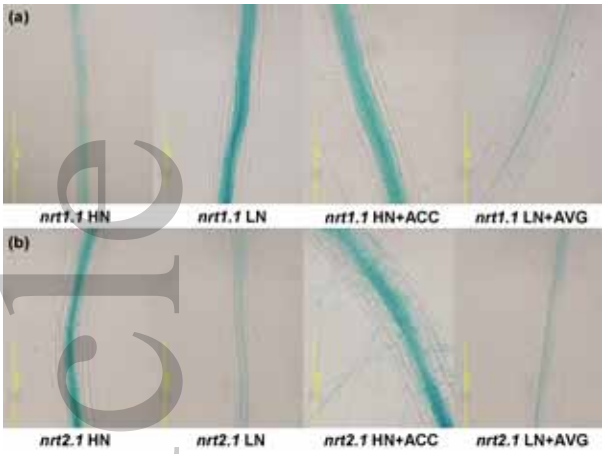

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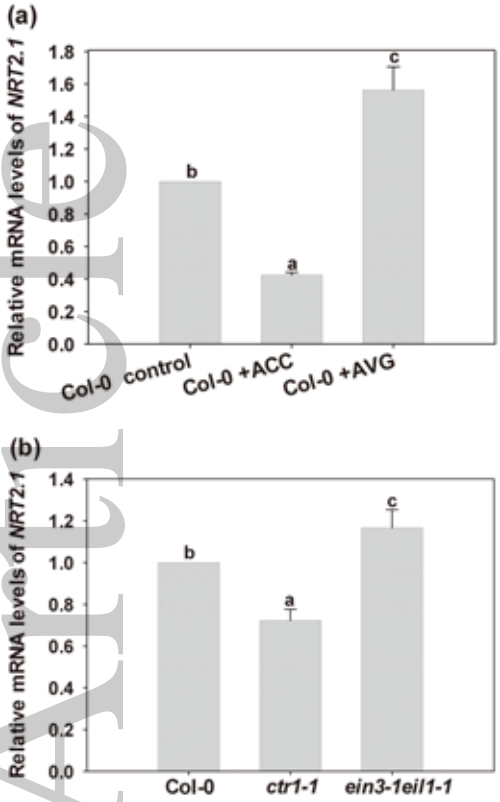

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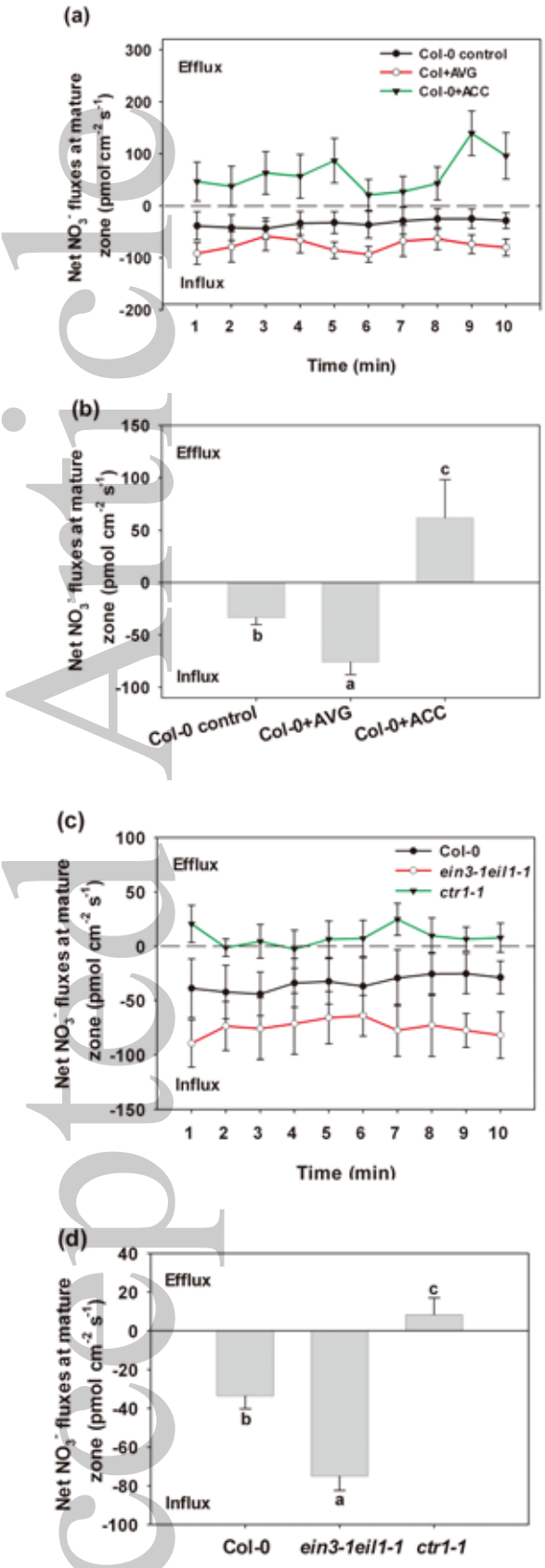

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