Ammonium stress in Arabidopsis: signaling, genetic loci, and physiological targets

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Ammonium (NH₄⁺) toxicity is a significant ecological and agricultural issue, and an important phenomenon in cell biology. As a result of increasing soil nitrogen input and atmospheric deposition, plants have to deal with unprecedented NH₄⁺ stress from sources below and above ground. In this review, we describe recent advances in elucidating the signaling pathways and identifying the main physiological targets and genetic loci involved in the effects of NH₄⁺ stress in the roots and shoots of Arabidopsis thaliana. We outline new experimental approaches that are being used to study NH₄⁺ toxicity in Arabidopsis and propose an integrated view of behavior and signaling in response to NH₄⁺ stress in the Arabidopsis system.

Ammonium toxicity in higher plants

Compromised plant growth and production as a result of NH₄⁺ accumulating in soils is a long-standing and serious problem in agriculture [1–3]. Over recent decades, excessive NH₄⁺/NH₃ deposition from the atmosphere has affected plant community composition and species viability in many environments, both natural and agricultural [4–14] (Box 1). At the cellular level, NH₄⁺ is a fundamental substrate for amino acid and protein synthesis in all living organisms, but it is toxic to cells when present in excess [15–18]. Even though NH₄⁺ is a preferred nitrogen source for many plants [3,15], NH₄⁺ toxicity should be seen as universal, even in species frequently labeled as ‘NH₄⁺ specialists’ [19,20], although toxicity thresholds tend to be shifted to higher NH₄⁺ concentrations in such specialists. Toxicity tends to be particularly pronounced when NH₄⁺ is supplied as the sole nitrogen source when potassium (K⁺) levels are low, or when pH is unbuffered [3,19]. A stunted root system and leaf chlorosis are among the most visible phenotypic manifestations of NH₄⁺ toxicity in higher plants [3].

In early studies on crop species, excretion of protons, leading to acidification of the rhizosphere, and a general suppression of cation uptake, were recognized as leading contributors to growth impairment [1–3,15]. This also appeared to offer an explanation for the reduction of toxicity symptoms by the co-presence of nitrate (NO₃⁻) because its uptake is associated with alkalinization of the root medium and stimulation of cation uptake, counteracting some of the effects of NH₄⁺ [15,21,22]. However, it has emerged more recently that these factors are not directly related. First, in several studies, NH₄⁺ toxicity was still observed even under conditions of pH buffering and in the presence of NO₃⁻, although the thresholds of toxic NH₄⁺ concentration were raised [23–25]. Second, sensitivities to NH₄⁺ vary among different plant species, independent of pH adaptations [3,26], which suggests the evolution of highly distinct mechanisms to deal with NH₄⁺ stress. Comparative studies of sensitive and tolerant species have provided much-needed insight into underlying mechanisms. For instance, it was found that excessive energy consumption was associated with rapid and futile NH₄⁺ transport across the plasma membranes of roots of sensitive barley (Hordeum vulgare) [27], concomitant with elevated NH₄⁺ accumulation in both roots and shoots [28]. Such NH₄⁺ overaccumulation is commonly observed in sensitive species, such as spinach (Spinacea oleracea L.), and tomato (Lycopersicon esculentum Mill.) [29,30]. Strong relations between futile NH₄⁺ cycling, NH₄⁺ tissue accumulation, respiratory rates, and growth have since been reported both under ‘control’ conditions and under conditions of elevated K⁺, when overall NH₄⁺ toxicity is reduced [19,28]. Overaccumulation of NH₄⁺ is contingent upon excessive NH₄⁺ uptake, and the mechanism responsible for this process has still to be defined at the molecular level. Indeed, uptake in the toxic range might occur both as cationic NH₄⁺ and gaseous NH₃ [31,32]. Recent studies have shown that phosphorylation of the threonine residue T460 of the high-affinity NH₄⁺ transporter AtAMT1.1 in response to an increase in exogenous NH₄⁺ leads to a loss of

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Keywords: ammonium toxicity; signaling location and response; molecular components; physiological behavior.
Box 1. Ammonium sources and risks for ecological systems

After carbon, nitrogen is the most important element for plant growth and production. Both nitrogen deficiency and toxicity are widespread problems in agricultural and natural ecological systems. NH$_4^+$ and NO$_3^-$ are the predominant nitrogen forms in most soils, and NH$_4^+$ concentrations can reach 40 mM in soils with low nitration rates or immediately following the application of fertilizer [3]. Because of the low mobility of NH$_4^+$ in soils and the higher availability of NH$_4^+$ for plants than NO$_3^-$ [19], NH$_4^+$-release fertilizers are commonly used in agriculture. Furthermore, approaches such as localized fertilizer application, ammonia volatilization inhibition, and foliar application of NH$_4^+$-release fertilizers are often used to improve nitrogen utilization efficiency (NUE). However, this can cause significant, and rapid, accumulation of NH$_4^+$ in tissues, resulting in stunted roots and leaf damage, commonly described as leaf ‘scorching’, ‘burning’, or ‘tipping’ [1,2,8–11]. NH$_4^+$/NH$_3$ deposition from the atmosphere can be an important additional nitrogen source for agricultural plants; however, this has exceeded plant demand in many terrestrial and aquatic ecosystems [5–7,14]. In China, anthropogenic NH$_4^+$/NH$_3$ deposition increased by approximately 60% between 1980 and 2010 [14] and, in Europe, it reached peak levels during the 1980s [5]. This has significantly increased plant foliar nitrogen concentrations in natural and seminatural ecosystems [14]. Over recent decades, excess NH$_4^+$ has caused the local disappearance of NH$_4^+$-sensitive species of trees, grasses, aquatic plants, and even fishes [4–13].

transport activity into Arabidopsis roots [33–36], showing that NH$_4^+$ transport AMT1.1 can be deactivated under NH$_4^+$ stress. Even though AMT1.1 is unlikely to be responsible for the excessive uptake of NH$_4^+$ under natural circumstances, it provides a precedent for how a sensing mechanism for the NH$_4^+$ ion may occur. In Lotus japonicus, overexpression of the high-affinity NH$_4^+$ transporter LjAMT1;3 resulted in inhibition of root elongation under high levels of NH$_4^+$ [37], underscoring a possible linkage of AMT regulation and toxicity.

Recently, several genetic regulators controlling sensitivity to NH$_4^+$ have been identified in Arabidopsis [24,25,38–49] (Table 1). Elucidation of the function of these genetic regulators in determining the sensitivity to NH$_4^+$ has not only offered insight into the molecular basis of historically described physiological responses to NH$_4^+$ stress, but also been instrumental in the identification of new physiological, biochemical, and signaling pathways involved in NH$_4^+$ toxicity. Experiments involving the localized application of an NH$_4^+$ supply in agar-plate media (Figure 1) have shown that NH$_4^+$ derived from belowground sources produces fundamentally different effects and signaling responses compared with NH$_4^+$ derived from aboveground sources [25,38].

In this review, we describe how root elongation, root gravitropism, lateral root formation, shoot biomass development, and chloroplastic function in Arabidopsis respond differently under NH$_4^+$ stress depending on whether the NH$_4^+$ is derived from aboveground or belowground sources. We discuss recent progress, emphasize key issues, and outline new experimental approaches in the study of NH$_4^+$ toxicity. Finally, we propose an integrated view of behavior and signaling in response to NH$_4^+$ stress in Arabidopsis, and discuss the relevance of new discoveries to managing NH$_4^+$ toxicity in the field.

### Root system development under NH$_4^+$ stress

#### Root elongation

Shortened roots are the most visible phenotype of NH$_4^+$ stress in most plants [1–3,23]. Given that root elongation rates are controlled through cell division and/or expansion along the longitudinal axis of the root [50,51], it is important to ask how NH$_4^+$ affects this growth and which cell growth process serves as the primary target. Based on experiments supplying NH$_4^+$ to different root zones (Figure 1), it was concluded that contact between the root tip and NH$_4^+$ is both necessary and sufficient for the inhibition of primary root elongation [38], and that the slight reduction in primary root growth seen under short exposure to shoot-supplied NH$_4^+$ (SSA) is a secondary effect [25]. Reduced cell expansion can account for approximately 70% of the NH$_4^+$-mediated inhibition of root elongation [38,41,48]. Auxin, known to have a vital role in root development [51,52], has for some time been linked to NH$_4^+$-mediated inhibition of root elongation, based on the finding that the root length of the auxin-resistant mutants aux1, axr1, and axr2 is less affected by high levels of NH$_4^+$ compared with wild type [53]. However, the primary root of aux1 was found to be as sensitive to root-supplied NH$_4^+$ (RSA) as wild type [38,48], but to be resistant to SSA [42].

### Table 1. Genetic loci related to NH$_4^+$ sensitivity in Arabidopsis thaliana

<table>
<thead>
<tr>
<th>Mutant name</th>
<th>Gene ID</th>
<th>Gene molecular function</th>
<th>Phenotype (NH$_4^+$ response)</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsn1/vtc1</td>
<td>AT2G39770</td>
<td>GMPase</td>
<td>Root elongation$^a$</td>
<td>[24,39]</td>
</tr>
<tr>
<td>trh1</td>
<td>AT4G23640</td>
<td>Potassium transporter</td>
<td>Root gravitropism$^b$</td>
<td>[44]</td>
</tr>
<tr>
<td>gsa-1/arg1</td>
<td>AT1G68370</td>
<td>DnaJ-like protein</td>
<td>Root gravitropism$^b$</td>
<td>[49]</td>
</tr>
<tr>
<td>amtl;3</td>
<td>AT3G24300</td>
<td>Ammonium transporter</td>
<td>Lateral root formation$^b$</td>
<td>[70]</td>
</tr>
<tr>
<td>aux1</td>
<td>AT2G38120</td>
<td>Auxin transporter</td>
<td>Lateral root formation$^b$ and root elongation$^b$</td>
<td>[25,42,53]</td>
</tr>
<tr>
<td>etr1</td>
<td>AT1G66340</td>
<td>Ethylene receptor</td>
<td>Lateral root formation$^b$</td>
<td>[47]</td>
</tr>
<tr>
<td>xbat32</td>
<td>AT5G57740</td>
<td>A negative regulator of ethylene biosynthesis</td>
<td>Lateral root formation$^a$</td>
<td>[47]</td>
</tr>
<tr>
<td>eto1-1</td>
<td>AT3G51770</td>
<td>A negative regulator of ethylene biosynthesis</td>
<td>Lateral root formation$^a$</td>
<td>[47]</td>
</tr>
<tr>
<td>dpms1</td>
<td>AT1G20575</td>
<td>Dolichol phosphate mannose synthase 1</td>
<td>Chlorophyll content$^a$ and root elongation$^a$</td>
<td>[40]</td>
</tr>
<tr>
<td>amos1/eey1</td>
<td>AT5G35220</td>
<td>Plastid metalloprotease</td>
<td>Chlorophyll content$^a$</td>
<td>[45]</td>
</tr>
<tr>
<td>amos2</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Leaf biomass$^a$, chlorophyll content$^a$, and lateral root formation$^a$</td>
<td>[43]</td>
</tr>
</tbody>
</table>

$^a$Sensitive to NH$_4^+$.

$^b$Resistant to NH$_4^+$.
Several lines of evidence support the notion that the RSA-mediated reduction of root elongation, associated with elevated NH$_4^+$ efflux in the elongation zone [38], is uncoupled from auxin pathways [38,48].

Root elongation is commonly used as a phenotypic indicator for plant adaptation to environmental stress, and its genetic control under stress conditions is of great interest [54,55]. Given that NH$_4^+$ sensitivity greatly varies among crop and wild plant species, and among accessions of Arabidopsis [3,7,23], the search for genetic loci controlling it has occupied much recent effort. This search has been helped by the recent identification of the Arabidopsis hypersensitive to NH$_4^+$ (hsn1-1) mutant based on root length assays [24]. The hsn1 mutant and its allelic mutant vtc1 are the result of a point mutation of the gene encoding the enzyme GDP-mannose pyrophosphorylase (GMPase), which synthesizes GDP-mannose [24], which is in turn essential for the biosynthesis of both L-ascorbic acid (AsA) and N-glycoproteins [56]. The activity of GMPase in hsn1 and vtc1 mutants is particularly sensitive to NH$_4^+$ [24]. Of the affected functions, defective protein glycosylation in roots, rather than decreased AsA synthesis, has been linked to the hypersensitivity response [24,39]. Defective N-glycosylation in vtc1-1 contributes to cell wall, membrane, and cell cycle defects, protein folding errors, and cell death in roots directly associated with NH$_4^+$-mediated root growth inhibition [24,41]. Interestingly, stimulation of NH$_4^+$ efflux in the elongation zone is coincident with the NH$_4^+$-mediated inhibition of root elongation, which is more pronounced in the vtc1-1 mutant [38]. However, NH$_4^+$ accumulation in the roots of vtc1 and hsn1 mutants is not significantly different from that in wild type [24,39]. These results indicate that a GMPase-dependent pathway participates in the regulation of futile NH$_4^+$ cycling [27,57,58], a hypothesis that has received recent support through the application of metabolomics approaches [59].

Clearly, GDP-mannose and N-glycosylation are important for NH$_4^+$ tolerance in plants. This conclusion is supported by the discovery of the involvement of dolichol phosphate mannose synthase 1 (DPMS1) in NH$_4^+$ sensitivity, which acts downstream of GMPase and mediates the biosynthesis of dolichol phosphate mannose (Dol-P-Man), which is required for the synthesis of N-glycoproteins, glycosylphosphatidylinositol (GPI)-anchored proteins, and arabinoxylan proteins [40]. However, some uncertainties remain regarding the role of GMPase and GDP-mannose in NH$_4^+$ sensitivity and tolerance. First, GMPase activity has been found to be similar in roots and shoots of vtc1-1 and hsn1-1 mutants [24], and shoots, unlike roots, do not show enhanced sensitivity to NH$_4^+$ [45]. However, both root elongation and chlorophyll content of the dpms1-1 mutant are sensitive to NH$_4^+$ [40]. Second, root elongation is not sensitive to NH$_4^+$ in mutants of phosphomannose isomerase (PMI) and phosphomannose mutase (PMM), which act upstream of GMPase in GDP-mannose biosynthesis [41], and whose decreased activities, indirectly lead to reduced levels of GDP-mannose [56,60,61]. Therefore, it was proposed that GDP-mannose deficiency is not the primary cause of NH$_4^+$ sensitivity [41]. However, the loss of function in the dpms1-1 mutant was achieved by transfer-DNA insertion [40], whereas the vtc1-1 [56], hsn1-1 [24], pmr1-1 [60], and pmm-12 [61] mutants are the results of point mutations and consequent partial functional defects in enzyme activities. Hence, it is possible that partial functional defects in enzyme activities might result in alterations of the distribution of GDP-mannose between subcellular compartments of tissues resulting in varying sensitivities.

Therefore, it must be asked how NH$_4^+$ inhibits GMPase activity. GMPase activity has also been observed to be responsive to alkaline pH [24]. Moreover, the sensitivity of root elongation in vtc1-1 is pH dependent [41]. Root elongation of vtc1-1 is less sensitive to NH$_4^+$ under neutral pH conditions [41]. Thus, it was proposed that the decrease
of GMPase activity in the presence of $\text{NH}_4^+$ may be caused by the alkalization of cytosolic pH, following the uptake of $\text{NH}_4^+$ [24,41]. However, root elongation of vtc1-1 was not significantly different from wild type when pH was shifted from 4.0 to 9.0 in growth media in the absence of $\text{NH}_4^+$ [41]. Therefore, alterations in GMPase activity could not be readily ascribed to pH changes alone. Instead, GMPase activity in vtc1-1 may be optimized under near-neutral pH conditions, and become potent in the antagonization of $\text{NH}_4^+$ toxicity under such conditions. Thus, root elongation of vtc1-1 shows greater sensitivity to $\text{NH}_4^+$ under acidic or alkaline pH conditions [41]. A recently isolated Arabidopsis svt2 suppressor of vtc1-1 exhibits root growth similar to the wild type in the presence of $\text{NH}_4^+$ [62], supporting earlier notions, and it is hoped that this will provide novel insights into the role of GMPase and GDP-mannose in $\text{NH}_4^+$ sensitivity in the near future.

**Root gravitropism**

In addition to elongation, root gravitropism is also affected by $\text{NH}_4^+$. Although moderate $\text{NH}_4^+$ can enhance root gravitropism in Arabidopsis, under high levels of $\text{NH}_4^+$, root gravitropism is reduced [44]. However, the effect of $\text{NH}_4^+$ on root gravitropism is independent of root elongation [44]. Furthermore, unlike in the case of root elongation, auxin distribution in the root tip is involved in the gravitropism response to $\text{NH}_4^+$ [44]. The K-carrier mutant trh1 shows different patterns of root gravitropism and DR5 (a synthetic auxin response element)::GUS (β-glucuronidase) signal intensity in root apex cells compared with wild type in response to $\text{NH}_4^+$ [44]. Based on this phenotype, a gravitropism-sensitive-to-ammonium 1 (gsa-1) mutant was identified in Arabidopsis [49]. GSA-1 is a mutation allelic to ALTERED RESPONSE TO GRAVITY 1 (ARG1), which encodes a DnaJ-like protein [63], and is required for establishment of the auxin exporter PIN3 (PIN-FORMED 3).-dependent lateral auxin gradient across the root cap following gravistimulation [64,65]. A recent study has shown that disruption of GSA-1/ARG1 can reduce basipetal auxin transport and the expression of the auxin influx carrier AUX1 in the lateral root-cap and epidermal cells of root apices [49]. However, $\text{NH}_4^+$ does not inhibit the expression of AUX1 but of the auxin exporter PIN2 in this region [49]. Therefore, it appears that ARG1/GSA-1 is required for the establishment of the PIN3-mediated lateral auxin gradient across the root cap and AUX1-mediated basipetal auxin transport to antagonize the reduction in PIN2-mediated auxin distribution, and protect root gravitropism under $\text{NH}_4^+$ stress [49]. PIN2 is only expressed in larger amounts in the transition zone. Indeed, it may be considered as a specific marker of the root apical zone [66]. Furthermore, PIN2 has emerged as both the target and response element when roots are subjected to diverse stresses [67]. For example, as in cases of salinity stress [68], $\text{NH}_4^+$ stress induces degradation of PIN2, which enables roots to override gravity to avoid areas of soil containing toxic concentrations of ions [49].

**Lateral root formation**

Lateral root elongation is suppressed in a similar way to the primary root [37,38,69]; however, lateral root formation is increased under RSA [38,70]. Lateral root initiation and higher-order lateral root branching are both enhanced by localized $\text{NH}_4^+$ supply [70]. Interestingly, the $\text{NH}_4^+$-induced promotion of lateral root formation is defective in a quadruple $\text{NH}_4^+$-transporter insertion line (qko, the amt1;1 amt1;2 amt1;3 amt2;1 mutant), but is independent of the cumulative uptake of $\text{NH}_4^+$ [70]. These results suggest that $\text{NH}_4^+$ acts as a signal to activate lateral root formation. Furthermore, the sensor function may be mediated by the $\text{NH}_4^+$ transporter AMT1;3 rather than by AMT1;1 in Arabidopsis because reconstitution of the expression of AMT1;3, but not of AMT1;1, in an amt1;3 or qko background restored higher-order lateral root development [70]. Primary root inhibition is accompanied by stimulation of lateral root formation in response to localized $\text{NH}_4^+$ supply. However, the molecular mechanism underlying the relation between primary root inhibition and lateral root formation is unknown.

Unlike RSA, SSA strongly suppresses lateral root formation, which can override the stimulation of lateral root formation by RSA [25]. Lateral root emergence, but not initiation, is reduced by SSA, independent of abscisic acid (ABA) signaling [25], which has been shown to be involved in the reduction of lateral root formation by high NO$_3^-$ and osmotic stress [71–73]. However, it is related to a reduced auxin response in roots as a result of impairment in long-distance auxin transport from shoots to roots [25]. The SSA-mediated reduction of both long-distance auxin transport and lateral root emergence are weakened in mutants that are defective in the auxin importer AUX1, but not in mutants that are defective in the auxin exporters PIN1 and PIN2. Furthermore, the expression of AUX1, particularly in vascular tissues, is repressed by SSA [25]. Thus, as part of the feedback loop between auxin levels and AUX1 expression [74], SSA appears to modulate local expression of AUX1 in shoots to reduce auxin influx and, consequently, lower auxin levels, resulting in a further decrease in AUX1-dependent long-distance auxin transport and auxin response in roots [25]. During early 2013, it was further reported that ethylene production in shoots, but not in roots, is enhanced by SSA, which results in the reduction of lateral root formation coupled to a suppression of AUX1 expression in shoots [47]. Under SSA, lateral root formation in the ethylene receptor-defective mutant etr1-3 is more resistant than wild type, whereas in ethylene-overproduction mutants, such as xba132 and eto1-1, it is less sensitive [47]. However, $\text{NH}_4^+$ content in shoots has been shown to increase linearly with SSA, but not with RSA, suggesting that it represents the internal trigger for SSA inhibition of root development [42]. Overall, the effect of SSA on lateral root formation is likely to be the result of systemic signaling. It is possible that, under SSA, accumulation of $\text{NH}_4^+$ triggers ethylene production that represses AUX1 function, resulting in reduction of auxin transport from shoots to roots and of auxin response in lateral root primordia, hindering lateral root formation.

**Effects of $\text{NH}_4^+$ stress on chloroplast function and shoot biomass**

Reduced shoot biomass and chlorosis of leaves are other important symptoms that have been frequently reported,
particularly in hydroponic studies of young plants suffering NH₄⁺ toxicity [3,75,76]. However, studies have shown that shoot biomass is not reduced by RSA in Arabidopsis grown on agar-plate systems [37] or in Lotus japonicus cultured with hydroponics [38], suggesting that RSA-mediated negative regulation of shoot development, where observed, is a secondary effect of an impaired root system. However, leaves are sensitive to NH₄⁺ when in direct contact with NH₄⁺ in agar-plate growth systems [25]. Leaf hypersensitivity to NH₄⁺ was also observed in the Dol-P-Man biosynthesis-defective dmps1 mutant when grown in agar medium [40]. Based on their chlorotic phenotype, ammonium overly sensitive 1 (amos1) [45] and amos2 [43] mutants were recently identified: these showed sensitivity to SSA but not to RSA. Gene clone analysis revealed that amos1 is an allelic mutation of EGY1 [45], which encodes a plastid metalloprotease and is required for normal chloroplast development and ethylene-dependent gravitropism of hypocotyls grown in the light [77]. Analysis of the amos1 mutant revealed the operation of an NH₄⁺-responsive, AMOS1/EGY1-dependent plastid retrograde signaling pathway, which is required for the expression of NH₄⁺-responsive genes in the nucleus and the maintenance of chloroplast functionality [34]. However, accumulation of NH₄⁺ and the expression of genes involved in NH₄⁺ transport and assimilation are not significantly altered in amos1 compared with wild type [45]. Therefore, it was suggested that AMOS1/EGY1 participated in the regulation of NH₄⁺-stress signaling. In addition, ABA was shown to be a downstream messenger of AMOS1/EGY1-dependent plastid retrograde signaling to regulate the NH₄⁺ responses [45]. Moreover, the generation of reactive oxygen species (ROS) was shown to be involved in AMOS1/EGY1-dependent plastid retrograde signaling [45,46]. Therefore, a reasonable proposal is that, under NH₄⁺ stress, the chloroplast receives the stress signal (the plasma membrane acting as the first site of perception of NH₄⁺ stress), triggering AMOS1/EGY1-dependent retrograde signaling (ROS are a plausible candidate for the signal) and recruiting downstream ABA signaling, to regulate the expression of NH₄⁺-responsive genes in the nucleus and prevent NH₄⁺ toxicity. Interestingly, the root development of amos1 mutants is similar to wild type [45]. These results also support the notion that SSA-mediated inhibition of lateral root formation is one involving systemic signaling rather than a simple secondary effect of impaired chloroplast functionality and photosynthesis production.

Different from amos1 and vte1/hsn1, both shoot and root development are impacted in the amos2 mutant, and symptoms are associated with excess accumulation of NH₄⁺ in shoots and a reduction in tissue K⁺ [43]. As emphasized above, applications of external K⁺ can alleviate NH₄⁺ toxicity in many plant species [19,28,32,53,78], a phenomenon that has been attributed to not only uptake competition with NH₄⁺ at the sites of channels and transporters [28,32,78], but also the optimized NH₄⁺ assimilation [19]. Improving plant performance by optimization of K⁺ in NH₄⁺ media is likely to be of substantial agronomic significance in crop species that are routinely grown in soils containing NH₄⁺, such as rice (Oryza sativa) [19]. However, as yet, little is known about the molecular mechanisms of interaction between NH₄⁺ and K⁺ and their regulation. Therefore, amos2, as a novel type of NH₄⁺-sensitive mutant that connects impairments in shoot development, lateral root formation, and NH₄⁺-K⁺ homeostasis, may provide a suitable tool to study these processes at the mechanistic level.

Towards an integrated view of NH₄⁺ stress responses

NH₄⁺ derived from shoot and root contact with a NH₄⁺ source entails strong modulation of Arabidopsis growth. However, details of the symptoms, signaling transduction, genetic regulation, and corresponding physiological processes differ significantly according to the sites of action (Figure 2). Recent studies suggest that RSA principally targets root system development, including elongation, gravitropism, and lateral root branching [38,49,70]. This event appears to occur in the root tip [38], but may involve different zones for these three localized effects. The target for the inhibition of root elongation and gravitropism may be in the elongation and transition zones, respectively [38,49]. However, reduced root elongation and increased lateral root number could plausibly involve NH₄⁺ transporters whose downregulatory response to elevated NH₄⁺ is now mechanistically understood [38,69]. PIN2 appears to be the target for inhibition of root gravitropism [49]. However, the regulatory networks are still unclear. For instance, the local NH₄⁺ sensing and signal transduction has still to be identified. Under SSA conditions, the signaling response is more complex and includes both local and systemic components. First, NH₄⁺-induced leaf chlorosis, as a shoot-localized behavior, is mediated by AMOS1/EGY1-dependent plastid retrograde signaling [45]. Second, the SSA-mediated reduction of lateral root formation is a typical systemic signaling process that involves local ethylene and auxin production in shoots, and also long-distance auxin transport and auxin response in roots [25,47]. However, these hormone signaling pathways do not appear to be involved in the early stages of the response to NH₄⁺, indicated by the recent identification using proteomics of fast-responding protein phosphorylation patterns in response to NH₄⁺ resupply [79]. Incidentally, both transcriptomic and proteomic analysis indicate that the signaling pathways in response to NH₄⁺ are distinct from pathways engaged in response to other nitrogen sources, such as NO₃⁻ [79,80].

Shoots are believed to be significantly more sensitive than roots when in direct contact with NH₄⁺ and, therefore, we suggest that it is crucial to prevent shoots coming into direct contact with NH₄⁺ when Arabidopsis seedlings are used in agar experiments. Plants may have evolved a more thorough and effective detoxification or toxicity avoidance mechanism in roots over time as an outcome of exposure to NH₄⁺ in soils, whereas the exposure of shoots to high NH₄⁺/NH₃ deposition via the atmosphere is mostly a recent, anthropogenic problem that has intensified only over the past several decades (Box 1). One suggestion is that compartmentalizing NH₄⁺ in roots can serve as a cost-effective strategy to avoid the occurrence of NH₄⁺ toxicity in the more sensitive shoot tissue.

Several new approaches have been used recently in the dissection of NH₄⁺ toxicity, such as a diversified local supply
system [25,38,69,70], ion-selective electrode techniques and isotopic labeling [38,81], forward genetics [24,43,45,49], metabolomics [59], transcriptomics [25,80], and proteomics [79]. In particular, we outline the utility of a novel supply device for the study of localized NH$_4^+$ supply in agar-grown Arabidopsis (Figure 1) and illustrate how its application has enabled the dissection of the fundamentally different consequences of RSA and SSA in terms of Arabidopsis growth. Our work suggests that studying the localized nutrient supply is necessary when the goal is to investigate precise nutrient effects in Arabidopsis in agar-plate systems.

Concluding remarks
Over the past few years, it has been demonstrated that the effects of RSA are highly localized, whereas SSA produces both local and systemic effects on plant growth via elaborate signaling networks (Figure 2). Several regulatory factors have been shown to take part in this signaling pathway. This simple working model, and the associated research methodology, should provide important new insight into NH$_4^+$ toxicity in plants while offering new experimental approaches to still outstanding mechanistic questions in the field. Our knowledge of the molecular components involved in the NH$_4^+$ stress response is in its infancy, and details of signal transduction, such as the precise identification of sensors and transcription factors, remain a challenge. Recent studies have demonstrated that NH$_4^+$ derived from aboveground and belowground sources suppresses plant growth differentially by targeting specific tissues. It is unclear whether differences in the behavior of specific tissues reflect different NH$_4^+$ accumulation patterns or sensitivity thresholds (tissue tolerance). It will be crucial to develop sensitive approaches that are able to map out NH$_4^+$ distribution in different tissues, and explore tissue-specific expression systems, which has led to advances in understanding in other ion stress fields, such as Na$^+$ stress [82]. Given that NH$_4^+$ is a major and, in many cases even ‘preferred’, nitrogen source for higher plants [20], much natural variation exists in sensitivity and tolerance traits [3], and the relation between NH$_4^+$ sensitivity and nitrogen-use efficiency in crops will be an important topic to explore, as recently illustrated for rice [83].

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