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

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## ABSTRACT

**The ethylene signalling 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 signalling 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 signalling 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 signalling 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 signalling under nitrate deficiency, which may contribute to finely tuning of plant nitrate acquisition during exploring dynamic soil conditions.**

*Key-words*: ethylene biosynthesis and signalling; high-affinity transport system; low nitrate.

## 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).

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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 $\mu$M 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; Zhuo *et al*. 1999; Okamoto, Vidmar & Glass 2003; Orsel *et al*. 2004). 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) (Zhuo *et al*. 1999; Vidmar *et al*. 2000).

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 (Johnson & Ecker 1998; Bleecker & Kende 2000). Ethylene biosynthesis and signalling have been well characterized at the molecular level, providing many genetic tools that can be used to determine how ethylene signalling 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-aminoethoxyvinylglycine (AVG) inhibits ACS and ethylene biosynthesis. Constitutive ethylene response 1 (CTR1) is a Raf-like kinase that acts as a negative regulator of ethylene signalling (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 (Chao *et al*. 1997; An *et al*. 2010) 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 defence 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 high nitrate (HN) conditions. However, there has been no detailed study that elaborated the molecular mechanism of the interaction between $NO_3^-$ deficiency and ethylene biosynthesis and signalling.

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 signalling. Moreover, we examined the role of *NRT2.1* in the ethylene-mediated response to $NO_3^-$ deficiency.

## MATERIALS AND METHODS

### Plant materials

All *A. 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 centre. 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 d, then transferred to plates containing HN or nitrogen-deficient [low nitrate (LN), 0.2 mM $NO_3^-$] medium in the absence or presence of 10 $\mu$M ACC (Sigma, St. Louis, MO, USA) or 10 $\mu$M 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 $\mu$M NaFeEDTA, 50 $\mu$M $H_3BO_3$, 12 $\mu$M $MnCl_2$, 1 $\mu$M $CuCl_2$, 1 $\mu$M $ZnCl_2$, and 0.03 $\mu$M $NH_4Mo_7O_{24}$, pH 5.8 (adjusted with NaOH), with 1% sucrose and 0.8% (w:v) agar was 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 d 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 $\mu$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 and 35 $\mu$m 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 micro-electrode. 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 $L^{-1}$ 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 $mL^{-1}$ 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, Carlsbad, CA, USA) and treated with RNase-Free DNase I (Promega, Madison, WI, USA). 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, Hercules, CA, USA) was used to run quantitative RT-PCR with the following primer pair combinations: *NRT2.1*: 5′-CTGGAGGGAACTTTG GATCAGGG-3′ and 5′-GTCACAGGTAACGTGCAAGC GACTA-3′; *EIN3*: 5′-GCATGTCCACATCGAGACAGT CG-3′ and GAGTTCACTGGCCTTGGCTGAG-3′; *EIL1*: 5′-TCTCCATCTCTGAAGTTGTGGGGAT-3′ and 5′- TCC ACCACAATCAAGAACAGAGCCT-3′; *CTR1*: 5′-CTACG CTTTCTGCGGCGGCT-3′ and 5′- GTCTGCTGCGCCCA GCTCTT-3′.

In addition, a housekeeping gene, *AtActin11*, was employed as a control: 5′-CCACATGCTATTCTGCGT TTGGACC-3′ and 5′-CATCCCTTACGATTTCACGCTCT GC-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.

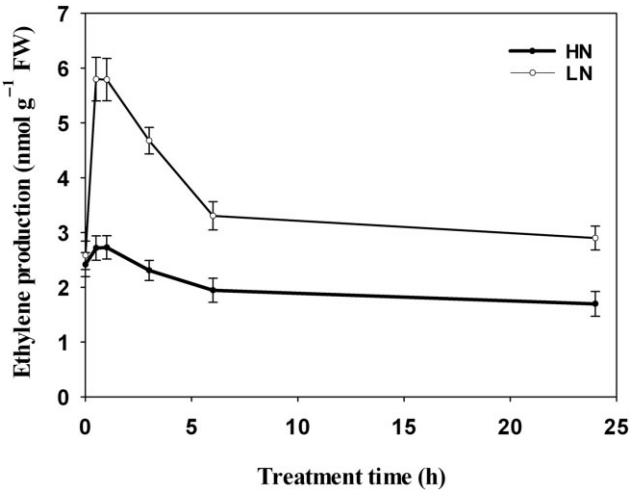


**Figure 1.** Effect of $NO_3^-$ deficiency on ethylene production in wild-type *Arabidopsis* (Col-0) seedlings. Ethylene production in Col-0 seedlings that were grown under high nitrate (HN) (10 mM $NO_3^-$) conditions for 7 d and then transferred to HN or low nitrate (LN) (0.2 mM $NO_3^-$) medium for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

## Statistical and graphical analyses

The data were statistically analysed using SPSS 13.0 (SPSS Inc., 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 signalling 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

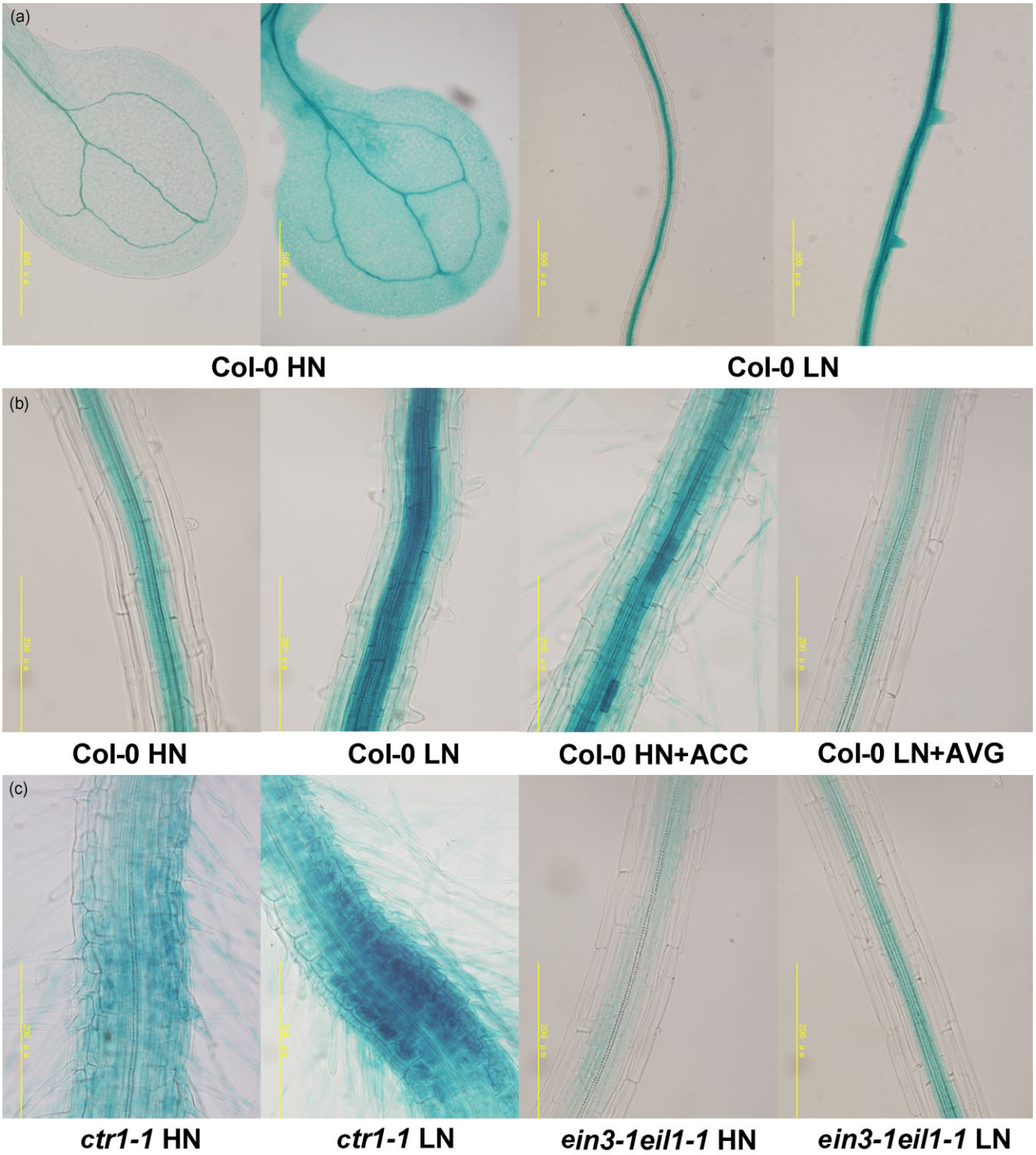


**Figure 2.** Effect of $NO_3^-$ deficiency on activity of the ethylene reporter *EBS:GUS* in *Arabidopsis*. (a) *EBS:GUS* activity in the leaf and root mature zone of Col-0/*EBS:GUS* seedlings that were grown under high nitrate (HN) (10 mM $NO_3^-$) conditions for 7 d and then transferred to HN or low nitrate (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 1-aminocyclopropane-1-carboxylic acid (ACC) (10 $\mu$M), and LN with 2-aminoethoxyvinylglycine (AVG) (10 $\mu$M) 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 $\mu$m, (b and c) 200 $\mu$m. The images are representative of at least three independent experiments, with >6 seedlings examined for each experiment.

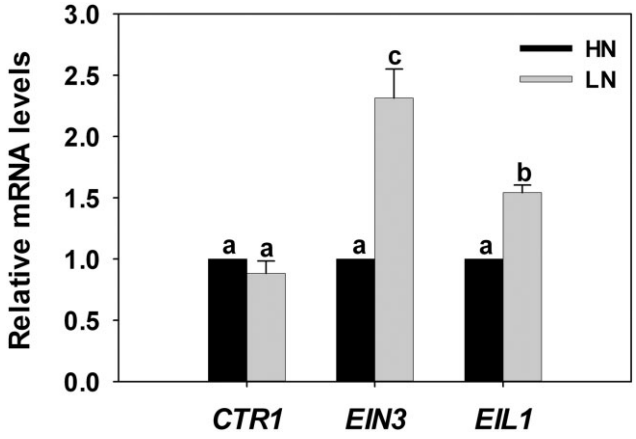


**Figure 3.** *CTR1*, *EIN3* and *EIL1* expression in Col-0 seedlings in response to $NO_3^-$ deficiency. Seedlings were grown under high nitrate (HN) (10 mM $NO_3^-$) conditions for 7 d and then transferred to HN or low nitrate (LN) (0.2 mM $NO_3^-$) medium for 24 h. The data represent the means ± SD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

that were grown under HN conditions for 7 d 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 seedlings were transferred from HN to LN medium; however, there was an approximately twofold 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 signalling 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,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 signalling. 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 signalling pathway in response to high and low $NO_3^-$ concentrations at molecular level, the expression of three critical genes in the ethylene signalling 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 signalling pathway is enhanced by $NO_3^-$ deficiency. Therefore, we confirmed that $NO_3^-$ deficiency may play a positive role in ethylene biosynthesis and signalling.

## 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 signalling 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 was different in Col-0 and *nrt2.1* seedlings (Fig. 5a,b). In Col-0 seedlings, the average $NO_3^-$ efflux was stimulated to be 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 threefold 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,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.

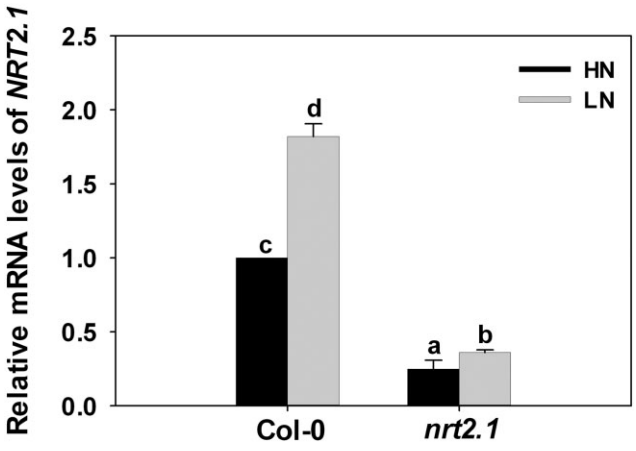


**Figure 4.** *NRT2.1* expression in Col-0 seedlings and the *nrt2.1* mutant. Seedlings were grown in high nitrate (HN) (10 mM $NO_3^-$) medium for 7 d and then transferred to HN or low nitrate (LN) (0.2 mM $NO_3^-$) medium for 24 h. The data represent the means ± SD of three replicates.

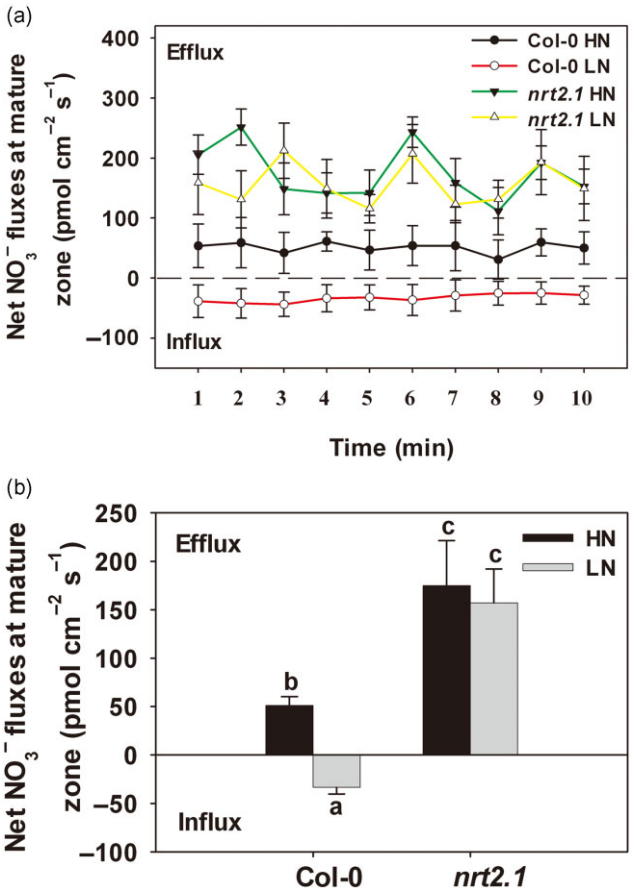


**Figure 5.** Influence of $NO_3^-$ deficiency on net plasma membrane $NO_3^-$ fluxes at the maturation zone in *Arabidopsis* primary roots. (a) High-affinity transport system (HATS) $NO_3^-$ fluxes in Col-0 and *nrt2.1* seedlings that were grown under high nitrate (HN) (10 mM $NO_3^-$) conditions for 7 d and then transferred to HN or low nitrate (LN) (0.2 mM $NO_3^-$) medium for 24 h were measured by ~~scanning ion-selective electrode technique (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 analysis of variance (ANOVA)].

## NRT2.1 is involved in the ethylene signalling 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 d 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 signalling 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 treatments. Similar to Col-0/*EBS:GUS*, enhanced *EBS:GUS* activity was observed in *nrt1.1/EBS:GUS* with LN treatment (Figs 2a,7a). 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 signalling 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 signalling pathway.

On the other hand, to determine the effect of ethylene signalling on *NRT2.1* expression under $NO_3^-$ deficiency, the impacts of ethylene biosynthesis precursor ACC and inhibitor AVG, and mutations of ethylene signalling 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 d and then transferred to LN medium with or without ACC/AVG (10 μM) for 24 h. Average HATS $NO_3^-$ influx in

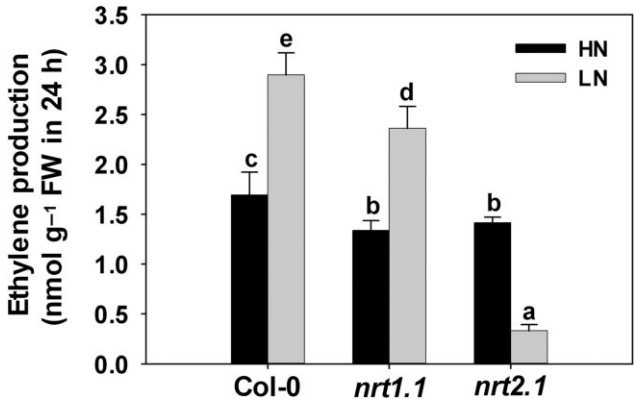


**Figure 6.** Ethylene production in Col-0, *nrt1.1* and *nrt2.1* *Arabidopsis* seedlings in response to $NO_3^-$ deficiency. Ethylene production in Col-0, *nrt1.1* and *nrt2.1* seedlings that were grown in high nitrate (HN) (10 mM $NO_3^-$) medium for 7 d and then transferred to HN or low nitrate (LN)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

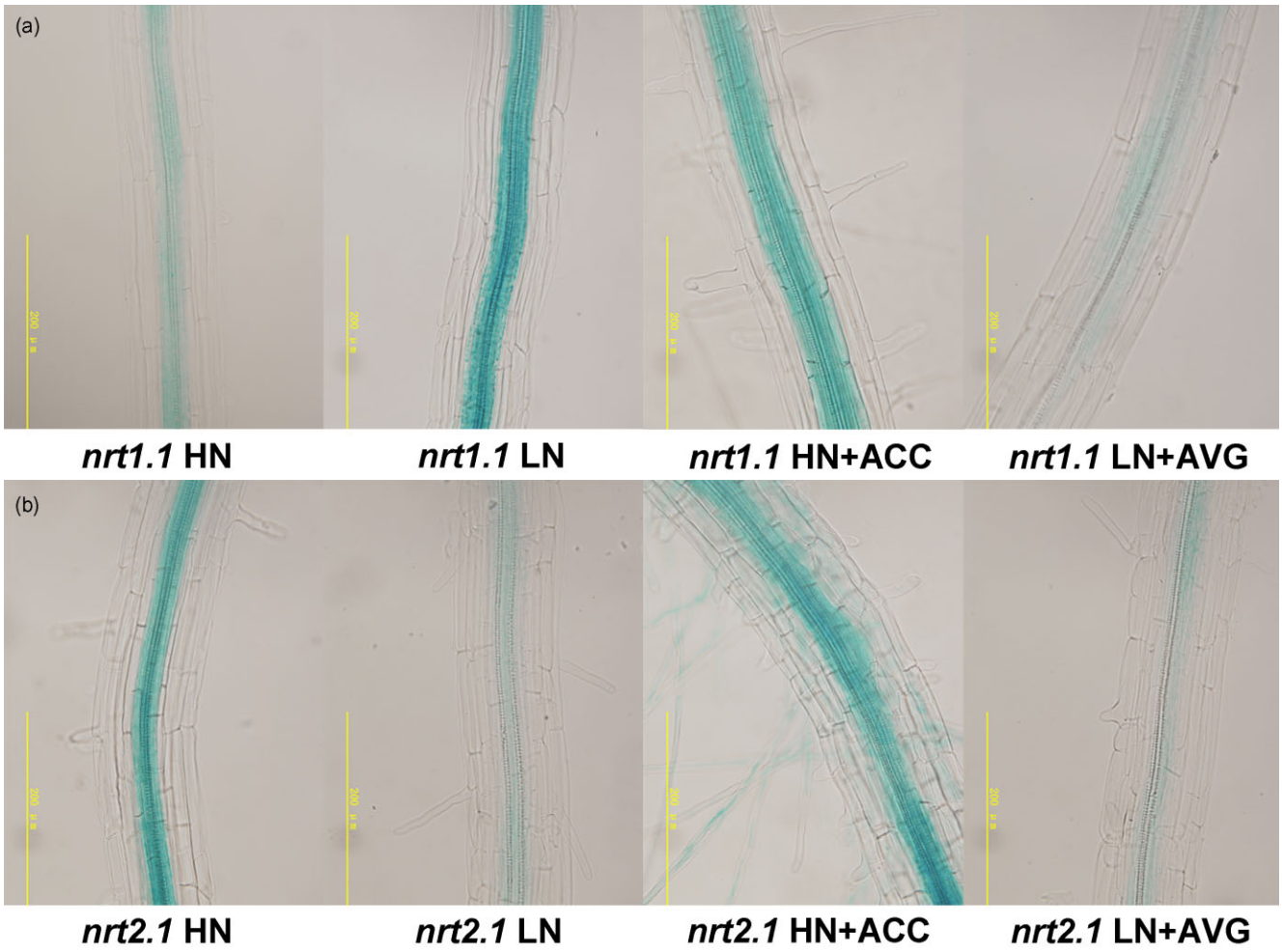


**Figure 7.** Effect of NRT2.1 on ethylene reporter *EBS:GUS* activity in response to $NO_3^-$ deficiency. *EBS:GUS* activity in *nrt1.1/EBS:GUS* (a) and *nrt2.1/EBS:GUS* (b) seedlings that were grown in high nitrate (HN) (10 mM $NO_3^-$) medium for 7 d and then transferred to HN or low nitrate (LN) (0.2 mM $NO_3^-$) medium in the absence or presence of 1-aminocyclopropane-1-carboxylic acid (ACC) or 2-aminoethoxyvinylglycine (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.

Col-0 seedlings under LN conditions was sharply enhanced to more than twofold with AVG treatment, whereas the net $NO_3^-$ flux switched to efflux with ACC treatment, assuming a mean value of 61.7 pmol $cm^{-2}$ $s^{-1}$ (Fig. 9a,b). Furthermore, the HATS $NO_3^-$ influx under LN conditions in the *ein3-1eil1-1* mutant was increased to approximately twofold 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 $cm^{-2}$ $s^{-1}$ (Fig. 9c,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 signalling (Shin & Schachtman 2004; Jung *et al.* 2009). In addition, roots from Fe-deficient cucumber, *tomato* and *pea* plants produce more ethylene than those from Fe-sufficient plants (Romera, Alcantara & De La Guardia 1999). Ethylene production is also associated 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

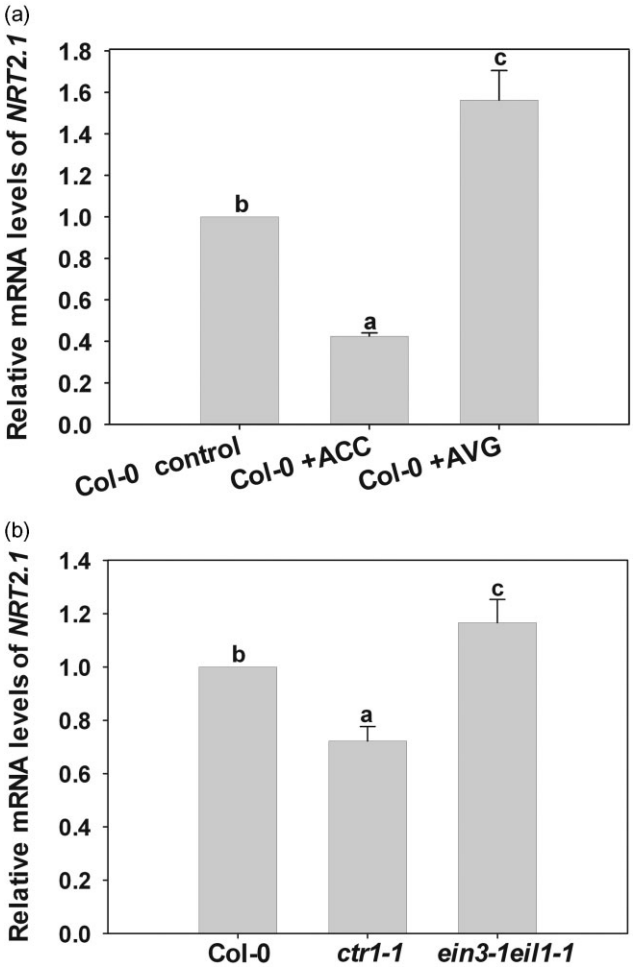


**Figure 8.** Effect of ethylene on *NRT2.1* expression. (a) *NRT2.1* transcript levels in Col-0 seedlings that were grown under high nitrate (HN) (10 mM $NO_3^-$) conditions for 7 d and then transferred to low nitrate (LN) (0.2 mM $NO_3^-$) medium upon the addition of 1-aminocyclopropane-1-carboxylic acid (ACC) or 2-aminoethoxyvinylglycine (AVG) (10 $\mu$M) 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.

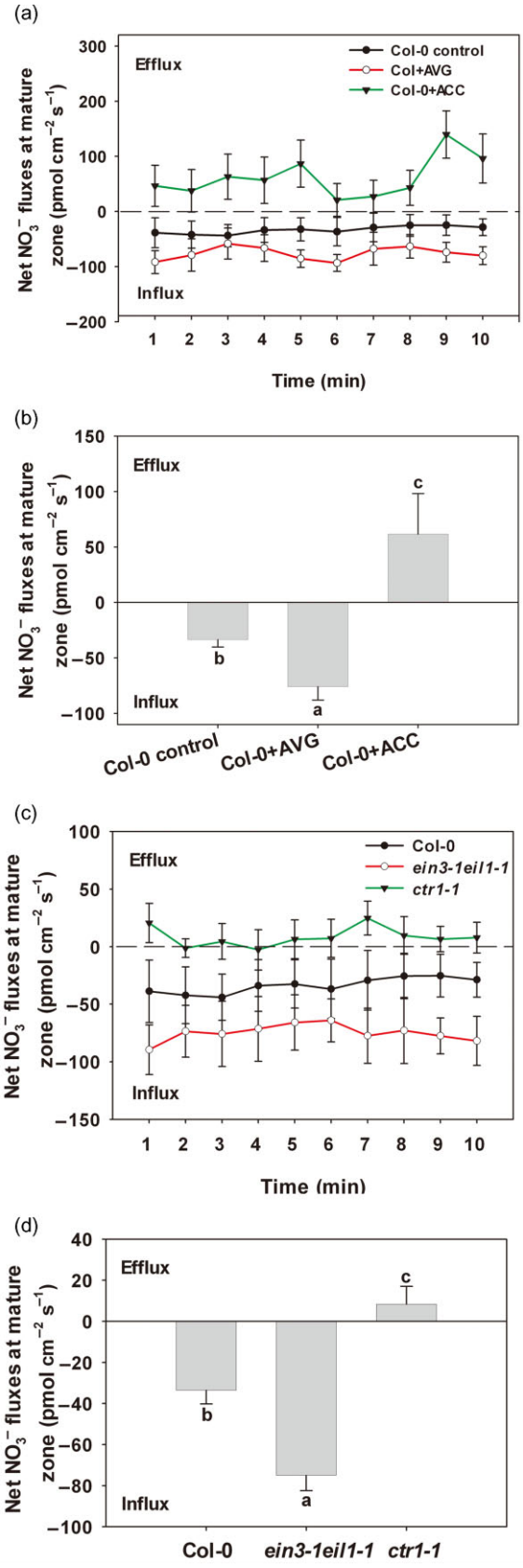


**Figure 9.** Influence of ethylene on net plasma membrane $NO_3^-$ fluxes at the maturation zone in *Arabidopsis* primary roots under $NO_3^-$ deficiency. (a) High-affinity transport system (HATS) $NO_3^-$ fluxes in Col-0 seedlings that were grown under high nitrate (HN) (10 mM $NO_3^-$) conditions for 7 d and then transferred to low nitrate (LN) (0.2 mM $NO_3^-$) medium with/without the addition of 1-aminocyclopropane-1-carboxylic acid (ACC) or 2-aminoethoxyvinylglycine (AVG) (10 $\mu$M) 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,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 analysis of variance (ANOVA)].

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 signalling pathway by comparing ethylene reporter *EBS:GUS* activity, which was previously used to monitor the reaction level of the ethylene signalling 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 signalling 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 signalling 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,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 signalling 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 signalling.

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 signalling 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,b); in comparison, it was decreased in *ctr1-1* and enhanced in *ein3-1eil1-1* in LN medium (Fig. 9c,d). This suggests that ethylene signalling 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 signalling 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 signalling 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

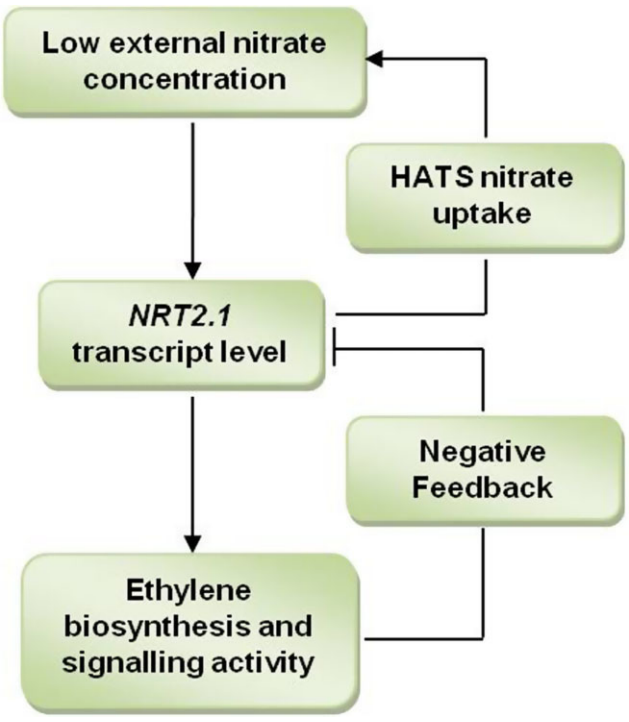


**Figure 10.** Proposed model illustrating the interaction among $NO_3^-$ deficiency, *NRT2.1* transcript level, and ethylene biosynthesis and signalling. Up arrow, increase. HATS, high-affinity transport system.

signalling 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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