Arabidopsis Plastid AMOS1/EGY1 Integrates Abscisic Acid Signaling to Regulate Global Gene Expression Response to Ammonium Stress[1][W][OA]

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Ammonium (NH_4^+) is a ubiquitous intermediate of nitrogen metabolism but is notorious for its toxic effects on most organisms. Extensive studies of the underlying mechanisms of NH_4^+ toxicity have been reported in plants, but it is poorly understood how plants acclimate to high levels of NH_4^+. Here, we identified an Arabidopsis (*Arabidopsis thaliana*) mutant, *ammonium overly sensitive1* (*amos1*), that displays severe chlorosis under NH_4^+ stress. Map-based cloning shows *amos1* to carry a mutation in *EGY1* (for ethylene-dependent, gravitropism-deficient, and yellow-green-like protein1), which encodes a plastid metalloprotease. Transcriptomic analysis reveals that among the genes activated in response to NH_4^+, 90% are regulated dependent on *AMOS1/EGY1*. Furthermore, 63% of *AMOS1/EGY1*-dependent NH_4^+-activated genes contain an ACGTG motif in their promoter region, a core motif of abscisic acid (ABA)-responsive elements. Consistent with this, our physiological, pharmacological, transcriptomic, and genetic data show that ABA signaling is a critical, but not the sole, downstream component of the *AMOS1/EGY1*-dependent pathway that regulates the expression of NH_4^+-responsive genes and maintains chloroplast functionality under NH_4^+ stress. Importantly, *abi4* mutants defective in ABA-dependent and retrograde signaling, but not ABA-deficient mutants, mimic leaf NH_4^+ hypersensitivity of *amos1*. In summary, our findings suggest that an NH_4^+-responsive plastid retrograde pathway, which depends on *AMOS1/EGY1* function and integrates with ABA signaling, is required for the regulation of expression of NH_4^+-responsive genes that maintain chloroplast integrity in the presence of high NH_4^+ levels.

Ammonium (NH_4^+) is a key intermediate of nitrogen metabolism in most living organisms (von Wirén and Merrick, 2004). For bacteria, fungi, and plants, NH_4^+ is also the preferred nitrogen source. However, sensitivity to NH_4^+ occurs widely in animals, plants, and microorganisms (Britto and Kronzucker, 2002; von Wirén and Merrick, 2004). Extensive studies have been carried out that dissect the underlying mechanisms of NH_4^+ toxicity in plants, animals, and microorganisms (Britto and Kronzucker, 2002; Hess et al., 2006; Norenberg et al., 2009). The paradox of NH_4^+ is particularly serious in plants, where stunted growth of roots and chlorosis of leaves are two major symptoms of toxicity (Britto and Kronzucker, 2002). Several physiological responses, including increased proton efflux, deficiency of other cations, carbon shortage, disruptions in hormonal homeostasis, damaged chloroplast ultrastructure, uncoupling of photophosphorylation, disruptions in photosynthesis, and energy depletion linked to futile transmembrane NH_4^+ cycling, have all been implicated in NH_4^+ stress in plants (Britto and Kronzucker 2002). Recently, the sites of action of NH_4^+ and underlying molecular mechanisms in plants have been addressed in more detail. For example, NH_4^+ inhibition of root growth was shown to target primarily cell elongation and require direct contact of the root tip with NH_4^+. Furthermore, root growth inhibition was directly linked to excessive NH_4^+ efflux in the root elongation zone (Li et al., 2010). The sensitivity of root elongation to NH_4^+ has been shown to be related to impaired functioning of the GDP-Man pyrophosphorylase (GMPase), which controls the synthesis of GDP-Man and is involved in the *N*-glycosylation of proteins, and, as such, is believed to influence general cellular stability and the regulation of NH_4^+ transport (Qin et al., 2008; Barth et al., 2010; Li

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et al., 2010). However, recently, defects in GMPase alone were found to be insufficient to explain root NH_4^+ sensitivity (Kempinski et al., 2011). For example, NH_4^+-induced reduction of lateral root formation results from contact of the shoot with NH_4^+ and decreased transport of shoot-derived auxin to roots, via the auxin influx carrier AUX1 (Li et al., 2011a, 2011b). Interestingly, defects in dolichol phosphate Man synthase1 result in both leaf chlorosis and the inhibition of root elongation in the presence of excess NH_4^+, not only linking to impaired *N*-glycosylation but also to a reduction in glycosyl phosphatidylinositol anchor attachment (Jadid et al., 2011). These studies, in combination, provide a significantly improved understanding of the process of NH_4^+ toxicity in plants.

Previous studies on the NH_4^+ stress response have focused largely on either a selected single gene or only a few genes. To gain insights into the global effects of NH_4^+ stress on plant gene expression, and to identify possible key regulators, we combined in this study the screening of mutants hypersensitive to NH_4^+ using forward genetics approaches with transcriptomics using the full genome microarray, along with bioinformatic analyses and physiological experiments. We identified the *ammonium overly sensitive1* (*amos1*) mutant in Arabidopsis (*Arabidopsis thaliana*), which displayed severe chlorosis under NH_4^+ stress and was affected in its global expression of NH_4^+-responsive genes. Map-based cloning revealed that *amos1* was an allelic mutation of the gene encoding *ETHYLENE-DEPENDENT GRAVITROPISM-DEFICIENT AND YELLOW-GREEN-LIKE PROTEIN1* (*EGY1*), which is known as a nucleus-encoded, plastid-localized, membrane-associated, and ATP-independent metalloprotease site-2 protease (S2P; Chen et al., 2005). It is required for normal chloroplast development, including the formation of thylakoid grana, the lamella system, and the accumulation of chlorophyll and chlorophyll *a*/*b*-binding proteins in chloroplast membranes. Additionally, *EGY1* is required for ethylene-dependent gravitropism of light-grown hypocotyls, a process linked to the regulation of endodermal plastid size and number (Chen et al., 2005; Guo et al., 2008). Our work furthermore explores the role of abscisic acid (ABA) signaling as a downstream component of an *AMOS1/EGY1*-dependent plastid retrograde signaling pathway, in the context of regulation of the gene expression and protection of chloroplast functionalities from NH_4^+ stress. Additionally, we examine the involvement of the reactive oxygen species (ROS) response in chloroplasts of guard cells as an upstream component of ABA signaling in the *AMOS1/EGY1*-dependent pathway during the NH_4^+ stress response.

Our findings not only document, to our knowledge for the first time, the global profile of NH_4^+-responsive genes under NH_4^+ stress but identify the critical role during NH_4^+ stress for the plastid metalloprotease EGY1-dependent retrograde signaling pathway in the control of nuclear gene expression through recruitment of ABA signaling.

RESULTS

Identification of the *amos1* Mutant

Based on enhanced leaf chlorosis on medium containing NH_4^+, we isolated three independent *amos* mutants from 5,160 lines of a chemical-inducible activation-tagging T-DNA insertion line library in the ecotype Columbia (Col-0) background on growth medium (GM) supplemented with 30 mM $(NH_4)_2SO_4$ and 10 μM of the inducer 1,7-β-estradiol (Supplemental Fig. S1, A and B). These mutant lines also displayed the chlorosis phenotype on NH_4^+ medium in the absence of 1,7-β-estradiol (Supplemental Fig. S1C), suggesting that the mutations in the three lines were a result of the position of insertion of the T-DNA rather than of the activation of adjacent genes. Genetic analyses indicated that the three identified mutants, named *amos1-1*, *amos1-2*, and *amos1-3*, were allelic and that the NH_4^+-induced leaf chlorosis phenotype in *amos1* seedlings is caused by single recessive mutations in a nuclear gene (Supplemental Table S1). In the following experiments, all further phenotypic analyses were based on *amos1-1*.

The $(NH_4)_2SO_4$-induced chlorosis in *amos1* seedlings was reversible upon transfer to medium without $(NH_4)_2SO_4$, on which green leaves were formed again (except cotyledons; Supplemental Fig. S1D). This supported the notion that the observed phenotype was indeed a consequence of exposure to high levels of $(NH_4)_2SO_4$. Because the screening medium contained not only NH_4^+ but also a relatively high concentration of SO_4^{2-}, it was necessary to analyze the specific factor that led to the observed phenotype of *amos1*. Our data show that leaf chlorosis in *amos1* was indeed specific to NH_4^+, rather than being caused by SO_4^{2-} or ionic stress (Supplemental Fig. S1E).

It was recently suggested that NH_4^+ accumulation in leaves via direct uptake into shoots from the medium can exceed the accumulation that results from root-to-shoot translocation following uptake into roots (Li et al., 2011a). Therefore, we asked as well whether chlorosis of *amos1* was induced by NH_4^+ contact of the root or the shoot. Our results show that *amos1* seedlings were able to grow normally when only the root was in contact with NH_4^+ following transfer, whereas they exhibited chlorotic leaves when the shoot was in direct contact with NH_4^+ following transfer (Supplemental Fig. S2). Generally, in addition to foliar application incurred from NH_4^+-releasing fertilizers, leaves of species such as Arabidopsis easily come into contact with NH_4^+ when seedlings are germinated and grown directly in soil or sterile medium. Our results show that *amos1* indeed displays chlorotic leaves when germinated and grown directly in NH_4^+ medium (Supplemental Fig. S2). These results, together with previous results (Li et al., 2011a, 2011b), suggest that seedlings in the germination and early developmental stages suffer NH_4^+ stress more readily because their leaves are able to contact NH_4^+ in the medium directly.

We further analyzed leaf chlorophyll content in *amos1* and wild-type seedlings in response to a range of NH_4^+ concentrations. In these solid agarose-based nutrient media, chlorophyll concentrations of Col-0 wild-type seedlings increased in the presence of NH_4^+ up to a concentration of 25 mM and decreased when NH_4^+ concentrations reached or exceeded 40 mM (Fig. 1A). By contrast, chlorophyll content in *amos1* was reduced by approximately 25% and 50%, respectively, when the NH_4^+ concentration was raised to 20 and 25 mM (Fig. 1A). The most sizable differences in chlorophyll accumulation in *amos1* and wild-type seedlings occurred at higher NH_4^+ concentrations, between 25 and 40 mM (Fig. 1A). We compared chlorophyll accumulation and chloroplast fluorescence between *amos1* and wild-type seedlings under identical concentrations of $(NH_4)_2SO_4$ and K_2SO_4. Chlorophyll accumulation and chloroplast fluorescence were severely affected in *amos1* seedlings when exposed to $(NH_4)_2SO_4$ but not K_2SO_4 (Fig. 1, B and C). Furthermore, we analyzed the phenotypes of *amos1* and wild-type seedlings in response to 25 and 40 mM NH_4^+ over time. In GM without NH_4^+, the chlorophyll content of *amos1* seedlings was between 66.3% and 55.5% of the wild type level (Supplemental Fig. S3, A and B). The difference in chlorophyll content between wild-type and *amos1* seedlings remained similar 24 h after NH_4^+ addition but was clearly exacerbated with prolonged NH_4^+ treatment. Chlorophyll contents in *amos1* were reduced to 24.5% or 12.7% of the wild type, respectively, in the presence of 25 and 40 mM NH_4^+ treatment for 7 d (Supplemental Fig. S3, A and B). Shoot fresh weight was 10% to 30% lower in *amos1* than in the wild type at these time points in GM containing NH_4^+ (Supplemental Fig. S3, C and D). The rate of shoot biomass production of *amos1* seedlings over time was not reduced upon short-term exposure to NH_4^+, then decreased with longer term NH_4^+ treatments, although the reduction was similar to the wild type (Supplemental Fig. S3, C and D). Furthermore, inhibition of root growth in response to NH_4^+, as assessed by primary root length and lateral root number, was not exacerbated in *amos1* seedlings when compared with the wild type (Supplemental Fig. S3, E and F). This is distinct from previously described NH_4^+-hypersensitive mutants of Arabidopsis, in which root morphological changes were the most visible phenotype (Qin et al., 2008; Jadid et al., 2011; Li et al., 2012). Considering these results jointly, *amos1* emerges as a novel NH_4^+-hypersensitivity mutant, displaying severe leaf chlorosis without any impairment of root growth or root development.

In addition, we determined the accumulation of NH_4^+ in shoots, which is considered an important factor contributing to NH_4^+ sensitivity in plants (Britto and Kronzucker, 2002; Balkos et al., 2010; Li et al., 2011a). Compared with mock treatment, NH_4^+ contents in shoots of *amos1* and wild-type seedlings were increased more than 150-fold upon exposure to 25 mM NH_4^+, but they were not significantly different between genotypes (Fig. 1D). Thus, the NH_4^+ sensitivity of *amos1* seedlings was not related to excess NH_4^+ accumulation when compared with the wild type. Given that the gene *VITAMIN C-1* (*VTC1*), encoding the enzyme GMPase, has been implicated as an important genetic locus for NH_4^+ sensitivity in roots of Arabidopsis (Qin et al., 2008; Barth et al., 2010), we asked whether the chlorosis phenotype under NH_4^+ exposure is related to this gene. However, *amos1/vtc1* double mutants displayed a similar leaf chlorosis response to

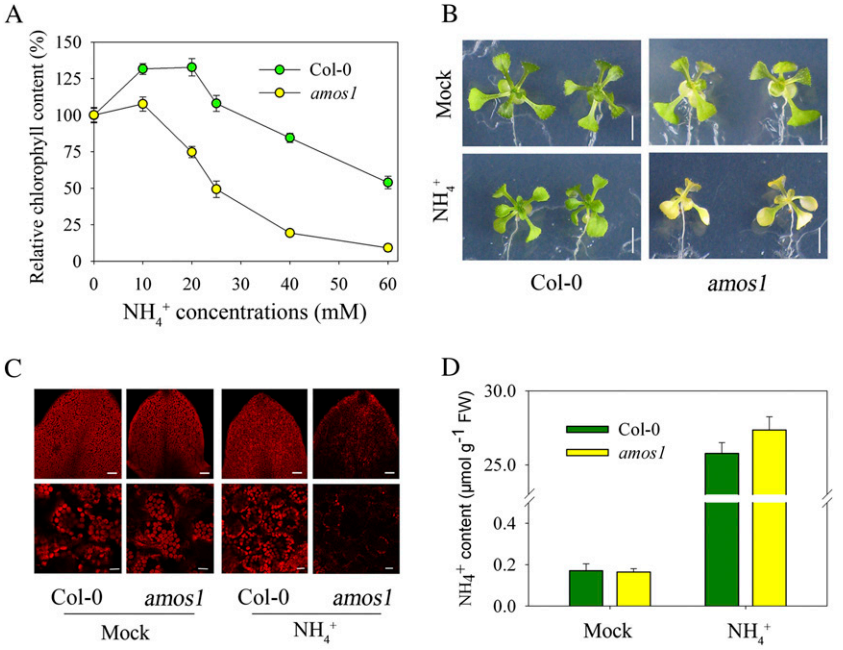

Figure 1. The *amos1* mutant is hypersensitive to excess NH_4^+. A, Relative chlorophyll concentrations in rosettes of 7-d-old wild-type (Col-0) and *amos1* seedlings exposed to serial concentrations of NH_4^+ [provided as $(NH_4)_2SO_4$] for 7 d, normalized to values for control medium (GM without NH_4^+) for each genotype. B, Leaf chlorosis (representative images) in 7-d-old wild-type and *amos1* seedlings, treated with 25 mM NH_4^+ or mock (12.5 mM K_2SO_4) for 7 d. Bars = 0.3 cm. C, Representative images of chlorophyll fluorescence of true leaves (top row) and chloroplasts (bottom row) in seedlings as described in B. Bars = 100 μm in the top row and 10 μm in the bottom row. D, NH_4^+ accumulation in shoots of *amos1* and wild-type seedlings. Treatment was as described in B. Values are means ± SE ($n \geq 4$). No significant differences were detected between the wild type and the mutant (Student's *t* test, $P > 0.05$). FW, Fresh weight.

amos1 seedlings while displaying a root growth response similar to *vtc1* seedlings (Supplemental Fig. S4), suggesting that the NH_4^+-induced leaf chlorosis of *amos1* seedlings was not related to the *GMPase* gene.

Identification of the AMOS1 Locus

Since T-DNA insertion loci isolated by thermal asymmetric interlaced PCR were not associated with the NH_4^+-hypersensitivity phenotype, a map-based cloning strategy was pursued to isolate the mutated gene. The *amos1* allele was located on chromosome 5 (Supplemental Fig. S5A) and was found to correspond to a segmental deletion of 1,071 bp in the gene *At5g35220* (Fig. 2A; Supplemental Fig. S5B), which encodes the previously described S2P metalloprotease EGY1. No expression of *EGY1* mRNA was detected in *amos1* seedlings (Supplemental Fig. S5C). Thus, *amos1* was a true null mutant. With exposure to 25 mM NH_4^+, chlorophyll accumulation in *egy1-1* and *egy1-2* seedlings was indeed similar to *amos1* seedlings (Fig. 2B). Furthermore, complementation of the *amos1* mutant with wild-type *EGY1* restored wild-type levels of chlorophyll accumulation in the presence of NH_4^+ (Fig. 2B). Collectively, these results show that the *amos1* mutant carries a loss-of-function mutation in the S2P metalloprotease gene *EGY1*. EGY1 has been identified as the first S2P metalloprotease homolog in plants (Chen et al., 2005; Chen and Zhang, 2010) and has been localized to chloroplasts (likely the thylakoid membrane system), based on the analysis of *EGY1-GFP* fusion constructs, and the defect arising from expressing the N-terminal transit peptide removed *EGY1* in *egy1* seedlings (Chen et al., 2005; Zhang et al., 2008).

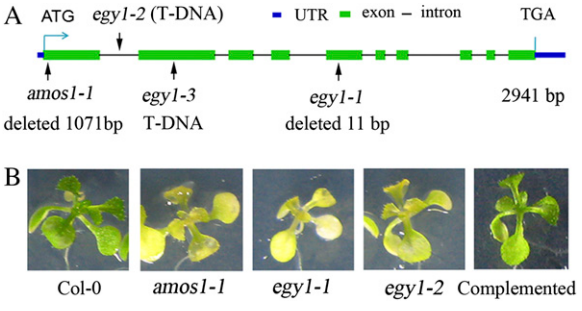

Figure 2. *AMOS1* identification. A, Diagram illustrating the genomic coding sequence of the Arabidopsis *AMOS1/EGY1* gene and the locations of the mutant alleles *amos1-1*, *egy1-1*, *egy1-2*, and *egy1-3*. UTR, Untranslated region. B, Phenotypes of 7-d-old seedlings of wild-type Col-0, *amos1-1*, *egy1-1*, *egy1-2*, and *amos1-1* complemented with *AMOS1/EGY1* (T3 generation), treated with 25 mM NH_4^+ for 7 d. One representative image is shown for each line. The experiments were reproduced at least twice.

Regulation of the Expression of NH_4^+ Stress-Responsive Genes by *AMOS1/EGY1*

To elucidate the molecular function of *AMOS1/EGY1* in the NH_4^+ stress response, we used full genomic microarray hybridization to analyze the difference of the Arabidopsis transcriptome between *amos1* and the wild type under both NH_4^+ and mock conditions. First, we analyzed the effect of the *AMOS1/EGY1* mutation on the transcriptional responses under both conditions. Only four genes were expressed at higher levels, but 888 genes were expressed at lower levels in *amos1* seedlings under both mock and NH_4^+ conditions, compared with the wild type ($q \leq 5$ and fold change ≥ 2 or ≤ 0.5, respectively; Fig. 3A; Supplemental Data Set S1). Of these, 867 (97.2%) were lower in *amos1* than in the wild type exclusively under NH_4^+ stress (Fig. 3A). Surprisingly, the most strongly enriched Gene Ontology (GO) categories in cellular components of these genes were in the nucleus, with only a small representation of chloroplasts (Fig. 3B). GO categories in biological processes indicated that genes in a broad range of pathways known to be associated with biotic and abiotic stress responses, such as "response to chitin," "response to heat," "cell redox homeostasis," "toxin catabolism," "autophagy," and "response to oxidative stress," were expressed at lower levels in the *amos1* mutant than in the wild type under NH_4^+ stress (Fig. 3C). Consistently, among the 10 genes showing the largest alterations in transcript levels in *amos1*, eight were repressed (Fig. 3D). Additionally, it is noteworthy that three small *HEAT SHOCK PROTEINs* (*HSPs*) were among the repressed genes (Fig. 3D). These results indicate that in the presence of NH_4^+, the full level of expression of nucleus-encoded genes associated with several biotic and abiotic stress-response pathways depends on plastid *AMOS1/EGY1*.

To test whether the genes showing lower expression in NH_4^+-exposed *amos1* plants than in the wild type are part of the transcriptional NH_4^+ response of Arabidopsis, we further analyzed the genome-wide transcriptional response of the wild type and *amos1* to NH_4^+ stress. Eighty-six activated genes and 42 repressed genes were found in the wild type. In *amos1*, the number of genes activated as a consequence of NH_4^+ exposure was reduced to 23, whereas the number of repressed genes was increased to 199 (Fig. 4A; Supplemental Data Set S2). Thus, accurate expression of 90% (78) of the NH_4^+-activated genes and 62% (26) of the NH_4^+-repressed genes depended upon *AMOS1/EGY1* (Fig. 4A). These results support the conclusion that *AMOS1/EGY1* is indeed required for regulation of the expression of NH_4^+-responsive genes, particularly those genes showing higher transcript levels in NH_4^+-exposed plants. The largest group of 183 genes, however, showed a decrease in transcript levels in response to NH_4^+ exposure in *amos1* without responding to NH_4^+ in the wild type.

A comparison of GO categories in biological processes of *AMOS1/EGY1*-dependent NH_4^+-responsive

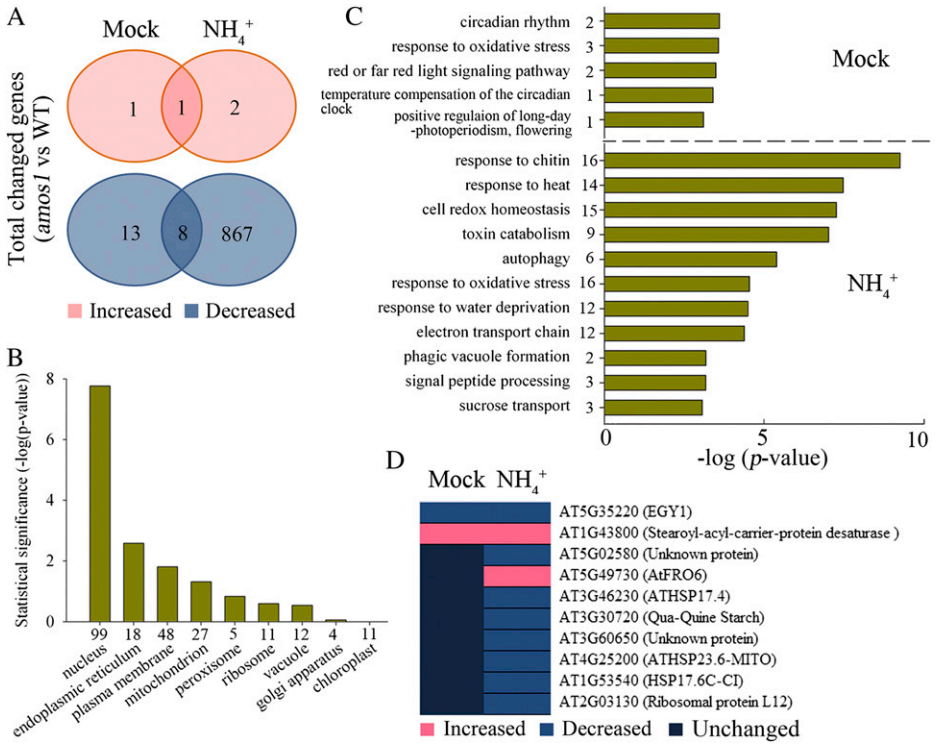

Figure 3. Transcriptional profile in *amos1* compared with the wild type (WT) under NH_4^+ stress. A, Total number of genes for which transcript levels were significantly changed in *amos1* relative to the wild type after mock treatment or NH_4^+ stress conditions imposed for 6 h ($q \leq 5$ and fold change ≥ 2 or ≤ 0.5). B, Enriched GO categories in cellular components of 867 genes expressed at significantly lower levels in *amos1* than in the wild type under NH_4^+ stress. C, Enriched GO categories for biological processes of 867 genes expressed at significantly lower levels in *amos1* than in the wild type under NH_4^+ stress ($P < 0.001$). Numbers in B and C indicate the counts of each GO category that appear in the 867 genes. D, The 10 genes exhibiting the largest difference in transcript levels in *amos1* (relative to the wild type) under NH_4^+ conditions (mock conditions given for reference).

genes (Fig. 4B) and the 867 genes repressed in *amos1* under NH_4^+ stress (Fig. 3C) revealed that "response to chitin" was the most enriched biological process in the category of NH_4^+-repressed genes in the wild type and, more pronouncedly, in *amos1*. However, "response to heat," "toxin catabolism," "response to oxidative stress," and "Suc transport" emerged as NH_4^+-activated biological processes in the wild type but not in *amos1*. The biological process "cell redox homeostasis" was only found among NH_4^+-repressed genes of *amos1*, but not the wild type. Other biological processes, such as "autophagy," were not enriched among NH_4^+ stress-

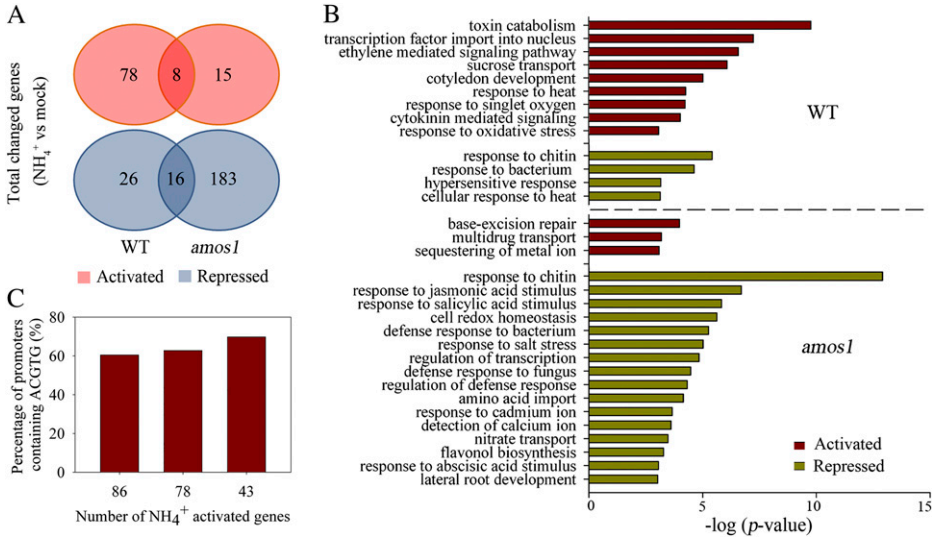

Figure 4. Genome-wide responses of transcript levels to NH_4^+ in shoots of *amos1* and the wild type (WT). A, Numbers of genes showing significant changes in transcript levels in response to NH_4^+ in the wild type and *amos1* according to the set thresholds (NH_4^+ relative to mock, q [%] ≤ 5 and fold change ≥ 2 or ≤ 0.5, respectively). B, Enriched GO categories for biological processes of the NH_4^+-activated (78 in the wild type and 15 in *amos1*) and NH_4^+-repressed (26 in the wild type and 183 in *amos1*) genes depending on *AMOS1/EGY1*. C, Percentage of promoters containing at least one occurrence of the ACGTG element in wild-type NH_4^+-activated genes: 86, NH_4^+-activated genes in the wild type; 78, *AMOS1/EGY1*-dependent NH_4^+-activated genes; 43, *AMOS1/EGY1*-dependent NH_4^+-activated genes in which transcript levels in *amos1* are 50% or lower of those in the wild type during NH_4^+ stress.

responsive genes. Additionally, "transcription factor import into nucleus," "ethylene-mediated signaling pathway," "cotyledon development," and "response to singlet oxygen" were enriched among NH_4^+-activated genes in the wild type and also appeared to depend on *AMOS1/EGY1* function (Fig. 4B), but they were not identified as significantly less active in *amos1* relative to the wild type (Fig. 3C). Unexpectedly, despite the severe leaf chlorosis, there were few known genes involved in chloroplast development or chlorophyll biosynthesis, except coproporphyrinogen III oxidase (*hemf2*), which was reduced to 22% at the transcript level in *amos1* but remained at 75% in the wild type under the NH_4^+ condition (relative to mock) among the NH_4^+-repressed genes in *amos1* (Fig. 4B; Supplemental Data Set S2).

The Involvement of ABA Signaling as a Downstream Component of the *AMOS1/EGY1*-Dependent Response to NH_4^+

To identify the potential signaling pathway involved in the *AMOS1/EGY1*-dependent NH_4^+ stress-responsive gene expression, we first analyzed the regulatory elements enriched in 500-bp regions of the sequence upstream of the promoters of the identified *AMOS1/EGY1*-dependent NH_4^+-activated and NH_4^+-repressed genes in the wild type (Fig. 4A). No significantly overrepresented regulatory element was found among NH_4^+-repressed genes. However, there were two motifs, ACGTG and CCCTCA, that were significantly overrepresented in the promoter regions of these *AMOS1/EGY1*-dependent activated genes (Supplemental Table S2). Moreover, only ACGTG remained enriched among the 43 *AMOS1/EGY1*-dependent NH_4^+-activated genes that were at least 2-fold down-regulated in *amos1* relative to the wild type (Supplemental Table S2). Additionally, we analyzed the number of genes with the occurrence of ACGTG at least once in the promoter regions among the 86 NH_4^+-activated genes, the 78 *AMOS1/EGY1*-dependent NH_4^+-activated genes, and the 43 *AMOS1/EGY1*-dependent NH_4^+-activated genes that were at least 2-fold down-regulated in *amos1* relative to the wild type. The data show that the percentage of genes with the promoter containing ACGTG was as high as 60% in the NH_4^+-activated genes and approached 70% in the latter category of 43 genes (Fig. 4C). The ACGTG motif is known as the core motif in both the light-responsive G-box (CACGTG; Terzaghi and Cashmore, 1995) and the ABA response element (Hattori et al., 2002; Himmelbach et al., 2003). This finding suggests that ABA, one of the principal phytohormones, might be involved in *AMOS1/EGY1*-mediated expression of NH_4^+-responsive genes and enhance NH_4^+ tolerance during NH_4^+ stress. Indeed, application of 1 μM ABA in the medium reversed the NH_4^+-induced suppression of chlorophyll accumulation and chloroplast fluorescence in *amos1* seedlings. However, ABA did not improve chlorophyll content in *amos1* under mock conditions (Fig. 5, A and B). The recovery of *amos1* seedlings by ABA was also confirmed in the other allelic mutants, *amos1-2* and *egy1-2* (Fig. 5B). These results indicate that ABA indeed rescues the NH_4^+ hypersensitivity of *amos1* seedlings.

We further asked whether the rescue of NH_4^+ hypersensitivity of *amos1* seedlings by ABA application is the consequence of a readjustment of the expression *AMOS1/EGY1*-dependent NH_4^+-responsive genes or of a reduction in NH_4^+ accumulation in both *amos1* and the wild type. To test these two hypotheses, we analyzed the global expression of *AMOS1/EGY1*-dependent NH_4^+-responsive genes and the NH_4^+ content of wild-type and *amos1* seedlings cultivated in NH_4^+ medium with or without ABA. Among the 78 *AMOS1/EGY1*-dependent NH_4^+-activated genes in the wild type, the majority of genes recovered to an expression level in *amos1* resembling that of the wild type following 1 μM ABA treatment for 6 h (Fig. 5C). Similarly, application of ABA also increased the expression of most *AMOS1/EGY1*-dependent NH_4^+-repressed genes (183 genes; Fig. 4A) in *amos1* to the level of the wild type (Supplemental Fig. S6A). However, it was interesting to find that there were three small *HSP* genes among a total of six *AMOS1/EGY1*-dependent NH_4^+-activated genes for which transcript levels were still at least 2-fold lower in *amos1* than in the wild type even following application of ABA (Supplemental Fig. S6B). In the wild type, the expression of these small *HSP* genes was almost identical with or without application of ABA under NH_4^+ stress (Supplemental Fig. S6B). These results indicate that under NH_4^+ stress, the small *HSP* response may occur in parallel with ABA signaling. In contrast to gene expression levels, NH_4^+ accumulation in shoots of *amos1* and wild-type seedlings was not affected by the application of 1 μM ABA (Fig. 5D). Collectively, these results show that during NH_4^+ stress, enhanced ABA signaling can compensate for the loss of the *AMOS1/EGY1*-dependent protection in *amos1* seedlings.

To investigate how ABA signaling is regulated by the *AMOS1/EGY1*-dependent pathway under NH_4^+ stress, we analyzed ABA levels in *amos1* and wild-type shoots. An approximately 2-fold increase in ABA content was observed in wild-type shoots under NH_4^+ exposure relative to mock treatment (Fig. 5E), consistent with previous observations (Peuke et al., 1994; Omarov et al., 1998). Unexpectedly, ABA content was about 5-fold higher in *amos1* shoots than in the wild type under mock treatment conditions. However, ABA content was dramatically decreased in *amos1* shoots upon NH_4^+ treatment (Fig. 5E), to levels that were 42.6% of those detected in the wild type under the same condition. These results suggest that the NH_4^+-induced ABA accumulation depends on *AMOS1/EGY1*, but NH_4^+ can also decrease ABA levels in *amos1* via another pathway. It is alternatively possible that exposure to inhibitory NH_4^+ exacerbates the chloroplast defect of *amos1* to an extent that ABA biosynthesis is compromised, which then leads to NH_4^+-induced

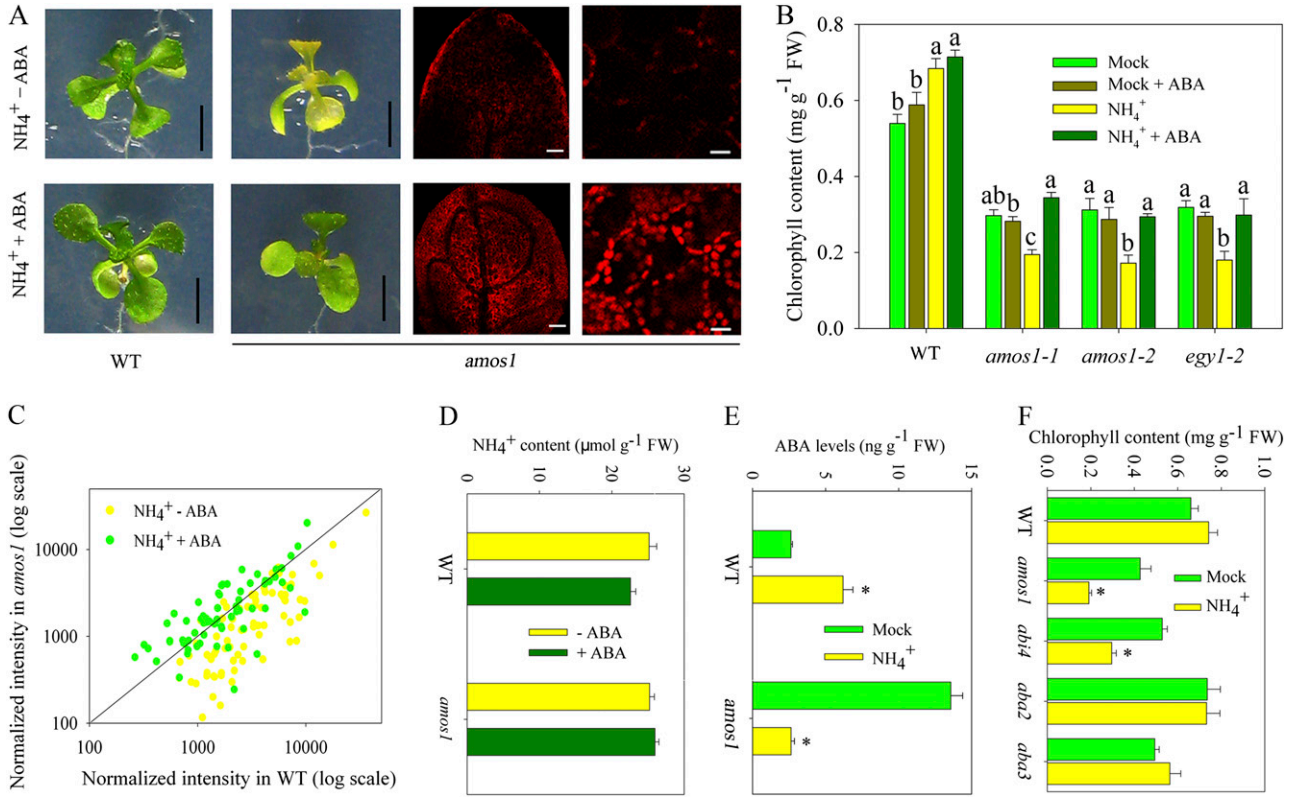

Figure 5. ABA treatment can readjust the expression of NH_4^+-responsive genes and alleviate NH_4^+ stress in *amos1* seedlings. A, Recovery of chlorophyll accumulation in leaves (left two; bar = 0.3 cm), chlorophyll fluorescence of true leaves (middle; bar = 100 μm), and chloroplasts (right; bar = 10 μm) in *amos1* seedlings grown in NH_4^+ medium through the application of external ABA for 7 d. One representative sample is shown for each type. B, Quantification of chlorophyll accumulation in 7-d-old wild-type (WT), *amos1-1*, *amos1-2*, and *egy1-2* seedlings with and without ABA treatment. Seedlings in A and B were treated with mock (12.5 mM K_2SO_4), mock + ABA (1 μM), NH_4^+ [12.5 mM $(NH_4)_2SO_4$], or NH_4^+ + ABA (1 μM) for 7 d ($n = 3$). Different letters indicate significantly different means between treatments within a given genotype (one-way ANOVA with Duncan's multiple comparison test, $P < 0.05$). FW, Fresh weight. C, Effects of external ABA on transcript levels of the 78 genes activated in response to NH_4^+ in an *AMOS1/EGY1*-dependent manner. Transcript levels of these selected genes are shown in scatterplots of *amos1* versus the wild type in medium containing 20 mM $(NH_4)_2SO_4$ with or without 1 μM ABA for 6 h. Each data point represents the arithmetic mean of three replicate experiments. D, Effects of external ABA on NH_4^+ accumulation in shoots of *amos1* and the wild type under exposure to NH_4^+ ($n = 4$; Student's *t* test, $P < 0.05$). Treatments were as in B. E, Endogenous ABA accumulation in shoots of 7-d-old seedlings of the wild type and *amos1* treated with mock and NH_4^+ (25 mM) for 1 d ($n = 4$). F, Chlorophyll accumulation in *abi4*, *aba2*, and *aba3* mutants exposed to NH_4^+. Seven-day-old seedlings of different Arabidopsis genotypes were treated with mock and NH_4^+ (25 mM) for 7 d. Asterisks indicate significant differences between mock and NH_4^+ treatment for the given genotype ($n = 4$; Student's *t* test, $P < 0.01$). All values represent means ± SE.

chlorosis in this genotype. We proceeded to test whether a defect of ABA signaling alone can bring about hypersensitivity of chloroplasts to NH_4^+ in the wild type. We analyzed chlorophyll accumulation in the ABA-insensitive *abi4* mutant and the ABA-deficient *aba2* and *aba3* mutants, in the Col-0 background, under NH_4^+ stress. Chlorophyll accumulation in *abi4* was decreased under NH_4^+ stress, as was found in *amos1*. By contrast, NH_4^+ stress did not result in a reduction of chlorophyll contents in the two ABA-deficient mutants (Fig. 5F). It has previously been reported that the ABA-insensitive mutant *abi4* is defective in retrograde signaling from plastid to nucleus, while the other ABA-insensitive or -deficient mutants are not impaired (Koussevitzky et al., 2007). These results indicated that a defect in ABA signaling that is possibly connected to plastid retrograde signaling, and not a defect in ABA biosynthesis, is responsible for the hypersensitivity of *amos1* to NH_4^+.

Recently, mild production of ROS in chloroplasts has been proposed as an important plastid retrograde signal triggering processes that protect the plant from moderate environmental stress (Galvez-Valdivieso and Mullineaux, 2010). Therefore, we tested whether the ROS response in chloroplasts is affected in *amos1* during NH_4^+ stress. To quantify the ROS response in *amos1* seedlings under NH_4^+ stress, we analyzed the accumulation of hydrogen peroxide (H_2O_2) using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescence. The signal of H_2DCFDA fluorescence in leaves was indeed enhanced in wild-type and *amos1*

seedlings when exposed to high levels of NH_4^+ for 6 h. It was interesting to find that the accumulation of H_2O_2 in guard cell chloroplasts of *amos1* seedlings was markedly lower than in the wild type upon exposure to high NH_4^+ (Fig. 6). Stomatal density was almost identical between the wild type and *amos1* (Supplemental Fig. S7), which suggests that the reduced H_2O_2 formation is not a consequence of reduced stomatal density. Furthermore, the H_2O_2 response in *amos1* seedlings could not be restored by the application of ABA (Fig. 6). This indicated that the ROS signaling capacity is compromised in chloroplasts of *amos1* seedlings under NH_4^+ stress and might play a role as a component of *AMOS1/EGY1*-dependent ABA signaling upstream, but not downstream, of ABA.

DISCUSSION

In the work presented here, we identified the Arabidopsis *amos1* mutant, characterized by hypersensitivity to excess NH_4^+ in leaves, manifest as severe chlorosis, whereas chlorophyll content remained normal in wild-type seedlings. We further revealed that the nuclear *AMOS1* locus is identical to *EGY1*, which encodes a membrane-bound and ATP-independent metalloprotease localized to plastids that is required for chloroplast biogenesis (Chen et al., 2005). The allelic *amos1* mutation did not disturb tissue NH_4^+ distribution or cause NH_4^+ hyperaccumulation in shoots, different from the previously characterized mutant *amos2* (Li et al., 2012). The analysis of the *amos1/vtc1* double mutant indicates that the NH_4^+-induced chlorosis in *amos1* is also not related to the *GMPase* gene, which has been implicated in the sensitivity of root development to NH_4^+ (Qin et al., 2008). Transcriptomic analysis identified 86 NH_4^+-activated genes, the expression of 90% of which depended on the function of *EGY1*. Furthermore, the number of NH_4^+-activated genes was decreased 3.7 times in *amos1* compared with the wild type, whereas the number of NH_4^+-repressed genes was increased almost five times in *amos1* relative to the wild type. Therefore, the metalloprotease *EGY1* in chloroplasts is an important component required for the correct regulation of NH_4^+-responsive transcript levels of nuclear genes.

ABA is a key hormone in the orchestration of stress signal transduction and defensive responses in plants (Xiong et al., 2002; Hubbard et al., 2010). ABA signaling may be involved in the NH_4^+ stress signal indicated by the enhanced ABA content in plants under the NH_4^+ condition (Peuke et al., 1994; Omarov et al., 1998). However, how exactly ABA signaling regulates NH_4^+ stress remains largely to be investigated. In this study, we have presented bioinformatic, pharmacological, physiological, genomic, and genetic data to show that ABA signaling acts as an important downstream component of the *AMOS1/EGY1*-dependent plastid retrograde signaling pathway to regulate the expression of NH_4^+ stress-responsive genes and to enhance chloroplast functionality under NH_4^+ stress. Furthermore, our results show that ABA signaling is not the sole downstream component of the *AMOS1/EGY1*-dependent NH_4^+ responses, because a very small portion of *AMOS1/EGY1*-dependent genes, such as small *HSP* genes, are still defective in *amos1* seedlings following the application of ABA. Recently, these small *HSP* genes have been shown to be required for the targeting of chloroplast outer membrane proteins (Kim et al., 2011). Furthermore, these *HSP* genes are activated by a chloroplast-ribosomal-protein-S1-dependent retrograde signaling during heat stress responses (Yu et al., 2012). These combined results indicate that, in addition to ABA-mediated signaling, an ABA-independent response (particularly small *HSP* genes) may act as another downstream component of the plastid *AMOS1/EGY1*-dependent retrograde pathway to maintain chloroplast functionality under NH_4^+ stress. Is defective ABA signaling alone sufficient to cause the hypersensitivity of chloroplasts to NH_4^+ in Arabidopsis?

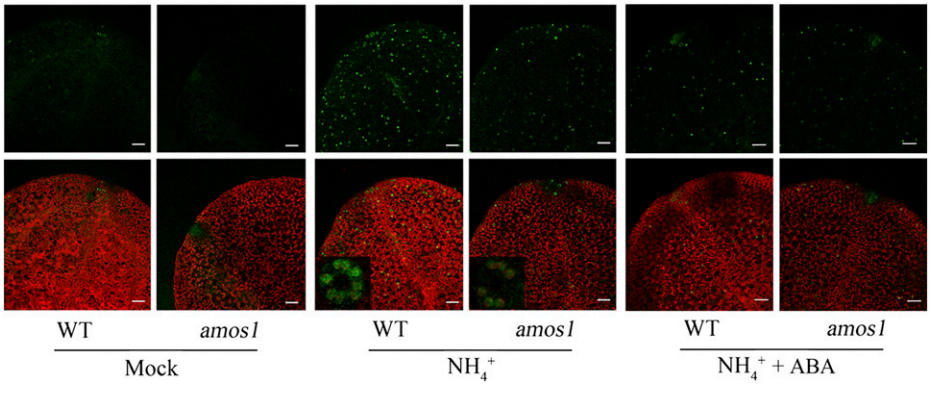

Figure 6. H_2O_2 response to NH_4^+ challenge and ABA treatment in guard cell chloroplasts of *amos1* and wild-type (WT) seedlings. Seven-day-old *amos1* and wild-type seedlings were treated with mock (12.5 mM K_2SO_4), NH_4^+ [12.5 mM $(NH_4)_2SO_4$], or NH_4^+ + ABA (1 μM) for 6 h, then H_2O_2 fluorescence (green, top row) and chloroplast fluorescence (red, bottom row) were observed. The insets show a single guard cell. A representative image from six individual leaves for each treatment is shown. The experiments were reproduced twice. Bars = 100 μm.

Our data show that ABA-deficient mutants were not hypersensitive to NH_4^+ stress, whereas *abi4* was sensitive to NH_4^+ stress. Considering that *ABI4* is implicated in both ABA and retrograde signaling (Koussevitzky et al., 2007), the sensitivity of the *abi4* mutant to NH_4^+ stress may be due to defects in both ABA and *ABI4*-mediated retrograde signaling. Therefore, a defect in ABA signaling that is possibly connected to plastid retrograde signaling, and not a defect in ABA biosynthesis, is responsible for the hypersensitivity of *amos1* to NH_4^+. The observations described here differ from those on ABA signaling in other abiotic stresses such as salinity or osmotic stress, in which the sensitivities of both ABA-insensitive and -deficient mutants are altered (Xiong et al., 2002; Hubbard et al., 2010).

The plastid-specific localization of the AMOS1/EGY1 protein, the specifically impaired chloroplast functionality, the defect in the H_2O_2 response of guard cell plastids, and the reduced expression of stress-associated nuclear genes occurring in *amos1* seedlings point to the importance of chloroplasts in the resistance to NH_4^+ stress and to their importance as a source of signals regulating nuclear gene expression during NH_4^+ stress. These results thus suggest the existence of a retrograde pathway in the regulation of cellular NH_4^+ stress responses. Plastid retrograde signaling is known to regulate nuclear gene expression, which serves not only to coordinate nucleus-encoded chloroplast protein levels but also to mediate plant stress responses such as those to high-light and drought stress (Fernández and Strand, 2008; Estavillo et al., 2011). ROS production in chloroplasts has frequently been proposed to act in plastid retrograde signaling (Kleine et al., 2009; Galvez-Valdivieso and Mullineaux, 2010). Recently, H_2O_2 in chloroplasts has been shown to trigger retrograde signaling, thus regulating the expression of nucleus-encoded genes involved in both biotic and abiotic stress responses, for example to pathogen attack, chilling, and high light (Maruta et al., 2012). Additionally, H_2O_2 in chloroplasts has been proposed to regulate the expression of nucleus-encoded *HSP* genes under heat stress (Yu et al., 2012). Therefore, H_2O_2 produced in guard cell chloroplasts may constitute an *AMOS1/EGY1*-dependent plastid retrograde signal, because the H_2O_2 response is defective in guard cell chloroplasts in *amos1* during NH_4^+ stress. As the H_2O_2 response in guard cell chloroplasts was not restored by ABA, it may be the upstream event of *AMOS1/EGY1*-dependent ABA signaling during the cellular NH_4^+ stress response. Interestingly, ABA signaling has been implicated in the H_2O_2-dependent plastid signal to activate high-light-responsive gene expression in leaves (Galvez-Valdivieso et al., 2009) and in the $^1O_2$-dependent plastid signal during late embryogenesis to predetermine plastid differentiation by reactivating relevant nucleus-encoded genes (Kim et al., 2009), although ABA is not regarded as a direct or actual candidate of the plastid signal (Koussevitzky et al., 2007). Thus, ABA signaling is proposed to integrate ROS-dependent plastid signaling (Kleine et al., 2009; Galvez-Valdivieso and Mullineaux, 2010). Therefore, this study provides evidence that ABA signaling is integrated into an *AMOS1/EGY1*-dependent chloroplast signal to regulate the expression of NH_4^+-responsive genes.

There have been two recent reports on primary retrograde signaling in plastids: first, a Mg-protoporphyrin IX/heme-dependent plastid retrograde signaling mechanism has been proposed to regulate nucleus-encoded chloroplast proteins (Koussevitzky et al., 2007); second, $^1O_2$-dependent plastid retrograde signaling has been proposed to play a central role in the activation of a stress-related signaling cascade rather than direct control of chloroplast biogenesis (Lee et al., 2007). Our work indicates that the primary function of the *AMOS1/EGY1*-mediated NH_4^+-related retrograde signaling lies in the activation of a stress-response signaling cascade during NH_4^+ stress that is more similar to $^1O_2$-dependent plastid retrograde signaling than to Mg-protoporphyrin IX/heme-dependent plastid retrograde signaling. Therefore, ROS release in chloroplasts is proposed to participate in *AMOS1/EGY1*-dependent plastid retrograde signaling. The H_2O_2 response defect in guard cell chloroplasts during NH_4^+ stress also supports this hypothesis. However, we cannot exclude the involvement of other ROS signals or precursors of chlorophyll in plastid signaling in the *amos1* seedling response to NH_4^+ stress. Clearly, the role of the dampened H_2O_2 response in *AMOS1/EGY1*-dependent chloroplast retrograde signaling and NH_4^+ hypersensitivity of *amos1* warrants future experimentation.

A further interesting question that arises from this study is how the NH_4^+-sensitive response and decrease in ABA and H_2O_2 accumulation are related to defective EGY1 metalloprotease activity in chloroplasts. The membrane-bound protease EGY1 is probably involved in chloroplast membrane protein metabolism, such as that of chlorophyll *a*/*b*-binding proteins, although the detailed mechanisms for this are as yet unknown (Chen et al., 2005). Excess NH_4^+ is well known for its harmful effects on chloroplast development and photosynthesis (Britto and Kronzucker, 2002; Drath et al., 2008). Therefore, considering *EGY1*'s role as a metalloprotease S2P homolog in plants (Chen et al., 2005; Chen and Zhang, 2010), the enzyme may be expected to be involved in cleaving chloroplast membrane proteins that suffered structural integrity in the process of NH_4^+ treatment and, thus, maintaining the turnover and assembly of the membrane-associated components of PSI and PSII. A key precursor for ABA synthesis is derived from carotenoids in chloroplasts (Cutler and Krochko, 1999), and H_2O_2 is generated by photoreduction of oxygen in PSI under many stress conditions (Asada 1999). Our results show that the NH_4^+-induced ABA and H_2O_2 accumulation depend on *AMOS1/EGY1*, suggesting that NH_4^+-induced ABA and H_2O_2 biogenesis regulate normal chloroplast functionality. However, NH_4^+ can also decrease ABA

levels in *amos1* shoots. It is alternatively possible that exposure to inhibitory NH_4^+ exacerbates the chloroplast defect of *amos1* to an extent that ABA biosynthesis is compromised. Thus, the defect in chloroplast functionality in the *amos1* mutant may impair the regulation of ABA metabolism and H_2O_2 production under NH_4^+ stress. However, important points to note in studies of this kind are that care must be taken with the interpretation of data based on even ostensibly highly specific mutations, as pleiotropic effects are common, especially in alterations of aspects as profound as nitrogen metabolism and response to nitrogen toxicity (for a recent case where pleiotropies have been documented, see that of the role of GMPase in root NH_4^+ sensitivity; Qin et al., 2008; Barth et al., 2010; Kempinski et al., 2011). Further investigation of the substrates of AMOS1/EGY1 metalloprotease may reveal the underlying relationship clearly.

Additionally, we should point out that future studies will need to be designed to address the effects of variable nitrogen source, especially the copresence of nitrate, and Suc level, both of which can have fundamental shift effects on thresholds of NH_4^+ toxicity (Kronzucker et al., 1999; Britto and Kronzucker 2002; Qin et al., 2008; Li et al., 2011b). It is reasonable to speculate that nitrate metabolism and Suc level/signaling might be altered in *amos1* mutants as a secondary effect of the impairment of chloroplast function. In this study, we focused on the frequently used combination of nitrate and NH_4^+ in the Arabidopsis nutrient medium and a constant level of Suc (Hirsch et al., 1998; Rawat et al., 1999; Li et al., 2010, 2011b), conditions for which NH_4^+ toxicity development is nevertheless clearly seen (Fig. 1). In summary, we have identified a previously unrecognized molecular connection between the function of plastid *AMOS1/EGY1* and the activation of NH_4^+-responsive genes in the nucleus, which is required for NH_4^+ tolerance in Arabidopsis. Furthermore, we found that ABA signaling acts as an important downstream component of an *AMOS1/EGY1*-dependent plastid retrograde signaling pathway to regulate the expression of NH_4^+ stress-responsive genes and to enhance chloroplast functionality under NH_4^+ stress. The work reported here identifies a new component of the physiological adaptation of plants to high NH_4^+ levels in the soil.

MATERIALS AND METHODS

Arabidopsis Lines and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) mutants *amos1-1*, *amos1-2*, and *amos1-3* were isolated in this study (Supplemental Materials and Methods S1); *egy1-1* and *egy1-2* were reported previously (Chen et al., 2005) and provided by Dr. Ning Li; *vtc1-1*, *abi4-1*, *aba3-1*, and *aba2-3* were obtained from the Arabidopsis Biological Resource Center at Ohio State University. All mutants are in Col-0 background, except *egy1-1* (ecotype Wassilewskija background). The *amos1/vtc1* double mutant lines were generated by crossing *amos1-1* and *vtc1-1* mutants. Seeds were surface sterilized for 5 min in 10% (v/v) chlorine bleach and 0.1% (w/v) SDS, followed by five rinses with sterile distilled water. Seeds were stored in an Eppendorf tube in the dark at 4°C for 2 to 3 d before sowing on GM on 13- × 13-cm square plates. The GM was composed of 2 mM KH_2PO_4, 5 mM $NaNO_3$, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM Fe-EDTA, 50 μM H_3BO_3, 12 μM $MnSO_4$, 1 μM $ZnC1_2$, 1 μM $CuSO_4$, 0.2 μM Na_2MoO_4, 1% (w/v) Suc, 0.5 g L^{-1} MES, and 0.8% (w/v) agar (pH 5.7 adjusted with 1 M NaOH). Seedlings were kept in a growth chamber at 23°C ± 1°C under a light intensity of 100 μmol photons m^{-2} s^{-1} and a photoperiod of 16 h of light and 8 h of dark. Unless otherwise stated, NH_4^+ and mock treatments were in GM supplemented with 12.5 mM $(NH_4)_2SO_4$ and 12.5 mM K_2SO_4, respectively. Other chemical compound treatments were also provided as additions to GM, as indicated.

amos1 Mapping and Complementation

The *amos1* mutant was crossed with the Landsberg *erecta* ecotype to generate the mapping population. Mapping was performed as described previously (Peters et al., 2003; Li et al., 2012). In brief, DNA was extracted from 300 mutant F2 individual seedlings, 30 individuals as a mixed DNA pool for preliminary mapping and others for further mapping, and genotyped with known insertion/deletion (InDel) molecular markers (Salathia et al., 2007; Supplemental Table S3). The candidate gene was mapped between the InDel marker C5P71 and the centromere. Known mutants in the identified region and the *egy1* mutants were tested in NH_4^+ medium (Fig. 2B). Then, amplification of the genomic sequences for *EGY1* and an analysis by reverse transcription-PCR of the levels of *EGY1* mRNA were carried out in the *amos1* mutant (Supplemental Materials and Methods S1; Supplemental Fig. S5). Finally, the pCAMBIA 35S::EGY1-GFP construct, identified previously (Chen et al., 2005) and provided by Dr. Ning Li, was introduced into the *amos1* seedlings to complement the *amos1* mutant function (Fig. 2B).

Chlorophyll Quantification

Chlorophyll content was assayed according to Wintermans and de Mots (1965). Shoots were extracted with 96% (v/v) ethanol at 23°C for 16 h. Absorbance was measured at 665 and 649 nm, and chlorophyll concentration was calculated as milligrams per gram of fresh weight.

Determination of H_2O_2

H_2DCFDA fluorescence was used to detect the H_2O_2 response. Seven-old-day *amos1* and Col-0 seedlings were treated with 12.5 mM $(NH_4)_2SO_4$ or 12.5 mM K_2SO_4 for 6 h. The leaves were transferred into 25 μM H_2DCFDA in 50 mM potassium phosphate buffer (pH 7.4) solution for 30 min in the dark at 23°C and washed with the potassium phosphate buffer for 15 min (Behnke et al., 2010). The leaves were observed with a Zeiss LSM710 confocal microscope as described below.

Fluorescence and Image Analysis

Images of whole leaves were taken using a Canon G7 camera. Chlorophyll fluorescence and H_2DCFDA fluorescence were observed by a Zeiss LSM710 confocal microscope with the following settings: 561- and 488-nm excitation wavelengths; 600- to 700-nm and 505- to 520-nm emission wavelengths, respectively. The images were captured and analyzed using Zeiss 2009 software, using representatives of at least 10 individual plants from each treatment. Experiments were repeated at least twice. Graphs were prepared using SigmaPlot 10.0 software.

Determination of NH_4^+

Shoots (30–50 mg fresh weight) of each sample were washed with 10 mM $CaSO_4$, frozen in liquid nitrogen, and then extracted with 1 mL of 10 mM formic acid for the NH_4^+ content assay by HPLC, following derivatization with *o*-phthaldialdehyde as described previously (Husted et al., 2000; Balkos et al., 2010).

Endogenous ABA Assay

ABA was quantified using a HPLC-electrospray ionization-tandem mass spectrometry method (Chen et al., 2011) in three independent biological replicates and two technical repeats. In brief, shoots (120–150 mg fresh biomass) of each sample were collected, frozen in liquid nitrogen, and then finely

ground, followed by extraction with 1.0 mL of methanol containing 20% water (v/v) at 4°C for 12 h. [$^2H_6$]ABA (50 ng g^{-1}) was added to plant samples as the internal standard prior to grinding. After centrifugation (10,000 rpm, 4°C, 20 min), the supernatant was collected and passed through a C-18 (100 mg) SPE cartridge, which was preconditioned with 8 mL of water, 8 mL of methanol, and 8 mL of methanol containing water (20%, v/v). Eluates were pooled and evaporated under a stream of nitrogen gas and reconstituted in 1 mL of water. The solution was acidified with 120 μL of 0.1 mol L^{-1} HCl and extracted with ethyl ether (4 × 1 mL). The ether phases were combined, dried under nitrogen gas, and reconstituted in 80 μL of acetonitrile. To the resulting solution, 10 μL of trimethylamine (20 μmol mL^{-1}) and 10 μL of 3-bromoactonyltrimethylammonium bromide (20 μmol mL^{-1}) were added. The reaction solution was vortex mixed for 30 min and evaporated under nitrogen gas, reconstituted with 200 μL of acetonitrile containing 20% (v/v) water, and 10 μL of the solution was subjected to HPLC-electrospray ionization-tandem mass spectrometry analysis.

Microarray Analysis and Data Mining

Seven-day-old *amos1* and Col-0 seedlings were treated with 20 mM K_2SO_4 (mock), 20 mM $(NH_4)_2SO_4$, and 20 mM $(NH_4)_2SO_4$ with 1 μM ABA for 6 h. Total RNA was extracted from shoots using Trizol reagent (Invitrogen) and was further purified using NucleoSpin RNA clean-up (Macherey-Nagel) according to the manufacturer's instructions. The RNA was used to prepare Cy_3-dCTP-labled cDNA. Three independent biological replicate experiments were carried out, composed of approximately 80 pooled seedlings for each treatment. The NimbleGen Arabidopsis thaliana Gene Expression 12×135K array was used in these microarray experiments, which were carried out according to the NimbleGen Expression user's guide and performed at CapitalBio in Beijing, China. Unless stated otherwise, the differentially expressed genes were determined by the microarray software (SAM, version 3.02), with a selection threshold of false discovery rate (q [%] ≤ 5; Storey and Tibshirani, 2003) and fold change ≥ 2.0 in the SAM output result. The differentially expressed genes were evaluated for enrichment of biological functions and cellular components in GO categories by use of the CapitalBio Molecule Annotation System (http://bioinfo.capitalbio.com/mas3/). The results were converted into corresponding graphs with SigmaPlot 10.0 software, based on statistical significance (P value) and count (the total number of times the GO category occurred in these genes). Promoter analysis (http://element.cgrb.oregonstate.edu/) was performed as described previously (Nemhauser et al., 2004).

Statistical and Graphical Analyses

For all experiments, statistical analyses of data and graph production were carried out using SPSS 13.0 and SigmaPlot 10.0 software. Details are presented in the figure legends. All the images and graphs were arranged with Adobe Photoshop 7.0 software.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *AMOS1/EGY1* (At5G35220) and *CBP20* (At5G44200).

Supplemental Data

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

Supplemental Figure S1. Isolation and characterization of the *amos1* mutant.

Supplemental Figure S2. NH_4^+-induced chlorosis in leaves of *amos1* seedlings dependent on the direct contact between NH_4^+-containing medium and shoot tissues.

Supplemental Figure S3. Concentration dependence of chlorophyll concentrations and morphological parameters in *amos1* and the wild type in response to NH_4^+ stress.

Supplemental Figure S4. Phenotypes of *amos1/vtc1* double mutants exposed to NH_4^+.

Supplemental Figure S5. Map-based cloning of *AMOS1* and confirmation.

Supplemental Figure S6. Effect of external ABA on the levels of transcripts responsive to NH_4^+ in *amos1*.

Supplemental Figure S7. Stomatal density of wild-type and *amos1* seedlings.

Supplemental Table S1. Genetic analysis of the *amos1* mutants.

Supplemental Table S2. The regulatory elements enriched in 500-bp promoter regions upstream of *AMOS1/EGY1*-dependent NH_4^+-activated genes.

Supplemental Table S3. InDel markers selected from the database (Salathia et al., 2007) used for map-based cloning as described in Supplemental Figure S5.

Supplemental Data Set S1. Comparison of genes differentially expressed in *amos1* relative to Col-0 under NH_4^+ and mock treatment.

Supplemental Data Set S2. Comparison of genes differentially expressed in NH_4^+ relative to mock treatment in *amos1* and Col-0.

Supplemental Materials and Methods S1.

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Supplemental data

Supplemental Materials and Methods

Arabidopsis Lines

The T3 populations (5160 lines) of chemical-inducible (1,7-β-estradiol) activation tagging T-DNA insertion lines in Col-0 background (Zuo et al., 2000; Zhang et al. 2005) were used in *amos1* mutant isolation. The pooled seeds of chemical-inducible (1,7-β-estradiol) activation tagging T-DNA insertion lines were kindly provided by Dr. Jianru Zuo (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences).

amos1 Mutant Screening

Five-day-old seedlings grown in GM were transferred to GM supplemented with 30 mM $(NH_4)_2SO_4$ and the inducer 10 μM 1,7-β-estradiol for another seven days. The putative mutants with chlorosis in leaves were selected and rescued. The seeds of candidate mutants were re-screened as the first time. *amos1* mutants that were backcrossed twice at minimum were used for the further experiments.

Genetic Analysis

The seedlings of F_1 generated from *amos1-1, amos1-2, amos1-3* crossed with each other were tested on NH_4^+ medium for allele mutation analysis. The seedlings of F_1 and F_2 populations generated from *amos1-1, amos1-2, amos1-3* backcrossed with Col-0 wild type were tested on NH_4^+ medium for genetic separation analysis (Supplemental Table SI).

Root and Shoot Growth Analysis

Plants grown vertically oriented on square Petri dishes were used for the measurement of root growth. The primary root of individual seedlings was carefully straightened along the side of a ruler, and root length was recorded. The number of visible lateral roots of individual seedlings was counted as described previously (Li et al., 2011). The shoot fresh biomass of two to five individuals as a sample was determined using a high-precision balance (0.000001) (XP105, Mettler Toledo).

Shoot- or Root-supplied NH_4^+ Experiments

The protocol for supplying NH_4^+ to shoot and root separately was as described previously (Li et al., 2010, 2011).

Analysis of Stomatal Density

Stomatal density was determined for the leaves of wild type (Col-0) and *amos1* mutant plants by light microscopy of nail polish imprints as described (Yu et al., 2008). Seven-day-old *amos1* and WT seedlings were treated with mock (12.5 mM K_2SO_4), NH_4^+ (12.5 mM $(NH_4)_2SO_4$) for 6 h. The number of stomata was counted in six individual leaves for each treatment. Stomatal density was described as the number of stomata per mm^2.

DNA, RNA Extraction and PCR Analysis

DNA was extracted with TPS [100 mM Tris-HCl (pH 8.0), 1 M KCl, 10 mM EDTA] method (Miura et al., 2009) and CTAB (Weigel and Glazebrook, 2002) for *amos1* mutant mapping and the genomic sequence of *EGY1* gene PCR amplification, respectively. Total RNA was isolated with RNAiso Reagent (TaKaRa). cDNA was synthesized from 1 μg total RNA with Superscript transcriptase M-MLV (TaKaRa) and used as template for PCR amplification with specific primers. The primers used for mapping are shown in Supplemental table III. The genomic sequence of the *EGY1* gene in *amos1* and Col-0 was amplified using the primers 5'- AGGATCGTAACCGAACCGTTTCT-3' and 5'-ACTCCGGTCAGGAAGAATCGACT-3', and the cDNA of EGY1 by using the primers 5'-TGCCCAAATGGCAAAAGAGACTCTG-3' and 5'-CAGCATGCAAAGCTGCATATCCC-3'. CBP20 was chosen as the housekeeping gene for mRNA expression with the primers 5'-ACCATCGGAAACGACAAAGAG-3' and 5'-CTTCACCATCGTCATCGGAGT-3'.

Arabidopsis Transformation

The pCAMBIA 35S::EGY1-GFP construct was transformed into *amos1* plants using the floral-dip method (Clough and Bent, 1998), and transgenic lines were selected on 50 mg l^{-1} hygromycin in MS medium.

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Supplemental Table SI. Genetic anaysis of the *amos1* mutants

Cross	Generation	Individuals response to NH_4^+		x^2
		No. (Resistant)	No.(Sensitive)	
amos1-1 x *amos1-2*	F_1		30	
amos1-1 x *amos1-3*	F_1		82	
amos1-2 x *amos1-3*	F_1		53	
amos1-1 x WT	F_1	52		
	F_2	128	41	0.018
amos1-2 x WT	F_1	55		
	F_2	114	31	0.083
amos1-3 x WT	F_1	50		
	F_2	111	40	0.27

NH_4^+-grown seedlings for which leaves appeared similar to the wild type (WT, Col-0) were scored as NH_4^+ resistant, and seedlings for which leaves appeared similar to *amos1* mutant were scored as NH_4^+ sensitive. The x^2 values are based on an expected ratio of 3:1 (resistant: sensitive) ($P > 0.05$).

Supplemental Table SII. The regulatory elements enriched in 500 base pair (bp) promoter regions upstream of *AMOS1/EGY1*-dependently NH_4^+-activated genes.

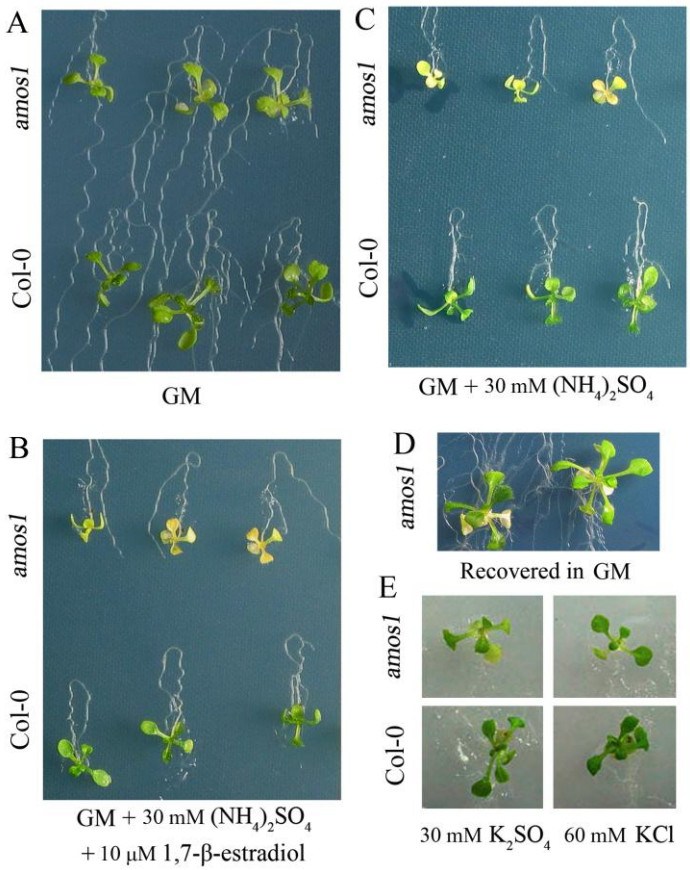
Genes	Word	Total count	Expected total count	Z-score	Promoter count	Expected promoter count	*p*-value
86	ACGTG	101	53	4.9	52	31	2.8e-06
	CCCTCA	22	7	5.4	20	7	1.1e-05
78	ACGTG	97	48	5.2	49	28	1.1e-06
	CCCTCA	20	7	5.2	19	6	8.8e-06
43	ACGTG	59	26	4.7	30	15	6.1e-06

Note: 86: NH_4^+-activated genes; 78: *AMOS1/EGY1*-dependent NH_4^+-activated genes; 43 *AMOS1/EGY1*-dependent NH_4^+-activated genes that in *amos1* were at least 2-fold down-regulated relative to wild type.

Supplemental Table SIII. InDel markers selected from the database (Salathia et al. 2007) used for map-based cloning as described in Supplemental Fig. S5.

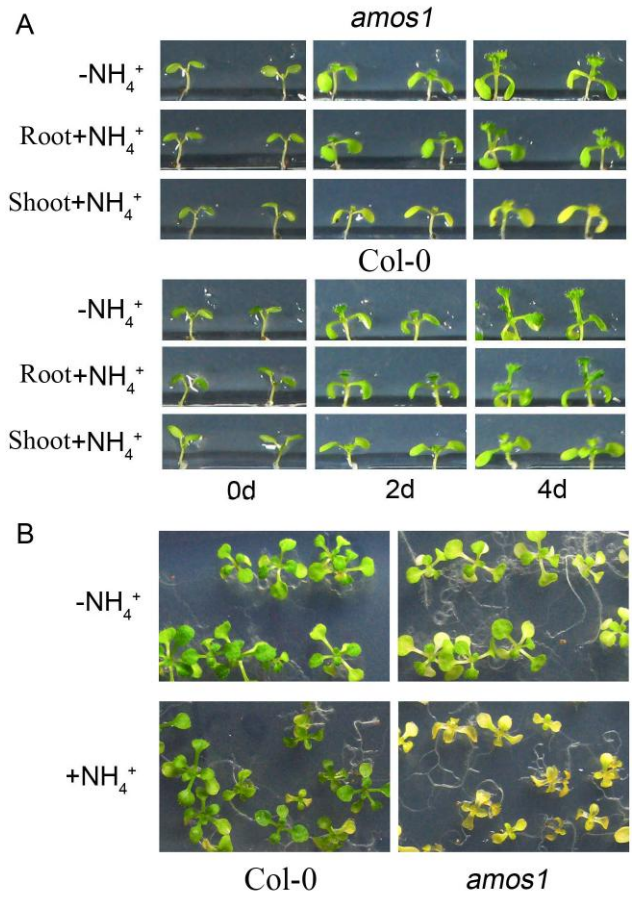
Marker Cereon NO. /rename	Size (bp) Col/Ler	Position (bp)	Primer (5'→3')
Cer456556 /C5P71	589/550	13718452	F:CCTCATTTTTATAAACCCAAACCA R: GAACCGGGCATAGAAGTCAA
Cer455569 /C5P73	550/507	14154436	F: CACCTTCCTTCCACCTCAGA R: TTGCCGAAGCTAGACTGGAT
Cer456453 /C5P76	580/534	14792975	F: CCTGATGAACAGAACGGAAGA R: TCGCTTCTTCTGGTGTTCCT
457357 /C5P81	608/494	15810692	F: GAAGTGTGGCTCTCCAATCC R: AAAGCACAAGCCATTTGACC

F: forward primer, R: reverse primer.

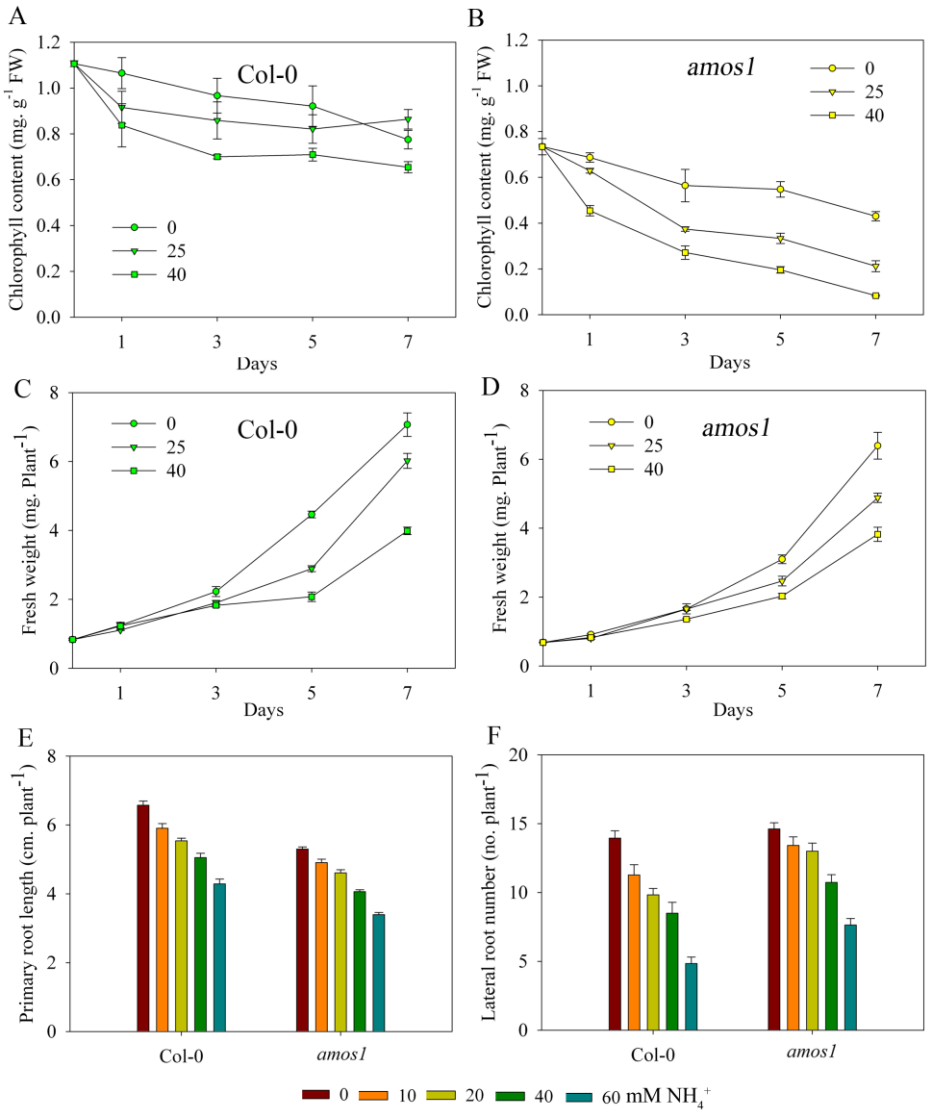


Supplemental Figure S1. Isolation and characterization of the ammonium overly sensitive *amos1* mutant.

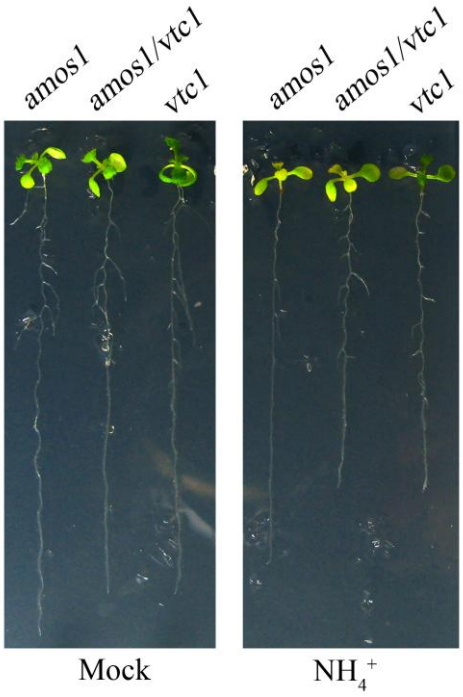
(A-C) Morphology of *amos1* and wild type (Col-0) seedlings germinated and grown on the growth medium (GM) for 5 days and then transferred to GM and GM supplemented with 30 mM $(NH_4)_2SO_4$ with or without inducer (10 μM 1,7-β-estradiol) for seven days, respectively. (D) The chlorotic *amos1* seedlings in (B) were transferred into the GM medium without $(NH_4)_2SO_4$ for another 10 days. (D) WT and *amos1* seedlings were grown for five days on GM and then transferred to GM supplemented with the given salts as indicated for seven days. Representative seedlings from 15 seedlings in total each treatment are shown. The experiments were reproduced at least twice.



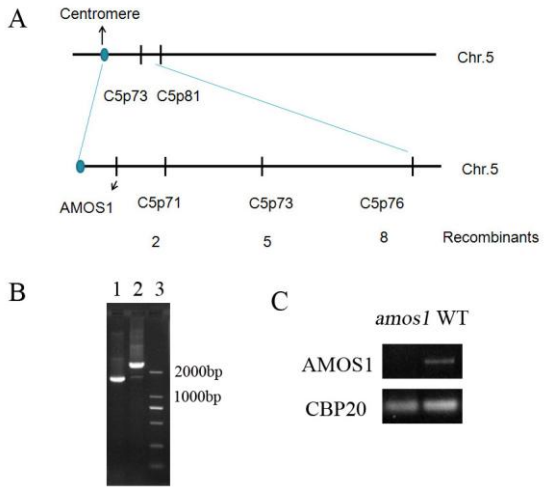
Supplemental Figure S2. NH_4^+-induced chlorosis in leaves of *amos1* seedlings dependent on the direct contact between NH_4^+-containing medium and shoot tissues. (A) Five-day-old *amos1* and WT seedlings in normal GM medium were transferred to control (-NH_4^+), root supplied NH_4^+ (root + NH_4^+) or shoot supplied NH_4^+ (shoot + NH_4^+), respectively. NH_4^+ was supplied as 30 mM $(NH_4)_2SO_4$. Two representative plants for each treatment are shown from three independent replicate experiments in total. (B) Photographs of *amos1* and WT germinated and grown on GM medium with or without 40 mM NH_4^+ for 14 days. Representative images are shown from two independent replicate experiments.



Supplemental Figure S3. Concentration-dependence of chlorophyll concentrations and morphological parameters in *amos1* and WT in response to NH_4^+ stress. (A-D) Total chlorophyll accumulation and fresh biomass of shoots. Seven-day-old seedlings were treated with 0, 25 and 40 mM NH_4^+ for 0, 1, 3, 5, 7 days. Values are the means ± SE ($n \geq 4$). (E-F) Primary root growth and lateral root number. Seven-day-old seedlings were transferred to serial concentrations of NH_4^+ for 3d. Error bars represent SE ($n \geq 12$).



Supplemental Figure S4. Phenotypes of *amos1/vtc1* double mutants exposed to NH_4^+. Seven-day-old *amos1*, *amos1/vtc1* and *vtc1* seedlings were treated with mock (12.5 mM K_2SO_4) or NH_4^+ (12.5 mM $(NH_4)_2SO_4$) for 3 days. A representative image for each genotype is shown, 12 seedlings in total each treatment. The experiments were reproduced twice.



Supplemental Figure S5. Map-based cloning of *AMOS1* and confirmation. (A) *AMOS1* is located between the centromere and Indel maker C5p71 (Supplemental Table SIII). (B) PCR amplified products of the partial genomic sequence of *EGY1* in *amos1* (1), Col-0 (2) and 2000bp DNA Marker (top two lanes are 2000 bp and 1000 bp respectively) (3). (C) The expression of *EGY1* mRNA in *amos1* and Col-0 (WT), CBP20 (At5G44200) as the housekeeping gene. The results of experiments (B) and (C) were repeated at least twice.

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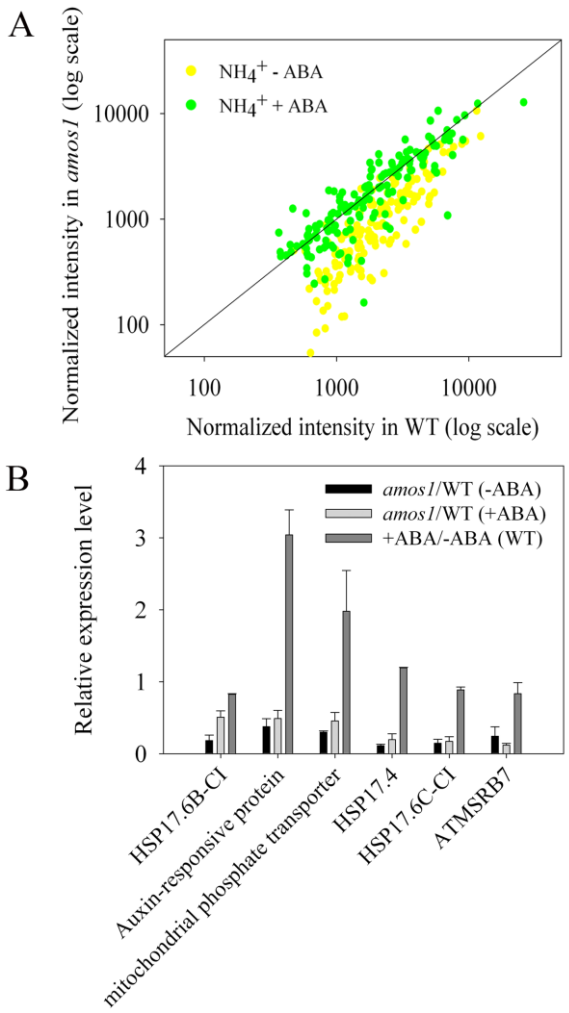
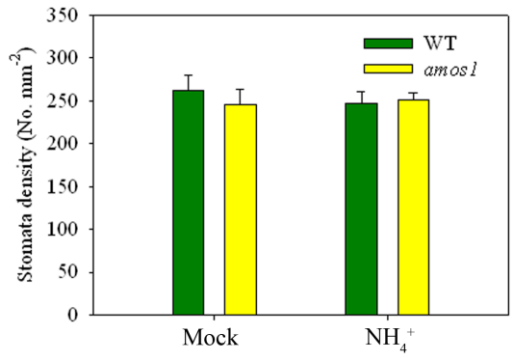


Supplemental Figure S6. Effect of external ABA on the levels of transcripts responsive to NH_4^+ in *amos1*. (A) Large partial of NH_4^+-repressed genes in *amos1* were restored to the similar expression level of wild type by ABA. Transcript levels of these 183 NH_4^+-repressed genes in *amos1* are shown in scatter plots of *amos1* versus wild type in NH_4^+ medium with or without 1 μM ABA. (B) Small subgroup of AMOS1/EGY1-dependent NH_4^+-activated genes that in *amos1* was not recovered to the wild type level by application of ABA during NH_4^+ stress. Relative expression levels of these genes are shown in bars of *amos1* versus wild type in NH_4^+ medium with or without 1 μM ABA, or in bars of NH_4^+ treatment with 1 μM ABA (+ABA) versus without ABA (-ABA) in wild type. The values (means ± SE, n = 3) in (B) are derived from the microarray data.



Supplemental Figure S7. Stomatal density of wild type (WT) and *amos1* seedlings. Seven-day-old *amos1* and WT seedlings were treated with mock (12.5 mM K_2SO_4), NH_4^+ (12.5 mM $(NH_4)_2SO_4$) for 6 h. Values are the means ± SE (n = 6).