SHORT COMMUNICATION

Ethylene signaling modulates *Arabidopsis thaliana* nitrate metabolism

Fiona Jamieson[1], Zhe Ji[1], Eric J. Belfield[1], Zhong Jie Ding[2], Shao Jian Zheng[2*], Nicholas P. Harberd[1*]

[1]Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, UK

[2]College of Life Sciences, Zhejiang University, Hangzhou 310058, P.R. China

[*]Correspondence: sjzheng@zju.edu.cn; nicholas.harberd@plants.ox.ac.uk

Received: xxx/Accepted: xxx

Abstract

Main conclusion Genetic analysis reveals a previously unknown role for ethylene signaling in regulating *Arabidopsis thaliana* nitrogen metabolism

Abstract Nitrogen (N) is essential for plant growth, and assimilation of soil nitrate (NO_3^-) and ammonium ions is an important route of N acquisition. Although N import and assimilation is subject to multiple regulatory inputs, the extent to which ethylene signaling contributes to this regulation remains poorly understood. Here, our analysis of *Arabidopsis thaliana* ethylene signaling mutants advances that understanding. We show that the loss of CTR1 function *ctr1-1* mutation confers resistance to the toxic effects of the NO_3^- analogue chlorate (ClO_3^-), and reduces the activity of the nitrate reductase (NR) enzyme of NO_3^- assimilation. Our further analysis indicates that lack of the downstream EIN2 component (conferred by novel *ein2* mutations) suppresses the effect of *ctr1-1*, restoring ClO_3^- sensitivity and NR activity to normal. Collectively, our observations indicate an important role for ethylene signaling in regulating *Arabidopsis thaliana* NO_3^- metabolism. We conclude that ethylene signaling enables environmentally responsive coordination of plant growth and N metabolism.

Keywords *Arabidopsis*; Chlorate Resistance; Ethylene; Nitrogen; Nitrate Reductase; Signaling

Abbreviations

CEND C-terminal end fragment of EIN2

CTR1 CONSTITUTIVE TRIPLE RESPONSE

ctr1-1 *constitutive triple response 1* (loss of function allele)
EIN2 ETHYLENE INENSITIVE 2
EIN3 ETHYLENE INSENSITIVE 3
ETR1 ETHYLENE RESISTANT 1 (Ethylene receptor)
N Nitrogen
NiR Nitrite reductase
NR Nitrate reductase
NRT1/NRT2 Nitrate transporters

Introduction

Nitrogen (N) is essential for the synthesis of the amino and nucleic acids that underpin plant growth (Miller and Cramer 2005; Smith et al. 2010). There are two main routes through which plants acquire N. Some can acquire atmospheric N via symbiotic relationships with N-fixing bacteria (Smith et al. 2010). In contrast, many rely exclusively upon regulated processes of absorption of soil NO_3^- ions, ammonium ions or amino acids. For example, in *Arabidopsis thaliana*, soil NO_3^- is imported through root nitrate transporters (NRT1 and NRT2; Wang et al. 2012), processed via a dedicated metabolic assimilatory pathway that includes nitrate (NR) and nitrite (NiR) reductases (Smith et al. 2010), and then fed into amino and nucleic acid biosynthesis.

Because plant growth is profoundly influenced by the environment, environmental signals modulate N assimilation. For example, phytochrome-mediated light signaling regulates NR and NiR activities (Lillo 2008). Here we further investigate the environmental regulation of N metabolism by asking if the evolutionarily conserved ethylene environmental stress-signaling pathway (Smith et al. 2010; Binder 2020) regulates NO_3^- assimilation in *Arabidopsis thaliana*. This pathway begins with the ETR1 ethylene receptor, which, in the absence of ethylene, maintains activation of the downstream CTR1 kinase, which in turn represses downstream ethylene signaling via phosphorylation of EIN2 (Binder 2020). This phosphorylation prevents the proteolytic cleavage of EIN2. In contrast, in the presence of ethylene, hormone binding to ETR1 inactivates CTR1, resulting in dephosphorylation and cleavage of EIN2, with subsequent migration of the EIN2 C-terminal (CEND) fragment into the cytosol and nucleus. The CEND fragment stabilizes the EIN3 transcription factor, thus causing ethylene-promoted downstream gene activation (Binder 2020).

Whilst previous studies indicated a role for ethylene in regulating N assimilation (Khan et al. 2008; Tian et al. 2009), they focused primarily on the molecular effects of short-term ethylene bursts. We therefore undertook a systematic study at the whole-plant level, and over longer time-scales. In particular, we used *Arabidopsis thaliana* mutants lacking CTR1 and EIN2. Lacking the CTR1 kinase, the *ctr1-1* mutant is dwarfed and displays constitutive ethylene responses (i.e., exhibits ethylene responses even in the absence of ethylene). First, using resistance to ClO_3^- as a proxy measure of altered NO_3^-

metabolism, we found that the *ctr1-1* mutation confers increased ClO_3^- resistance, suggesting that *ctr1-1* confers a reduction in NO_3^- uptake or assimilation. Accordingly, we next found that *ctr1-1* confers a reduction in the activity of the NR enzyme of NO_3^- assimilation. Second, we mutagenized *ctr1-1* and screened for novel mutants carrying secondary mutations conferring suppression of *ctr1* phenotype. DNA sequencing of such mutants identified multiple independent mutations in the *EIN2* gene. Further analysis showed that these novel *EIN2* mutants displayed normal ClO_3^- resistance and NR activity, despite retaining *ctr1-1*. We conclude that ethylene signaling plays a role in modulating *Arabidopsis thaliana* NO_3^- metabolism.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (Col-0 genetic background unless stated otherwise) seeds were surface sterilised with 70% (v/v) ethanol and left to dry on filter paper, then sown on agar plates or the surface of sieved compost (ICL Levington Advanced F2 compost, particles < 1 cm) and subsequently stratified in the dark at 4 °C for 2 – 4 days. After stratification plants were moved to controlled environment rooms (CERs) with a long-day photoperiod (16 h light/ 8 h dark) at 22 °C, irradiance 120 µmol of photons m^{-2} s^{-1}.

ClO_3^- resistance assays and measurements of green shoot area

4-5-week-old *Arabidopsis thaliana* plants were left for ~7 days without watering, following which 2.5 mM $KClO_3$ was applied. Any solution not absorbed into the soil after 2h was discarded. Control plants were treated with water. A second $KClO_3$ application occurred once the soil surface had become visibly dry (variable time between the two applications). Plants were imaged 7 days following the first application.

In quantitating susceptibility to $KClO_3$, photographic images were analyzed using imageJ (http://www.imagej.nih.gov/ij/) for determination of the proportion of each rosette displaying green versus yellow hues. Individual rosette images were cropped, and any leaves falling into the frame from other plants manually removed by selecting and clearing that area. Using the Image > Adjust > Color Threshold command, all areas of green-hued tissue were selected by setting the following parameters: Hue 41 - 90; Saturation 0 - 255 and Brightness 60 - 255. This area was measured (Analyse > Measure), and compared to the yellow-hued area of tissue, measured by setting the parameters: Hue 1 - 40; Saturation 0 - 255; and Brightness 60 - 255. If necessary the brightness threshold was adjusted to ensure that no background, and all tissue, was captured. The proportion of the total area measured occupied by green hues gave an indication of resistance to ClO_3^-, and was calculated as follows:

$$\%\ rosette\ tissue\ remaining\ green = \frac{Area\ of\ green\ tissue}{Area\ of\ green\ tissue + Area\ of\ yellow\ tissue}\ x\ 100$$

NR activity assays, determination of NR mRNA and protein abundances

0.5 g rosette leaf was harvested, ground to fine powder in liquid N_2 and homogenised in 5 ml/g extraction buffer (50 mM potassium phosphate (KH_2PO_4 pH 7.6), 1 mM EDTA, 5 mM 1,4-dithiothreitol (DTT), 0.5% Triton and 1x HALT^{TM} Protease Inhibitor Cocktail (ThermoFisher)) until thawed. 2 ml of protein extract was transferred to a separate Eppendorf tube and centrifuged at 17,115 *g* for 15 minutes, before supernatant was collected and centrifugation repeated. 1 ml of protein extract was collected and kept on ice for NR Activity Assays and protein quantification.

To determine NR activity, 200 µl protein extract was added to 800 µl reaction buffer (50 mM KH_2PO_4 (pH 7.6), 5 mM EDTA, 10 mM KNO_3 and 0.2 mM NADH). The reaction was allowed to proceed for 30 minutes in the dark at room temperature, then stopped by adding 1 ml of 0.36 M Zinc Acetate (ZnAc), followed by centrifugation at 14,674 *g* 12,000 r.p.m for 5 minutes. 500 µl of supernatant was transferred to a new tube, to which 1 ml of colour developer reagent (made up of equal volumes of 1% sulphanilamide in 3 M HCl and 0.02% N-(1-napthyl) ethylenediamine dihydrocholoride) was added. The reaction mixture was left in the dark for 30 minutes to allow colour development. The concentration of nitrite was determined by measuring the absorbance at 540 nm (OD_{540}) using an Evolution 260 BIO UV-Visible Spectrophotometer. Nitrate reductase activity was expressed as the absorbance of the reaction mixture at 540 nm (A540) relative to the sample protein concentration. Protein was quantified using the Bio-Rad DC colorimetric protein assay kit (Bio-Rad) according to the manufacturer's instructions.

RNA was extracted from 3rd and 4th youngest rosette leaves using Trizol (ThermoFisher) using the standard protocol. cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen). For semi-quantitative analysis of gene expression, RT-PCR reactions were set up using 1 µl of cDNA and 5 µM of intron-spanning primers for genes of interest in a 47 µl reaction mixture. The *CBP20* (At5g44200) gene was used as an internal normalisation and cDNA loading control. The amount of PCR product was quantified by measuring band intensities on 1% agarose gels stained with ethidium bromide and analysed using VisionWorksLS software.

For immunoblot analysis, total protein was extracted from WT and *ctr1* plants, either from rosette leaves of 5-week-old plants grown on soil or from 2-week-old seedling shoots grown on half-strength Murashige & Skoog salt (Merck Life Science, Dorset, UK) media (pH 5.8) containing 0.5% sucrose and 1% agarose, using extraction buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% NP–40 detergent, 10% glycerol, 1 mM DTT and protease inhibitor cocktail (Roche LifeScience, Penzberg, Germany). Protein samples were heated at 70 °C for 10 minutes before being subjected to SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) and transferred to a nitrocellulose membrane. The membrane was stained in Ponceau S solution (Sigma, St Louis, MO, USA; P7170) following manufacturer's instructions

before blocking. Actin and NR proteins were detected using anti-Actin (Agrisera, Vännäs, Sweden; AS13 2640) and anti-NR (Agrisera, Vännäs, Sweden; AS08 310) antibodies, respectively. The membranes were visualised on the iBright FL1500 Imaging System (Thermo Fisher Scientific, Waltham, MA, USA; A44241) and the band intensities quantified with on-board image analysis.

Mutagenesis of *ctr1-1*

ctr1-1 seeds were initially bulked up from laboratory stocks in a CER, following which ~12,500 collected seeds were washed with 0.1% Tween-20 for 15 minutes. This solution was removed and the seeds rinsed three times with dH_2O prior to adding 50 ml of 0.4% ethyl methane sulfonate (EMS) solution, followed by mixing on a benchtop shaker for 8 hours in darkness. Next, the seeds were washed 10 times with dH_2O, then soaked in 50 ml dH_2O and agitated on a benchtop shaker for 1 hour to allow diffusion of residual EMS out of the seeds. The seeds were subsequently left to dry overnight on filter paper. The seeds were then mixed with ~130 g of sand, and 10 g of sand/ seed mixture sown on trays of Levington's F2 compost mix. Seeds were grown under standard conditions and allowed to self-fertilize. The resultant M_2 seeds were bulk harvested.

DNA sequence analysis

DNA fragments spanning *CTR1* were amplified using the primers (CTR1-F: 5'-AAAGTGGAGCAAGGGAGCAA and CTR1-R: 5'-ACCGCAGCTACAACCTATGG), and then sequenced by Sanger-sequencing to confirm the genetic background of each *ctr1-1* suppressor. Full-length *EIN2* gene fragments were amplified using the primers (EIN2-F: 5'-ATGGAAGCTGAAATTGTGAATGTGA and EIN2-R: 5'-TCAACCCAATGATCCGTACGC), and were also analysed with Sanger-sequencing.

Results and discussion

***ctr1-1* confers ClO_3^- resistance and reduced NR activity**

The *Arabidopsis thaliana ctr1-1* mutant maintains constitutive ethylene signaling, and displays a characteristic seedling ethylene 'triple response' phenotype (short root; short and thick hypocotyl; exaggerated apical hook) in the absence of ethylene (Kieber et al. 1993). Mature *ctr1-1* plants are dwarfed, have small rosette leaves, short stems, and delayed flowering (Achard et al. 2007). We first determined the possible effect of *ctr1-1* on NO_3^- metabolism, via the frequently used ClO_3^- resistance assay (treatment with $KClO_3$; see **Materials and methods**). Because the ClO_3^- ion is a structural analogue of NO_3^-, ClO_3^- is readily taken up by root NO_3^- uptake systems and then transported to the shoot. Here, NR converts ClO_3^- to chlorite (Cove 1976; Murphy and Imbrie 1981). Because chlorite is toxic, plants grown on $KClO_3$-treated soil display leaf chlorosis. This assay was previously used to screen for *Arabidopsis thaliana* mutants displaying increased ClO_3^- resistance, including *chl1*, subsequently identified as containing a loss of function *nrt1.1* mutation in *NRT1.1*, the gene encoding the NRT1.1 nitrate transporter

(Ho et al. 2009).

Whilst ClO_3^- treatment of wild-type (WT) plants caused leaf chlorosis, treatment of control *nrt1.1* plants (where lack of the NRT1.1 impairs ClO_3^- uptake) did not (Ho et al. 2009; Fig. 1a). Interestingly, *ctr1-1* also confers ClO_3^- resistance (Fig. 1a): the vegetative rosette tissue of *ctr1-1* plants remained green following ClO_3^- treatment (Fig. 1a). We next quantitatively compared ClO_3^- resistances, using ImageJ (see **Materials and methods**). These comparisons revealed no significant differences between control-treated WT, *ctr1-1* and *nrt1.1* plants (Fig. 1b). In contrast, whilst the green tissue percentage of ClO_3^--treated *nrt1.1* plants was not detectably different from that of control, only ~25% of WT rosette tissue remained green following ClO_3^- treatment (Fig. 1b). ~80% of *ctr1-1* rosette tissue remained green following ClO_3^- treatment, indicating that *ctr1-1* confers relatively strong ClO_3^- resistance (albeit not as strong as conferred by *nrt1.1*; Fig. 1b), and hence that ethylene signaling might impact ClO_3 resistance.

We next studied a broader range of ethylene-related mutants, first using ethylene over-producing and receptor mutants. The ethylene over-producing *eto1*, *eto2* and *eto3* mutants all display enhanced ethylene production (Chae et al. 2003; Wang et al. 2004), whilst *etr1-1* contains a mutation in the gene encoding the ETR1 ethylene receptor, thus conferring ethylene insensitivity (Binder 2020). ImageJ analysis showed that whilst the percentage remaining green rosette tissue of ClO_3^--treated *eto1* and *etr1* was not significantly different to that of WT (~17%), thus indicating a relative lack of resistance, both *eto2* and *eto3* ClO_3^--treated rosettes retained ~40% green tissue (thus indicating partial resistance versus the stronger *ctr1* (~62%) and *nrt1.1* (~98 %) resistances; Fig. 1c). Next, we assayed *ein2-1* and *ein3-1* mutants, which respectively lack the downstream EIN2 and EIN3 components of ethylene signaling, finding no significant difference between WT, *ein2-1* and *ein3-1*, in conditions where *ctr1-1* and *nrt1.1* exhibited obvious ClO_3^- resistance (Fig. 1d). These observations support the following conclusions. First, the constitutively active ethylene signaling of *ctr1-1* promotes ClO_3^- resistance. Accordingly, increased ethylene signaling in *eto2* and *eto3* (due to ethylene overproduction) also promotes ClO_3^- resistance. However, the ClO_3^- resistance of *eto2* and *eto3* is less than that of *ctr1-1* (and is undetectable in *eto1*), perhaps because their ethylene overproduction is largely a seedling (rather than adult plant) phenomenon (Chae et al. 2003). Second, the ethylene insensitivity conferred by *etr1-1*, *ein2-1* and *ein3-1* has no obvious effect on ClO_3^- resistance, suggesting that there is a threshold level of ethylene signaling below which further reductions do not detectably affect ClO_3^- resistance.

We next compared leaf extract NR activities, finding that *ctr1* plants retain ~50% of WT activity (Fig. 1e). To determine if this difference in NR activity was due to changes at transcriptional or post-transcriptional levels, we measured the relative abundances of mRNAs transcribed from the *NIA1* and *NIA2* genes that encode NR in *Arabidopsis thaliana*. As expected, *NIA2* mRNA was more abundant than *NIA1* (Fig. 1f), as it encodes ~90% of *Arabidopsis thaliana* NR (Yu et al. 1998). Whilst there was no

detectable difference between *NIA1* mRNA abundances in WT and *ctr1-1*, NIA2 mRNA abundance was partially reduced (Fig. 1f). Furthermore, use of an anti-NR antibody revealed that detectable NR protein abundance was also reduced in *ctr1-1* versus WT (Fig. 1g). We conclude that reductions in NR-encoding transcript and NR protein abundances may contribute to the reduced NR activity and increased ClO_3^- resistance of *ctr1-1*. However, it is possible that CTR1 also regulates NR activity via post-translational mechanisms.

Lack of EIN2 function correlates with suppression of the ClO_3^- resistance and NR activity reduction conferred by *ctr1-1*

In order to further understand the role of CTR1 and the ethylene signaling pathway in regulating N metabolism, we next performed genetic suppression analysis of *ctr1-1*. Tall plants (in which the dwarfism conferred by *ctr1-1* was suppressed) were selected from an EMS-mutagenized *ctr1-1* M_2 population (see **Materials and methods**; Fig. 2a). Single tall plants were isolated, allowed to self-pollinate, shown to have 100% tall progeny (indicating homozygosity for new suppressing mutations; data not shown), and shown by DNA sequencing to retain the progenitor *ctr1-1* allele (indicating that they were not WT contaminants; data not shown). Four independent homozygous tall lines (9-11, 12-9, 16-3 and 22-10) were next selected for further analysis (Fig. 2b). For example, we found that line 22-10, despite retaining *ctr1-1*, lacked the ClO_3^- resistance conferred by *ctr1-1* (Fig. 2c). Furthermore, all of 9-11, 12-9, 16-3 and 22-10, again despite retaining *ctr1-1*, no longer displayed the reduced NR activity (Fig. 2d) conferred by *ctr1-1*. Therefore, each of lines 9-11, 12-9, 16-3 and 22-10 was carrying an unknown novel ('second-site') mutation suppressing the effects of *ctr1-1* on NO_3^- metabolism. Because mutations in *EIN2* have previously been shown to suppress *ctr1-1* phenotypes (Alonso et al. 1999), we determined the DNA sequences of *EIN2* in each of 9-11, 12-9, 16-3 and 22-10, finding independent *EIN2* nucleotide substitution mutations in each line (Fig. 2e).

A plausible potential explanation for the ClO_3^- resistance of *ctr1-1* was that this resistance was an inherent property of the dwarfism conferred, rather than a specific consequence of constitutive ethylene signaling. Accordingly, mutations at *EIN2* would suppress the ClO_3^- resistance conferred by *ctr1-1* because they suppress dwarfism, rather than because of an effect on ethylene-regulated N metabolism. To test this potential explanation we used a range of dwarf mutants (*min-1* is taller, whilst *cp3-1* and *dw1-1* are both shorter than *ctr1-1*; Fig. S1a; Fig. 2b). Nevertheless, all three of *min-1*, *cp3-1* and *dw1-1* exhibit a degree of ClO_3^- resistance not detectably different from that of WT (Supplemental Fig. 1b). We conclude that the ClO_3^- resistance conferred by *ctr1-1* is specifically due to constitutive ethylene signaling, rather than to a more generalized effect of dwarfism on ClO_3^- uptake or metabolism.

Whilst future analyses might benefit from mutant screens more directly assaying

suppression of N-related (rather than plant height) phenotypes, our present initial observations indicate that multiple independent loss-of-function mutations at *EIN2* confer increased ClO_3^--sensitivity on *ctr1-1*. These observations in turn suggest that lack of EIN2 function suppresses the effects of *ctr1-1* on NO_3^- metabolism, and that ethylene regulates plant N metabolism via the CTR1 and EIN2 ethylene signaling components. Being sessile organisms, plants need to respond to environmental variability by coordinating the regulation of growth and metabolism. Whilst ethylene signaling has long been known to coordinate growth in response to environmental stress, the involvement of this pathway in metabolic regulation has been less clear. Our results reveal that the ethylene signaling pathway enables regulatory coordination of plant growth, environmental response and N metabolism.

Author contribution statement FJ, ZJD and NPH conceived the project. FJ, ZJD and NPH designed the experiments. FJ, ZJ and ZJD performed the experiments. FJ, ZJ, EJB, SJZ, ZJD and NPH contributed to data analysis. FJ and NPH wrote the manuscript with input from all authors.

Acknowledgements. FJ was supported by Biological and Biotechnological Sciences Research Council (BBSRC) grant BB/M011224/1. This work was additionally supported by UK Biotechnology and Biotechnological Research Council grants (BB/N013611/1 and BB/S013741/1) to EJB and NPH and Natural Science Foundation of Zhejiang Province grant LY21C020001 to ZJD.

Conflict of interest We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

Figure Legends

Fig. 1 The *ctr1-1* mutation confers chlorate resistance and reduced NR activity. **a** Soil-grown 6-week-old WT, *nrt1.1* and *ctr1-1* plants 8 days after treatment with water (control) or 2.5mM $KClO_3$ (chlorate-treated), bolt-stems removed. Scale bars = 50mm. **b-d** Proportion (%) of rosette tissue remaining green following treatment (as in **a**) with water (control, dark) or $KClO_3$ (chlorate-treated, light). Plant genotypes as indicated. Values are means ± SD. Different letters denote statistically significant differences (one-way ANOVA/Tukey HSD post hoc test, $P < 0.05$, $n = 4$). **e** NR activity in shoot tissue of 5-week-old WT and *ctr1-1* plants determined relative to protein content (* denotes statistically significant difference, student's t-test, $P<0.05$, $n = 4$). **f** Semi-quantitative RT-PCR determined abundances of *NIA1* and *NIA2* mRNAs (relative to *CAP BINDING PROTEIN 20* (*CBP20*) control mRNA in WT (dark) and *ctr1-1* (light) shoots (* denotes statistically significant difference, student's t-test, $P<0.05$, $n = 3$). **g** NR protein abundance in WT versus *ctr1-1*, with

immunodetectable NR quantified against Actin control (arbitrarily set at 1.00 in WT), showing a reduction (0.82) in *ctr1-1*. A single representative of an experiment performed three times is shown.

Fig. 2 Lack of EIN2 suppresses *ctr1-1*-conferred chlorate resistance and reduced NR activity. **a** Suppressor screen: tall plants putatively carrying novel mutations suppressing *ctr1-1* phenotype are marked with adjacent plastic pipette tips. **b** Heights of 5-week-old control (WT, *ctr1-1*) and suppressor line (9-11, 12-9, 16-3 and 22-10) plants. * denotes statistically significant difference compared to WT (student's t-test, $P<0.05$, $n = 10$). **c** 5-week-old WT, *nrt1.1*, *ctr1-1* and suppressor line 22-10 plants treated either with water (control, left panel) or with 2.5mM $KClO_3$ (right panel). Plants were imaged 7 days after treatment. Scale bar = 50mm. **d** NR activity (relative to protein content) in shoot tissue of 4-week-old control (WT, *ctr1-1*) and suppressor line (9-11, 12-9, 16-3 and 22-10) plants (* denotes statistically significant difference compared to WT, student's t-test, $P<0.05$, $n = 4$). Error bars denote SD. **e** Diagram of the *Arabidopsis thaliana EIN2* gene structure: shaded bars denote exons, lines denote introns, unshaded bars denote untranslated regions. The grey region indicates the position of the EIN2 C-END domain (Binder 2020), the red bar indicates the position of the predicted nuclear translocation sequence, and the black region the EIN2 N-terminus transmembrane domain (Binder 2020). Positions and nature of nucleotide substitution mutations in each of suppressor lines 9-11, 12-9, 16-3 and 22-10 are as indicated. Scale bar = 100 base pairs.

References

Achard P, Baghour M, Chapple A, Hedden P, Van der Straeten D, Genschik P, Moritz T, Harberd NP (2007) The plant stress hormone ethylene controls floral transition via DELLA-dependent regulation of floral meristem-identity genes. Proc Natl Acad Sci USA 104: 6484-6489. https://doi.org/10.1073/pnas.0610717104

Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis.* Science 284: 2148-2152. https://doi.org/10.1126/science.284.5423.2148

Binder BM (2020) Ethylene signaling in plants. J Biol Chem 295: 7710-7725.

Chae HS, Faure F, Kieber JJ (2003) The *eto1*, *eto2*, and *eto3* mutations and cytokinin treatment increase ethylene biosynthesis in *Arabidopsis* by increasing the stability of ACS protein. Plant Cell 15: 545-559. https://doi.org/10.1105/tpc.006882

Cove DJ (1976) Chlorate toxicity in *Aspergillus nidulans*: The selection and characterization of chlorate resistant mutants. Heredity 36: 191-203. https://doi.org/10.1038/hdy.1976.24

Ho C-H, Lin S-H, Hu H-C, Tsay Y-F (2009) CHL1 functions as a nitrate sensor in plants. Cell 138: 1184-1194. https://doi.org/10.1016/j.cell.2009.07.004

Lillo C (2008) Signalling cascades integrating light-enhanced nitrate metabolism. Biochem J 415:11-19. https://doi.org/10.1042/BJ20081115

Khan NA, Mir MR, Nazar R, Singh S (2008) The application of ethephon (an ethylene releaser) increases growth, photosynthesis and nitrogen accumulation in mustard

(*Brassica juncea* L.) under high nitrogen levels. Plant Biol 10: 534-538. https://doi.org/10.1111/j.1438-8677.2008.00054.x
Kieber JJ, Rothenberg M, Roman G, Feldmann K, Ecker JR (1993) *CTR1*, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. Cell 72: 427-441. https://doi.org/10.1016/0092-8674(93)90119-b
Koornneef M, van Eden J, Hanhart CJ, Stam P, Braaksma FJ, Fenstra WJ (1983) Linkage map of *Arabidopsis thaliana*. J Hered 74: 265-272 https://doi.org/10.1093/oxfordjournals.jhered.a109781
Miller AJ, Cramer MD (2005) Root nitrogen acquisition and assimilation. Plant Soil 274: 1-36. https://doi.org/10.1007/s11104-004-0965-1
Murphy TM, Imbrie CW (1981) Induction and characterization of chlorate-resistant strains of *Rosa damascena* cultured cells. Plant Phys 67: 910-916. https://doi.org/10.1104/pp.67.5.910
Quinn MH, Oliverio K, Yanovsky MJ, Casal JJ (2002) CP3 is involved in negative regulation of phytochrome A signalling in *Arabidopsis*. Planta 215: 557-564. https://doi.org/10.1007/s00425-002-0784-7
Smith AM, Coupland G, Dolan L, Harberd N, Jones J, Martin C, Sablowski R, Amey A (2010) Plant Biology, Garland Science, New York.
Tian QY, Sun P, Zhang WH (2009) Ethylene is involved in nitrate-dependent root growth and branching in *Arabidopsis thaliana*. New Phytol 184: 918-931. https://doi.org/10.1111/j.1469-8137.2009.03004.x
Wang KLC, Yoshida H, Lurin C, Ecker JR (2004) Regulation of ethylene gas biosynthesis by the *Arabidopsis* ETO1 protein. Nature 428: 945-950. https://doi.org/10.1038/nature02516
Wang Y-Y, Hsu P-K, Tsay Y-F (2012) Uptake, allocation and signaling of nitrate. Trends Plant Phys 17: 458-467. https://doi.org/10.1016/j.tplants.2012.04.006
Youn JH, Kim TW, Joo SH, Son SH, Roh J, Kim S, Kim TW, Kim SK (2018) Function and molecular regulation of DWARF1 as a C-24 reductase in brassinosteroid biosynthesis in *Arabidopsis*. Journal of Experimental Botany 69: 1873-1886. https://doi.org/10.1093/jxb/ery038
Yu X, Sukumaran S, Marton L (1998) Differential expression of the *Arabidopsis Nia1* and *Nia2* genes. Plant Phys 116: 1091-1096. https://doi.org/10.1104/pp.116.3.1091