The Plant Cell, Vol. 26: 3984–3998, October 2014, www.plantcell.org

The *Arabidopsis* Ethylene/Jasmonic Acid-NRT Signaling Module Coordinates Nitrate Reallocation and the Trade-Off between Growth and Environmental Adaptation[W][OPEN]

Guo-Bin Zhang, Hong-Ying Yi, and Ji-Ming Gong[1]

National Key Laboratory of Plant Molecular Genetics and National Center for Plant Gene Research (Shanghai), Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, People's Republic of China

ORCID ID: 0000-0003-2105-0587 (J.-M.G.)

Stresses decouple nitrate assimilation and photosynthesis through stress-initiated nitrate allocation to roots (SINAR), which is mediated by the nitrate transporters NRT1.8 and NRT1.5 and functions to promote stress tolerance. However, how SINAR communicates with the environment remains unknown. Here, we present biochemical and genetic evidence demonstrating that in *Arabidopsis thaliana*, ethylene (ET) and jasmonic acid (JA) affect the crosstalk between SINAR and the environment. Electrophoretic mobility shift assays and chromatin immunoprecipitation assays showed that ethylene response factors (ERFs), including OCTADECANOID-RESPONSIVE *ARABIDOPSIS* AP2/ERF59, bind to the GCC boxes in the *NRT1.8* promoter region, while ETHYLENE INSENSITIVE3 (EIN3) binds to the EIN3 binding site motifs in the *NRT1.5* promoter. Genetic assays showed that cadmium and sodium stresses initiated ET/JA signaling, which converged at EIN3/EIN3-Like1 (EIL1) to modulate *ERF* expression and hence to upregulate *NRT1.8*. By contrast, ET and JA signaling mediated the downregulation of *NRT1.5* via EIN3/EIL1 and other, unknown component(s). SINAR enhanced stress tolerance and decreased plant growth under nonstressed conditions through the ET/JA-NRT1.5/NRT1.8 signaling module. Interestingly, when nitrate reductase was impaired, SINAR failed to affect either stress tolerance or plant growth. These data suggest that SINAR responds to environmental conditions through the ET/JA-NRT signaling module, which further modulates stress tolerance and plant growth in a nitrate reductase-dependent manner.

INTRODUCTION

In higher plants, nitrate assimilation occurs by the reduction of nitrate to nitrite, then ammonium, which is then assimilated to glutamate via the glutamine synthase/glutamate oxyglutarate aminotransferase cycle (Crawford, 1995). These metabolic intermediates act as important signaling molecules or precursors of further nitrogen derivatives, which sustain plant growth, development, and responses to biotic and abiotic stresses (Stitt, 1999; Forde and Lea, 2007; Wang et al., 2007; Vidal and Gutiérrez, 2008; Mur et al., 2012). Nitrate assimilation is energy-intensive and occurs preferentially in foliar chloroplasts, where carbon skeletons, energy, and reducing power derived from photosynthesis can be easily accessed. Thus, the direct coupling of nitrate assimilation and photosynthesis in chloroplasts is believed to be energy-efficient and is known as nitrate photoassimilation (Searles and Bloom, 2003), a process adopted by most herbaceous plants and requiring long-distance root-to-shoot transport of nitrate taken up by plant roots (Smirnoff and Stewart, 1985; Andrews, 1986).

Adverse conditions such as low light intensity and heavy metal stresses induce SINAR, in which the assimilation of nitrate in the roots becomes prevalent (Smirnoff and Stewart, 1985; Andrews, 1986; Hernandez et al., 1997) and the direct coordination between nitrate assimilation and photosynthesis decouples, leading to decreased energy efficiency. Despite this loss of efficiency, plants frequently use SINAR and the root assimilation machinery. One explanation is that through SINAR, nitrate assimilation in roots will not compete directly for the reductants and energy generated by photosynthetic electron transport, thus optimizing plant growth in unfavorable environments (Canvin and Atkins, 1974). Thus, elucidation of the mechanisms underlying SINAR might help improve our understanding of nitrogen use efficiency.

SINAR is mediated by two nitrate transporter genes, *NRT1.5* and *NRT1.8*, which encode low-affinity nitrate uptake transporters. *NRT1.5* is mainly expressed in root pericycle cells and functions to load nitrate into the xylem, and *NRT1.8* is expressed predominantly in xylem parenchyma cells and functions primarily to unload nitrate from the xylem sap (Lin et al., 2008; Li et al., 2010). Under normal conditions, nitrate in roots is loaded into xylem vessels by NRT1.5 and then transferred to aerial tissues, where NRT1.8 unloads it into xylem parenchyma cells. The unloaded nitrate then translocates to mesophyll cells via symplastic diffusion. Under stress conditions, *NRT1.5* expression in

[1] Address correspondence to jmgong@sibs.ac.cn.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Ji-Ming Gong (jmgong@sibs.ac.cn).
[W] Online version contains Web-only data.
[OPEN] Articles can be viewed online without a subscription.
www.plantcell.org/cgi/doi/10.1105/tpc.114.129296

roots decreases, so much less nitrate is loaded into xylem vessels. Meanwhile, *NRT1.8* expression in roots increases strongly, thus unloading nitrate back into the root symplasm. This highly coordinated regulation of *NRT1.5* and *NRT1.8* fine-tunes nitrate allocation between roots and shoots in response to environmental signals. Moreover, plants with impaired SINAR are more sensitive to cadmium (Cd), salt (Na), and drought stresses (Li et al., 2010; Chen et al., 2012). These observations suggest that SINAR might enhance stress tolerance at the cost of energy efficiency. Interestingly, the coordinated, opposite expression of *NRT1.5* and *NRT1.8* occurs under diverse stress conditions (Zimmermann et al., 2004), indicating that SINAR might represent a universal response mechanism (Gojon and Gaymard, 2010; Li et al., 2010; Chen et al., 2012), although how it crosstalks with such a wide range of stresses and mediates stress tolerance remains to be determined.

Stresses induce the biosynthesis of ethylene (ET), jasmonic acid (JA), and other stress hormones (Fujita et al., 2006). Once produced, ET binds to and inactivates its receptors, which consequently alleviates the CONSTITUTIVE TRIPLE RESPONSE1 (CTR1)-derived phosphorylation of ETHYLENE-INSENSITIVE2 (EIN2). The C terminus of the nonphosphorylated EIN2 is then cleaved and enters the nuclei, where it activates EIN3/EIN3-Like1 (EIL1) and the downstream transcriptional cascades (Solano et al., 1998; Ju et al., 2012) to mediate a wide range of stress responses (Berrocal-Lobo et al., 2002; Achard et al., 2006; Zhang et al., 2011; Li et al., 2013). Likewise, the stress-induced JA is perceived by CORONATINE INSENSITIVE1 (COI1), which recruits and

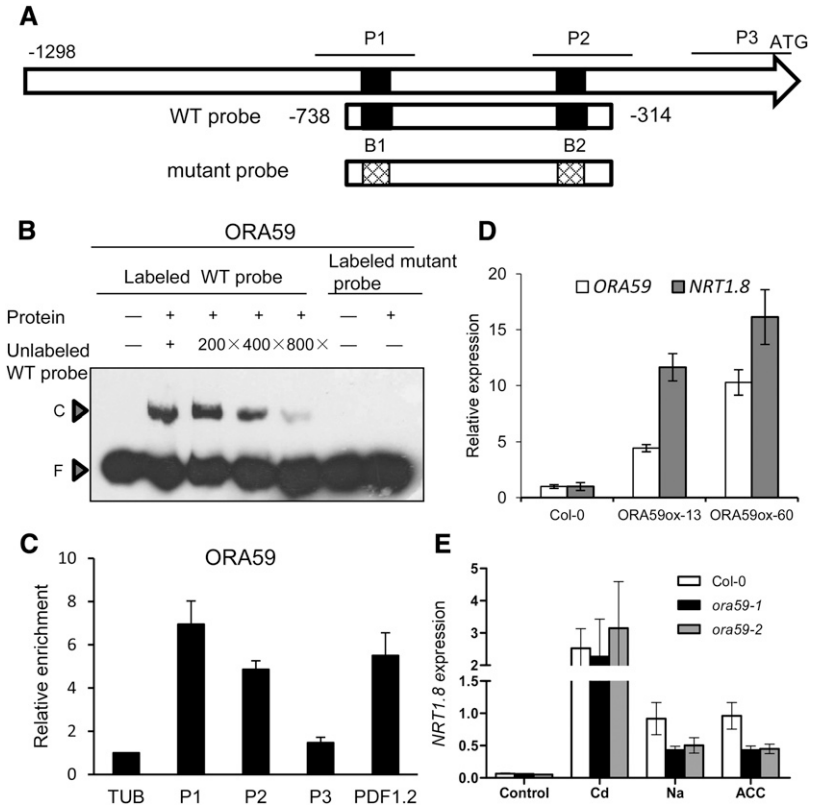

Figure 1. ORA59 Binds to the Promoter of *NRT1.8* and Modulates Its Expression.

(A) Schematic diagram of the 1298-bp promoter fragment upstream of the *NRT1.8* start codon. P1 (−795 to −634) and P2 (−410 to −233), covering the predicted GCC boxes B1 and B2, as well as P3 (−160 to −7), serving as a negative control, indicate promoter regions subjected to the ChIP-qPCR assay in **(C)**. The wild-type probe used for the EMSA in **(B)** contains the wild-type GCC boxes (5′-AGCAGCC), while the mutant probe contains the mutagenized GCC boxes (5′-ATCATCC) where G was mutagenized to T.

(B) EMSA determination of complex formation between ORA59 and the *NRT1.8* promoter. Wild-type and mutant probes, as indicated in **(A)**, were amplified using the primers listed in Supplemental Table 1. Competition assay for the labeled wild-type probe was performed by adding 200-/400-/800-fold excesses of unlabeled wild-type probe. C, DNA-protein complex; F, free probe.

(C) ChIP assay using IgG beads to precipitate ORA59-TAP protein followed by qPCR detection of P1, P2, and P3 regions as indicated in **(A)**. *TUB2* and *PDF1.2* were used as internal and positive controls, respectively. Data are means ± SD, $n = 3$.

(D) Quantitative RT-PCR analysis of *NRT1.8* and *ORA59* expression in roots of wild-type and *ORA59*-overexpressing plants grown in hydroponics. Values are means ± SD, $n = 3$.

(E) Quantitative RT-PCR analysis of *NRT1.8* expression in roots of 4-week-old wild-type, *ora59-1*, and *ora59-2* plants treated with 200 μM $CdCl_2$, 150 mM NaCl, or 20 μM ACC for 6 h. *NRT1.8* expression levels were normalized to those of *SAND*.

Values are means ± SD; $n = 3$.

degrades JASMONATE ZIM-DOMAIN proteins, thus derepressing the downstream transcriptional factors to mediate a series of stress responses (Vijayan et al., 1998; Dombrecht et al., 2007; Kazan and Manners, 2012; Hu et al., 2013). Recent work also showed that ET and JA signaling pathways crosstalk via EIN3/EIL1 and synergistically regulate the expression of a number of downstream genes (Zhu et al., 2011). Other studies further proposed that ET and JA posttranslationally modulate ethylene response factors (ERFs) independent of EIN3/EIL1 (Bethke et al., 2009; Van der Does et al., 2013).

In this study, we provide biochemical, genetic, and physiological evidence to demonstrate that ET and JA signaling pathways crosstalk to fine-tune the SINAR process via the coordinated upregulation of *NRT1.8* and downregulation of *NRT1.5*. Our results further suggest that SINAR mediates the trade-off between stress tolerance and plant growth dependent on the activity of nitrate reductase.

RESULTS

ORA59 Is an Immediate Upstream Regulator of *NRT1.8*

Enhanced *NRT1.8* expression was proposed to function as an essential step in the regulation of SINAR (Gojon and Gaymard, 2010; Li et al., 2010). In the effort to identify the upstream regulators that integrate diverse signals to mediate *NRT1.8* expression in *Arabidopsis thaliana*, we noticed that *NRT1.8* transcript levels were

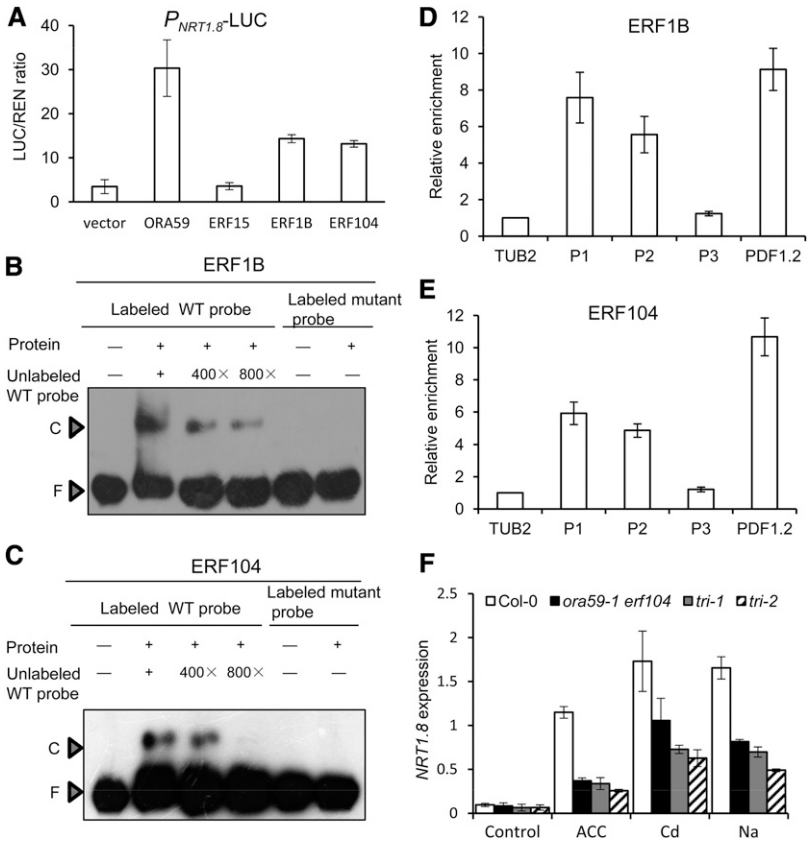

Figure 2. Other ERFs Function Redundantly with ORA59 to Regulate *NRT1.8* Expression.

(A) Transient transcriptional activity assay of $P_{NRT1.8}$:LUC. The $P_{NRT1.8}$*:LUC-35S:REN* reporter construct was transiently expressed in *Arabidopsis* protoplasts together with the control vector, 35S:ORA59, 35S:ERF15, 35S:ERF1B, or 35S:ERF104 effector. REN was used as an internal control. The LUC:REN ratio represents the relative activity of the *NRT1.8* promoter.

(B) and **(C)** EMSA of ERF1B **(B)** or ERF104 **(C)** binding to the *NRT1.8* promoter regions. Wild-type and mutant probes as indicated in Figure 1A were amplified. Competition for the labeled wild-type probe was performed by adding 400-/800-fold excess of unlabeled wild-type probe. C, DNA-protein complex; F, free probe.

(D) and **(E)** ChIP assay using IgG beads to precipitate ERF1B-TAP protein **(D)** or anti-GFP antibody against ERF104-GFP protein **(E)**, followed by qPCR detection of P1, P2, and the negative control P3 regions as indicated in Figure 1A. *TUB2* and *PDF1.2* were used as internal and positive controls, respectively.

(F) Quantitative RT-PCR analysis of *NRT1.8* expression in roots of 4-week-old wild-type, *ora59-1 erf104*, *tri-1*, and *tri-2* plants exposed to 200 μM $CdCl_2$, 150 mM NaCl, or 20 μM ACC for 6 h. Data were normalized to that of *SAND*.

Values are means ± SD; $n = 3$.

constitutively elevated in transgenic plants overexpressing OCTADECANOID-RESPONSIVE *ARABIDOPSIS* AP2/ERF59 (*ORA59*; Pré et al., 2008), an ERF family member. To test the idea that ORA59 might be one of the upstream regulators of *NRT1.8*, the promoter sequence of *NRT1.8* was scanned; this identified two putative GCC boxes, B1 (−719 to −713) and B2 (−347 to −341) (Figure 1A), which are known ERF binding motifs (Solano et al., 1998). Gel-shift assays indicated that incubation of the His-ORA59 recombinant protein with a wild-type probe containing this sequence (−738 to −314) resulted in a significant protein-DNA complex. Also, increasing amounts of unlabeled wild-type probe gradually decreased the complex levels, while no complex was observed when His-ORA59 was incubated with labeled mutant probe (Figure 1B). These results indicated that ORA59 binds to the promoter of *NRT1.8*. Chromatin immunoprecipitation (ChIP) assays using plants overexpressing *ORA59* from a 35S:*ORA59-TAP* construct (*ORA59ox*) showed significant ORA59 enrichment in the P1 and P2 regions in the *NRT1.8* promoter (Figure 1C). Corresponding to the elevated levels of *ORA59*, *NRT1.8* expression was also enhanced in *ORA59ox-13* and *ORA59ox-60* plants (Figure 1D). These data suggest that ORA59 acts as an immediate upstream regulator of *NRT1.8*.

Given that *NRT1.8* is upregulated by various stresses and ORA59 binds to its promoter region, we then determined if ORA59 functions in stress-induced *NRT1.8* expression. As shown in Figure 1E, *NRT1.8* was induced in the wild-type Columbia-0 (Col-0) when exposed to Cd or Na and the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC), while in the loss-of-function mutants *ora59-1* and *ora59-2* (Supplemental Figures 1A and 1B), the induction upon treatment with Na and ACC decreased significantly compared with Col-0 ($P < 0.05$), although changes upon treatment with Cd were not observed (Figure 1E). These results suggest that ORA59 is involved in the upregulation of *NRT1.8*, but factors other than ORA59 might function redundantly in this induction process, especially when under Cd stress.

ERF1B and ERF104 Function Redundantly with ORA59 to Mediate *NRT1.8* Expression

Considering that other factors might regulate *NRT1.8* expression, we further characterized another eight reported members of the ERF B-3 subfamily, to which ORA59 belongs (Nakano et al., 2006), including *ERF1A* (accession number At4g17500, National Center for Biotechnology Information reference sequence NP_567530.4; also known as *ATERF1* and referred to as At-ERF#100 by Nakano et al., 2006) and *ERF1B* (accession number At3g23240, National Center for Biotechnology Information reference sequence NP_188965.1; also known as *ERF1* and referred to as At-ERF#092 by Nakano et al., 2006). The results showed that transient overexpression of *ORA59*, *ERF1B*, and *ERF104* in *Arabidopsis* protoplasts activated the expression of LUC driven by the *NRT1.8* promoter (*NRT1.8:LUC*; Figure 2A), while overexpression of *ERF1A* only slightly activated *NRT1.8:LUC* (Supplemental Figure 1C). Other tested *ERFs* did not show any significant activation of the *NRT1.8* promoter (Figure 2A; Supplemental Figure 1C; $P > 0.2$). These

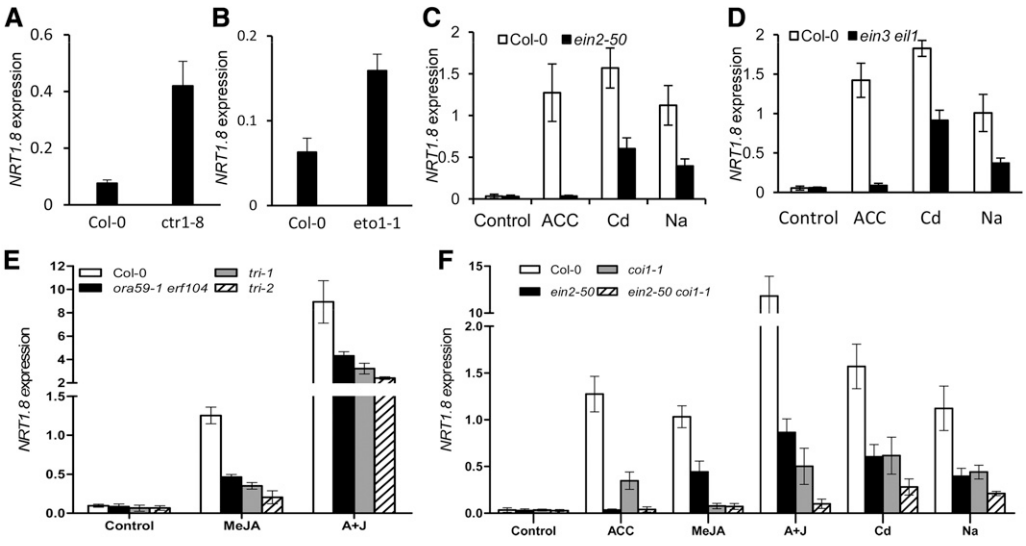

Figure 3. Regulation of *NRT1.8* Expression in ET/JA Signaling Mutants.

NRT1.8 expression levels in the roots of 4-week-old plants were determined by quantitative RT-PCR, treatments were as indicated, and data were normalized to those of *SAND*.

(A) and **(B)** *NRT1.8* expression in roots of *ctr1-8* **(A)**, *eto1-1* **(B)**, and Col-0.

(C) and **(D)** *ein2-50* **(C)**, *ein3 eil1* **(D)**, and the wild-type control were exposed to 20 μM ACC, 200 μM $CdCl_2$, or 150 mM NaCl for 6 h.

(E) *ora59-1 erf104*, *tri-1*, *tri-2*, and wild-type plants were treated with 50 μM MeJA or 20 μM ACC + 50 μM MeJA (A+J) for 6 h.

(F) *ein2-50*, *coi1-1*, *ein2-50 coi1-1*, and wild-type plants were treated with 20 μM ACC, 50 μM MeJA, 20 μM ACC + 50 μM MeJA (A+J), 200 μM $CdCl_2$, or 150 mM NaCl for 6 h.

Values are means ± SD; $n = 3$ in **(A)** to **(E)** and $n = 4$ in **(F)**.

data suggest that, in addition to ORA59, ERF1B and ERF104 might also participate in the transcriptional regulation of *NRT1.8*.

Consistent with this, electrophoretic mobility shift assay (EMSA) and ChIP analyses showed that ERF1B and ERF104 bind directly to the GCC boxes in the *NRT1.8* promoter (Figures 2B to 2E). In *ora59-1 erf104* and the *ora59-1 erf104 erf1b* triple mutants (*tri-1* and *tri-2*), which showed significantly decreased *ERF1B* transcript levels (Supplemental Figure 1F), *NRT1.8* expression upon treatment with ACC or NaCl was further decreased compared with *ora59-1*, and significant decrease was also observed for Cd treatment (compare Figures 1E and 2F; $P < 0.05$). In the *ERF104* and *ERF1B* overexpression lines, *NRT1.8* expression was increased (Supplemental Figures 1D and 1E). These results together indicate that ERF1B and ERF104 function redundantly with ORA59 to mediate *NRT1.8* expression in response to Cd/Na. Note, however, that *NRT1.8* expression in *tri-1* and *tri-2* was not apparently different from that in *ora59-1 erf104* (Figure 2F), which might be attributable to the fact that *ERF1B* was still detectable in the triple mutant plants (Supplemental Figure 1F), or *ERF1B* might just play a minor role in Cd/Na-induced *NRT1.8* expression.

The Stress-Induced Expression of *NRT1.8* Is Mediated by ET/JA Signaling

Given that *NRT1.8* is greatly induced by ACC (Figure 2F) and that ORA59, ERF1B, and ERF104 are immediate upstream regulators of *NRT1.8* (Figures 1 and 2), we questioned to what extent the ET signaling pathway is involved in the SINAR process mediated by *NRT1.8*. In the *ctr1-8* and *ethylene overproducing1* (*eto1-1*) mutant plants, *NRT1.8* levels were steadily elevated compared with the wild-type Col-0 (Figures 3A and 3B). Consistent with this, upregulation of *NRT1.8* upon Cd/Na treatment decreased in the ET-insensitive mutant *ein2-50* compared with Col-0, and its induction by ACC did not occur (Figure 3C). Similar results were obtained in the ET-insensitive *ein3 eil1* double mutants (Figure 3D). These data suggest that the ET signaling pathway might be extensively involved in the regulation of *NRT1.8* through the major signaling components that have been characterized.

However, given that the induction of *NRT1.8* upon Cd/Na treatment did not decrease to the control level (as observed for the ACC treatment) in the ET signaling mutants (Figures 3C and 3D) and that *ORA59* and *ERF1B* are also regulated by JA (Lorenzo et al., 2003; Pré et al., 2008), we further measured *NRT1.8* expression upon treatment with methyl jasmonate (MeJA) or ACC plus MeJA. The results showed that MeJA greatly induced *NRT1.8* expression in the wild-type Col-0. Also, the induction upon treatment with ACC plus MeJA was even more dramatic than treatment with either ACC or MeJA alone (Figure 3E), as would be expected for a synergistic effect of ACC and MeJA (Lorenzo et al., 2003; Pré et al., 2008). Moreover, in *ora59-1 erf104* double mutants and the *tri-1* and *tri-2* triple mutants, *NRT1.8* expression in response to both treatments was decreased significantly compared with Col-0 (Figure 3E; $P < 0.05$). These results suggest that ET and JA signaling pathways interact to mediate *NRT1.8* expression.

Consistent with this, in *ein2-50* and *coi1-1* mutants, where ET or JA signaling is essentially prevented, *NRT1.8* expression decreased upon treatment with ACC, MeJA, or ACC plus MeJA, and the expression in the double mutant *ein2-50 coi1-1* even decreased to levels comparable to those observed under control conditions (Figure 3F). A similar result was obtained for *NRT1.8* expression upon Cd/Na treatment, except that the levels in *ein2-50*

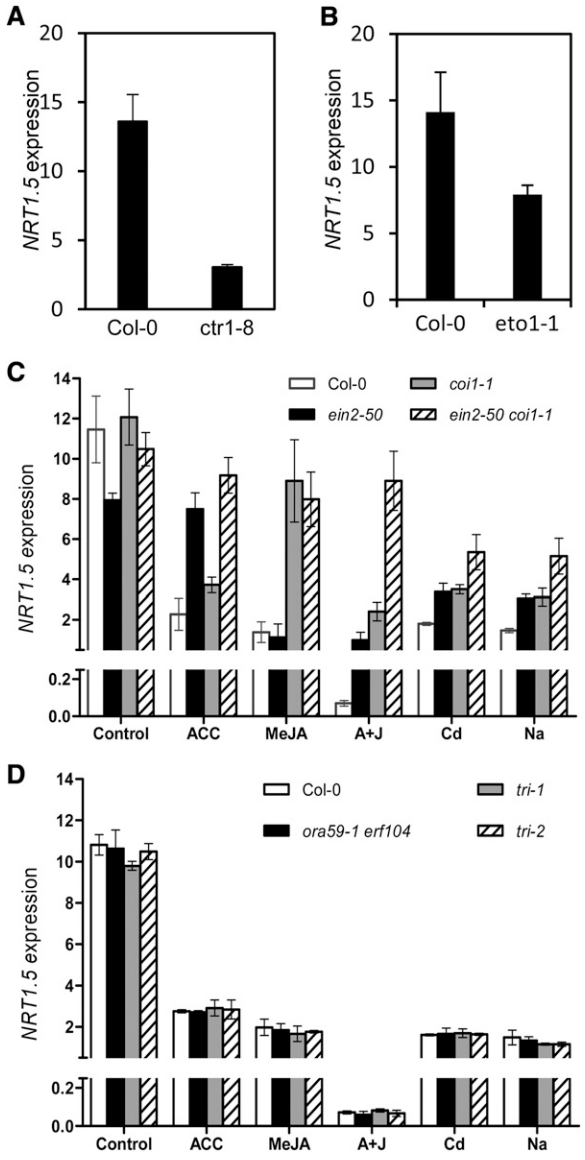

Figure 4. Stresses Inhibit *NRT1.5* Expression through ET/JA Signaling.

Four-week-old plants under control conditions or exposed to 20 μM ACC, 50 μM MeJA, 20 μM ACC + 50 μM MeJA (A+J), 200 μM $CdCl_2$, or 150 mM NaCl for 6 h as indicated. The expression levels of *NRT1.5* were determined by quantitative RT-PCR. Data were normalized to those of *SAND*. Values are means ± SD; $n = 3$ to 4.

(A) and **(B)** *NRT1.5* expression in roots of *ctr1-8* **(A)**, *eto1* **(B)**, and their wild-type control Col-0.

(C) *NRT1.5* expression in roots of *ein2-50*, *coi1-1*, *ein2-50 coi1-1*, and Col-0 plants.

(D) *NRT1.5* expression in roots of *ora59-1 erf104*, *tri-1*, *tri-2*, and wild-type control plants.

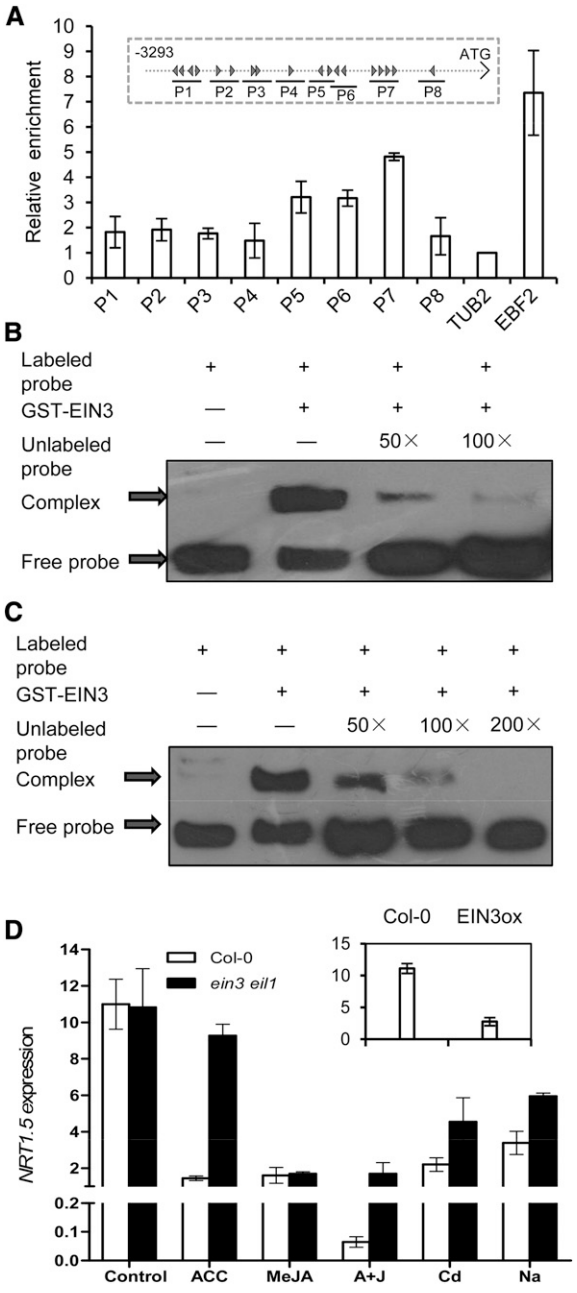

Figure 5. EIN3 Acts as the Immediate Upstream Regulator of *NRT1.5*.

(A) ChIP assay using anti-GFP antibody followed by qPCR detection of the enrichment of P1 to P8 regions as indicated in the inset, where a schematic diagram of the 3293-bp promoter fragment upstream of the *NRT1.5* start codon is shown and the black arrowheads indicate putative EIN3 binding motifs. Chromatin was isolated from root samples of 4-week-old *35S:EIN3-GFP/ein3 eil1* plants exposed to 20 μM ACC + 50 μM MeJA for 6 h. *TUB2* and *EBF2* were used as internal and positive controls, respectively. Values are means ± SD; $n = 3$.

(B) and **(C)** Representative EMSA for EIN3 binding to the fragments of P5 **(B)** and P7 **(C)** in the *NRT1.5* promoter.

(D) *NRT1.5* expression in roots of 4-week-old wild-type and *ein3 eil1* plants treated with 20 μM ACC, 50 μM MeJA, 20 μM ACC + 50 μM MeJA (A+J), 200 μM $CdCl_2$, or 150 mM NaCl for 6 h. The inset shows *NRT1.5* expression in 6-d-old wild-type Col-0 and *EIN3ox* seedlings. Data were normalized to those of *SAND*. Values are means ± SD; $n = 3$.

coi1-1 were low but still significant compared with the control condition (Figure 3F; $P < 0.01$). Comparable expression patterns were also observed for *ORA59*, *ERF1A*, *ERF1B*, and *ERF104* (Supplemental Figure 2), the immediate upstream regulators of *NRT1.8*. These results indicate that ET/JA signaling plays a dominant role in the regulation of *NRT1.8* expression, but other mechanisms such as abscisic acid (ABA) signaling might still contribute (Supplemental Figure 3A). Note that *ERF104* did not show an apparent response to stress hormones (Supplemental Figure 2D), which might be due to the fact that *ERF104* regulation by ACC occurs preferentially at the post-translational level (Bethke et al., 2009).

Further analysis showed that in *ein3 eil1* mutants, *ORA59* expression upon treatment with stress hormones or Cd/Na dramatically decreased to the control level (Supplemental Figure 4A), whereas a similar decrease for other *ERF*s was observed only after treatment with stress hormones (Supplemental Figures 4B to 4D). These results suggest that among the characterized *ERF*s, *ORA59* expression upon Cd/Na treatment is regulated exclusively via EIN3/EIL1, while others are regulated by additional factors. Taking into account the expression pattern of *ERF*s in *ein2-50*, *coi1-1*, and *eni2-50 coi1-1* (Supplemental Figure 2), these results further indicate that EIN3/EIL1 act downstream of EIN2 and COI1 to relay signals derived from Cd/Na stresses.

NRT1.5 Inhibition upon Cd/Na Treatments Is Mediated through the ET/JA Signaling Pathway, and EIN3 Acts as an Immediate Upstream Regulator

Given that *NRT1.8* and *NRT1.5* are coordinately but oppositely regulated by various stresses to mediate nitrate reallocation to roots (Li et al., 2010; Chen et al., 2012), we further determined if the *NRT1.5* inhibition under stress conditions was also regulated by ET/JA signaling. As expected, *NRT1.5* expression was steadily downregulated in the *ctr1-8* and *eto1-1* mutants (Figures 4A and 4B). Exposure to Cd/Na as well as stress hormones decreased *NRT1.5* expression in the wild-type Col-0, and functional disruption of *EIN2* or *COI1* significantly alleviated the inhibition of *NRT1.5* by the corresponding treatments, although an apparent reciprocal alleviation of *NRT1.5* suppression upon ACC or MeJA treatment was not observed in *coi1-1* or *ein2-50* mutants (Figure 4C). In the *ein2-50 coi1-1* double mutant, *NRT1.5* expression upon stress hormone treatment was even restored to control levels, and significant restoration was also observed for Cd/Na treatments (Figure 4C), similar to the regulation of *NRT1.8*, but in an opposite manner (Figure 3F). Interestingly, ABA decreased *NRT1.5* expression while salicylic acid did not show any significant effect (Supplemental Figure 3B), as was observed for *NRT1.8*, and also in an opposite manner (Supplemental Figure 3A). These results together suggest that the coordinated regulation of *NRT1.8* and *NRT1.5* under stress conditions is predominantly mediated by ET/JA signaling.

However, further analyses did not show alleviation of the *NRT1.5* suppression upon treatments in *ora59-1 erf104* double mutants or the triple mutants *tri-1* and *tri-2* (Figure 4D), indicating that *NRT1.5* and *NRT1.8* are regulated by different immediate upstream regulators. Consistent with this postulation, scan of the *NRT1.5* promoter sequence did not reveal any GCC box, as in the *NRT1.8* promoter, but instead identified several EIN3 binding site motifs (Figure 5A, inset). ChIP analysis using *35S:EIN3-GFP/ein3 eil1* plants showed enrichment of EIN3 in the P5, P6, and P7 regions in the *NRT1.5* promoter (Figure 5A; $P < 0.05$ versus TUB2), suggesting that EIN3 binds to the *NRT1.5* promoter in vivo. EMSA further indicated in vitro binding of EIN3 to P5 and P7 fragments (Figures 5B and 5C). Consistent with this, suppression of *NRT1.5* upon ACC treatment was almost abolished in *ein3 eil1* mutants, and the suppression by Cd/Na was also alleviated ($P < 0.05$), whereas apparent alleviation was not observed upon exposure to MeJA (Figure 5D). *NRT1.5* expression decreased in *EIN3ox* plants (Figure 5D, inset). These results together suggest that EIN3 acts as an immediate upstream regulator of *NRT1.5*, but the interaction between them appears to be established only when environmental signals are transduced through the ET cascade. Notably, *35S:EIN3-GFP/ein3 eil1* serves as a complementation line and behaves just like a wild-type control (He et al., 2011).

The Interaction between Stress Tolerance and Nitrate Reallocation Is Predominantly Coordinated by the ET/JA-*NRT1.5*/*NRT1.8* Signaling Module

Previous studies revealed that under Cd/Na stresses, *NRT1.5* and *NRT1.8* respond together to fine-tune SINAR (Li et al., 2010; Chen et al., 2012). Also, our research here further revealed that ET/JA signaling pathways transduce signals to coordinate the regulation of *NRT1.5* and *NRT1.8* (Figures 1 to 5), indicating that ET/JA would enhance nitrate allocation to roots as Cd/Na do. This hypothesis was tested using the *nia1 nia2* double mutant, in which nitrate reduction is almost abolished (Wilkinson and Crawford, 1993), and the results showed that nitrate reallocation to roots increased upon treatment with stress hormones or Cd/Na (Figure 6A; $P < 0.05$). In *ein2-50* and *coi1-1* mutants, nitrate allocation to roots upon treatment with stress hormones or Cd/Na decreased correspondingly compared with Col-0 (Figure 6B; $P < 0.05$). In the *ein2-50 coi1-1* double mutant, nitrate reallocation decreased further (Figure 6B), supporting the conclusion that SINAR is predominantly mediated by ET/JA signaling.

Given that SINAR enhances stress tolerance and that ET/JA signaling mediates SINAR, we further determined the stress tolerance mediated by the interaction between nitrate reallocation and ET/JA signaling. As shown in Figure 7A, when nitrate concentration increased from 2.25 to 25 mM in the medium, enhanced tolerance to Na/Cd was observed in the wild-type Col-0. By contrast, the ET-insensitive *ein3 eil1* mutants did not respond. In the triple mutant *ein3 eil1 nrt1.5-4*, however, steadily enhanced tolerance was observed despite the external nitrate concentration (Figure 7A), which is reasonable, as loss of NRT1.5 function tended to show constitutively promoted nitrate reallocation to roots and stress tolerance (Chen et al., 2012). Similar results were obtained for the JA-insensitive mutant *coi1-2* and the double mutant *coi1-2 nrt1.5-4* (Figure 7B). The positive relationship

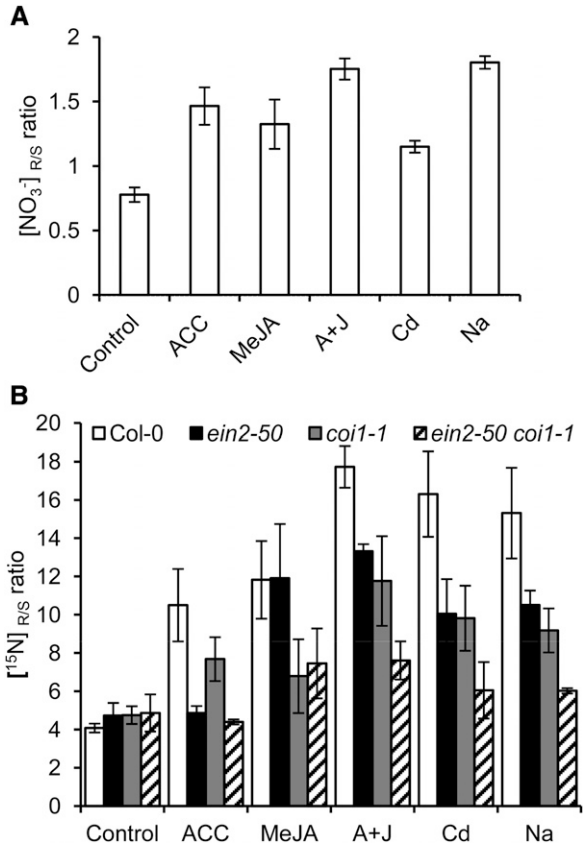

Figure 6. Stress-Induced Nitrate Reallocation Mediated by ET/JA Signaling.

(A) The *nia1 nia2* mutant plants were grown hydroponically with NH_4HCO_3 as the sole nitrogen source. At 4 weeks of age, plants were transferred to 4.5 mM NO_3^- solution and treated with 20 μM ACC, 50 μM MeJA, 20 μM ACC + 50 μM MeJA (A+J), 200 μM $CdCl_2$, or 150 mM NaCl for 12 h. The nitrate ratio between roots and shoots ($[NO_3^-]_{R/S}$) was calculated.

(B) Four-week-old Col-0, *ein2-50*, *coi1-1*, and *ein2-50 coi1-1* plants grown in quarter-strength hydroponics medium were treated with 20 μM ACC, 50 μM MeJA, 20 μM ACC + 50 μM MeJA (A+J), 200 μM $CdCl_2$, or 150 mM NaCl for 12 h and then labeled with 3 mM $K^{15}NO_3$ for 30 min. The nitrate ratio between roots and shoots ($[^{15}N]_{R/S}$) was calculated. Values are means ± SD; $n = 3$.

between nitrate concentration and Na/Cd tolerance was also suppressed in *tri-2* mutants (Figure 7C), which imitate the functional impairment of *NRT1.8*, thus causing stress sensitivity (Li et al., 2010). In *ctr1-8* mutants, enhanced tolerance to Na/Cd was observed with increased nitrate concentration, while the double mutant *ctr1-8 nrt1.8-2* showed a decreased response (Figure 7D). These results together suggest that the interaction between stress tolerance and nitrate reallocation is predominantly coordinated by the ET/JA-*NRT1.5*/*NRT1.8* signaling cascade. Note that plant tolerance was quantified here using relative root growth, because ET/JA signaling mutants always show pleotropic effects that might interfere with each other under given conditions, as indicated by the growth of *ein3 eil1* and *coi1-2*

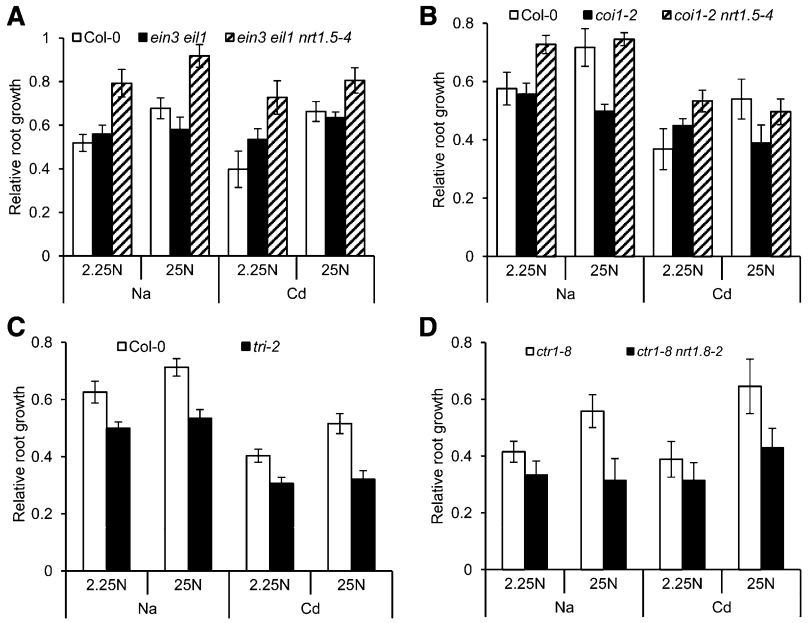

Figure 7. Stress Tolerance Correlates with Nitrate Allocation via the ET/JA-*NRT1.5/NRT1.8* Signaling Module.

Four-day-old seedlings germinated on ammonium plates as described in Methods were transferred to medium with either 2.25 mM nitrate (2.25 N) or 25 mM NO_3^- (25 N) plus $CdCl_2$/NaCl and allowed vertical growth for 6 d (**[A]** to **[C]**) or 9 d **(D)** before the determination of root elongation. Cd, 40 μM $CdCl_2$ in **(A)** to **(C)** and 50 μM $CdCl_2$ in **(D)**; Na, 100 mM NaCl in **(A)** to **(C)** and 125 mM NaCl in **(D)**. Relative root growth is defined as the ratio of root elongation rates in the presence of Cd/Na against those in the same medium without Cd/Na. Values are means ± SD; n = 7 to 12.

mutants exposed to a combination of Cd and low-level nitrate (Figures 7A and 7B).

Nitrate Reallocation to Roots Decreases Plant Regular Growth under Nonstressed Conditions

In *nrt1.5* mutants, when the nitrate supply increased, stress tolerance was enhanced while plant regular growth was suppressed (Lin et al., 2008; Chen et al., 2012), indicating that nitrate reallocation to roots possibly mediates the trade-off between stress tolerance and plant growth. To test this hypothesis, we used ET/JA signaling-related mutants to mimic nitrate reallocation without exposure to stresses, thus decoupling the two important steps in SINAR. In *ctr1-8* mutants, where upregulation of *NRT1.8* and downregulation of *NRT1.5* were steadily activated (Figures 3A and 4A), suppression of root growth was enhanced when nitrogen was switched from ammonium to nitrate, and further suppression was observed when the nitrate concentration increased (Figure 8A). Consistent data were obtained in *eto1-1* mutants (Figure 8B), the MeJA-treated Col-0 plants (Figure 8C), and the *EIN3*-overexpressing seedlings (Supplemental Figure 5). These results together indicate that nitrate reallocation to roots decreases plant regular growth under nonstressed conditions. Note that the relative growth for JA signaling mutants was defined as Col-0/*coi1-1*, and, as a constitutive JA response mutant is not available, MeJA was applied to mimic a constitutive JA response in the wild-type Col-0.

To further determine how much this process was attributable to the NRT components in the ET/JA-*NRT1.8/NRT1.5* signaling pathway, *nrt1.8-2* was crossed with *ctr1-8* and *eto1-1*. As expected, similar growth was observed in *ctr1-8* and the double mutant *ctr1-8 nrt1.8-2* when exposed to ammonium, while increased growth was observed in *eto1-1 nrt1.8-2* mutants with nitrate as the nitrogen source (Figure 8D). Although further elevation of nitrate concentration decreased the absolute growth of both *ctr1-8* and *ctr1-8 nrt1.8-2* mutants, *ctr1-8 nrt1.8-2* still showed better growth than *ctr1-8* (Figure 8D). Similar results were consistently obtained in *eto1-1 nrt1.8-2* and *eto1-1* mutants (Figure 8E). Given that *nrt1.8* is stress-sensitive (Li et al., 2010), while a cross of *nrt1.8-2* with *ctr1-8* and *eto1-1* increased their regular growth, these observations together support a postulation that the NRT components contribute to the trade-off between stress tolerance and plant growth through nitrate reallocation. Note, though, that the contribution of NRT1.5 was not genetically determined, due to the technical difficulty of alleviating the downregulation of *NRT1.5* in *ctr1-8* and *eto1-1* mutants. Nevertheless, the conclusion should stand as indicated by other lines of evidence and previous studies (Figures 4, 7A, and 7B; Lin et al., 2008; Chen et al., 2012).

SINAR Functions in Mediating Plant Growth and Stress Tolerance Require Nitrate Reductase

Nitrate reallocation was proposed to act as a signal to mediate stress tolerance (Gojon and Gaymard, 2010; Li et al., 2010; Chen et al., 2012), and here it was further identified to balance stress tolerance and plant growth (Figures 7 and 8). To address the question of whether nitrate, nitrite, nitric oxide (NO), or another nitrogen metabolite is perceived as the signal for nitrate reallocation, *nrt1.8-2* and *nrt1.5-4* were crossed to *nia1 nia2*, where typical nitrate reallocation was induced upon treatment with Na/Cd (Supplemental Figures 6A to 6D). However, the results

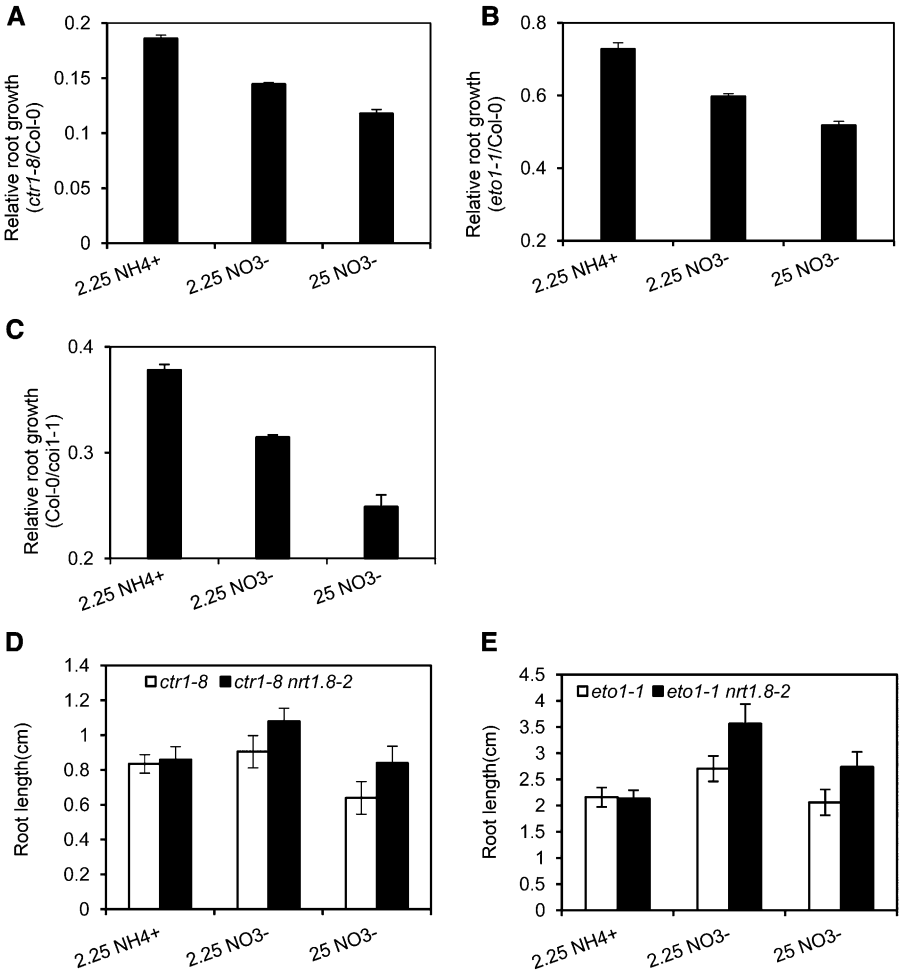

Figure 8. Nitrate Reallocation Mediated by the ET/JA-NRT Signaling Module Reduces Root Growth under the Nonstressed Condition.

Seedlings of *ctr1-8*, *eto1-1*, *coi1-1*, *ctr1-8 nrt1.8-2*, and *eto1-1 nrt1.8-2* mutants and their wild-type control Col-0 were germinated and allowed to grow vertically for 7 to 9 d on medium supplemented with 2.25 mM ammonium (2.25 NH_4^+), 2.25 mM nitrate (2.25 NO_3^-), or 25 mM nitrate (25 NO_3^-), followed by measurement of root growth. Relative root growth of each mutant was calculated by normalizing the data to those of their wild-type control, except for **(C)**.

(A) and **(B)** Relative root growth of *ctr1-8* **(A)** and *eto1-1* **(B)** against the wild-type Col-0 under different conditions. Values are means ± SE; $n = 5$.

(C) Seeds from heterologous *coi1-1*(+/−) plants were plated on medium with 10 μM MeJA to screen homozygous seedlings, which together with Col-0 were incubated with different nitrogens plus 10 μM MeJA to mimic the constitutive JA response; correspondingly, the relative growth is defined as the ratio of *coi1-1*(+/+) to Col-0. Values are means ± SE; $n = 5$.

(D) and **(E)** Root growth of *ctr1-8* and *ctr1-8 nrt1.8-2* **(D)** or *eto1-1* and *eto1-1 nrt1.8-2* **(E)** under different conditions. Values are means ± SD; $n = 9$ to 11.

unexpectedly showed that the *nia1 nia2 nrt1.8-2* and *nia1 nia2 nrt1.5-4* triple mutants responded to Na/Cd treatments similarly to the *nia1 nia2* double mutants (Figure 9A; $P > 0.2$), in contrast with what we observed in *nrt1.8-2* and *nrt1.5-4* with normal nitrate reductase function (Figure 7; Chen et al., 2012). Furthermore, the previously observed differential plant growth in response to nitrate reallocation (Figure 8; Lin et al., 2008; Chen et al., 2012) was also impaired in the *nia1 nia2 nrt1.8-2* and *nia1 nia2 nrt1.5-4* mutants under nonstressed conditions (Figure 9B; $P > 0.1$). These results suggest that nitrate reallocation functions to mediate stress tolerance and plant growth, dependent on nitrate reductase.

Given that *nia1 nia2* mutants show a substantial decrease in nitrate reduction and thus in subsequent assimilates and derivatives, we wondered if NO might play a role in SINAR signal perception, as proposed (Gojon and Gaymard, 2010). However, when the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) was applied, the differential response to Na/Cd was still observed in *nrt1.8-2* and *nrt1.5-4* mutants compared with Col-0 (Figure 9C; $P < 0.01$). When under nonstressed conditions, consistent phenotypes were observed (Figure 9D). These results appeared to indicate that NO might not serve as the signal molecule in SINAR signal perception. Note, though, that *nrt1.8-2* showed enhanced growth under control conditions (no cPTIO), as expected, and application of cPTIO appeared to impair its growth (Figure 9D), which might be due to the interaction between cPTIO toxicity and the increased stress sensitivity in *nrt1.8-2*.

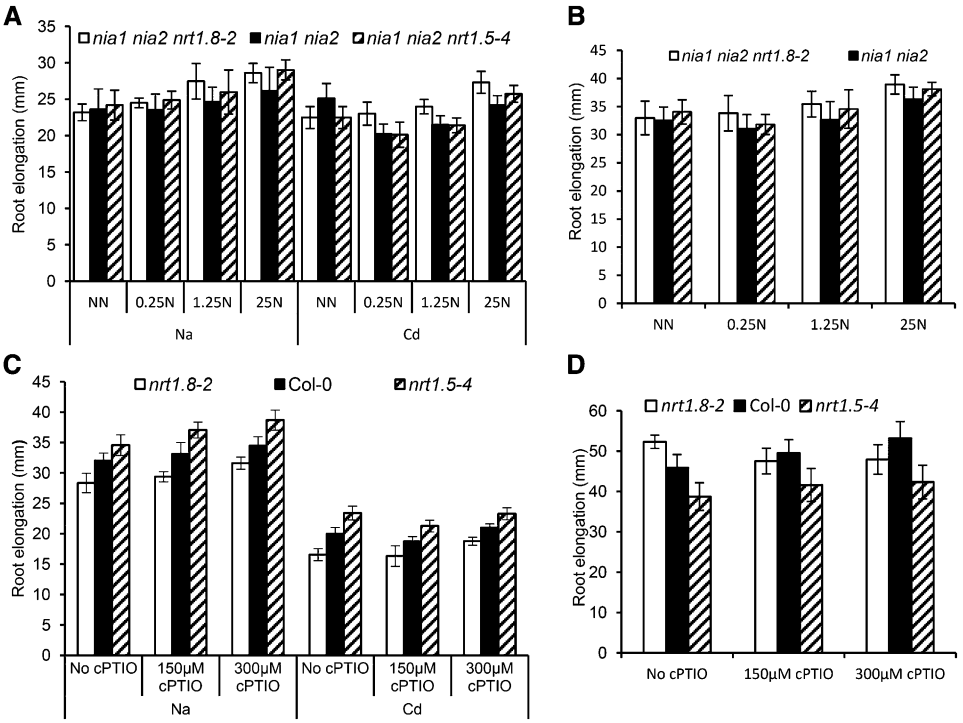

Figure 9. Nitrate Reallocation Mediates Stress Tolerance and Plant Growth Dependent on Nitrate Reductase.

(A) and **(B)** Stress response and regular growth of *nrt1.8-2* and *nrt1.5-4* in the *nia1 nia2* background. *Arabidopsis* seeds were germinated on 2.25 mM ammonium medium for 4 d, transferred to the same ammonium medium spiked with various levels of nitrate (NN, no nitrate; 0.25N, 0.25 mM KNO_3; 1.25N, 1.25 mM KNO_3; 25N, 25 mM KNO_3) and 40 μM $CdCl_2$ or 100 mM NaCl **(A)** or no stress treatment **(B)**, and allowed vertical growth for 6 d before the determination of root elongation.

(C) and **(D)** Stress response and regular growth of *nrt1.8-2* and *nrt1.5-4* treated with a NO scavenger. Four-day-old seedlings were transferred from 2.25 mM ammonium medium to 25 mM nitrate medium with **(C)** or without **(D)** the combined treatment of Cd/Na (Cd, 40 μM $CdCl_2$; Na, 100 mM NaCl) and cPTIO and allowed vertical growth for 6 d before the determination of root elongation.

Values are means ± SD; $n = 7$.

DISCUSSION

Recent studies indicated that SINAR serves as a universal mechanism in response to diverse stresses (Li et al., 2010; Chen et al., 2012). However, how SINAR responds to the environment remains unknown. This study demonstrates that, in response to Cd/Na treatment, the ET/JA signaling pathways are activated to mediate EIN3 and its induction of *NRT1.5*, as well as several *ERFs* including *ORA59*, which further induce *NRT1.8*, thus establishing a dynamic coordination of the essential components NRT1.5 and NRT1.8 in SINAR. Our data also revealed that SINAR mediates stress tolerance and plant growth, dependent on nitrate reductase.

The ET/JA-NRT Signaling Module Presents a Primary Mechanism to Coordinate Crosstalk between SINAR and the Environment

Stresses induce the biosynthesis of ET and JA (Fujita et al., 2006), which further activate downstream signaling pathways to mediate a wide range of stress responses (Vijayan et al., 1998; Berrocal-Lobo et al., 2002; Achard et al., 2006; Dombrecht et al., 2007; Zhang et al., 2011; Kazan and Manners, 2012; Hu et al., 2013; Li et al., 2013). A recent study further discovered that ET and JA signaling pathways crosstalk via EIN3/EIL1 and function synergistically (Zhu et al., 2011). In our research, we showed that ET/JA signaling might function conservatively, via known signaling components, to regulate the communication between SINAR and the environment (Figure 10). One point of supporting evidence is that in the mutants of major ET or JA signaling components, such as *ctr1-8*, *ein2-50*, and *coi1-1*, *NRT1.8*/*NRT1.5* expression altered correspondingly as expected (Figures 3 to 5), and this alteration was enhanced in the double mutant *ein2-50 coi1-1*, where both ET and JA signaling are essentially blocked (Figures 3F and 4C). Further supporting evidence came from the results that *NRT1.8* induction upon ET in *coi1-1* or upon JA in *ein2-50* still occurred (Figure 3F) and that nitrate reallocation to *coi1-1* roots upon ACC or to *ein2-50* roots upon JA was still significant (Figure 6B), in line with previous observations that the expression of *ERF1B* and *ORA59* in response to JA or ET requires intact ET and JA signaling pathways (Lorenzo et al., 2003; Pré et al., 2008). These data support the model that ET/JA signaling is involved in Cd/Na-mediated SINAR via important, well-characterized components (Figure 10; Zhao and Guo, 2011; Zhu et al., 2011). Note that in *ein3 eil1*, the inhibition of *NRT1.5* upon JA treatment was not

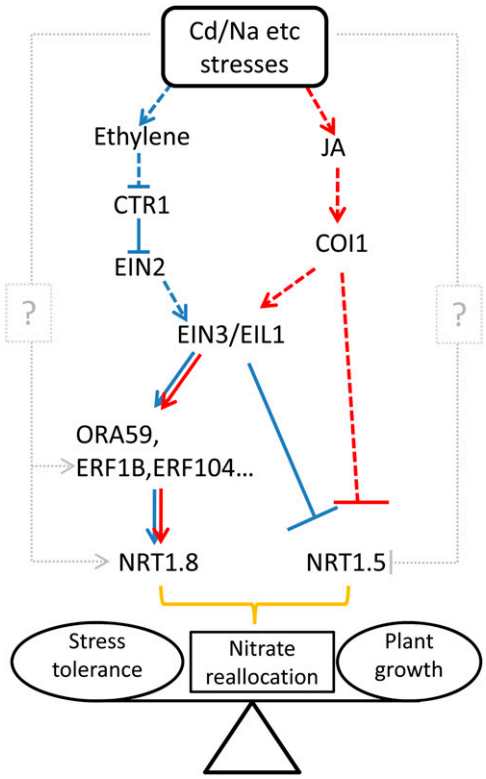

Figure 10. A Simplified Model for the Interaction between ET/JA Signaling and Nitrate Reallocation to Mediate Plant Adaptation to the Environment.

Stresses such as cadmium or salt induce the production of ET and JA, which further activates the ET and JA signaling pathways, respectively. EIN3/EIL1 acts to converge signals transduced by ET and JA cascades, and further transduces the signal down to ERFs, which induce *NRT1.8* expression by binding to its promoter, or EIN3/EIL1 decodes signals via the ET pathway by binding to the promoter of *NRT1.5* and suppresses its expression. The JA pathway also participates in the downregulation of *NRT1.5* via COI1. The coordinated upregulation of *NRT1.8* and downregulation of *NRT1.5* fine-tunes nitrate reallocation to roots, which modulates the balance between stress tolerance and plant growth. Blue lines display the route for signals going through the ET signaling pathway, while red lines indicate those through the JA signaling pathway. Dashed lines indicate steps not shown or possible unidentified components. Gray dotted lines and question marks indicate alternative pathways/components that contribute, but to a much lesser extent, in the regulation of *NRT1.5* and *NRT1.8*.

alleviated as it was upon ET treatment (Figure 5D), suggesting that the stress-derived JA signaling cascade mediates *NRT1.5* expression via new component(s) other than EIN3/EIL1, the integrator of JA and ET signals (Figure 10; Zhu et al., 2011; Kazan and Manners, 2012).

Moreover, although *NRT1.8/NRT1.5* expression upon ET/JA was not induced in corresponding ET/JA signaling mutants, a significant regulation upon Cd/Na treatment still occurred (Figures 3 and 4), indicating that alternative signaling pathway(s) might work with ET/JA signaling to mediate SINAR. The results that *NRT1.8* and *NRT1.5* were coordinately regulated by ABA supported this postulation (Supplemental Figure 3). However, given that nitrate reallocation to roots of *ein2-50 coi1-1* under Cd/Na exposure was restored to levels comparable to those under control conditions (Figure 6B), the alternative pathway(s) might have a subtle contribution. Note that we do not exclude the possibility that nitrate transporters other than NRT1.5/NRT1.8 might contribute, but they are likely to be controlled by ET/JA signaling (Figure 6B).

Furthermore, given that SINAR was proposed as a universal adaptive response due to the highly coordinated *NRT1.5/NRT1.8* expression under diverse conditions (Gojon and Gaymard, 2010; Li et al., 2010; Chen et al., 2012) and that ET/JA signaling acts genetically upstream of *NRT1.5/NRT1.8* to mediate SINAR under Cd/Na stresses, another concern is if ET/JA-NRT signaling represents a common mechanism in the regulation of SINAR. This possibility was only investigated in a limited set of conditions here, but the fact that ET/JA are produced under a wide range of stresses might suggest so (Glazebrook, 2005; Maksymiec et al., 2005; Arteca and Arteca, 2007; Cao et al., 2007; Zhang et al., 2011; Ismail et al., 2012; Poltronieri et al., 2013), as ET/JA production would eventually activate the downstream signaling cascades, although new components specific to certain stimuli might exist, as was observed for the JA-mediated *NRT1.5* expression (Figure 10).

How SINAR Balances Plant Growth and Stress Tolerance

Previous studies proposed that reallocated nitrate per se might act as a signal to coordinate responses (Li et al., 2010). A more recent study further excluded the possibility of a nutritional role by substituting nitrate with ammonium and provided evidence supporting the signal hypothesis (Chen et al., 2012). However, when investigating the stress response in *nia1 nia2 nrt1.8-2* and *nia1 nia2 nrt1.5-4* mutants, where nitrate reduction is fundamentally disrupted, we surprisingly found that they responded to Cd/Na similarly and did not show any apparent difference from the control *nia1 nia2* (Figures 9A and 9B). These observations appear to be contradictory to the hypothesis that nitrate per se might be the signal molecule.

Gojon and Gaymard (2010) proposed an alternative hypothesis where NO serves as the signal molecule, because Cd promotes NO synthesis in roots (Besson-Bard et al., 2009), and nitrate reductase represents an important way to synthesize NO. However, application of the NO scavenger cPTIO did not alter the varied growth rates or differential responses of *nrt1.8-2* and *nrt1.5-4* mutants to Cd/Na (Figures 9C and 9D), implying that NO is unlikely to be the signal molecule. In this respect, we hypothesize that Glu/Gln or other nitrogen assimilates might be the candidate signaling molecules, as they are known to function as signaling molecules in mediating various biological processes (Stitt, 1999; Walch-Liu et al., 2006; Forde and Lea, 2007; Szabados and Savouré, 2010). A more likely model is that nitrate per se does act as the signal molecule to mediate the balance between stress tolerance and plant growth, but its function requires the activity of nitrate reductase.

SINAR Is a Dynamic Mechanism to Modulate Plant Growth and Environmental Adaptation

SINAR decouples the direct correlation between nitrate assimilation and photosynthesis; thus, it is believed to decrease energy

use efficiency and evolutionary competitiveness (Canvin and Atkins, 1974; Smirnoff and Stewart, 1985; Andrews, 1986). One major concern about this phenomenon is what exactly the physiological importance is. Recent studies discovered that when SINAR was impaired, plants became sensitive to Cd stress (Li et al., 2010), while promotion of this process enhanced tolerance to diverse stresses (Li et al., 2010; Chen et al., 2012), indicating that SINAR decreases energy efficiency but increases stress tolerance. This study further discovered that in mutant plants with enhanced SINAR, regular root growth decreased under non-stressed conditions (Figures 8A to 8C; Supplemental Figure 5) and vice versa (Figures 8D and 8E). These observations support a model in which SINAR partitions energy within plants in response to environmental cues, thus allowing a dynamic balance between plant growth and stress tolerance (Figure 10).

ET/JA signaling regulates the trade-off between plant growth and stress tolerance (Achard et al., 2003, 2006; Yang et al., 2012), and here we showed that SINAR also regulates this and functions downstream of the ET/JA signaling cascade (Figures 1 to 8). This suggests that SINAR contributes significantly to the overall balancing mechanism mediated by ET/JA, although the exact contribution might remain to be determined due to the technical difficulties in concomitantly modulating *NRT1.5* and *NRT1.8* expression. It is worth noting that plant growth in this context was evaluated only with roots, considering the pleiotropic effects of ET/JA signaling mutants, but the conclusion is likely also applicable to shoot growth, mainly because *nrt1.5-2* mutants showed dramatic growth reduction in the whole plant (Lin et al., 2008).

Additionally, given that nitrate assimilation requires a lot of energy, an intriguing strategy to improve nitrogen use efficiency is to block SINAR, thus enhancing nitrate photoassimilation. This strategy is of special importance in China, where eutrophication is widespread (Ju et al., 2009); however, considering the frequency of environmental stress, a more plausible strategy might be to enhance the basal long-distance root-to-shoot nitrate transport, retaining the SINAR mechanism but decreasing it to a minimal level that will require further study to be determined. This hypothesis is supported by the growth promotion in nonstressed *ctr1-8 nrt1.8-2* or *eto1-1 nrt1.8-2* mutants (Figures 8D and 8E), where the ET/JA-derived SINAR was partially impaired by *nrt1.8*. Considering that NRT1.8/NRT1.5 are two essential components in SINAR, further promotion of plant growth might be obtained by constitutive elevation of *NRT1.5* and concomitant disruption of *NRT1.8* in roots.

In conclusion, we revealed that the ET/JA-NRT signaling module is involved in the regulation of SINAR and that EIN3/EIL1 act as pivotal signal integrators to modulate *NRT1.5* and *NRT1.8* expression. Components other than EIN3/EIL1 might exist in the signaling pathway to relay the stress-initiated JA signal, and SINAR mediates plant adaption to environments in a nitrate reductase-dependent manner.

METHODS

Plant Materials

Arabidopsis thaliana mutant or transgenic lines were gifts from colleagues (*ein2-50*, *coi1-1*, *ein3 eil1*, *coi1-2*, *nia1 nia2*, *EIN3ox*, *ctr1-8*, *eto1-1*, and *35S:EIN3-GFP/ein3 eil1*) or generated in our laboratory (*nrt1.8-2* and *nrt1.5-4*) as described (Chen et al., 2012). The mutant lines *ora59-1* (CS405772), *ora59-2* (CS855464), and *erf104* (SALK_057720C) were ordered from the ABRC and the European Arabidopsis Stock Centre. All double or triple mutant lines were generated by crossing the single mutants and subsequent PCR genotyping, except that an additional screen with 50 μM MeJA in plate medium was performed to identify the *coi1-2 nrt1.5-4* homozygote (Xu et al., 2002) or 50 μM MeJA plus 10 μM ACC for *ein2-50 coi1-1*.

DNA Constructs and Transformation into Plants

To make the ERF1B-RNAi construct, a cDNA fragment specific to *ERF1B* was amplified using the PCR primers listed in Supplemental Table 1, and the fragment was further introduced into the modified binary vector pFGC5941 (a gift from Hai Huang) by a two-step cloning strategy, according to the instructions (http://www.chromdb.org/rnai/vector_info.html). The resulting construct, ERF1B-RNAi, was then transformed into *ora59-1 erf104* to generate the *ora59-1 erf104 erf1b* triple mutants *tri-1* and *tri-2*. To make the *35S:ORA59-TAP* and *35S:ERF1B-TAP* constructs, the coding sequences of *ORA59* and *ERF1B* were amplified and introduced into the pCambias1300-TAP vector, as described (Luo et al., 2012). The *35S:ERF104-GFP* construct was generated by introducing green fluorescent protein (*GFP*) into the vector pCambias1300 and then fusing the coding sequence of *ERF104* to the 5′ terminus of *GFP*. All constructs were transformed into the indicated plants by the floral dip method (Clough and Bent, 1998). Transgenic lines with 3:1 segregation on selection plates were used for further isolation of homozygotes with strong expression of target genes.

Growth Conditions and Phenotyping

Arabidopsis plants were generally grown in quarter-strength minimal medium or hydroponics as described (Gong et al., 2003). At 4 weeks of age, plants were treated with the indicated hormones or stresses and tissues were sampled and subjected to further assays. Alternatively, the minimal medium was supplemented with elevated nitrate [24 mM KNO_3, 0.5 mM $Ca(NO_3)_2$] or substituted nitrogen sources [2.25 mM ammonium:1.125 mM $(NH_4)_2$ succinate, 1.25 mM KCl, 0.5 mM $CaCl_2$]. Surface-sterilized seeds were plated on the above media as indicated and incubated at 4°C for 4 d, then allowed to grow vertically at 22°C for 7 to 9 d before root elongation was measured. Or, as indicated, *Arabidopsis* seeds were germinated on minimal medium-containing plates supplemented with 2.25 mM ammonium for 4 d, transferred to medium with the indicated nitrogen plus $CdCl_2$/NaCl, and allowed to grow vertically for the indicated time before the determination of root elongation.

RT-PCR and Real-Time Quantitative RT-PCR

Total RNA from plants grown under the indicated conditions was prepared using TRIzol reagent (Invitrogen). First-strand cDNA synthesis, RT-PCR, and quantitative RT-PCR were performed as described previously (Li et al., 2010). The primers used in these assays are listed in Supplemental Table 1, and the expression levels were normalized to those of the *SAND* control.

Protein Purification and EMSA

Fragments of *ORA59*, *ERF1B*, and *ERF104* coding sequences were cloned into the vector pET-30a(+), and the resulting constructs were further transformed into *Escherichia coli* BL21(DE3) pLysS. Recombinant proteins were induced by isopropyl β-D-1-thiogalactopyranoside and purified using Ni-NTA agarose (Qiagen). For *EIN3*, a truncated cDNA fragment representing the EIN3 DNA binding domain (amino acids 1 to 314) was cloned into pGEX 4T-1, and the recombinant protein was purified by glutathione *S*-transferase-agarose affinity chromatography (GE Healthcare).

EMSA was performed using the Light Shift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's instructions. All DNA

fragments used as wild-type probes were amplified using the PCR primers listed in Supplemental Table 1, then labeled with biotin or left unlabeled and used as competitors. Mutant probes were generated using the Multipoints Mutagenesis Kit (Takara).

Transient Transcriptional Activity Assay

A 1298-bp promoter fragment upstream of the *NRT1.8* start codon (ATG) was cloned into pGreen II 0800 to generate the reporter plasmid, and the coding sequences of selected *ERF* genes were cloned into pGreen II 62-SK to make effector plasmid (Hellens et al., 2005). Once transformed with 12 μg of effector plasmids and 3 μg of reporter plasmids, the *Arabidopsis* protoplasts were incubated at 20 to 25°C overnight and then harvested and lysed (Yoo et al., 2007). Firefly luciferase (LUC) and Renilla luciferase (REN) activities were assayed using the dual-luciferase reporter assay system (Promega).

ChIP-Quantitative PCR

At 4 weeks of age, root samples from hydroponically grown transgenic plants (ERF-overexpressing or *35S:EIN3-GFP/ein3 eil1* lines) with or without treatments (20 μM ACC + 50 μM MeJA for 6 h) were fixed in formaldehyde crosslinking solution. ChIP was performed as described previously (Aggarwal et al., 2010) using anti-GFP antibody (for ERF104-GFP or EIN3-GFP protein fusions; Invitrogen) or IgG beads (for ERF1B-TAP or ORA59-TAP protein fusions; GE Healthcare). The enriched DNA fragments were subjected to quantitative PCR (qPCR) determination using the primers listed in Supplemental Table 1. qPCR was performed using SYBR Premix Ex Taq (Takara) according to the manufacturer's instructions, and relative enrichment of fragments was calculated by comparing samples treated with or without antibodies. *TUB2* was used as an internal control.

Determination of Nitrate Content

The *nia1 nia2* plants were grown hydroponically with ammonium as the sole nitrogen source; the original 1.25 mM KNO_3 and 0.5 mM $Ca(NO_3)_2$ in the hydroponic solution (Gong et al., 2003) were replaced with 2.25 mM NH_4HCO_3, 1.25 mM KCl, and 0.5 mM $CaCl_2$. At 4 weeks of age, plants were subjected to treatments as indicated and tissues were sampled. Nitrate was extracted and determined as described (Li et al., 2010).

Analysis of Root-to-Shoot Nitrate Allocation Using $^{15}NO_3^-$

Four-week-old Col-0, *ein2-50*, *coi1-1*, and *ein2-50 coi1-1* plants grown in quarter-strength hydroponics medium were treated with 20 μM ACC, 50 μM MeJA, 20 μM ACC + 50 μM MeJA, 200 μM $CdCl_2$, and 150 mM NaCl for 12 h, washed in 0.1 mM $CaSO_4$ for 1 min, labeled for 30 min in 3 mM $K^{15}NO_3$ medium (99.15% atom excess of ^{15}N; pH 6.0), and washed again in 0.1 mM $CaSO_4$ for 1 min (Wang and Tsay, 2011). The root and shoot tissues were sampled separately and dried at 80°C for 3 d. ^{15}N contents were determined using a continuous-flow isotope ratio mass spectrometer coupled to a carbon nitrogen elemental analyzer (Vario EL III/Isoprime; Elementar).

Statistical Analysis

Two-tailed Student's *t* tests were performed. Differences were deemed significant at $P < 0.05$ and extremely significant at $P < 0.01$.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *NRT1.5* (At1g32450), *NRT1.8* (At4g21680), *ERF1B/ERF1* (At3g23240), *ORA59* (At1g06160), *ERF104* (At5g61600), *ERF1A/ATERF1* (At4g17500), *ERF2* (At5g47220), *ERF6* (At4g17490), *ERF13* (At2g44840), *ERF15* (At2g31230), *EIN3* (At3g20770), *EIL1* (At2g27050), *CTR1* (At5g03730), *EIN2* (At5g03280), *COI1* (At2g39940), *ETO1* (At3g51770), *NIA1* (At1g77760), *NIA2* (At1g37130), *SAND* (At2g28390), and *TUB2* (At5g62690). Additional sequence data are available in Supplemental Table 1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. ERF Family Members Function Redundantly in the Regulation of *NRT1.8* Expression.

Supplemental Figure 2. *ERF* Expression upon Stress/Hormone Treatment in the ET/JA Mutants.

Supplemental Figure 3. *NRT1.5* and *NRT1.8* Expression in Response to Salicylic Acid or ABA Treatment.

Supplemental Figure 4. The Expression of *ERFs* in the *ein3 eil1* Mutant in Response to Different Treatments.

Supplemental Figure 5. Root Growth of *EIN3*-Overexpressing Lines under Nonstressed Conditions.

Supplemental Figure 6. Nitrate Reallocation upon Stresses in *nrt1.8* and *nrt1.5* Mutants in the *nia1 nia2* Background.

Supplemental Table 1. List of Primer Sequences.

ACKNOWLEDGMENTS

We thank Chi-Kuang Wen (Shanghai Institutes for Biological Sciences), Hongwei Guo (Peking University), Chuanyou Li (Chinese Academy of Sciences), Daoxin Xie (Tsinghua University), and Nigel Crawford (University of California San Diego) for kindly providing the seeds of *ein2-50*, *ctr1-8*, *eto1-1*, *EIN3ox*, *ein3 eil1*, *35S:EIN3-GFP/ein3eil1*, *coi1-1*, *coi1-2*, and *nia1 nia2*; Hai Huang (Shanghai Institutes for Biological Sciences) and Roger P. Hellens (Mt. Albert Research Centre) for the vectors of pFGC5941, pGreen II 0800, and pGreen II 62-SK; Jingli Hou and Li Zhang (Instrumental Analysis Center, Shanghai Jiao Tong University) for helping with ^{15}N analysis; Chi-Kuang Wen for careful reading of the article; and Yan-Lei Fu for helping with ChIP analysis. This work was supported by the National Science Foundation of China (Grants 31325003 and 31121063), the Ministry of Agriculture of China (Grant 2014ZX08009-003-005), and the SA-SIBS scholarship program.

AUTHOR CONTRIBUTIONS

J.G. and G.Z. designed the research. G.Z. and H.Y. performed the research. J.G. and G.Z. analyzed the data and wrote the article.

Received June 26, 2014; revised September 16, 2014; accepted October 9, 2014; published October 17, 2014.

REFERENCES

Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., Van Der Straeten, D., Peng, J., and Harberd, N.P. (2006). Integration of plant responses to environmentally activated phytohormonal signals. Science **311:** 91–94.

Achard, P., Vriezen, W.H., Van Der Straeten, D., and Harberd, N.P. (2003). Ethylene regulates *Arabidopsis* development via the modulation of DELLA protein growth repressor function. Plant Cell **15:** 2816–2825.

Aggarwal, P., Vaites, L.P., Kim, J.K., Mellert, H., Gurung, B., Nakagawa, H., Herlyn, M., Hua, X., Rustgi, A.K., McMahon, S.B., and Diehl, J.A. (2010). Nuclear cyclin D1/CDK4 kinase regulates *CUL4* expression and triggers neoplastic growth via activation of the PRMT5 methyltransferase. Cancer Cell **18:** 329–340.

Andrews, M. (1986). The partitioning of nitrate assimilation between root and shoot of higher plants. Plant Cell Environ. **9:** 511–519.

Arteca, R.N., and Arteca, J.M. (2007). Heavy-metal-induced ethylene production in *Arabidopsis thaliana*. J. Plant Physiol. **164:** 1480–1488.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in *Arabidopsis* confers resistance to several necrotrophic fungi. Plant J. **29:** 23–32.

Besson-Bard, A., Gravot, A., Richaud, P., Auroy, P., Duc, C., Gaymard, F., Taconnat, L., Renou, J.P., Pugin, A., and Wendehenne, D. (2009). Nitric oxide contributes to cadmium toxicity in Arabidopsis by promoting cadmium accumulation in roots and by up-regulating genes related to iron uptake. Plant Physiol. **149:** 1302–1315.

Bethke, G., Unthan, T., Uhrig, J.F., Pöschl, Y., Gust, A.A., Scheel, D., and Lee, J. (2009). Flg22 regulates the release of an ethylene response factor substrate from MAP kinase 6 in *Arabidopsis thaliana* via ethylene signaling. Proc. Natl. Acad. Sci. USA **106:** 8067–8072.

Canvin, D.T., and Atkins, C.A. (1974). Nitrate, nitrite and ammonia assimilation by leaves: Effect of light, carbon dioxide and oxygen. Planta **116:** 207–224.

Cao, W.H., Liu, J., He, X.J., Mu, R.L., Zhou, H.L., Chen, S.Y., and Zhang, J.S. (2007). Modulation of ethylene responses affects plant salt-stress responses. Plant Physiol. **143:** 707–719.

Chen, C.Z., Lv, X.F., Li, J.Y., Yi, H.Y., and Gong, J.M. (2012). *Arabidopsis* NRT1.5 is another essential component in the regulation of nitrate reallocation and stress tolerance. Plant Physiol. **159:** 1582–1590.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Crawford, N.M. (1995). Nitrate: Nutrient and signal for plant growth. Plant Cell **7:** 859–868.

Dombrecht, B., Xue, G.P., Sprague, S.J., Kirkegaard, J.A., Ross, J.J., Reid, J.B., Fitt, G.P., Sewelam, N., Schenk, P.M., Manners, J.M., and Kazan, K. (2007). MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. Plant Cell **19:** 2225–2245.

Forde, B.G., and Lea, P.J. (2007). Glutamate in plants: Metabolism, regulation, and signalling. J. Exp. Bot. **58:** 2339–2358.

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006). Crosstalk between abiotic and biotic stress responses: A current view from the points of convergence in the stress signaling networks. Curr. Opin. Plant Biol. **9:** 436–442.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. **43:** 205–227.

Gojon, A., and Gaymard, F. (2010). Keeping nitrate in the roots: An unexpected requirement for cadmium tolerance in plants. J. Mol. Cell Biol. **2:** 299–301.

Gong, J.M., Lee, D.A., and Schroeder, J.I. (2003). Long-distance root-to-shoot transport of phytochelatins and cadmium in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **100:** 10118–10123.

He, W., et al. (2011). A small-molecule screen identifies L-kynurenine as a competitive inhibitor of TAA1/TAR activity in ethylene-directed auxin biosynthesis and root growth in *Arabidopsis*. Plant Cell **23:** 3944–3960.

Hellens, R.P., Allan, A.C., Friel, E.N., Bolitho, K., Grafton, K., Templeton, M.D., Karunairetnam, S., Gleave, A.P., and Laing, W.A. (2005). Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. Plant Methods **1:** 13.

Hernandez, L.E., Garate, A., and Carpena-Ruiz, R. (1997). Effects of cadmium on the uptake, distribution and assimilation of nitrate in *Pisum sativum*. Plant Soil **189:** 97–106.

Hu, P., Zhou, W., Cheng, Z., Fan, M., Wang, L., and Xie, D. (2013). JAV1 controls jasmonate-regulated plant defense. Mol. Cell **50:** 504–515.

Ismail, A., Riemann, M., and Nick, P. (2012). The jasmonate pathway mediates salt tolerance in grapevines. J. Exp. Bot. **63:** 2127–2139.

Ju, C., et al. (2012). CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **109:** 19486–19491.

Ju, X.T., Xing, G.X., Chen, X.P., Zhang, S.L., Zhang, L.J., Liu, X.J., Cui, Z.L., Yin, B., Christie, P., Zhu, Z.L., and Zhang, F.S. (2009). Reducing environmental risk by improving N management in intensive Chinese agricultural systems. Proc. Natl. Acad. Sci. USA **106:** 3041–3046.

Kazan, K., and Manners, J.M. (2012). JAZ repressors and the orchestration of phytohormone crosstalk. Trends Plant Sci. **17:** 22–31.

Li, J.Y., et al. (2010). The *Arabidopsis* nitrate transporter NRT1.8 functions in nitrate removal from the xylem sap and mediates cadmium tolerance. Plant Cell **22:** 1633–1646.

Li, Z., Peng, J., Wen, X., and Guo, H. (2013). *Ethylene-insensitive3* is a senescence-associated gene that accelerates age-dependent leaf senescence by directly repressing *miR164* transcription in *Arabidopsis*. Plant Cell **25:** 3311–3328.

Lin, S.H., Kuo, H.F., Canivenc, G., Lin, C.S., Lepetit, M., Hsu, P.K., Tillard, P., Lin, H.L., Wang, Y.Y., Tsai, C.B., Gojon, A., and Tsay, Y.F. (2008). Mutation of the *Arabidopsis* NRT1.5 nitrate transporter causes defective root-to-shoot nitrate transport. Plant Cell **20:** 2514–2528.

Lorenzo, O., Piqueras, R., Sánchez-Serrano, J.J., and Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15:** 165–178.

Luo, D., Bernard, D.G., Balk, J., Hai, H., and Cui, X. (2012). The DUF59 family gene *AE7* acts in the cytosolic iron-sulfur cluster assembly pathway to maintain nuclear genome integrity in *Arabidopsis*. Plant Cell **24:** 4135–4148.

Maksymiec, W., Wianowska, D., Dawidowicz, A.L., Radkiewicz, S., Mardarowicz, M., and Krupa, Z. (2005). The level of jasmonic acid in *Arabidopsis thaliana* and *Phaseolus coccineus* plants under heavy metal stress. J. Plant Physiol. **162:** 1338–1346.

Mur, L.A.J., Mandon, J., Persijn, S., Cristescu, S.M., Moshkov, I.E., Novikova, G.V., Hall, M.A., Harren, F.J.M., Hebelstrup, K.H., and Gupta, K.J. (2012). Nitric oxide in plants: An assessment of the current state of knowledge. AoB Plants **5:** pls052.

Nakano, T., Suzuki, K., Fujimura, T., and Shinshi, H. (2006). Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol. **140:** 411–432.

Poltronieri, P., Taurino, M., De Domenico, S., Bonsegna, S., and Santino, A. (2013). Activation of the jasmonate biosynthesis pathway in roots in drought stress. In Climate Change and Plant Abiotic Stress Tolerance, N. Tuteja and S.S. Gill, eds (Weinheim, Germany: Wiley-VCH), pp. 325–342.

Pré, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C.M.J., and Memelink, J. (2008). The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. Plant Physiol. **147:** 1347–1357.

Searles, P.S., and Bloom, A.J. (2003). Nitrate photo-assimilation in tomato leaves under short-term exposure to elevated carbon dioxide and low oxygen. Plant Cell Environ. **26:** 1247–1255.

Smirnoff, N., and Stewart, G.R. (1985). Nitrate assimilation and translocation by higher plants: Comparative physiology and ecological consequences. Physiol. Plant. **64:** 133–140.

Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene signaling: A transcriptional cascade mediated by

ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev. **12:** 3703–3714.

Stitt, M. (1999). Nitrate regulation of metabolism and growth. Curr. Opin. Plant Biol. **2:** 178–186.

Szabados, L., and Savouré, A. (2010). Proline: A multifunctional amino acid. Trends Plant Sci. **15:** 89–97.

Van der Does, D., Leon-Reyes, A., Koornneef, A., Van Verk, M.C., Rodenburg, N., Pauwels, L., Goossens, A., Körbes, A.P., Memelink, J., Ritsema, T., Van Wees, S.C.M., and Pieterse, C.M.J. (2013). Salicylic acid suppresses jasmonic acid signaling downstream of SCF^{COI1}-JAZ by targeting GCC promoter motifs via transcription factor ORA59. Plant Cell **25:** 744–761.

Vidal, E.A., and Gutiérrez, R.A. (2008). A systems view of nitrogen nutrient and metabolite responses in *Arabidopsis*. Curr. Opin. Plant Biol. **11:** 521–529.

Vijayan, P., Shockey, J., Lévesque, C.A., Cook, R.J., and Browse, J. (1998). A role for jasmonate in pathogen defense of *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95:** 7209–7214.

Walch-Liu, P., Liu, L.H., Remans, T., Tester, M., and Forde, B.G. (2006). Evidence that L-glutamate can act as an exogenous signal to modulate root growth and branching in *Arabidopsis thaliana*. Plant Cell Physiol. **47:** 1045–1057.

Wang, R., Xing, X., and Crawford, N. (2007). Nitrite acts as a transcriptome signal at micromolar concentrations in Arabidopsis roots. Plant Physiol. **145:** 1735–1745.

Wang, Y.Y., and Tsay, Y.F. (2011). *Arabidopsis* nitrate transporter NRT1.9 is important in phloem nitrate transport. Plant Cell **23:** 1945–1957.

Wilkinson, J.Q., and Crawford, N.M. (1993). Identification and characterization of a chlorate-resistant mutant of *Arabidopsis thaliana* with mutations in both nitrate reductase structural genes *NIA1* and *NIA2*. Mol. Gen. Genet. **239:** 289–297.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D. (2002). The SCF^{COI1} ubiquitin-ligase complexes are required for jasmonate response in *Arabidopsis*. Plant Cell **14:** 1919–1935.

Yang, D.L., et al. (2012). Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. Proc. Natl. Acad. Sci. USA **109:** E1192–E1200.

Yoo, S.D., Cho, Y.H., and Sheen, J. (2007). *Arabidopsis* mesophyll protoplasts: A versatile cell system for transient gene expression analysis. Nat. Protoc. **2:** 1565–1572.

Zhang, L., Li, Z., Quan, R., Li, G., Wang, R., and Huang, R. (2011). An AP2 domain-containing gene, *ESE1*, targeted by the ethylene signaling component EIN3 is important for the salt response in *Arabidopsis*. Plant Physiol. **157:** 854–865.

Zhao, Q., and Guo, H.W. (2011). Paradigms and paradox in the ethylene signaling pathway and interaction network. Mol. Plant **4:** 626–634.

Zhu, Z., et al. (2011). Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **108:** 12539–12544.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol. **136:** 2621–2632.