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ORIGINAL ARTICLE

Isolation and characterization of a novel ammonium overly sensitive mutant, *amos2*, in *Arabidopsis thaliana*

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Abstract Ammonium (NH_4^+) toxicity is a significant agricultural problem globally, compromising crop growth and productivity in many areas. However, the molecular mechanisms of NH_4^+ toxicity are still poorly understood, in part due to a lack of valuable genetic resources. Here, a novel *Arabidopsis* mutant, *amos2* (*am*monium *o*verly *s*ensitive *2*), displaying hypersensitivity to NH_4^+ in both shoots and roots, was isolated. The mutant exhibits the hallmarks of NH_4^+ toxicity at significantly elevated levels: severely suppressed shoot biomass, increased leaf chlorosis, and inhibition of lateral root formation. *Amos2* hypersensitivity is associated with excessive NH_4^+ accumulation in shoots and a reduction in tissue potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}). We show that the lesion is specific to the NH_4^+ ion, is independent of NH_4^+ metabolism, and can be partially rescued by elevated external K^+. The *amos2* lesion was mapped to a 16-cM interval on top of chromosome 1, where no similar mutation has been previously mapped. Our study identifies a novel locus controlling cation homeostasis under NH_4^+ stress and provides a tool for the future identification of critical genes involved in the development of NH_4^+ toxicity.

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Abbreviations

Col	Colombia ecotype
GM	Growth medium
Ler	Landsberg erecta ecotype
Mes	2-Morpholinoethanesulfonic acid
TAIR	The *Arabidopsis* information resource
WT	Wild type

Introduction

Ammonium (NH_4^+) is a major source of nitrogen for plant growth and development (Gerendas et al. 1997; Kronzucker et al. 1997; Britto and Kronzucker 2002). To satisfy the high nitrogen demand of agricultural crops, farmers often add nitrogen in large quantities. However, this excessive use of nitrogen fertilizer leads to nitrogen volatilization and subsequent transport and deposition of NH_3/NH_4^+ via the atmosphere, resulting in undesirable accumulation of ammonium in many soils. As a consequence, NH_4^+ concentrations can range from 2 to 20 mM in agricultural soil solutions, with levels as high as 40 mM having been measured (Glass et al. 2002; Kronzucker et al. 2003a). Growth inhibition and limitation of crop yield frequently result from such soil accumulations (Gerendas et al. 1997; Britto and Kronzucker 2002). A thorough understanding of the mechanisms underlying NH_4^+ toxicity is essential to confront this agronomic problem.

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Only few mechanisms underlying NH_4^+ toxicity have been elucidated. The majority of investigations have dealt with key physiological changes, such as NH_4^+-induced disorders in pH regulation, uncoupling of photophosphorylation, carbon consumption by roots, futile and energetically intensive transmembrane cycling of the NH_4^+ ion, and impairments in the N-glycosylation of proteins (Britto et al. 2001; Britto and Kronzucker 2002; Qin et al. 2008; Balkos et al. 2010; Barth et al. 2010; Li et al. 2010; Kempinski et al. 2011). One particularly well-documented effect of NH_4^+ is that of disruption of cation nutrition, especially that of K^+ (Speer et al. 1994; Kronzucker et al. 2003b; Szczerba et al. 2006). Similarly, addition of K^+ can alleviate NH_4^+ toxicity (Szczerba et al. 2006, 2008a; Balkos et al. 2010). Although the exact mechanism behind the interaction of K^+ and NH_4^+ toxicity is not known, it has been suggested that membrane passage of NH_4^+ via multiple channels (K^+ channels, non-selective cation channels, and possibly aquaporins) could play a role (Szczerba et al. 2008a, b; Britto and Kronzucker 2008; Balkos et al. 2010; ten Hoopen et al. 2010), and there may be both K^+-sensitive and K^+-insensitive pathways associated with NH_4^+ uptake (Szczerba et al. 2008a; Balkos et al. 2010; ten Hoopen et al. 2010). It is well established that NH_4^+ can inhibit K^+ uptake (Gerendas et al. 1997; Hirsch et al. 1998; Szczerba et al. 2006; ten Hoopen et al. 2010), and that, in turn, NH_4^+ uptake can be inhibited by K^+ (Balkos et al. 2010), possibly competitively (ten Hoopen et al. 2010). These observations may partially explain the disruption of potassium homeostasis under ammonium exposure and the rescue from toxicity when K^+ is raised (Britto and Kronzucker 2002).

One approach to elucidating mechanisms of NH_4^+ toxicity in plants is to use mutant lines. Their employment is important, because responses to NH_4^+ stress between species, and even among ecotypes within species, are highly heterogeneous (Schortemeyer et al. 1997; Li et al. 2011; Cruz et al. 2011). Mutant lines, against established wild-type (WT) genetic backgrounds, partially avoid this difficulty. To date, only one NH_4^+-sensitive mutant, *vtc1*, has been characterized in *Arabidopsis* (Qin et al. 2008; Barth et al. 2010; Li et al. 2010). The mutant is disrupted in GDP-mannose pyrophosphorylase (GMPase), and increased NH_4^+ sensitivity has been interpreted to result from defective N-glycosylation of proteins (Barth et al. 2010; Li et al. 2010). Accumulation of the NH_4^+ ion itself in plant tissue remained unaffected (Barth et al. 2010). As symptoms of NH_4^+ toxicity most often include NH_4^+ hyperaccumulation in tissues (Britto et al. 2001; Szczerba et al. 2008a; Balkos et al. 2010), and are coupled to a disruption in cation homeostasis, leaf chlorosis, root growth inhibition, and lower plant biomass (Gerendas et al. 1997; Britto and Kronzucker 2002), the isolation and characterization of NH_4^+-responsive mutants displaying alterations in a combination of these traits in *Arabidopsis* represents a critical step in our effort to understand NH_4^+ tolerance.

Therefore, we have here isolated and characterized a novel *Arabidopsis* mutant, *amos2* (*am*monium *o*verly *s*ensitive *2*). Physiological and phenotypical analyses of WT and *amos2* in response to NH_4^+ stress are described, and the genetic locus responsible for the *amos2* mutation is identified using map-based cloning.

Materials and methods

Plant materials and growth conditions

Plant materials used in this work included WT *Arabidopsis thaliana* L. (Col-0 and Ler ecotypes) and genetic mutants derived from the Col-0 background. All seeds were surface-sterilized and cold-treated for 2–3 days at 4°C in the dark to synchronize germination. Seed germination and seedling growth were accomplished by using the *A. thaliana* normal growth medium (GM) (Li et al. 2010), containing 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 $ZnCl_2$, 1 μM $CuSO_4$, 0.2 μM Na_2MoO_4, 0.5 g/l Mes, 1% sucrose, 0.8% agarose (pH 5.7, adjusted with 1 M NaOH). The day of sowing was considered day 0. Seedlings were grown, oriented vertically on the surface of the media in a growth chamber, set at a 16-h light/8-h dark photoperiod, an irradiance of 100 μmol m^{-2} s^{-1}, and a constant temperature of 23 ± 1°C. For ammonium and specific nutrient treatments, seedlings were used for experiments at 5 days after sowing (primary root length was approximately 2 cm at this stage of development), and subsequently transferred to fresh agar plates containing varying concentrations of $(NH_4)_2SO_4$, NH_4Cl, K_2SO_4, KNO_3, NaCl, mannitol or 4% (w/w) sucrose for an additional 9 days. In the K^+ "rescue" experiments, GM was supplemented with two concentrations (4 and 8 mM) of external K^+ (as K_2SO_4), to provide total K^+ concentrations in media of 2 mM (GM control), 6 mM and 10 mM. Hydroponic culture was prepared as described by Xu and Shi (2008), using modified Hoagland solution.

Isolation of the *amos2* mutant plants

Arabidopsis thaliana plants (Col-0) were mutagenized with T-DNA transformation (kindly provided by Professor Zuo of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences; Zuo et al. 2000). Seeds were surface-sterilized and sown on normal GM plates for 5 days, and then transferred to GM supplemented with 15 mM $(NH_4)_2SO_4$ (ammonium concentration 30 mM), to

screen for the presence of shoot- and lateral root (LR)-sensitive phenotypes. After treatment for 9 days, putative mutants with reduced numbers of LRs and inhibited shoot growth were selected and rescued, transferred to soil, and allowed to self-fertilize. The homozygous M_4 *amos2* mutant was backcrossed to the WT Col-0, and the resulting F_1 generation was crossed with WT Col-0 two times to remove unlinked mutations caused by the mutagenesis.

Growth assays

The lengths of primary roots and LRs of individual seedlings were measured directly with a ruler and with Image J software (National Institutes of Health; http://rsb.info.nih.gov/ij) from digital images captured with a Canon G7 camera, respectively. LR number and LR density were determined by counting the LRs present in the primary root from the tip to the root/stem transition under a magnifying glass. LR density was determined by dividing the LR number by the primary root length and expressed as LR density per centimeter. Average LR length was determined by dividing the total LR length of individual seedlings by the LR numbers and expressed as average LR length per LR number. The fresh weight of each individual shoot was measured immediately after harvest. For observation of inflorescence phenotypes and flowering time, WT and *amos2* plants were first germinated and grown for 18 days on GM and then transferred to soil with a 16-h light/8-h dark cycle at 23°C in a growth chamber. The development of the main inflorescence was measured daily after bolting, and flowering time was measured by counting the number of days from sowing until the first flower bud was visible.

Chlorophyll quantification

Plant chlorophyll concentration was measured with a method modified after Wintermans and De Mots (1965). Fresh leaves of *Arabidopsis* plants were soaked in 96% ethanol, kept at 4°C in darkness for 12 h and then centrifuged at 1,000*g*, 4°C for 10 min. The supernatant was used for determining absorbance at 665 and 649 nm to obtain chlorophyll *a*, chlorophyll *b*, and total chlorophyll concentration. Total chlorophyll concentration was expressed as milligrams per gram fresh weight.

Mineral analysis

5-day-old WT and *amos2* seedlings grown on GM were transferred to fresh media with 0 or 15 mM $(NH_4)_2SO_4$ for an additional 9 days of growth and then harvested. To measure tissue NH_4^+ content, WT and *amos2* seedlings were desorbed for 10 min in 10 mM $CaSO_4$ to remove extracellular NH_4^+. Roots and shoots were weighed separately and extracted at a ratio 1:10 (w/v) with 10 mM formic acid on ice, centrifuged at 2°C for 10 min at 25,000*g*, and filtered with 0.45 µm nylon filters (Costar, Corning Inc., Lowell, MA, USA) at 2°C for 5 min at 5,000*g*. Ammonium was analyzed by the *o*-phthalaldehyde (OPA) method using a high-performance liquid chromatography (HPLC) system (Waters Corp., Milford, MA, USA, equipped with a Phenomenex Gemini C18 analytical column 4.6 mm × 150 mm; particle size 5 µm). The analytical principle was based on detection of fluorescence upon reaction between the fluorochrome OPA and ammonium as described by Husted et al. (2000).

For other mineral analyses, the shoots of the seedlings were dried at 75°C prior to analysis, and samples were digested with HNO_3 and subjected to ICP-AES (IRIS Advantage, Thermo Electron, Waltham, MA, USA).

Activity of glutamine synthetase

The enzyme assay for glutamine synthetase was conducted according to the method described by Shi et al. (2010). Samples (0.2 g) were homogenized in the extraction buffer composed of 100 mM Tris–HCl (pH 7.6), 1 mM $MgCl_2$, 1 mM EDTA, 1% 2-mercaptoethanol, and 1% PVP. The homogenate was centrifuged at 12,000*g* for 15 min at 4°C. The supernatant fraction was used as the crude extract for protein content and enzyme activity measurements. Protein contents were determined by the Bradford method (Bradford 1976). GS activity was assayed as the ADP-dependent conversion of L-glutamine to γ-glutamylhydroxamate and measured by spectrophotometric absorbance at 540 nm (Taira et al. 2004). The reaction was carried out in 1.5 ml GS assay mixture composed of 40 mM imidazole-Cl (pH 7.0), 30 mM L-glutamine, 3 mM $MnCl_2$, 0.4 mM ADP, 20 mM sodium arsenate, and 60 mM NH_2OH.

Genetic analysis of *amos2* mutant

5-day-old plants of the parents, F_1, and F_2 progenies derived from the crosses of Col × *amos2* and Ler (Landsberg erecta ecotype) × *amos2* were grown under $(NH_4)_2SO_4$ medium for 9 days. Sensitive individuals were scored and compared statistically (χ^2-test). Segregation of LR reduced in normal GM of the cross progenies were also analyzed.

Genetic mapping of *amos2*

The F_2 generation of the cross Ler × *amos2* was selected based on the mutant phenotype. To confirm the phenotypes of F_2 individuals, the F_3 progenies were further tested for both reduced lateral root phenotype and sensitivity to $(NH_4)_2SO_4$. For the initial mapping analysis, primers for

InDel (insertion/deletion) markers were used (Salathia et al. 2007). PCR was performed in a 20-µl volume containing 10–100 ng/µl genomic DNA (1 µl), 10 µM of each primer (1 µl), 2.5 mM dNTPs (1.6 µl), 25 mM Mg^{2+} (1.2 µl), 10× PCR buffer (2.0 µl), and 0.2 U of *Taq* DNA polymerase (TaKaRa). The following PCR program was used: 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and final extension at 72°C for 7 min. PCR products were resolved on 3% agarose gels in TAE buffer.

Statistical and graphical analyses

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

Results

Isolation of the *amos2* mutant

To investigate the genetic basis of plant responses to ammonium, we screened a T-DNA-tagged *A. thaliana* population on agar plates containing 15 mM $(NH_4)_2SO_4$. Five mutants displaying hypersensitive features conditional on NH_4^+ were isolated. Here we describe one mutant, designated *amos2* (*am*monium *o*verly *s*ensitive *2*). Characterization of the other mutants is ongoing and will be described elsewhere. The *amos2* mutant sensitivity was characterized by (1) increased chlorosis, (2) decreased shoot fresh weight, and (3) inhibition of lateral root (LR) formation compared with WT, in media containing NH_4^+ (Fig. 1a). Prior to phenotypical and physiological analysis, we crossed the *amos2* mutant with WT (Col-0) three times, to purify the genetic background. The hybrid plants (F_1), of *amos2* × Col-0, displayed the same phenotypes as WT. The F_2 populations revealed a 3:1 segregation ratio of the wild type and mutant (Table 1), indicating that the *amos2* was a recessive mutation in a single nuclear locus.

To further study the phenotypic alterations induced by NH_4^+ in *amos2*, we grew *amos2* mutant and WT seedlings side-by-side on vertical plates containing a range of NH_4^+ contents. In the absence of NH_4^+, primary root growth and development of aerial parts of *amos2* were indistinguishable from WT, but *amos2* exhibited decreased LR growth, indicating that AMOS2 is important for normal LR development under normal growth conditions (Fig. 1b). Upon transfer to the medium supplemented with 10, 15, 20, and 25 mM $(NH_4)_2SO_4$, shoot growth of *amos2* seedlings was more severely inhibited and leaves were smaller and chlorotic, whereas the leaves of WT were still partially green even at 25 mM $(NH_4)_2SO_4$. Unlike the WT, *amos2* plants showed almost complete cessation of LR emergence under high $(NH_4)_2SO_4$ concentrations (≥15 mM) (Fig. 1b). More than 85% of the NH_4^+-stressed *amos2* seedlings displayed the high-sensitivity phenotype.

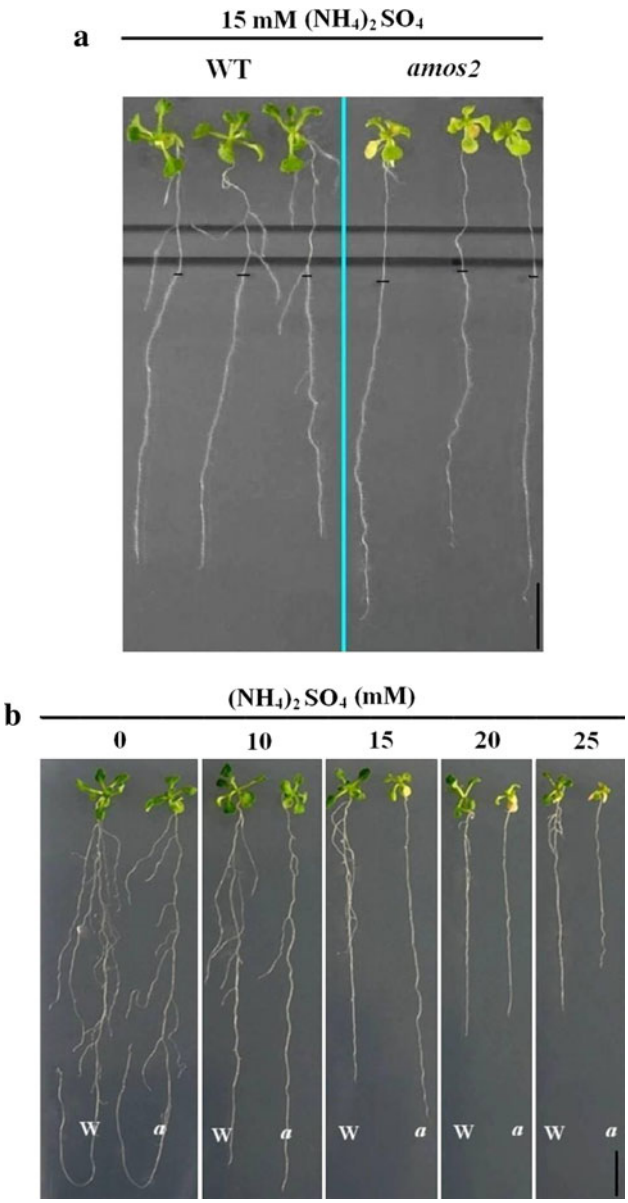

Fig. 1 Conditional phenotypes of wild-type (Col-0) and *amos2* plants in response to ammonium supply. **a** Photograph of an agar plate supplied with 15 mM $(NH_4)_2SO_4$ (NH_4^+ concentration of 30 mM) showing the reduced size, leaf chlorosis, and impaired lateral root (LR) growth in the *amos2* mutant. The positions of primary root tips are marked (*black line*). **b** Five-day-old WT (*W*) and *amos2* (*a*) seedlings grown on normal GM were transferred to GM medium containing 0, 10, 15, 20 and 25 mM $(NH_4)_2SO_4$. The photographs were taken 9 days after transfer to $(NH_4)_2SO_4$. *Bars* 1 cm

Table 1 Genetic analysis of the *amos2* mutant

Cross	Generation	Total	Phenotype		χ^2 ($P > 0.05$)
			WT	Mutant	
Col × *amos2*	F1	31	31	0	
	F2	152	110	42	0.43
Ler × *amos2*	F1	22	22	0	
	F2	312	241	71	0.72

The χ^2 values are based on an expected ratio of 3:1 (WT:*amos2* mutant)

High NH_4^+ differentially affects shoot growth and root system architecture in wild-type and *amos2* mutant

To more clearly define the alterations in both aerial parts and root system architecture in response to ammonium in relation to the mutation in AMOS2, we performed temporal and single-point measurements of shoot fresh weight, chlorophyll content, primary root length, average LR length, LR number, and LR density in WT and *amos2* mutants treated with varying concentrations of NH_4^+. Ammonium-treated WT and *amos2* plants showed a dose-dependent inhibitory effect of NH_4^+ on the growth of aerial parts, but shoot fresh weight of *amos2* was inhibited more than that of WT under all the experimental concentrations [71% reduction in *amos2* versus 43% in WT, respectively, at 20 mM $(NH_4)_2SO_4$] (Fig. 2a). In the absence of NH_4^+ treatment, no significant difference in total chlorophyll content was detected between the WT control and the mutant. Lower-concentration $(NH_4)_2SO_4$ treatment (10 and 15 mM) significantly increased the chlorophyll content in WT, consistent with previous reports (Zhou et al. 2006; Li and Shi 2007), while the transfer of WT seedlings to higher NH_4^+ concentrations caused reductions in chlorophyll content. Unlike WT, *amos2* chlorophyll content was reduced at and above 10 mM $(NH_4)_2SO_4$, and decreased linearly as NH_4^+ concentration increased in the medium (Fig. 2b). The results suggest that the photosynthetic capacity of WT plants remained higher during NH_4^+ treatment than that of *amos2* mutants.

Excess amounts of NH_4^+ are known to inhibit root growth (Britto and Kronzucker 2002; Cruz et al. 2006; Li et al. 2010). When seedlings were exposed to media with increasing levels of NH_4^+, a clear trend of decreasing primary root and average LR length was observed in both WT and *amos2*. Even though no significant difference was found between WT and mutants for primary and average root length (Fig. 2c, d), the number of emerged LRs of *amos2* seedlings was significantly reduced by treatment with NH_4^+ compared with WT seedlings, at all concentrations tested (Fig. 2e, f). At 15 mM $(NH_4)_2SO_4$, lateral root formation in *amos2* was inhibited by ~85%, more than double the inhibition seen in the WT (Fig. 2e). Compared with untreated control, the LR density of WT plants was slightly increased, to coincide with the decrease in primary root length, at 10 mM $(NH_4)_2SO_4$. Higher levels of $(NH_4)_2SO_4$ (≥15 mM) inhibited LR density. By contrast, NH_4^+ sharply reduced LR density in *amos2* at all tested concentrations. The difference in inhibition profiles between WT and *amos2* plants was starkest at the highest NH_4^+ concentrations (Fig. 2f). Overall, our results suggest that the mutation in AMOS2 does not alter the NH_4^+-mediated inhibition of LR elongation, but alters specific developmental traits related to lateral root formation under high NH_4^+ conditions.

The *amos2* mutant is specifically sensitive to NH_4^+

Since the mutant identified in this screen exhibited a sensitivity phenotype in response to high applications of $(NH_4)_2SO_4$, it was necessary to determine the role of the sulfate ion, high nitrogen level per se, and osmotic potential on the mutant phenotype, to establish whether the phenotypic response was ammonium-specific. Thus, the WT and *amos2* seedlings were also treated on media enriched with, or depleted of, a variety of ions and molecules, and then shoot fresh weight was determined. The results are shown in Fig. 3. *amos2* seedlings were very sensitive to both $(NH_4)_2SO_4$ and NH_4Cl, but did not show a significant difference in shoot growth and development compared with WT on medium containing 10 and 15 mM K_2SO_4, or 20 and 30 mM KNO_3. *amos2* seedlings were also equal to WT on 20 and 30 mM NaCl, 40 and 60 mM mannitol, or 4% (w/w) sucrose. This shows that the *amos2* mutation results in a hypersensitivity that is specific to NH_4^+, rather than to equivalent concentrations of other ions, or to simple osmotic stress.

Morphological characterization of the *amos2* mutant

When seedlings were grown in GM lacking NH_4^+, the most striking phenotype of *amos2* was its lack of lateral roots (Fig. 4a), whereas no difference was found in primary root growth compared with WT (Fig. 1b). In addition, a notable phenotype of soil-grown *amos2* mutant plants was the delay in floral stem development (Fig. 4b, c). For instance, at the 26th day, the floral stem of WT was four times longer than that of the *amos2* mutant (Fig. 4b). The mutant also exhibited a delayed flowering time, on average by about 7 days, relative to WT grown under similar conditions (Fig. 4c). Unlike many dwarf mutants, *amos2* plants were similar in size to WT at the mature stage (Fig. 4b, c). Furthermore, the mutant displayed additional phenotypes, including low fertility rates in the early stage of development and a larger number of bud clusters

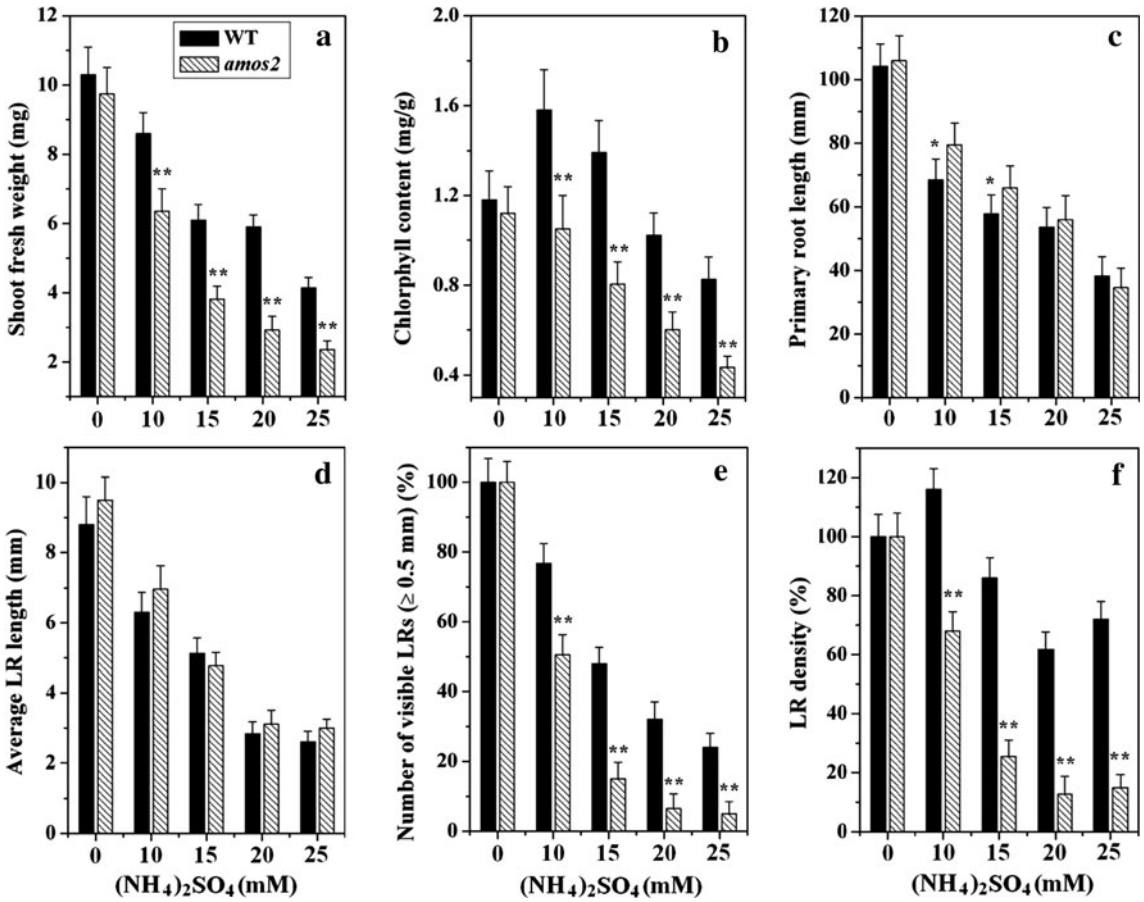

Fig. 2 Effects of high NH_4^+ on shoot growth and root system architecture in wild-type and *amos2* mutant. Five-day-old seedlings, grown vertically on GM, were subjected to increasing ammonium ($(NH_4)_2SO_4$) supply for 9 days. **a** Shoot fresh weight. **b** Chlorophyll content. **c** Primary root length. **d** Average lateral root (LR) (≥0.5 mm) length, expressed as total LR length divided by total LR number per plant. **e** Number of visible LRs (≥0.5 mm) per plant. **f** LR density expressed as the number of LRs per centimeter. Values are the mean ± SE, $n = 18$. *Asterisks* indicate statistical differences between the mutant and wild type ($0.01 < {*}P < 0.05$, $**P < 0.01$, independent samples *t* test). The experiment was repeated three times

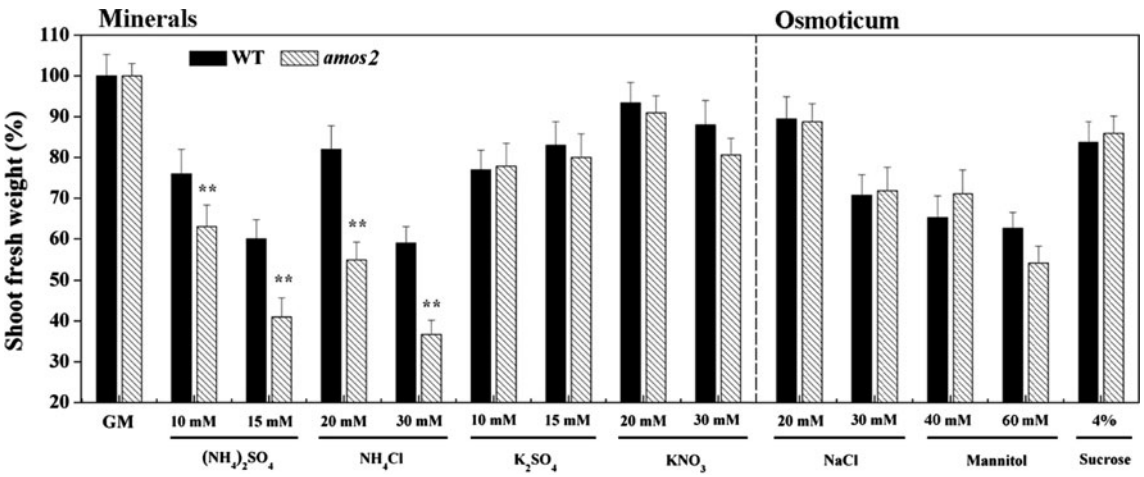

Fig. 3 Specificity of the *amos2* mutant to NH_4^+. WT and *amos2* seedlings were grown for 5 days on GM and then transferred to GM supplemented with salts and osmotica as indicated. Shoot fresh weight was measured 9 days after transfer. Growth on GM nutrient was considered as 100%. Values are the mean ± SE, $n = 12$–15. *Asterisks* indicate statistical differences between the mutant and wild type ($**P < 0.01$, independent samples *t* test). The experiment was repeated three times

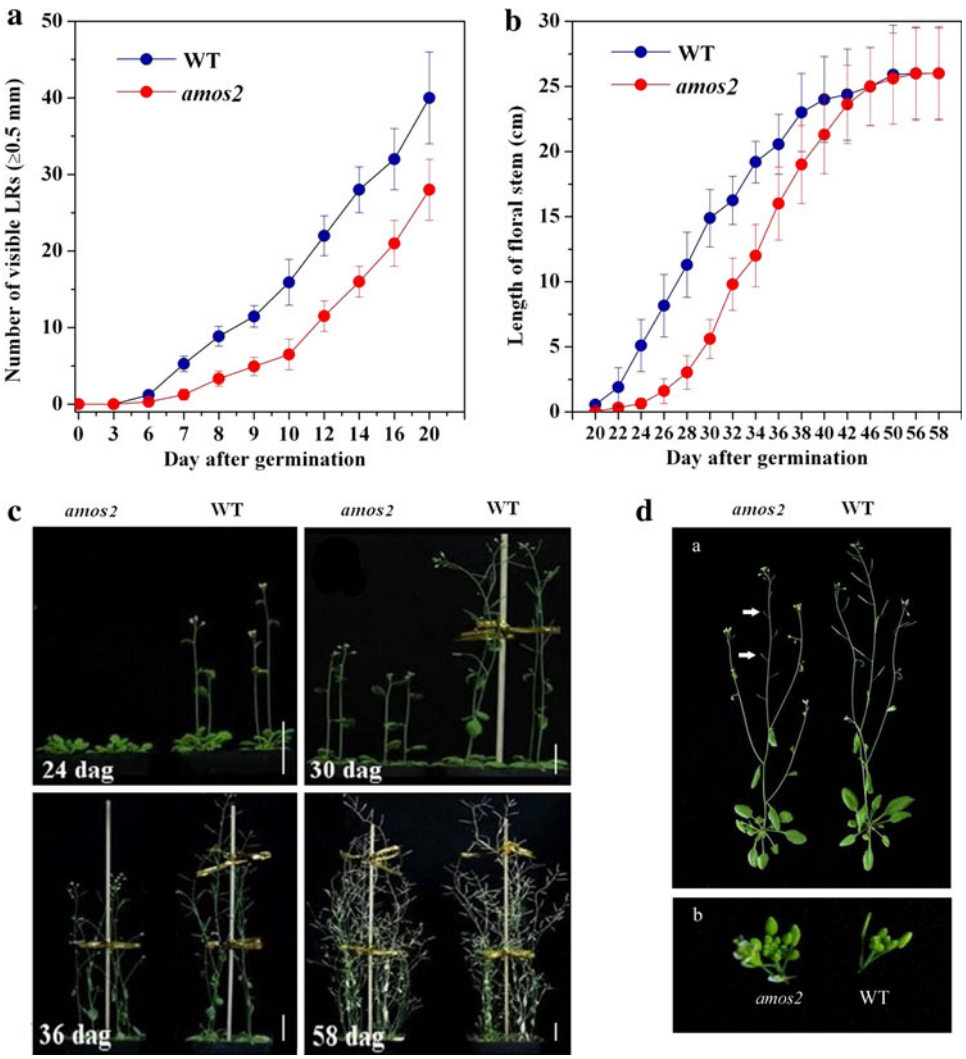

Fig. 4 Growth of wild type and *amos2* under normal growth conditions and in hydroponic culture. **a** Number of visible LRs (≥0.5 mm) of the wild-type and the *amos2* mutant seedlings grown on GM lacking NH_4^+ as in Fig. 1b. **b**, **c** Floral stem development and flowering time phenotype of *amos2* plants grown in soil. WT and *amos2* plants were first germinated and grown for 18 days on growth medium and then transferred to soil with a 16-h light/8-h dark cycle at 23°C in a growth chamber. Floral stem development (**c**) and its quantification (**b**) from 20 to 58 days after germination. **d** Forty-five-day-old plants of WT and *amos2* mutant in hydroponic culture. *a amos2* plants showed reduced fertility (see *arrows*). *b amos2* plants produced a higher number of floral buds at the shoot apex. Values are the mean ± SE, $n = 15$–20. *Bars* 1 cm. The experiment was repeated three times. *dag* days after germination (see online article for color version of this figure)

(Fig. 4d). These results suggest that AMOS2 plays a role in the regulation of lateral root and flower development under normal growth conditions.

The *amos2* mutant accumulates excessive NH_4^+ in shoot tissue

To investigate whether the NH_4^+-sensitive phenotype of the *amos2* mutant is associated with increased internal accumulation of NH_4^+, the aerial and root tissue NH_4^+ content of WT and *amos2* seedlings was determined. When plants were grown on medium from which NH_4^+ had been omitted, the NH_4^+ content did not differ significantly between WT and *amos2* mutant in either shoot or root tissues, and root NH_4^+ content exceeded shoot content in both WT and mutant (Fig. 5a). However, NH_4^+ accumulation in shoots was threefold greater in *amos2* seedlings than in WT seedlings when cultivated in the presence of 15 mM $(NH_4)_2SO_4$, while there was no difference in root NH_4^+ content of WT and *amos2* (Fig. 5a). Treated with 15 mM $(NH_4)_2SO_4$, root NH_4^+ content was also higher than shoot content in WT, whereas shoot NH_4^+ content exceeded that of roots in *amos2* (Fig. 5a).

Activities of the enzyme glutamine synthetase (GS), centrally involved in the NH_4^+ assimilation processes, was determined in shoots of WT and *amos2*, to test whether a limitation in assimilatory rates may be responsible for the NH_4^+ overaccumulation in the shoots of *amos2*. In normal GM lacking NH_4^+, GS activity was similar in WT and *amos2*. GS activity was induced by external NH_4^+ in ammonium-fed WT and *amos2* plants, but this increase was not significantly different in the two genotypes (Fig. 5b). This indicated that NH_4^+ metabolism was not affected by the mutation in AMOS2.

The NH_4^+ sensitivity of *amos2* involves imbalanced ion homeostasis

Changes in cation composition, specifically a reduction in tissue K^+, have been frequently reported in connection

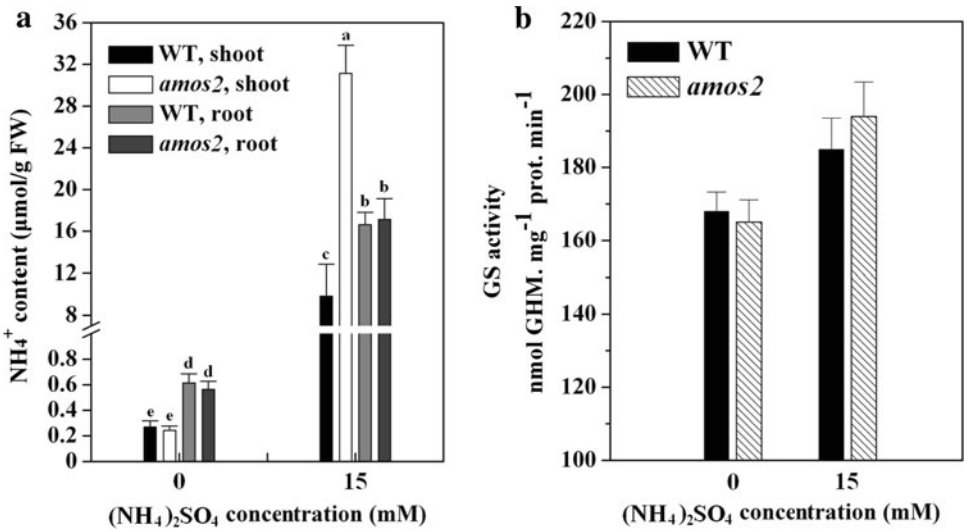

Fig. 5 NH_4^+ tissue contents and shoot GS activities in wild type and *amos2*. Five-day-old WT and *amos2* seedlings were grown on GM and transferred to fresh media with 0 or 15 mM $(NH_4)_2SO_4$ for an additional 9 days of growth, and then NH_4^+ tissue content and GS activity were determined. **a** NH_4^+ contents (expressed as μmol/g fresh weight) in the aerial and root tissues of WT and *amos2* seedlings. **b** GS activities in the shoot of WT and *amos2* seedlings. Values are mean ± SE of three replicates of three experiments. *Different letters* indicate statistical differences at $P < 0.01$ (one-way ANOVA analysis with Duncan post hoc test). *FW* fresh weight, *GS* glutamine synthetase

with NH_4^+ toxicity (Britto and Kronzucker 2002; ten Hoopen et al. 2010). Therefore, to assess whether the mutation in AMOS2 affects ion homeostasis under NH_4^+ stress, the mineral content was analyzed in shoots of seedlings grown on agar medium with 0 and 15 mM $(NH_4)_2SO_4$. Potassium (K) content of *amos2* under control conditions (0 mM NH_4^+) was not found to be significantly different from that of WT. However, when high NH_4^+ was supplied, K content of shoot tissue in both WT and *amos2* plants decreased, but the K content of *amos2* was inhibited significantly more than that of WT (68 vs. 39% inhibition, respectively; Fig. 6). A significant decrease in shoot calcium (Ca) and magnesium (Mg) content was also observed in *amos2* plants compared with WT when grown on GM without NH_4^+, but there was no difference between shoot Ca and Mg content of WT and *amos2* plants when grown on medium containing 15 mM $(NH_4)_2SO_4$ (Fig. 6). *amos2* mutant seedlings, in contrast to WT, had lower concentrations of sodium (Na) than unstressed seedlings, but slightly increased Na content in shoot tissue under high-NH_4^+ conditions than under control condition (Fig. 6). No significant differences in shoot content of phosphorus (P), iron (Fe), manganese (Mn), or zinc (Zn) between WT and *amos2* plants grown on either medium were detected (Fig. 6). These results suggest that the AMOS2 gene may play a role in the disruption of ion homeostasis, especially K^+ homeostasis, under conditions of high NH_4^+.

To investigate the function of potassium on *amos2* hypersensitivity, we examined the effect of external K^+ addition on plant growth and shoot NH_4^+ content under NH_4^+ stress in both genotypes. The growth suppression induced by NH_4^+ was alleviated by K^+ addition in both WT and *amos2* plants, but the fresh weight was increased more significantly in *amos2*. Compared with plants grown in GM only supplemented with high NH_4^+, *amos2* mutant shoot fresh weight increased more than 70%, as compared with ~15% in the WT, in GM supplemented with high NH_4^+ plus 4 mM K^+ (providing a total K^+ concentration of 6 mM) (Fig. 7). External K^+ also reduced tissue NH_4^+ content in both *amos2* and wild-type plants. Again, this effect was more pronounced in *amos2* plants (Fig. 7b). Further increasing $[K^+]_{ext}$ to 10 mM (GM plus 8 mM K^+) did not significantly decrease the shoot NH_4^+ content in the genotypes additionally, and both genotypes also experienced no additional benefit in shoot weight (Fig. 7b). These data indicate that external K^+ alleviates *amos2* NH_4^+ toxicity symptoms more significantly than WT ($P < 0.01$), further suggesting that *amos2* hypersensitivity was at least partially related to K^+ deficiency.

Map position of *amos2*

The kanamycin resistance assay is an effective method for screening transgenic plants expressing the kanamycin resistance gene (Bechtold and Pelletier 1998). To determine whether the *amos2* phenotypes resulted from T-DNA insertion into the locus, the progeny from crosses between *amos2* and WT were scored for kanamycin resistance, but the *amos2* mutant does not show resistance to kanamycin (data not shown), revealing that the mutation was not

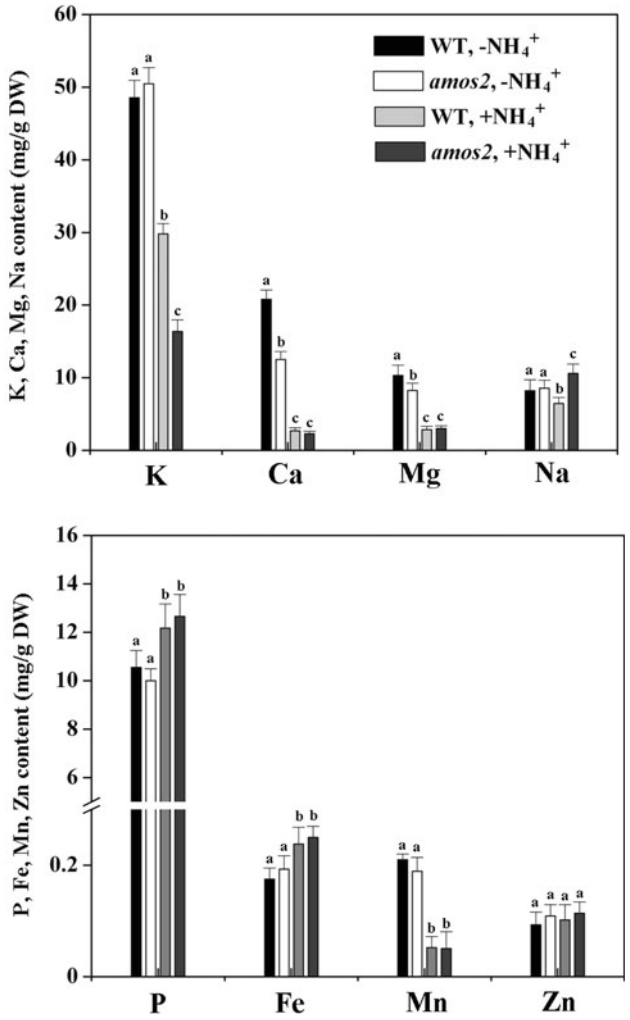

Fig. 6 Mineral contents in shoots of WT and *amos2* seedlings. Five-day-old seedlings grown on GM were transferred to the same medium ±15 mM $(NH_4)_2SO_4$ for an additional 9 days and then mineral content was determined. Values are mean ± SE of three replicates of two experiments. *Different letters* indicate statistical differences between treatments for a given mineral (one-way ANOVA analysis with Duncan post hoc test, $P < 0.05$). *DW* dry weight

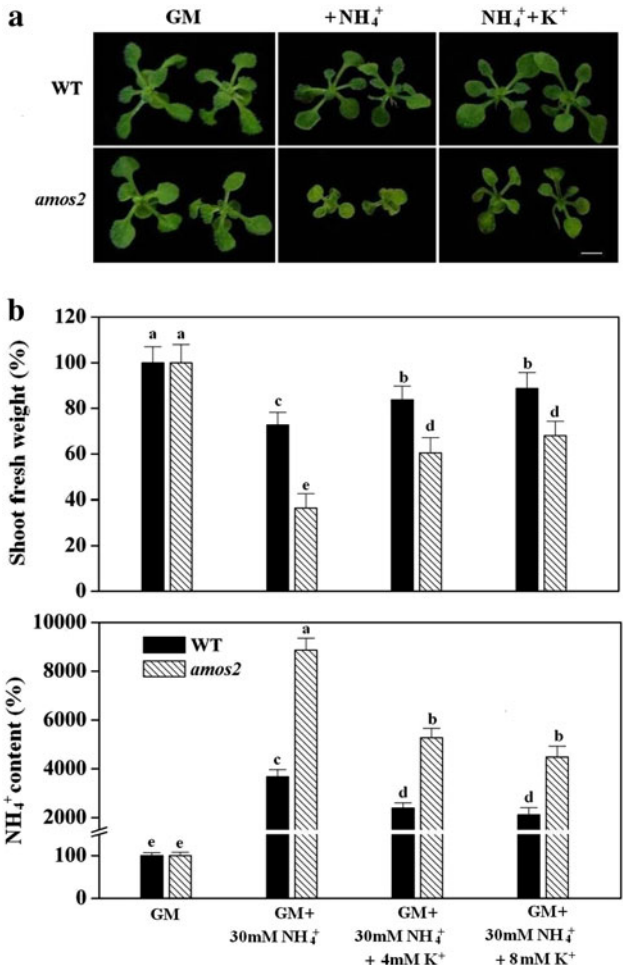

Fig. 7 Effects of external K^+ on plant growth and shoot NH_4^+ content of wild-type and *amos2* seedlings. Five-day-old WT and *amos2* seedlings grown on GM (containing 2 mM K^+) were transferred to GM supplemented with 15 mM $(NH_4)_2SO_4$ alone or in combination with varying concentrations of K^+ (provided as K_2SO_4). Shoot fresh weight and NH_4^+ tissue content were measured 9 days after transfer. **a** The aerial phenotype of wild type (*upper panels*) and *amos2* (*lower panels*) grown on GM, Plants were grown on GM, and GM containing 30 mM NH_4^+ (from $(NH_4)_2SO_4$) and GM containing 30 mM NH_4^+ plus 4 mM K^+ (GM supplemented with K_2SO_4) (total K^+ concentration of 6 mM) for 9 days. *Bars* 2 mm. **b** Shoot fresh weight and shoot NH_4^+ contents (expressed as percentage) in control, GM + 30 mM NH_4^+ (2 mM K^+), 30 mM NH_4^+ plus 4 mM K^+ (total K^+ concentration of 6 mM), and 30 mM NH_4^+ plus 8 mM K^+ (total K^+ concentration of 10 mM). Shoot NH_4^+ contents (expressed as μmol/g fresh weight) of WT and *amos2* seedlings in control were 0.37 ± 0.1, 0.35 ± 0.15, respectively. Values are the mean ± SE, $n = 18$–25. *Different letters* indicate statistical differences at $P < 0.01$ (one-way ANOVA analysis with Duncan post hoc test) (see online article for color version of this figure)

properly tagged by T-DNA, similar previous reports (e.g. Liu et al. 2007; El Kassis et al. 2007; Zheng et al. 2010).

To identify the genetic determinant responsible for the phenotypes of *amos2*, the *amos2* locus was determined by map-based cloning. For this approach, the *amos2* mutant was crossed with Ler ecotype. The F_2 populations derived from the cross of Ler × *amos2* showed a 3:1 segregation ratio for the WT and mutant phenotypes (Table 1), confirming that *amos2* is a recessive mutation at a single nuclear locus. A total of 67 F_2 plants were subjected to initial mapping of the *amos2* mutation using InDel markers (Table 2). The initial mapping procedure revealed that the gene was positioned in a 16-cM region at the top of chromosome 1, between the markers CER459662 and 464650 (Fig. 8). To check whether there are any known mutant(s) in this particular region that could be allelic to the *amos2* mutant, we referred to the TAIR website database (http://www.arabidopsis.org/) and previous published reports on mutant phenotypes in this region. To our

Table 2 The InDel markers used for cloning experiments

Marker name	Size (Col/Ler)	Position (bp)	Primer sequence (5′→3′)
474009	510/478	6,988,148	F: CCTGTGTTGGTCATTTCC
			R: ACCAATTGCAACAATCATC
CER459662	544/495	9,203,766	F: GCGATGGAAAATGAGATTAG
			R: CATTTACGGCCCATTATGCT
CER458676	616/560	9,559,825	F: TGGTGCTCTTTTTGGCTTCT
			R: ATGCTCCCATTTCAAGAACG
464650	811/607	12,228,509	F: AAGCGGAAAGGGACGTAGAT
			R: TGGTAGTACGGGTTTTGGTC
CER464787	311/267	13,724,956	F: TCTGTGGAGTGAAACGCGACTTGA
			R: TCCGTCAGAGACGTGAAGGCATTA
CER465523	496/442	18,748,314	F: TGGATCATCGAGGGACTCAT
			R: AGGCCAAAATACAGCTGACG
CER464751	596/498	26,628,510	F: AAACCCCTTCCAGGATGAAC
			R: ACGTTTTGAACCACCGCTAC

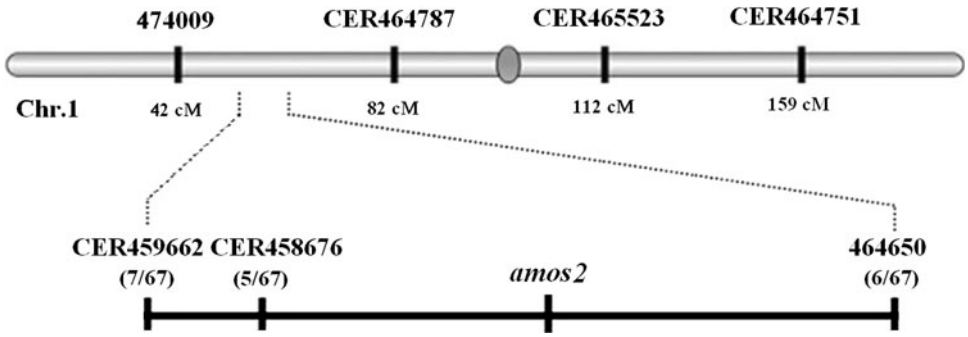

Fig. 8 Genetic map of the *amos2* locus. Genetic map was constructed with 67 recessive individuals, with the chromosome orientation indicated. *Numbers* indicate the recombination events detected between the corresponding markers and the *amos2* locus

knowledge, *amos2* places within a region where no similar mutation has been reported hitherto.

Discussion

To date, aside from the reported growth suppressions on NH_4^+ substrates in mutants defective in the enzyme glutamine synthetase (Lothier et al. 2011) and in low-affinity potassium transport (Hirsch et al. 1998), only one NH_4^+-sensitivity mutant, *vtc1*, has been characterized in *Arabidopsis* (Qin et al. 2008; Barth et al. 2010). Its lesion has been linked to the enzyme GDP-mannose pyrophosphorylase, and not to NH_4^+ accumulation itself. Indeed, the direct connection with NH_4^+ exposure has recently been questioned, and proton effects have been invoked instead (Kempinski et al. 2011). To expand our ability to understand the mechanisms of NH_4^+ toxicity, we have here identified and characterized a novel *Arabidopsis* mutant, *amos2*, hypersensitive to NH_4^+ in both roots and shoots (Fig. 1). Key features of the *amos2* mutant grown under high NH_4^+ condition as described in this report include decreased shoot biomass, increased leaf chlorosis, and inhibition of lateral root (LR) formation (Figs. 1, 2). This is the first example of an *Arabidopsis* mutant showing the combination of these traits. Genetic analysis indicated that *amos2* is a single recessive mutation (Table 1), and maps to a locus on a 16-cM region on top of chromosome 1 (Fig. 8), where no previous mutant has been described.

A pronounced characteristic of the *amos2* mutant is that it accumulates excessive ammonium in its shoot tissue, exceeding the concentrations found in its roots (Fig. 5a). By contrast, wild-type plants grown on high NH_4^+ show high accumulation of ammonium in the root compared with the shoot (Fig. 5a). Our finding that the *amos2* mutant displays significantly decreased shoot biomass and increased leaf chlorosis (Fig. 2) is consistent with this observation. The accumulation of free NH_4^+ in the shoot is widely considered to be critical to the development of NH_4^+ toxicity (Gerendas et al. 1995; Lasa et al. 2001; Szczerba et al. 2008a). Glutamine synthetase, the enzymatic entry point to NH_4^+ metabolism, when inhibited, has been shown to result in elevated NH_4^+ levels and higher toxicity (Lee et al. 1992; Kronzucker et al. 1995; Hirano et al. 2008). Indeed, the general view has emerged that plants with higher GS activity are more tolerant to NH_4^+

(Magalhaes and Huber 1989; Gerendas et al. 1997; Balkos et al. 2010; Lothier et al. 2011). Likewise, optimization of GS activity, along with that of the anaplerotic enzyme PEP-carboxylase, has been observed to coincide with alleviation from NH_4^+ toxicity and growth stimulation in cucumber and rice (Roosta and Schjoerring 2008; Balkos et al. 2010). However, our results indicate that GS activity was not affected by the mutation in AMOS2 (Fig. 5b), showing that impaired glutamine synthetase function is not responsible for the NH_4^+ overaccumulation in the *amos2* mutant. This dual observation, that of elevated tissue NH_4^+ level and of unaltered glutamine synthetase, sets the *amos2* mutant apart from *vtc1*, whose conditional short-root phenotype did not show any change in tissue NH_4^+ while displaying significantly lowered GS activity (Barth et al. 2010). In directly impacting upon NH_4^+ accumulation, the *amos2* lesion appears to link more directly to primary NH_4^+ homeostasis than *vtc1*.

Many physiological studies have shown that lower uptake of cations, especially of K^+, is one of the key aspects of the NH_4^+ toxicity syndrome (Wilcox et al. 1973; Gerendas et al. 1997; Cao and Tibbitts 1998; Szczerba et al. 2006; Roosta and Schjoerring 2007), and increased supply of K^+ has been shown to alleviate NH_4^+ toxicity in some cases (Gerendas et al. 1997; Britto and Kronzucker 2002; Kronzucker et al. 2003b; Szczerba et al. 2006; Roosta and Schjoerring 2007, 2008; Balkos et al. 2010; ten Hoopen et al. 2010). We found that shoot K content in both WT and *amos2* plants decreased as a function of NH_4^+ dose, but that the K^+ content of *amos2* was inhibited significantly more than that of WT under high-NH_4^+ (Fig. 6). These results are consistent with the above-described shoot-sensitive phenotype of the *amos2* mutant. In support of this observation, elevated external K^+ alleviated *amos2* NH_4^+ toxicity symptoms and reduced tissue NH_4^+ content more significantly than in WT (Fig. 7).

On account of the diffusion limitations inherent in agar media as opposed to hydroponic culture (see Li and Shi 2007; Li et al. 2010, for extensive discussion), we had to apply larger concentrations of both NH_4^+ and K^+ than is customary in hydroponic studies (Gerendas et al. 1995; Cao and Tibbitts 1998; Kronzucker et al. 2003b; Roosta and Schjoerring 2007, 2008; Szczerba et al. 2008a; Balkos et al. 2010). To achieve similar growth suppressions as seen in the latter studies, typically with 5–10 mM NH_4^+, 30 mM, i.e. approximately three- to sixfold higher concentrations, were necessary (see detailed inhibitory study in Li et al. 2010). Likewise, (3- to 6-fold) higher K^+ concentrations were necessary to achieve relief from NH_4^+ toxicity: symptoms were pronounced at 2 mM K^+, but were at least partially alleviated by stepping up to 6 mM K^+ (and no further improvement was seen at 10 mM K^+) in *amos2* (Fig. 7). This corresponds to a shift in the NH_4^+/K^+ ratio from 15 to 5, in excellent agreement with previous studies in sensitive species, which indicate an NH_4^+/K^+ ratio of 4–8 must typically be exceeded for NH_4^+ toxicity to be established (Gerendas et al. 1995; Cao and Tibbitts 1998; Kronzucker et al. 2003b; Roosta and Schjoerring 2007, 2008; Szczerba et al. 2008a). Shifting external K^+ concentrations into the operation range of the ammonium-insensitive low-affinity transport system ("mechanism 2") for potassium uptake (Britto and Kronzucker 2008; Coskun et al. 2010) appears to be furthermore key to alleviation from toxicity (Kronzucker et al. 2003b; Roosta and Schjoerring 2008); in our study this is clearly achieved at 6 and 10 mM K^+ (corresponding to concentrations at, or exceeding, 1 mM in hydroponic studies). It is of interest that once NH_4^+/K^+ ratios are sufficiently lowered, no further benefit is seen with additional increases in external K^+. Szczerba et al. (2008a) and Balkos et al. (2010) in barley and rice, respectively, have shown that optimizations of growth, NH_4^+ fluxes, and shoot NH_4^+ contents are already achieved by raising external K^+ from 0.1 to 1.5 mM, against a background of 10 mM NH_4^+, with no additional benefit at 5 and 40 mM K^+. Gerendas et al. (1995) and Roosta and Schjoerring (2008) have reported similar results, in corn and cucumber, respectively. We also noted that wild-type and mutant *Arabidopsis* did not receive additional benefit at 10 mM K^+, compared with 6 mM (Fig. 7b). Interestingly, in highly NH_4^+-tolerant species, such as rice, NH_4^+ toxicity is typically only observed at external K^+ concentrations below 0.1 mM and at NH_4^+/K^+ ratios exceeding 400 (Balkos et al. 2010), underscoring the central importance of NH_4^+–K^+ interaction to determining NH_4^+ toxicity and tolerance.

Mechanistically, it is now well established that the presence of elevated external NH_4^+ directly suppresses high-affinity K^+-influx systems of the KUP/HAK/KT family (Hirsch et al. 1998; Szczerba et al. 2006, 2008b; Britto and Kronzucker 2008; ten Hoopen et al. 2010). A further target point of NH_4^+ has been shown to be in the long-distance translocation of K^+ from root to shoot, which can be suppressed as much as 90% in the presence of high NH_4^+ (Kronzucker et al. 2003b), while an additional factor may be the enhancement of unidirectional K^+ efflux that has been observed upon NH_4^+ exposure (Coskun et al. 2010). A decline in cytosolic K^+ concentrations, normally held within narrow limits, can furthermore be the result of long-term exposure to NH_4^+ (Kronzucker et al. 2003b). Which of these mechanisms contribute to the decreased K^+ content of *amos2* plants in the presence of NH_4^+ is a matter for future investigations. Our results from the current mutant analysis support the strong mechanistic relationship between the accumulation of free NH_4^+ and the disruption of K^+ homeostasis in particular in the shoot (Gerendas et al. 1997; Britto and Kronzucker 2002; Szczerba et al.

2006, 2008b; ten Hoopen et al. 2010), and the fact that this disruption arises by virtue of a single recessive mutation in *amos2* provides a highly valuable genetic resource for future study of this important relationship in the context of plant NH_4^+ toxicity.

In the absence of NH_4^+, the *amos2* mutant exhibits pronounced developmental phenotypes that affect both LR and floral stem (Fig. 4), suggesting that AMOS2 plays an important role in the regulation of lateral root and flower development under normal growth conditions. It has to be kept in mind, however, that NH_4^+ stress per se has been frequently reported to promote early flowering and a shortened life cycle (Claussen and Lenz 1995; Britto and Kronzucker 2002), so that the addition of NH_4^+ is expected to aggravate the phenotype. In line with this phenotype, a significant decrease in shoot Ca^{2+} was also observed in *amos2* plants compared with WT when grown on GM, while, in the presence of NH_4^+, suppressions in Ca^{2+} levels were similar in WT and mutant (Fig. 6). Decreases in leaf-tissue Ca^{2+} with elevated NH_4^+ supply have been documented in a variety of experimental systems (Wilcox et al. 1973; Gerendas et al. 1995, 1997; Cao and Tibbitts 1998; Roosta and Schjoerring 2007), and these are seen both in WT and *amos2*, reflecting no difference in this target of ammonium toxicity. Calcium is widely known to regulate growth and development of plants (Hepler 1988; Mahalakshmi et al. 2007), including the control of flowering and vegetative organogenic pathways (Capitani and Altamura 2004; Iwano et al. 2009), and adventitious root formation (Bellamine et al. 1998; Lanteri et al. 2006; Li et al. 2008). In addition to effects on Ca^{2+} status in the mutant, we observed significant suppressions in leaf Mg^{2+} and Mn^{2+} in both WT and mutant, to a similar extent. Both cations are critical to photosynthesis function (Pittman 2005; Cakmak and Kirkby 2008). Since photosynthesis is nearly always affected as part of exposure to high external NH_4^+ (Gerendas et al. 1997; Britto and Kronzucker 2002), interference with the homeostasis of these two cations is likely critical to NH_4^+ toxicity in general. While leaf status of manganese has been rarely examined in the context of ammonium toxicity, effects of NH_4^+ nutrition on leaf magnesium status, while not universal (Gerendas et al. 1995, 1997), have been observed in several studies (Wilcox et al. 1973; Cao and Tibbitts 1998; Roosta and Schjoerring 2007). However, as in the case with Ca^{2+}, suppressions in Mg^{2+} and Mn^{2+} were not affected by the mutation, singling out K^+ as the principal target to explain the enhanced NH_4^+ sensitivity of the mutant. Nevertheless, based on the above, AMOS2 might, in addition to lending itself as an investigatory tool for questions on NH_4^+–K^+ interactions, provide a useful resource for the study of mechanisms of Ca^{2+}-dependent regulation of plant morphogenesis.

In summary, we have identified and characterized a novel NH_4^+-sensitivity mutant, *amos2*. We believe the mutant will provide a valuable genetic model for the study of the mechanisms underpinning NH_4^+ toxicity and serve as a starting point toward the isolation of novel genes in pathways of NH_4^+ sensing. Currently, work is underway to isolate the gene for the *amos2* mutation, using a map-based cloning approach in our laboratories, and subsequently to introduce this into an agriculturally important crop species such as rice or barley to determine whether the observed alteration in ammonium tolerance in *Arabidopsis* can be replicated in commercially important crop systems. By taking this approach, it is hoped that a better understanding of plant NH_4^+ toxicity will be gained, and that new molecular genetic approaches to address it will be generated.

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