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 NH$_4^+$-responsive genes and maintains chloroplast functionality under NH$_4^+$ stress. Importantly, ab14 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.
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 AMOSI/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 AMOSI/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 amos1 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$_4$)$_2$SO$_4$ 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$_4$)$_2$SO$_4$-induced chlorosis in amos1 seedlings was reversible upon transfer to medium without (NH$_4$)$_2$SO$_4$, 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$_4$)$_2$SO$_4$. 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, 2011b). 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$_4$)$_2$SO$_4$ and K$_2$SO$_4$. Chlorophyll accumulation and chloroplast fluorescence were severely affected in amos1 seedlings when exposed to (NH$_4$)$_2$SO$_4$ but not K$_2$SO$_4$ (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 GM$\mathrm{P}$ase, 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$_4$)$_2$SO$_4$] 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$_2$SO$_4$) 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 $\mu$m in the top row and 10 $\mu$m 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 ≥ 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 NH₄⁺-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 NH₄⁺-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 NH₄⁺, 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 NH₄⁺ (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 NH₄⁺ for 7 d. One representative image is shown for each line. The experiments were reproduced at least twice.

**Regulation of the Expression of NH₄⁺ Stress-Responsive Genes by AMOS1/EGY1**

To elucidate the molecular function of AMOS1/EGY1 in the NH₄⁺ stress response, we used full genomic microarray hybridization to analyze the difference of the Arabidopsis transcriptome between amos1 and the wild type under both NH₄⁺ 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 NH₄⁺ conditions, compared with the wild type (q ≤ 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 NH₄⁺ 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 NH₄⁺ 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 NH₄⁺, 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 NH₄⁺-exposed amos1 plants than in the wild type are part of the transcriptional NH₄⁺ response of Arabidopsis, we further analyzed the genome-wide transcriptional response of the wild type and amos1 to NH₄⁺ 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 NH₄⁺ 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 NH₄⁺-activated genes and 62% (26) of the NH₄⁺-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 NH₄⁺-responsive genes, particularly those genes showing higher transcript levels in NH₄⁺-exposed plants. The largest group of 183 genes, however, showed a decrease in transcript levels in response to NH₄⁺ exposure in amos1 without responding to NH₄⁺ in the wild type.

A comparison of GO categories in biological processes of AMOS1/EGY1-dependent NH₄⁺-responsive
genes (Fig. 4B) and the 867 genes repressed in *amos1* under NH₄⁺ stress (Fig. 3C) revealed that “response to chitin” was the most enriched biological process in the category of NH₄⁺-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 NH₄⁺-activated biological processes in the wild type but not in *amos1*. The biological process “cell redox homeostasis” was only found among NH₄⁺-repressed genes of *amos1*, but not the wild type. Other biological processes, such as “autophagy,” were not enriched among NH₄⁺ stress.

**Figure 3.** Transcriptional profile in *amos1* compared with the wild type (WT) under NH₄⁺ stress. A, Total number of genes for which transcript levels were significantly changed in *amos1* relative to the wild type after mock treatment or NH₄⁺ stress conditions imposed for 6 h (q ≤ 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 NH₄⁺ stress. C, Enriched GO categories for biological processes of 867 genes expressed at significantly lower levels in *amos1* than in the wild type under NH₄⁺ 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 NH₄⁺ conditions (mock conditions given for reference).

**Figure 4.** Genome-wide responses of transcript levels to NH₄⁺ in shoots of *amos1* and the wild type (WT). A, Numbers of genes showing significant changes in transcript levels in response to NH₄⁺ in the wild type and *amos1* according to the set thresholds (NH₄⁺ relative to mock, q [%] ≤ 5 and fold change ≥ 2 or ≤ 0.5, respectively). B, Enriched GO categories for biological processes of the 867 genes expressed at significantly lower levels in *amos1* than in the wild type under NH₄⁺ stress. C, Percentage of promoters containing at least one occurrence of the ACGTG element in wild-type NH₄⁺-activated promoters: 86, NH₄⁺-activated genes in the wild type; 78, AMOS1/EGY1-dependent NH₄⁺-activated genes; 43, AMOS1/EGY1-dependent NH₄⁺-activated genes in which transcript levels in *amos1* are 50% or lower of those in the wild type during NH₄⁺ 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 (hemeF2), 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 CCACTA, 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 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...
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$_2$O$_2$) using 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) fluorescence. The signal of H$_2$DCFDA 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$_2$O$_2$ 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$_2$O$_2$ formation is not a consequence of reduced stomatal density. Furthermore, the H$_2$O$_2$ 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/otc1 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 plastid retrograde signaling pathway to regulate the expression of NH$_4^+$ stress-responsive genes and to enhance 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$_2$O$_2$ 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$_2$SO$_4$), NH$_4^+$ [12.5 mM (NH$_4$)$_2$SO$_4$], or NH$_4^+$ + ABA (1 μM) for 6 h, then H$_2$O$_2$ 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 AB14 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 AB14-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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ in chloroplasts has been proposed to regulate the expression of nucleus-encoded HSP genes under heat stress (Yu et al., 2012). Therefore, H$_2$O$_2$ produced in guard cell chloroplasts may constitute an AMOS1/EGY1-dependent plastid retrograde signal, because the H$_2$O$_2$ response is defective in guard cell chloroplasts in amos1 during NH$_4$+-stress. As the H$_2$O$_2$ 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$_2$O$_2$-dependent plastid signal to activate high-light-responsive gene expression in leaves (Galvez-Valdivieso et al., 2009) and in the O$_2$-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, O$_2$-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 O$_2$-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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ accumulation depend on AMOS1/EGY1, suggesting that NH$_4$+-induced ABA and H$_2$O$_2$ 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$_2$O$_2$ 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); ceg1-1 and ceg1-2 were reported previously (Chen et al., 2005) and provided by Dr. Ning Li; vtc1-1, abk1-1, abk3-1, and abu2-3 were obtained from the Arabidopsis Biological Resource Center at Ohio State University. All mutants are in Col-0 background, except ceg1-1 (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 mEq KH$_2$PO$_4$, 5 mEq NaNO$_3$, 2 mEq MgSO$_4$, 1 mm CaCl$_2$, 0.1 mm Fe-EDTA, 50 µM H$_2$BO$_3$, 12 µM MnSO$_4$, 1 µM ZnCl$_2$, 1 µM CuSO$_4$, 0.2 µEq Na$_2$MoO$_4$, 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 mEq (NH$_4$)$_2$SO$_4$ and 12.5 mEq K$_2$SO$_4$, 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 CS5017 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$_2$O$_2$**

H$_2$DCFDA fluorescence was used to detect the H$_2$O$_2$ response. Seven-old-day *amos1* and Col-0 seedlings were treated with 12.5 mm (NH$_4$)$_2$SO$_4$ or 12.5 mEq K$_2$SO$_4$ for 6 h. The leaves were transferred into 25 µm H$_2$DCFDA 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$_2$DCFDA 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-phthalaldehyded 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. [3H]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-bromocorannyltrimethylammonium 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 K₂SO₄ (mock), 20 mM (NH₄)₂SO₄, and 20 mM (NH₄)SO₄ 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 Cy₃-dCTP labeled 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 fold change value of 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 (Nemhauer 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 (AtSG35220) and CBP20 (AtSG44220).

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. NH₄⁺-induced chlorosis in leaves of amos1 seedlings dependent on the direct contact between NH₄⁺-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 NH₄⁺ stress.

Supplemental Figure S4. Phenotypes of amos1/ebc1 double mutants exposed to NH₄⁺.

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

Supplemental Figure S6. Effect of external ABA on the levels of transcripts responsive to NH₄⁺ 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 NH₄⁺-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 NH₄⁺ and mock treatment.

Supplemental Data Set S2. Comparison of genes differentially expressed in NH₄⁺ relative to mock treatment in amos1 and Col-0.

Supplemental Materials and Methods S1.

ACKNOWLEDGMENTS

We thank the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, for providing the T-DNA lines and Dr. Ning Li (Hong Kong University of Science and Technology) for providing the seeds of egyl-1 and egyl-2 and the pCAMBIA 35S: EGY1-GFP construct. We also thank Dr. Zhubua He and Dr. Muyang Wang (Institute of Plant Physiology and Ecology, Chinese Academy of Sciences) for their guidance in amos1 mutant mapping. We are sincerely grateful to Dr. Yanhua Su (Institute of Soil Science, Chinese Academy of Sciences) and other members in our group for their suggestions and work in this study. Additionally, we thank Mingruan Chen and Dr. Yuqi Feng (Department of Chemistry, Wuhan University) for the determination of ABA content.

Received August 29, 2012; accepted October 8, 2012; published October 12, 2012.

LITERATURE CITED


Plastid and Abscisic Acid Signaling Modulate NH$_4^+$ Responses


Li Q, Li BH, Kronzucker HJ, Shi WM (2010) Root growth inhibition by NH$_4^+$ in Arabidopsis is mediated by the root tip and is linked to NH$_4^+$ efflux and GMPase activity. Plant Cell Environ 33: 1529–1542


Zhang J (2008) The degradation of EGY1 metalloprotease. MPhil thesis. Hong Kong University of Science and Technology, Hong Kong
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 (NH₄)₂SO₄ 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 F₁ generated from amos1-1, amos1-2, amos1-3 crossed with each other were tested on NH₄⁺ medium for allele mutation analysis. The seedlings of F₁ and F₂ populations generated from amos1-1, amos1-2, amos1-3 backcrossed with Col-0 wild type were tested on NH₄⁺ 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$_2$SO$_4$), NH$_4^+$ (12.5 mM (NH$_4$)$_2$SO$_4$) 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'-AGGATCGTAACCGAACGTTTCT-3' and 5'-ACTCCGGTCAGGAAGAATCGACT-3', and the cDNA of EGY1 by using the primers 5'-TGCCCAAATGGCAAAAGAGACTCTG-3' and 5'-CAGCATGCAAAGCTGCTATTCC-3'. CBP20 was chosen as the housekeeping gene for mRNA expression with the primers 5'-ACCATCGAAACACGAAAGAG-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.

LITERATURE CITED


### Supplemental Table SI. Genetic analysis of the *amos1* mutants

<table>
<thead>
<tr>
<th>Cross</th>
<th>Generation</th>
<th>Individuals response to $\text{NH}_4^+$</th>
<th>$x^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. (Resistant)</td>
<td>No. (Sensitive)</td>
</tr>
<tr>
<td><em>amos1-1</em> x <em>amos1-2</em></td>
<td>F$_1$</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td><em>amos1-1</em> x <em>amos1-3</em></td>
<td>F$_1$</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td><em>amos1-2</em> x <em>amos1-3</em></td>
<td>F$_1$</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td><em>amos1-1</em> x WT</td>
<td>F$_1$</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F$_2$</td>
<td>128</td>
<td>41</td>
</tr>
<tr>
<td><em>amos1-2</em> x WT</td>
<td>F$_1$</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F$_2$</td>
<td>114</td>
<td>31</td>
</tr>
<tr>
<td><em>amos1-3</em> x WT</td>
<td>F$_1$</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F$_2$</td>
<td>111</td>
<td>40</td>
</tr>
</tbody>
</table>

$\text{NH}_4^+$-grown seedlings for which leaves appeared similar to the wild type (WT, Col-0) were scored as $\text{NH}_4^+$ resistant, and seedlings for which leaves appeared similar to *amos1* mutant were scored as $\text{NH}_4^+$ 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 \textit{AMOS1/EGY1}-dependently NH\textsubscript{4}+-activated genes.

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

Note: 86: NH\textsubscript{4}+-activated genes; 78: \textit{AMOS1/EGY1}-dependent NH\textsubscript{4}+-activated genes; 43 \textit{AMOS1/EGY1}-dependent NH\textsubscript{4}+-activated genes that in \textit{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.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Size (bp)</th>
<th>Position (bp)</th>
<th>Primer (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereon NO.</td>
<td>Col/Ler</td>
<td>/rename</td>
<td></td>
</tr>
</tbody>
</table>
| Cer456556 /C5P71 | 589/550 | 13718452 | F: CCTCATTATTTATAAACCCAAACCA  
| | | | R: GAACGGGATAGAAGTCAA |
| Cer455569 /C5P73 | 550/507 | 14154436 | F: CACCTTCCTCCACCTCAGA  
| | | | R: TTGCCGAAGCTAGACTGGAT |
| Cer456453 /C5P76 | 580/534 | 14792975 | F: CCTGAAGACAGACGGAAGA  
| | | | R: TCGCTTCCTCTGGGTGTCC |
| 457357 /C5P81 | 608/494 | 15810692 | F: GAAGTGTTGGCTCTCCAATCC  
| | | | 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$_4$)$_2$SO$_4$ 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$_4$)$_2$SO$_4$ 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$_4$)$_2$SO$_4$. 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$_2$SO$_4$) or NH$_4^+$ (12.5 mM (NH$_4$)$_2$SO$_4$) 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.
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$_2$SO$_4$), NH$_4^+$ (12.5 mM (NH$_4$)$_2$SO$_4$) for 6 h. Values are the means ± SE ($n = 6$).