RESEARCH PAPER

The Arabidopsis AMOT1/EIN3 gene plays an important role in the amelioration of ammonium toxicity

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Abstract

Ammonium (NH₄⁺) toxicity inhibits shoot growth in Arabidopsis, but the underlying mechanisms remain poorly characterized. Here, we show that a novel Arabidopsis mutant, ammonium tolerance 1 (amot1), exhibits enhanced shoot growth tolerance to NH₄⁺. Molecular cloning revealed that amot1 is a new allele of EIN3, a key regulator of ethylene responses. The amot1 mutant and the allelic ein3-1 mutants show greater NH₄⁺ tolerance than the wild type. Moreover, transgenic plants overexpressing EIN3 (EIN3ox) are more sensitive to NH₄⁺ toxicity. The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) increases shoot sensitivity to NH₄⁺, whereas the ethylene perception inhibitor Ag⁺ decreases sensitivity. NH₄⁺ induces ACC and ethylene accumulation. Furthermore, ethylene-insensitive mutants such as etr1-3 and ein3eil1 display enhanced NH₄⁺ tolerance. In contrast, the ethylene overproduction mutant eto1-1 exhibits decreased ammonium tolerance. AMOT1/EIN3 positively regulates shoot ROS accumulation, leading to oxidative stress under NH₄⁺ stress, a trait that may be related to increased expression of peroxidase-encoding genes. These findings demonstrate the role of AMOT1/EIN3 in NH₄⁺ tolerance and confirm the strong link between NH₄⁺ toxicity symptoms and the accumulation of hydrogen peroxide.

Keywords: Ammonium stress, amot1 mutant, Arabidopsis, AMOT1/EIN3, H₂O₂, peroxidases.

Introduction

Ammonium (NH₄⁺), an important source of nitrogen for many species (Kronzucker et al., 1997; Balkos et al., 2010), is frequently present in soils and in the atmosphere in significant quantities (Britto and Kronzucker, 2002; Dupre et al., 2009). However, NH₄⁺ is toxic at moderate levels, frequently achieved in soils, to most plants, in particular those in temperate agriculture, with stunted root and leaf growth as major symptoms of toxicity (Britto and Kronzucker, 2002; Coskun et al., 2013). Several important physiological processes have been linked to excessive NH₄⁺ exposure, such as ionic imbalance, relationships with carbon biochemistry, energy consumption, and modifications of hormonal balance (Britto and Kronzucker, 2002; Coskun et al., 2013). Ethylene production has been shown to increase linearly with tissue NH₄⁺ accumulation (Barker,
However, the detailed mechanisms of ethylene biosynthesis in Arabidopsis (Davletova et al., 2005; Xing et al., 2008), including APX1 (ascorbate peroxidase 1) and CAT1 (catalase 1) in Arabidopsis (Davletova et al., 2005; Xing et al., 2008), also affects ROS levels. Peroxidases (PODs) have been proposed as alternative producers of ROS (Apel and Hirt, 2004; Bindschedler et al., 2006). PODs catalyze the oxidoreduction of various substrates using H2O2. PODs, rather than NADPH oxides and respiratory burst oxidase homologs (RBOHs) have been identified as alternative producers of ROS (Apel and Hirt, 2004; Bindschedler et al., 2006). However, data regarding the pathways involved in NH4+ regulation of H2O2 production are still rare. ROS can be generated in the apoplast via the activity of NADPH oxidases under stress (Mittler et al., 2004). A group of NADPH oxidases and respiratory burst oxidase homologs (RBOHs) have been identified in Arabidopsis (Sagi and Fluhr, 2006). ROS are tightly regulated via a production/scavenging equilibrium. The expression of genes involved in the ROS regulatory network, including APX1 (ascorbate peroxidase 1) and CAT1 (catalase 1) in Arabidopsis (Davletova et al., 2005; Xing et al., 2008), also affects ROS levels. Peroxidases (PODs) have been proposed as alternative producers of ROS (Apel and Hirt, 2004; Bindschedler et al., 2006). PODs catalyze the oxidoreduction of various substrates using H2O2. PODs, rather than NADPH oxidases, have been proposed as the major ROS producer in French bean (Phaseolus vulgaris) treated with a cell wall elicitor of Colletotrichum lindemuthianum, the fungus that causes anthracnose (Bolwell et al., 1998). Kim et al. (2010) found that a POD contributes to ROS production during the Arabidopsis root response to potassium deficiency, showing the POD to be a component of the low potassium signal transduction pathway. Recently, Balzergue et al. (2017) showed that –Pi induces root tip ROS accumulation, indicating that PODs play a role. Further, the POD inhibitor salicylhydroxamic acid (SHAM) restored root growth and reduced ROS accumulation under –Pi conditions (Balzergue et al., 2017). Although ethylene and ROS have been reported to be involved in NH4+ sensitivity, there has been no study to evaluate the role of ethylene in high-NH4+-induced ROS production in leaves, and further research is necessary to clarify the circumstances under which NH4+ causes oxidative stress in plants.

One approach to elucidating mechanisms of NH4+ toxicity in plants is to use mutant lines. Qin et al. (2008) isolated the first NH4+-sensitive root elongation mutant, vct1 (vitamin C defective 1), disrupted in GDP-mannose pyrophosphorylase (GMPase). Recently, several genetic regulators controlling root sensitivity to NH4+ have been identified in Arabidopsis, such as aux1 (auxin resistant 1) (Cao et al., 1993; Li et al., 2011), thl1 (tiny root hair 1) (Zou et al., 2012), dpms1 (dolichol phosphate mannose synthase1) (Jadid et al., 2011), and gsa1 (gravitropism sensitive to ammonium1) (Zou et al., 2013). Elucidation of the function of these genetic regulators in determining the root sensitivity to NH4+ offered insight into the molecular basis of historically described physiological responses to NH4+ toxicity. In contrast, the underlying mechanisms of impaired leaf growth under NH4+ toxicity are still largely unknown. Reduced shoot biomass and leaf chlorosis are important symptoms (Britto and Kronzucker, 2002). Based on their chlorotic phenotypes, ammonium-overly-sensitive 1 (amos1) (B. Li et al., 2012) and amos2 (G. Li et al., 2012) mutants were recently identified: the AMOS1 locus is identical to EGY1 (ethylene-dependent gravitropism-deficient and yellow-green-like protein1), which encodes a membrane-bound, ATP-independent metalloprotease localized to plastids, required for chloroplast biogenesis (Chen et al., 2005). However, the genetic locus responsible for the amos2 mutation has not been identified. These studies, in combination, provide a significantly improved understanding of the process of NH4+ toxicity in plants.

Here, we report a novel Arabidopsis thaliana mutant, amot1 (ammonium tolerance 1), which displays enhanced shoot growth in response to NH4+ stress. Gene cloning shows amot1 to be allelic to EIN3. Our results demonstrate that the disruption of AMOT1/EIN3 reduces high-NH4+-induced ROS accumulation in leaves, leading to reduced oxidative stress in the shoot. Moreover, AMOT1/EIN3 up-regulates shoot expression of the genes coding for PODs, previously shown to correlate positively with NH4+-induced changes in ROS content and cell growth inhibition.

Materials and methods

Plant materials and growth conditions

Plant materials used in this work included wild-type (WT) A. thaliana L. (Col-0 ecotype) and genetic mutants derived from the Col-0 background. The mutants ein3-1 (Chao et al., 1997), ein1-1 (Alonso et al., 2003), ein3-1eil1-1 (Alonso et al., 2003), EIN3ox (35S:EIN3) (Chao et al., 1997), and 5×EBS:GUS/Col-0 transgenic plants (He et al., 2011) were described previously. The eto1-1 (Alonso and Stepanova, 2004), etr1-3 and Ecker, 1990), and nth1 mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds were surface-sterilized and cold-treated at 4 °C for 48 h prior to being sown onto standard growth medium. The standard growth medium was described previously (G. Li et al., 2013) and was composed of 2 mM KH2PO4, 5 mM NaNO3, 2 mM MgSO4, 1 mM CaCl2, 0.1 mM Fe–EDTA, 50 μM Fe–EDTA, 50 μM
H$_2$BO$_3$, 12 μM MnSO$_4$, 1 μM ZnCl$_2$, 1 μM CuSO$_4$, 0.2 μM Na$_2$MoO$_4$, 0.5 g l$^{-1}$ MES, 1% sucrose, and 0.8% agarose (pH 5.7, adjusted with 1 M NaOH). The day of sowing was considered day 0. Seedlings were grown, oriented vertically on the surface of the medium in a growth chamber, under a 16 h light/8 h dark photoperiod, an irradiance of 100 μmol m$^{-2}$ s$^{-1}$, and a constant temperature of 23±1 °C. Other chemical treatments were provided as additions to the growth medium, as indicated.

Screening conditions

Transfer DNA (T-DNA) lines were constructed in the laboratories of D. Weigel and C. Somerville using the pSK115 vector. Approximately 7500 independent lines (stock no. N21995) were produced by the ABRc. After surface sterilization, seeds were sown and grown on vertically-oriented growth medium plates. After 5 d, seedlings were transferred to growth medium plates supplemented with 20 mM (NH$_4$)$_2$SO$_4$. Potential NH$_4^+$ tolerance mutants were selected after 6 d and rescued, transferred to soil, and allowed to self-fertilize. The homozygous M$_4$ mutant was backcrossed to the WT Col-0, and the resulting F$_1$ generation was crossed with WT Col-0 twice to remove unlinked mutations caused by the mutagenesis.

Thermal asymmetric interlaced PCR

DNA for PCR amplification was extracted according to Weigel and Glazebrook (2002). Plant T-DNA-flanking sequences were amplified by PCR according to the protocols of Rodrigues et al. (2009). The following primers were used: SKI1, 5′-AATTGGTAATCTTCTTTCTCCTCCATATT-GA-3′; SKI2, 5′-AATTGGACATCAACATTGCTGAT-CAT-3′; SKI3, 5′-TGATCCATGATTGATCCCGACCAT-3′; AD1, 5′-TG(TG)TG(TG)TG(TG)TG(TG)-3′, AD2, 5′-(ACGT)T CGA(GC)(AT)G(AT)G(TG)(AT)(AT)-3′; AD3, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD4, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD5, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD6, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′. The following primers were used: SKI1, 5′-AATTGGTAATCTTCTTTCTCCTCCATATT-GA-3′; SKI2, 5′-AATTGGACATCAACATTGCTGAT-CAT-3′; SKI3, 5′-TGATCCATGATTGATCCCGACCAT-3′; AD1, 5′-TG(TG)TG(TG)TG(TG)TG(TG)-3′, AD2, 5′-(ACGT)T CGA(GC)(AT)G(AT)G(TG)(AT)(AT)-3′; AD3, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD4, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD5, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD6, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′. The following primers were used: SKI1, 5′-AATTGGTAATCTTCTTTCTCCTCCATATT-GA-3′; SKI2, 5′-AATTGGACATCAACATTGCTGAT-CAT-3′; SKI3, 5′-TGATCCATGATTGATCCCGACCAT-3′; AD1, 5′-TG(TG)TG(TG)TG(TG)TG(TG)-3′, AD2, 5′-(ACGT)T CGA(GC)(AT)G(AT)G(TG)(AT)(AT)-3′; AD3, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD4, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD5, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD6, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′. The following primers were used: SKI1, 5′-AATTGGTAATCTTCTTTCTCCTCCATATT-GA-3′; SKI2, 5′-AATTGGACATCAACATTGCTGAT-CAT-3′; SKI3, 5′-TGATCCATGATTGATCCCGACCAT-3′; AD1, 5′-TG(TG)TG(TG)TG(TG)TG(TG)-3′, AD2, 5′-(ACGT)T CGA(GC)(AT)G(AT)G(TG)(AT)(AT)-3′; AD3, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD4, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD5, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′; AD6, 5′-(ACGT)TCGA(GC)(AT)(AT)(AT)(AT)-3′.

Growth assays

For high-NH$_4^+$ stress experiments, 5-day-old seedlings were transferred onto growth medium containing various concentrations of (NH$_4$)$_2$SO$_4$. Following 6 d of treatment, photographs were taken, and relative rosette size and shoot biomass were measured. To study the effect of precursors or inhibitors, the medium was supplemented with NH$_4^+$ plus the indicated concentrations of ACC (Sigma), AgNO$_3$, (Shanghai yuanye biotechnology Co. Ltd, Shanghai, China), H$_2$O$_2$ (Shanghai yuanye biotechnology Co. Ltd), or SHAM (Shanghai yuanye biotechnology Co. Ltd). The fresh weight of each individual shoot was measured immediately after harvest using a high-precision balance (0.000001 g). The fresh weight of each individual shoot was measured immediately after harvest using a high-precision balance (0.000001 g).

Histochemical staining and image analysis

Histochemical staining of H$_2$O$_2$ was performed as previously described (Dong et al., 2009) with minor modifications. Shoots were vacuum-infiltrated with 0.1 mg ml$^{-1}$ 3,3′-diaminobenzidine (DAB) in 50 mM Tris-acetate buffer, at pH 5.0. Samples were incubated for 24 h at room temperature in the dark prior to transfer to 80% ethanol. Histochemical analysis of β-glucuronidase (GUS) reporter enzyme activity was performed as described by Weigel and Glazebrook (2002). The specific enzyme activity was calculated as the amount of enzyme units catalyzing the transformation of 1 μM substrate per minute by the amount of fresh weight in grams.

Ethylene measurements

After seedling exposure to 40 mM NH$_4^+$ for varying durations, as indicated, shoots from the control and treatments were weighed and put into 5 ml gas-tight vials containing 1 ml of agar medium (0.7% agar). Headspace samples (1 ml) were withdrawn and analyzed using a GC-6850 gas chromatograph (Agilent Technologies Japan, Ltd), which was equipped with an FID detector.

Yeast one-hybrid (Y1H) analysis

Promoter fragments from At1g49570 (2217 bp) and At5g19890 (1692 bp) were cloned into the pAbAi vector to produce the bait constructs pAbAi-At1g49570 and pAbAi-At5g19890, respectively. The coding sequence (CDS) of AMOT1/EIN3 was fused to the pGADT7 vector to generate a prey construct, AD-EIN3. The bait construct and the empty vector (AD) served as the negative control; p53-AbAi/pGAD-p53 were used as a positive control and transformed separately into yeast cells. Transformed yeast cells were diluted with a 10× dilution series and dotted onto SD plates lacking Ura and Leu (with or without antibiotic).
Statistical and graphical analyses

For all experiments, data were statistically analyzed using the SPSS 13.0 program (SPSS Chicago, IL, USA). Details are shown in the figure legends. Graphs were produced using Origin 8.0. All graphs and images were arranged using Adobe Photoshop 7.0.

Results

Enhanced tolerance of the amot1 mutant to ammonium toxicity

Under our growth conditions, \( \text{NH}_4^+ \), at concentrations of 20–30 mM, caused slight reductions in shoot size and biomass (Supplementary Fig. S1). A concentration of 40 mM significantly inhibited shoot size and biomass (Supplementary Fig. S1). To explore the mechanisms of \( \text{NH}_4^+ \)-induced shoot growth inhibition, we performed a forward genetic screen for seedlings that show a shoot phenotype that was more resistant than WT plants when grown on medium containing 40 mM \( \text{NH}_4^+ \). Seedlings that appeared similar to the WT without \( \text{NH}_4^+ \) but displayed significantly higher resistance of shoot growth to \( \text{NH}_4^+ \) were \( \text{amot} \) (ammonium tolerance) mutants. We present the characterization of the \( \text{amot1} \) mutant.

In agar plates without \( \text{NH}_4^+ \), the \( \text{amot1} \) shoot growth phenotype was indistinguishable from that of the WT (Fig. 1A). However, when grown in the presence of high \( \text{NH}_4^+ \), \( \text{amot1} \) shoot growth displayed greater resistance to \( \text{NH}_4^+ \) than the WT (Fig. 1A).

\( \text{NH}_4^+ \)-treated WT and \( \text{amot1} \) plants showed a dose-dependent inhibitory effect of \( \text{NH}_4^+ \) on the growth of aerial parts in response to a range of \( \text{NH}_4^+ \) concentrations, but WT shoot growth was inhibited more than in \( \text{amot1} \) at the concentrations used (Fig. 1B, C). Furthermore, we analyzed the shoot phenotypes of \( \text{amot1} \) and WT seedlings in response to 40 mM \( \text{NH}_4^+ \) over time. The shoot growth between WT and \( \text{amot1} \) seedlings remained similar 2 d after \( \text{NH}_4^+ \) addition, but the difference was clearly accentuated under prolonged \( \text{NH}_4^+ \) treatment, with the mutant maintaining significantly higher growth rates (Fig. 1D, E). Considering these results together, \( \text{amot1} \) emerges as the first \( \text{NH}_4^+ \)-resistant mutant, displaying superior shoot growth.

The WT and \( \text{amot1} \) seedlings were also treated on medium enriched with a variety of ions and molecules, and the results indicate that \( \text{amot1} \) seedlings are highly resistant to both (\( \text{NH}_4 \))$_2$SO$_4$ and NH$_4$Cl, but responded to 15 mM and 20 mM K$_2$SO$_4$ or 60 mM and 80 mM mannitol in a similar pattern to the WT (Fig. 2).

The \( \text{amot1} \) mutant is a novel \( \text{ein3} \) allele, and loss of \( \text{EIN3} \) function enhances ammonium tolerance

The WT as female parent was crossed with the homozygous mutant as the pollen donor. F$_1$ plants were selfed to obtain

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**Fig. 1.** Isolation and characterization of the ammonium-tolerant \( \text{amot1} \) mutant. (A) Appearance of *Arabidopsis thaliana* wild-type (WT) and \( \text{amot1} \) mutant plants following treatment with \( \text{NH}_4^+ \). Five-day-old plants were transferred to control and 40 mM \( \text{NH}_4^+ \) concentration for 6 d, and then pictures were taken. Scale bars=0.5 cm. (B and C) Relative rosette diameter and fresh shoot weight of *A. thaliana* WT and \( \text{amot1} \) mutant plants following treatment with various \( \text{NH}_4^+ \) concentrations for 6 d. The rosette diameter on control nutrient solution was considered as 1. Values are the means ±SD, n=8–11. Different letters indicate statistical differences at P<0.05 (one-way ANOVA with Duncan post-hoc test). (D and E) Relative rosette diameter and fresh shoot weight of *A. thaliana* WT and \( \text{amot1} \) mutant plants following treatment with 40 mM \( \text{NH}_4^+ \) for 0, 2, 4, and 6 d. Values are the means ±SD, n=6–10. Asterisks indicate statistical differences between the WT and \( \text{amot1} \) under \( \text{NH}_4^+ \) treatment at the indicated times (independent samples t-test, "P<0.05). (This figure is available in color at JXB online.)
F₂ seeds. Both F₁ and F₂ seeds were assayed for growth on NH₄⁺ medium. All examined F₁ progeny (45 seedlings) displayed the same phenotypes as the WT. In the F₂ population, the amot1 phenotype segregated at an approximate 1:3 ratio (amot1:WT=54:142; x²=0.55, P=0.05), indicating that amot1 is a recessive mutation at a single nuclear locus. T-DNA-flanking sequences were isolated from the mutant by thermal asymmetric interlaced PCR, and sequence analysis revealed that the pSKI15 T-DNA was inserted into the exon of the EIN3 gene (At3g20770) in amot1, 192 bp downstream of the start codon, ATG [Fig. 3A(a)]. EIN3 gene transcripts were greatly reduced in amot1 compared with the WT [Fig. 3A(b) and (c)]. To ascertain further whether the NH₄⁺-resistant phenotype in amot1 is due to the mutation in the EIN3 gene, we analyzed the previously reported allele ein3-1 (Chao et al., 1997) and crossed the amot1 mutant with ein3-1 plants. With exposure to 40 mM NH₄⁺, shoot size and biomass in ein3-1 seedlings were indeed similar to those of amot1 seedlings (Fig. 3B). Furthermore, the amot1 mutant was crossed to ein3-1, and the F₁ progeny showed a phenotype similar to that of the parents in the presence of NH₄⁺ (Fig. 3B). Collectively, these results show that the amot1 mutant is a new loss-of-function allele of the EIN3 gene.

AMOT1/EIN3 is a member of a protein family that includes EIN3-like (EIL) proteins (Chao et al., 1997) and initiates transcriptional re-programming in various ethylene responses (Guo and Ecker, 2004; Peng et al., 2014). We sought to determine the role of AMOT1/EIN3 in NH₄⁺ resistance. Because AMOT1/EIN3 and its close homolog EIL1 functionally overlap (Chao et al., 1997; An et al., 2010), we examined the ein3-1eil1-1 double mutant seedling response under various NH₄⁺ concentrations. Under high NH₄⁺, ein3-1eil1-1 had superior tolerance to NH₄⁺ compared with the WT (26% reduction in ein3-1eil1-1 versus 52% in the WT at 40 mM NH₄⁺) (Fig. 3C). Furthermore, in contrast to the ein3 single mutant’s tolerant phenotype, the ein3-1eil1-1 double mutant exhibited a more tolerant phenotype than the amot1 mutant (Fig. 3C). We also examined the phenotype of the eil1 single mutant upon treatment with high NH₄⁺; however, the eil1-1 mutant was similar to the WT under high NH₄⁺ (Supplementary Fig. S2). Next, we examined the NH₄⁺-responsive phenotype of a transgenic line overexpressing EIN3 under the control of the 35S promoter (35S:EIN3/Col-0 or EIN3ox), which displays an enhanced ethylene response (Chao et al., 1997; An et al., 2010; Z. Li et al., 2013). The transcripts of AMOT1/EIN3 were significantly increased in the EIN3ox seedlings [Z. Li et al., 2013; Fig. 3A(b) and (c)]. Under high NH₄⁺, EIN3ox plants displayed increased sensitivity, based on shoot size and biomass, when compared with their WT counterparts (73% shoot biomass reduction in EIN3ox plants versus 52% in the WT at 40 mM NH₄⁺) (Fig. 3C). Together, these results suggest that constitutive overexpression of AMOT1/EIN3 leads to elevated shoot NH₄⁺ sensitivity in Arabidopsis. Consistent with a previous report (G. Li et al., 2013), the ein3eil1 lateral root number was also more resistant than that of the WT to high-NH₄⁺ stress (Supplementary Fig. S3).

Enhanced shoot ethylene evolution is involved in ammonium-mediated inhibition of shoot growth

Aerial tissue NH₄⁺ content was determined, and the NH₄⁺ content increased gradually with treatment time compared with that in untreated shoots (Fig. 4A). Shoot ethylene production under NH₄⁺ exposure was also significantly greater than without NH₄⁺ and increased linearly with NH₄⁺ treatment time (Fig. 4B), consistent with Barker (1999b). Ethylene is synthesized from SAM via ACC, which is catalyzed by the enzyme ACS (Adams and Yang, 1979). ACC amounts in untreated and treated seedlings are presented in Fig. 4C. Consistent with previous reports, ACC amounts increased linearly with NH₄⁺ treatment time. As ACS and ACO are key enzymes of the ethylene biosynthetic pathway in plants, AtACS2, AtACS7, AtACS11, and AtACO2 expression was examined. Expression of the four genes was rapidly up-regulated in response to high NH₄⁺ (Fig. 4D).

We further investigated the activity of AMOT1/EIN3 in response to NH₄⁺ in shoot. A transgenic reporter line harboring the GUS gene driven by five tandem repeats of the EIN3-binding site (EBS) followed by a minimal 35S promoter (5×EBS:GUS/Col-0) has been used to monitor the transcriptional activity of EIN3 (Stepanova et al., 2007; He et al., 2011). Following NH₄⁺ treatment, GUS staining became intensified in the cotyledons of 5×EBS:GUS/Col-0 plants (Fig. 4F),
indicative of elevated levels of AMOT1/EIN3 activity. We also observed that the expression of the ethylene-responsive gene ERF1 was up-regulated by NH₄⁺ in the WT (Fig. 4G). In keeping with the results on AMOT1/EIN3 activity, expression of ERF1, a direct target gene of EIN3 (Solano et al., 1998), was lower in the NH₄⁺-treated ein3eil1 mutant, but higher in EIN3ox lines, compared with the WT (Fig. 4G).

The WT plants treated with the ethylene biosynthetic precursor ACC displayed decreased tolerance to NH₄⁺ (Fig. 4E). Consistent with this, the ethylene overproduction mutant eto1-1 (ethylene overproducer 1) also showed reduced NH₄⁺ tolerance compared with the WT (Fig. 4G). In the presence of the ethylene receptor antagonist Ag⁺, shoot growth of the WT was significantly increased when the plants were exposed to NH₄⁺ stress (Fig. 4E). As ethylene is known to activate downstream signaling pathways by binding to ethylene receptors (e.g. ETR1), we examined whether ethylene regulates shoot growth sensitivity to NH₄⁺ in such a way. Shoot growth in the ethylene-insensitive (ethylene receptor) mutant etr1-3 and positive regulator mutants in ethylene signaling, amot1 and ein3-1, was more tolerant to NH₄⁺ than that of the WT; consistent with this, EIN3ox lines displayed increased shoot growth sensitivity (Fig. 4H). These results indicate that ethylene has a negative effect on NH₄⁺ tolerance in Arabidopsis shoot growth.

**AMOT1/EIN3 regulates ammonium-induced ROS accumulation in shoots**

High NH₄⁺ induces an increase in ROS in plants; however, the biological mechanism of NH₄⁺-induced ROS accumulation remains largely unknown. Here, we examined the levels of endogenous H₂O₂ in the WT, ein3eil1, and EIN3ox in response to high-NH₄⁺ treatment. NH₄⁺ stress increased H₂O₂ accumulation in the cotyledons of the WT, indicated by DAB staining (Fig. 5A, B). We further found higher levels of DAB staining in EIN3ox but lower levels in ein3eil1 than in the WT following NH₄⁺ stress (Fig. 5A, B), in accordance with the NH₄⁺ tolerance phenotypes of these genotypes (Fig. 3C). We also measured the H₂O₂ contents in shoots. As inferred from DAB staining assays, high-NH₄⁺ treatment induced accumulation of H₂O₂ in the WT. However, the level was lower in ein3eil1 and higher in EIN3ox shoots compared with Col-0 under NH₄⁺ stress (Fig. 5C). The H₂O₂ content under control conditions was not significantly different among the three ecotypes.
Fig. 4. Effects of ethylene on shoot growth tolerance to NH₄⁺. (A) NH₄⁺ content in Arabidopsis shoots for the duration of the NH₄⁺ treatment. (B) Ethylene evolution in Arabidopsis shoots for the duration of the NH₄⁺ treatment. (C) 1-Aminocyclopropane-1-carboxylic acid (ACC) content in Arabidopsis shoots for the duration of the NH₄⁺ treatment. Seedlings at 5 d after germination were exposed to NH₄⁺ for varying treatment times, and NH₄⁺ content (A), ethylene evolution (B), and ACC content were determined. Values are means ±SD of three replicates. Different letters indicate statistical differences at P<0.05 (one-way ANOVA with Duncan post-hoc test). (D) Effect of NH₄⁺ treatment on shoot ACS and ACO gene expression by qRT-PCR for 6 h. Values are means ±SD of three replicates. CBP20 was used as the internal reference gene, and the control was considered as 1. (E) Effect of supplied ethylene inhibitors 30 μM AgNO₃ and 25 μM ACC on shoot biomass of WT seedlings grown in 40 mM NH₄⁺ treatment medium. Values are the means ±SD, n=10–12. (F) Schematic diagram of the EIN3 activity reporter system showing the EIN3 protein, five tandem repeats of the EBS (5×EBS), and the GUS gene. Expression of 5×EBS:GUS in leaves of the WT under control conditions and 24 h NH₄⁺ treatment. One representative sample from each treatment (10 plants) is shown. GUS staining intensity was quantified using Image J software, and the control was considered as 1. Values are means ±SD of three replicates. (G) Effect of NH₄⁺ treatment on shoot ERF1 gene expression of WT, ein3eil1, and EIN3ox lines by qRT-PCR for 6 h. Values are means ±SD of three replicates. ACTIN2 was used as the internal reference gene, and the WT control was considered as 1. (H) Effect of NH₄⁺ treatment for 6 d on shoot fresh weight of WT, amot1, ein3-1, EIN3ox, etr1-3, and eto1-1 seedlings. Values are the means ±SD, n=12. Different letters indicate statistical differences at P<0.05 of control and NH₄⁺ treatment, respectively (one-way ANOVA with Duncan post-hoc test).

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( Supplementary Fig. S4). A split-shoot experiment was devised to examine further the relationship between NH₄⁺-induced AMOT1/EIN3 transcriptional activity and ROS accumulation (Supplementary Fig. S5A). The cotyledon supplied with NH₄⁺ displayed significantly increased EBS:GUS expression compared with the untreated cotyledon (Fig. S5B). Consistent with EBS:GUS inductive sites, a higher intensity of DAB staining was also detected in the NH₄⁺-treated cotyledon (Supplementary Fig. S5C).

In this study, the concentration of MDA equivalents was increased in NH₄⁺-treated leaves (Fig. 6A). However, we observed a higher MDA level in EIN3ox, and a lower level in ein3eil1 than in the WT following NH₄⁺ stress (Fig. 6A). The effects of NH₄⁺ on shoot growth were also examined in combination with external H₂O₂. The combined treatment with NH₄⁺ and H₂O₂ in the medium markedly inhibited shoot growth compared with NH₄⁺ alone, and the combined treatment inhibited shoot growth more significantly in amot1 and ein3eil1 (Fig. 6B).

We further examined the expression of genes encoding antioxidant metabolic enzymes, such as APX1, APX2, CAT1, CAT2, and CAT3. NH₄⁺ stress did not induce APX and CAT gene expression in WT shoots, and these genes were also not much affected in ein3eil1 and EIN3ox seedlings under NH₄⁺ stress (Fig. 6C), suggesting that AMOT1/EIN3 regulation of NH₄⁺-induced ROS accumulation might not be related to APX1-, APX2-, CAT1-, CAT2-, and CAT3-mediated antioxidant activity. Previous studies showed that drought stress increases RBOH transcript levels (Lee et al., 2012). However, the expression patterns of RBOHA, RBOHB, RBOHD, and RBOHF under control and high-NH₄⁺ stress were similar, and they were also not much affected in the ein3eil1 mutant and in EIN3ox lines compared with the WT (Fig. 6C). Furthermore, DAB staining and shoot growth in response to high-NH₄⁺ stress were also similar between the WT and the rbohd mutant (Supplementary Fig. S6).

AMOT1/EIN3 induces the transcription of peroxidases and increases their activity

Podgóriska et al. (2015) proposed that higher POD levels are positively correlated with NH₄⁺-induced ROS generation and cell growth inhibition. We found that the expression of two of the genes encoding PODs, At5g19890 and At1g48570, was induced by NH₄⁺ treatment in the WT, and expression was more elevated in EIN3ox while it was reduced in ein3eil1 with or without NH₄⁺ (Fig. 7A, B). The transcript levels of other POD-encoding genes, such as At5g42180, At2g18980, At4g11290, and At3g49960, were not increased by NH₄⁺ treatment in the WT, and were also not significantly altered in EIN3ox and ein3eil1 seedlings, under either normal or NH₄⁺-
stress conditions (Supplementary Fig. S7). The POD activity assay also showed that POD activity was significantly elevated in EIN3ox seedlings compared with the WT and ein3eil1 under NH4+ stress (Fig. 7C). POD activity in EIN3ox seedlings under control conditions was also slightly elevated compared with the WT and with ein3eil1 (Supplementary Fig. S8). We next analyzed the promoter regions of two POD genes (At5g19890 and At1g48570) and found EBSs (ATGTA) in each promoter (data not shown). To test the interaction between the AMOT1/EIN3 protein and the At5g19890 and At1g48570 promoters, a Y1H assay was performed. As shown in Fig. 7D, bait yeast cells co-transformed with the empty vector (AD) or the fusion vector (AD-EIN3) grew well on synthetic dropout medium (SD) without Ura and Leu. However, only the yeast cells co-transformed with the fusion vector AD–EIN3 survived on the selective medium supplemented with 400 ng ml⁻¹ aureobasidin A (AbA; Fig. 7D). The data suggest that the AMOT1/EIN3 protein interacts with the At5g19890 and At1g48570 promoters in the yeast system. SHAM is a widely used POD inhibitor (Balzergue et al., 2017). The shoot biomass of the WT and EIN3ox lines was increased under NH4+ stress after SHAM treatment, but no effects on ein3eil1 lines were observed (Fig. 7E, F). Furthermore, the POD inhibitor SHAM could decrease NH4+–induced DAB staining, indicating H2O2 accumulation in both WT and EIN3ox leaves (Fig. 7G; Supplementary Fig. S9).
Role of AMOT1/EIN3 in ammonium toxicity tolerance

The ammonium mutant accumulates less NH$_4^+$ in shoot tissue under NH$_4^+$ toxicity

There was no difference in shoot NH$_4^+$ content of the WT, amot1, and ein3eil1 with 3 d of NH$_4^+$ treatment (Fig. 8A), although ROS accumulation was greater in WT shoots than in those of ein3eil1 at this treatment time (Fig. 5). However, higher levels of NH$_4^+$ in the WT, but lower levels in amot1 and ein3eil1, than in the WT were recorded following high-NH$_4^+$ stress during a 6 d treatment (Fig. 8A). Consistently, NH$_4^+$ accumulation was slightly higher in EIN3ox shoots than in the WT following the treatment (Fig. 8B). Activities of the enzyme GS, centrally involved in the NH$_4^+$ assimilation process (Kronzucker et al., 1995; Hirano et al., 2008), was determined in shoots of the WT and amot1. GS activity was induced by external NH$_4^+$ in NH$_4^+$-fed WT and amot1 plants, but this increase was not significantly different in the two genotypes (Fig. 8C). This indicates that NH$_4^+$ metabolism was not affected by the AMOT1 mutation.

Discussion

Stunted root system and decreased leaf biomass are among the most visible phenotypic manifestations of NH$_4^+$ toxicity in higher plants (Britto and Kronzucker, 2002). Several genetic regulators controlling root sensitivity to NH$_4^+$ have been identified in Arabidopsis (Li et al., 2014); however, little is known about the specific targets and pathways that lead to impaired leaf growth in the context of NH$_4^+$ toxicity. To gain insight into the mechanisms of the effects of NH$_4^+$ on shoot growth, we employed a molecular genetics approach, based on a mutant screen for altered response to NH$_4^+$. In the current work, enhanced NH$_4^+$ tolerance of shoot growth was found in amot1. We further revealed that the nuclear AMOT1 locus is identical to EIN3, which encodes a transcriptional activator required for initiating transcriptional re-programming in various ethylene responses (Guo and Ecker, 2004; Yoo et al., 2009). It was found that amot1 and ein3-1, a reported allele, showed enhanced shoot growth tolerance compared with the WT, but the transgenic line overexpressing EIN3 (EIN3ox) was more sensitive. The activity of AMOT1/EIN3, indicated by using EBS:GUS in shoots, was markedly enhanced on NH$_4^+$ stress during a 6 d treatment (Fig. 4F). These results suggest that AMOT1/EIN3 plays an important role in the NH$_4^+$-induced impairment of shoot growth. It was demonstrated furthermore that this inhibitory effect is related to enhanced shoot ACC and ethylene accumulation. More importantly, it was found that AMOT1/EIN3 positively regulates shoot ROS accumulation, which leads to oxidative stress in Arabidopsis shoots under NH$_4^+$ stress, and up-regulates the shoot expression of the genes coding for PODs previously shown to correlate positively with NH$_4^+$ (Fig. 4F).
increased treatment time (Fig. 4A). Furthermore, a previous observation showed high ethylene evolution to correlate with high tissue NH$_4^+$ but to be independent of nitrogen form and pH regime (Feng and Barker, 1992c). Hence, together with previous reports, our study suggests that greatly increased shoot NH$_4^+$ content may be the intrinsic trigger leading to enhanced ethylene evolution under NH$_4^+$ stress. The rate-limiting step in ethylene biosynthesis lies in the production of ACC by ACS (Schellingen et al., 2014). Shoot ACC amounts also increased linearly with NH$_4^+$ treatment time (Fig. 4C). Furthermore, we show here that AtACS2, AtACS7, AtACS11, and AtACO2, which encode ACS and ACO, the two key enzymes responsible for ethylene synthesis, are transcriptionally up-regulated by NH$_4^+$ treatment (Fig. 4D). Therefore, it is conceivable that the increased ACC biosynthesis resulting from up-regulation of ACS and ACO gene expression is involved in NH$_4^+$-induced

![Fig. 8.](image)

**Fig. 8.** Effects of NH$_4^+$ treatment on shoot NH$_4^+$ content and GS activity. (A) NH$_4^+$ contents in the shoot tissues of WT, ein3eil1, and amot1 seedlings. Five-day-old WT, ein3eil1, and amot1 seedlings were grown on growth medium and transferred to fresh medium with control or NH$_4^+$ for 3 d and 6 d of growth, and then NH$_4^+$ tissue content was determined. Values are means ±SD of three replicates. (B) NH$_4^+$ contents in the shoot tissues of WT and EIN3ox seedlings. Five-day-old WT and EIN3ox seedlings were grown on growth medium and transferred to fresh medium with control or NH$_4^+$ for 3 d and 6 d of growth, and then NH$_4^+$ tissue content was determined. Values are means ±SD of three replicates. (C) GS activities in the shoots of WT and EIN3ox seedlings. Five-day-old seedlings were exposed to 40 mM NH$_4^+$ for 5 d. Values are means ±SD of three replicates. ACTIN2 was used as the internal reference gene, and the WT control was considered as 1. Different letters indicate statistical differences at P<0.05 of control and NH$_4^+$ treatment, respectively (one-way ANOVA with Duncan post-hoc test). (G) Effect of SHAM on the NH$_4^+$-induced H$_2$O$_2$ accumulation in shoots of WT and EIN3ox. Seedlings at 5 d were exposed to 40 mM NH$_4^+$ with or without 10 μM SHAM for 3 d, and then DAB staining of shoots was performed. Scale bars=200 μm.
ethylene evolution. Further study on the detailed mechanisms of NH$_4^+$-regulated ethylene evolution is warranted.

In our study, we provide several lines of evidence supporting the notion that ethylene biosynthesis and signaling play a negative role in the adaptation of Arabidopsis shoot growth to NH$_4^+$ stress. ACC content, ethylene production, and AMOT1/EIN3 activity, and the expression of genes encoding key enzymes responsible for ethylene synthesis in the Arabidopsis shoot showed dramatic increases after NH$_4^+$ treatment. The dual evidence that shoot biomass was inhibited by NH$_4^+$ to a greater extent in the ethylene overproduction mutant eto1-1 and the EIN3ox line, compared with the WT, and that mutations in ethylene receptors (e.g. etr1-3) and key positive regulators in ethylene signaling (e.g. amot1 and ein3-1) showed increased shoot growth compared with the WT under NH$_4^+$ stress (Figs 3, 4H), supports this notion. The observations that the externally supplied ethylene inhibitor Ag$^+$ alleviated the phenotypic manifestation of toxicity, but that ACC, a precursor of ethylene, aggravated NH$_4^+$-suppressed shoot growth in the WT (Fig. 4E), further demonstrate the important role of shoot ethylene signaling. Our findings are in excellent agreement with a previous study showing that Ag$^+$ improved plant shoot ethylene signaling. Our findings are in excellent agreement with a previous study showing that Ag$^+$ improved plant shoot ethylene signaling.

The underlying mechanisms determining ROS accumulation by NH$_4^+$ in leaves are only partially understood (Bittsánzszyk et al., 2015). Our results show that AMOT1/EIN3 is involved in H$_2$O$_2$ metabolism in leaves under NH$_4^+$ stress. First, a higher level of H$_2$O$_2$ cytochemical staining in EIN3ox was found, while a lower level of H$_2$O$_2$ staining was seen in ein3eil1 than in the WT following NH$_4^+$ stress (Fig. 5A, B), in accordance with the NH$_4^+$ tolerance phenotypes and lipid peroxidation profiles of these genotypes (Figs 3, 6A). In agreement with the above results, NH$_4^+$ stress increased leaf H$_2$O$_2$ concentrations in the WT, while these were lower in ein3eil1 and higher in EIN3ox under identical treatment conditions (Fig. 5C). Moreover, our split-shoot experiment showed that a higher DAB staining intensity was detected in the components of EBS/GUS cotyledons exposed to NH$_4^+$ (Supplementary Fig. S5). These results suggest that AMOT1/EIN3 positively regulates NH$_4^+$-induced leaf H$_2$O$_2$ accumulation. Investigations on whether oxidative stress is involved in NH$_4^+$ stress regeneration and the POD inhibitor SHAM could restore root growth and reduce ROS accumulation under –Pi (Balzergue et al., 2017). Podgórska et al. (2015) showed that higher POD levels positively correlate with NH$_4^+$-induced ROS content and cell growth inhibition. Consistent with this, by examining POD gene expression and POD activity under NH$_4^+$ conditions, we show that NH$_4^+$ stress increases expression of some POD genes (At5g19890 and At1g48570) and POD activity in WT shoots (Fig. 7A–C). Moreover, the peroxidase inhibitor SHAM could indeed alleviate NH$_4^+$-induced shoot ROS accumulation (Fig. 7G; Supplementary Fig. S9) and growth inhibition (Fig. 7E, F). This result, together with previous reports, confirms that NH$_4^+$ induces accumulation of ROS and suggests that POD expression and activity may play an important role. Our qRT-PCR analyses show that two POD genes (At5g19890 and At1g48570) were constitutively up-regulated in EIN3ox but down-regulated in the ein3eil1 double mutant, regardless of NH$_4^+$ (Fig. 7A). Moreover, NH$_4^+$-induced POD activity was positively correlated with the expression of EIN3 genes, as shown for ein3eil1 and EIN3ox seedlings (Fig. 7B).

Further studies revealed that the key transcription factor AMOT1/EIN3 may directly target the two POD genes (At5g19890 and At1g48570), as the AMOT1/EIN3 protein could specifically bind to the promoters of the At5g19890 and At1g48570 genes, as revealed by the Y1H assay. Further supporting our finding of increased POD activity and DAB staining, indicating H$_2$O$_2$ accumulation in EIN3ox, the POD inhibitor SHAM was shown to enhance EIN3ox shoot growth and reduce DAB staining used to indicate H$_2$O$_2$ accumulation under NH$_4^+$ stress (Fig. 7; Supplementary Fig. S9). Collectively, these data indicate that the ethylene signaling-mediated NH$_4^+$...
response of Arabidopsis shoot growth is brought about, at least partially, through POD genes (e.g. At5g19890 and At1g48570), via AMOT1/EIN3.

The accumulation of free shoot NH$_4^+$ is widely considered to be critical to the development of NH$_4^+$ toxicity (Gerendas et al., 1997; Szcerba et al., 2008). Because H$_2$O$_2$ accumulation was greater in WT shoots than in ein3eil1, but higher in EIN3ox than in the WT; after a 3 d treatment with high NH$_4^+$, we hypothesized that loss of function or overexpression of AMOT1/EIN3 may entail reduced or enhanced NH$_4^+$ content in the shoot at the 3 d treatment time, respectively. However, there was no difference in shoot NH$_4^+$ content between the WT, amot1, ein3eil1, and EIN3ox (Fig. 8A). These results further highlight that AMOT1/EIN3 plays an important role in regulating NH$_4^+$-induced shoot ROS accumulation and rules out that reduced shoot ROS accumulation during the early phase of exposure (within 3 d in our study) by loss of function of AMOT1/EIN3 resulted from reduced NH$_4^+$ content. However, a higher NH$_4^+$ content in EIN3ox and the WT, and a lower content in amot1 and ein3eil1 were observed following high-NH$_4^+$ stress for prolonged treatment times (6 d) (Fig. 8A, B). Our results indicate that GS activity was not affected by the mutation in AMOT1/EIN3 (Fig. 8C), showing that GS is not responsible for the lower NH$_4^+$ accumulation in the amot1 mutant. Alternatively, oxidative stress itself, induced by H$_2$O$_2$, can increase cellular NH$_4^+$ concentrations by inducing proteolytic activity (Sweetlove et al., 2002). It is not clear how AMOT1/EIN3 mediates shoot NH$_4^+$ accumulation over longer periods of treatment, and more research is warranted to examine this.

In summary, we have identified and characterized a novel gene, AMOT1/EIN3, that controls shoot NH$_4^+$ sensitivity and propose a model for ethylene–AMOT1/EIN3 functions in shoot NH$_4^+$ sensitivity (Fig. 9). Our study shows the importance of EIN3 in shoot inhibition under high-NH$_4^+$ stress, providing strong genetic evidence in support of the role of the ethylene biosynthesis and signaling pathway in regulating shoot NH$_4^+$ sensitivity. Under NH$_4^+$ stress, ethylene is perceived and transduced, affecting the transcription factor EIN3, and initiating the ethylene response. EIN3 regulates ROS accumulation, which leads to oxidative stress in Arabidopsis shoots under NH$_4^+$ stress. The expression of EIN3-mediated POD genes (e.g. At5g19890 and At1g48570) is involved in NH$_4^+$-induced shoot ROS accumulation. (A) In the wild type, NH$_4^+$ stress enhanced the expression of ACS and ACO genes, encoding ACS and ACO, the two key enzymes responsible for ethylene synthesis. Under NH$_4^+$ stress, ethylene is perceived and transduced, affecting the transcription factor EIN3, and initiating the ethylene response. EIN3 regulates ROS accumulation, which leads to oxidative stress in Arabidopsis shoots under NH$_4^+$ stress. The expression of EIN3-mediated POD genes (e.g. At5g19890 and At1g48570) in the shoot is blocked under NH$_4^+$ stress. Ethylene, regulation of ROS accumulation and oxidative stress is lowered. Orthogons in orange represent known EIN3 functions, and orthogons in gray with dashed lines represent the inhibition of EIN3 functions due to the amot1/ein3 mutation. Red arrows indicate increased POD gene expression, ROS accumulation, and oxidative stress, and thick and thin red arrows indicate, respectively, a high or low ROS accumulation and oxidative stress.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Rosette diameter and fresh shoot weight of Arabidopsis thaliana wild-type (WT, Col-0) plants following treatment with various NH$_4^+$ concentrations.

Fig. S2. Relative rosette diameter (A) and fresh shoot weight (B) of Arabidopsis thaliana WT and ein1 mutant plants following treatment with NH$_4^+$ for 6 d.

Fig. S3. Lateral root number of Arabidopsis thaliana WT and ein3eil1 mutant plants following treatment with high NH$_4^+$ for 6 d.

Fig. S4. H$_2$O$_2$ content in WT, EIN3ox, and ein3eil1 shoot tissue under control conditions.

Fig. S5. EBS:GUS expression and DAB staining in the split-root experiment.

Fig. S6. Effect of NH$_4^+$ treatment on shoot DAB staining and biomass of the WT and the Atbbohd mutant.

Fig. S7. qRT-PCR analysis of POD gene expression in WT, EIN3ox, and ein3eil1 shoot tissue under NH$_4^+$ treatment for 6 h.

Fig. S8. Measurement of POD activity of WT, EIN3ox, and ein3eil1 shoot tissue under control conditions.
Fig. S9. Mean relative DAB staining intensity in WT (A) and EIN3ox (B) shoots treated with NH$_4^+$ and NH$_4^+$ plus SHAM. Table S1. Gene-specific primers used for qRT-PCR.

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