Review

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

Baohai Li[1], Guangjie Li[1], Herbert J. Kronzucker[2], František Baluška[3], and Weiming Shi[1]

[1] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, No. 71 East Beijing Road, Nanjing 210008, China
[2] Department of Biological Sciences, University of Toronto, 1265 Military Trail, Toronto, Ontario, M1C 1A4, Canada
[3] Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, Bonn, D-53115, Germany

Ammonium (NH_4^+) 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 NH_4^+ 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 NH_4^+ stress in the roots and shoots of *Arabidopsis thaliana*. We outline new experimental approaches that are being used to study NH_4^+ toxicity in *Arabidopsis* and propose an integrated view of behavior and signaling in response to NH_4^+ stress in the *Arabidopsis* system.

Ammonium toxicity in higher plants

Compromised plant growth and production as a result of NH_4^+ accumulating in soils is a long-standing and serious problem in agriculture [1–3]. Over recent decades, excessive NH_4^+/NH_3 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, NH_4^+ 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 NH_4^+ is a preferred nitrogen source for many plants [3,15], NH_4^+ toxicity should be seen as universal, even in species frequently labeled as 'NH_4^+ specialists' [19,20], although toxicity thresholds tend to be shifted to higher NH_4^+ concentrations in such specialists. Toxicity tends to be particularly pronounced when NH_4^+ 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 NH_4^+ 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 (NO_3^-) because its uptake is associated with alkalinization of the root medium and stimulation of cation uptake, counteracting some of the effects of NH_4^+ [15,21,22]. However, it has emerged more recently that these factors are not directly related. First, in several studies, NH_4^+ toxicity was still observed even under conditions of pH buffering and in the presence of NO_3^-, although the thresholds of toxic NH_4^+ concentration were raised [23–25]. Second, sensitivities to NH_4^+ vary among different plant species, independent of pH adaptations [3,26], which suggests the evolution of highly distinct mechanisms to deal with NH_4^+ 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 NH_4^+ transport across the plasma membranes of roots of sensitive barley (*Hordeum vulgare*) [27], concomitant with elevated NH_4^+ accumulation in both roots and shoots [28]. Such NH_4^+ overaccumulation is commonly observed in sensitive species, such as spinach (*Spinacea oleracea L.*), and tomato (*Lycopersicon esculentum Mill.*) [29,30]. Strong relations between futile NH_4^+ cycling, NH_4^+ tissue accumulation, respiratory rates, and growth have since been reported both under 'control' conditions and under conditions of elevated K^+, when overall NH_4^+ toxicity is reduced [19,28]. Overaccumulation of NH_4^+ is contingent upon excessive NH_4^+ 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 NH_4^+ and gaseous NH_3 [31,32]. Recent studies have shown that phosphorylation of the threonine residue T460 of the high-affinity NH_4^+ transporter AtAMT1.1 in response to an increase in exogenous NH_4^+ leads to a loss of

Corresponding author: Shi, W. (wmshi@issas.ac.cn).
Keywords: ammonium toxicity; signaling location and response; molecular components; physiological behavior.

1360-1385/$ – see front matter
 http://dx.doi.org/10.1016/j.tplants.2013.09.004

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 nitrification 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^- [15], 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' [1,2,84–86]. 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 chloroplast 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*

Mutant name	Gene ID	Gene molecular function	Phenotype (NH_4^+ response)	Refs
hsn1/vtc1	AT2G39770	GMPase	Root elongation[a]	[24,39]
trh1	AT4G23640	Potassium transporter	Root gravitropism[b]	[44]
gsa-1/arg1	AT1G68370	DnaJ-like protein	Root gravitropism[a]	[49]
amt1;3	AT3G24300	Ammonium transporter	Lateral root formation[b]	[70]
aux1	AT2G38120	Auxin transporter	Lateral root formation[b] and root elongation[b]	[25,42,53]
etr1	AT1G66340	Ethylene receptor	Lateral root formation[b]	[47]
xbat32	AT5G57740	A negative regulator of ethylene biosynthesis	Lateral root formation[a]	[47]
eto1-1	AT3G51770	A negative regulator of ethylene biosynthesis	Lateral root formation[a]	[47]
dpms1	AT1G20575	Dolichol phosphate mannose synthase 1	Chlorophyll content[a] and root elongation[a]	[40]
amos1/egy1	At5G35220	Plastid metalloprotease	Chlorophyll content[a]	[45]
amos2	Unknown	Unknown	Leaf biomass[a], chlorophyll content[a], and lateral root formation[a]	[43]

[a]Sensitive to NH_4^+.

[b]Resistant to NH_4^+.

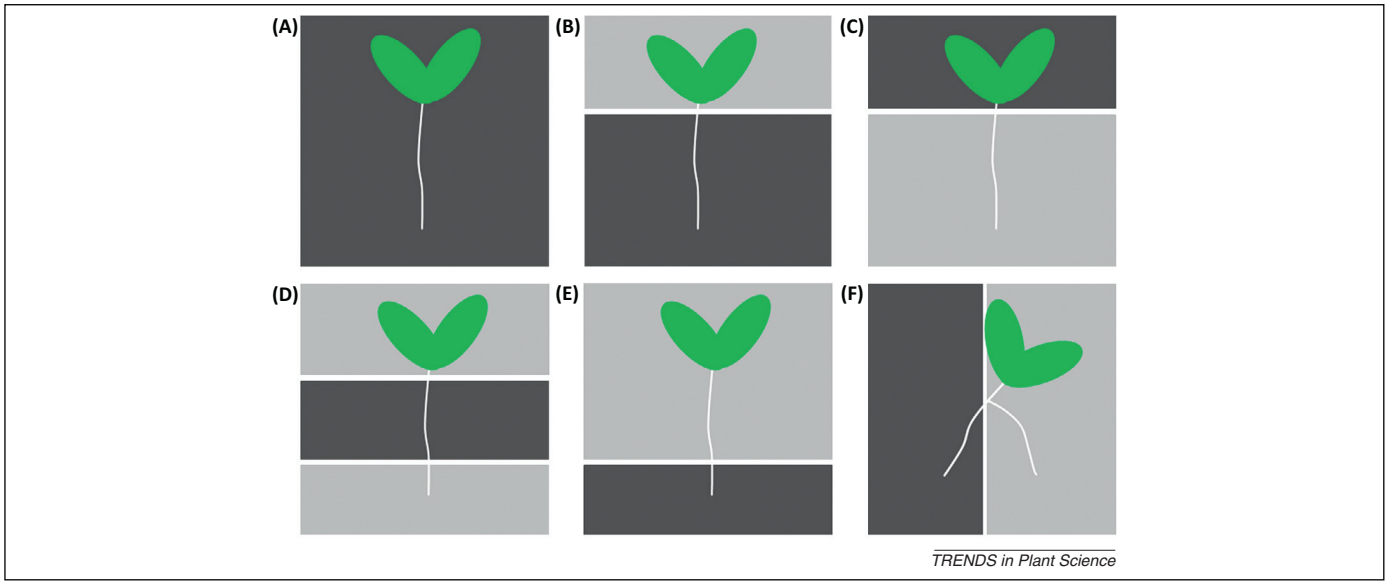

Figure 1. Schematic representation of six types of ammonium (NH_4^+) supply in a novel agar-plate growth system: **(A)** whole plant, **(B)** root, **(C)** shoot, **(D)** mature root, **(E)** root tip, and **(F)** split-root. Dark areas represent zones with elevated NH_4^+ and light areas represent normal growth medium. The separator gap between normal growth medium and medium with elevated NH_4^+ is approximately 3 mm thick to avoid nutritional contamination of the different zones [25,38]. Custom-made segmented plates (13 × 13 cm) can be separated into two or three parts using a movable glass strip (3 mm in width and 1.5 cm in height) plugged into a fixed plastic groove (2 mm in height) in the plate [25], which can be used to create various nutrient supply zones.

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 arabinogalactan 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], *pmi-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 or tissues resulting in varying sensitivities.

Therefore, it must be asked how NH_4^+ represses 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 NH_4^+ may be caused by the alkalinization of cytosolic pH, following the uptake of 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 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 NH_4^+ toxicity under such conditions. Thus, root elongation of *vtc1-1* shows greater sensitivity to 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 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 NH_4^+ sensitivity in the near future.

Root gravitropism

In addition to elongation, root gravitropism is also affected by NH_4^+. Although moderate NH_4^+ can enhance root gravitropism in *Arabidopsis*, under high levels of NH_4^+, root gravitropism is reduced [44]. However, the effect of 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 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 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, 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 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], 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 NH_4^+ supply [70]. Interestingly, the NH_4^+-induced promotion of lateral root formation is defective in a quadruple NH_4^+-transporter insertion line (*qko*, the *amt1;1 amt1;2 amt1;3 amt2;1* mutant), but is independent of the cumulative uptake of NH_4^+ [70]. These results suggest that NH_4^+ acts as a signal to activate lateral root formation. Furthermore, the sensor function may be mediated by the 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 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 *xbat32* and *eto1-1*, it is less sensitive [47]. However, 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 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 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 NH_4^+ 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 NH_4^+ when in direct contact with NH_4^+ in agar-plate growth systems [25]. Leaf hypersensitivity to NH_4^+ was also observed in the Dol-P-Man biosynthesis-defective *dpms1* mutant when grown in agar medium [40]. Based on their chlorotic phenotypes, *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 NH_4^+-responsive, AMOS1/EGY1-dependent plastid retrograde signaling pathway, which is required for the expression of NH_4^+-responsive genes in the nucleus and the maintenance of chloroplast functionality [34]. However, accumulation of NH_4^+ and the expression of genes involved in NH_4^+ 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 NH_4^+-stress signaling. In addition, ABA was shown to be a downstream messenger of AMOS1/EGY1-dependent plastid retrograde signaling to regulate the NH_4^+ 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 NH_4^+ stress, the chloroplast receives the stress signal (the plasma membrane acting as the first site of perception of NH_4^+ 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 NH_4^+-responsive genes in the nucleus and prevent NH_4^+ 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 vtc1/hsn1*, both shoot and root development are impacted in the *amos2* mutant, and symptoms are associated with excess accumulation of NH_4^+ in shoots and a reduction in tissue K^+ [43]. As emphasized above, applications of external K^+ can alleviate NH_4^+ toxicity in many plant species [19,28,32,53,78], a phenomenon that has been attributed to not only uptake competition with NH_4^+ at the sites of channels and transporters [28,32,78], but also the optimized NH_4^+ assimilation [19]. Improving plant performance by optimization of K^+ in NH_4^+ media is likely to be of substantial agronomic significance in crop species that are routinely grown in soils containing NH_4^+, such as rice (*Oryza sativa*) [19]. However, as yet, little is known about the molecular mechanisms of interaction between NH_4^+ and K^+ and their regulation. Therefore, *amos2*, as a novel type of NH_4^+-sensitive mutant that connects impairments in shoot development, lateral root formation, and NH_4^+–K^+ homeostasis, may provide a suitable tool to study these processes at the mechanistic level.

Towards an integrated view of NH_4^+ stress responses

NH_4^+ derived from shoot and root contact with a NH_4^+ 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 NH_4^+ transporters whose downregulatory response to elevated NH_4^+ 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 NH_4^+ 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, NH_4^+-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 NH_4^+, indicated by the recent identification using proteomics of fast-responding protein phosphorylation patterns in response to NH_4^+ resupply [79]. Incidentally, both transcriptomic and proteomic analysis indicate that the signaling pathways in response to NH_4^+ are distinct from pathways engaged in response to other nitrogen sources, such as NO_3^- [79,80].

Shoots are believed to be significantly more sensitive than roots when in direct contact with NH_4^+ and, therefore, we suggest that it is crucial to prevent shoots coming into direct contact with NH_4^+ 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 NH_4^+ in soils, whereas the exposure of shoots to high NH_4^+/NH_3 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 NH_4^+ in roots can serve as a cost-effective strategy to avoid the occurrence of NH_4^+ toxicity in the more sensitive shoot tissue.

Several new approaches have been used recently in the dissection of NH_4^+ toxicity, such as a diversified local supply

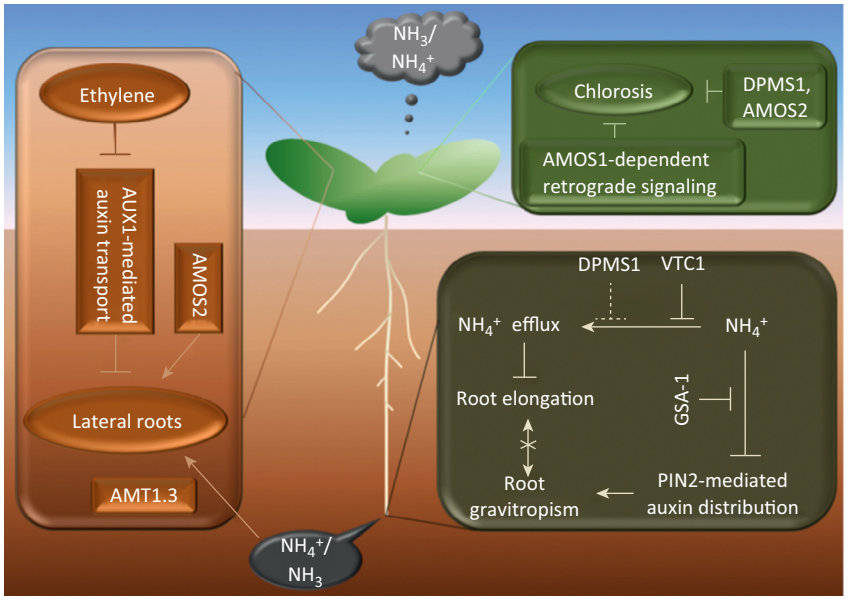

Figure 2. Model of behavior and signaling responses under ammonium (NH_4^+) stress in *Arabidopsis thaliana* showing signal locations, genetic loci, and physiological targets of NH_4^+ stress. NH_4^+ stress may be derived from belowground (root-supplied NH_4^+, RSA) or aboveground (shoot-supplied NH_4^+, SSA) sources. Under RSA conditions, root elongation is inhibited, root gravitropism is reduced, and lateral root numbers increase. These responses seem to be controlled by independent genes and pathways and are believed to be highly localized responses that occur in the root tip. However, the inhibition of root elongation and reduced root gravitropism responses are likely to be localized in different zones in the root tip, such as the elongation zone (associated with NH_4^+ efflux) and the transition zone (degradation of PIN2), respectively. By contrast, the shoot is less affected by RSA. Under SSA conditions, both local responses (leaf chlorosis) and systemic responses (reduced lateral root emergence) are observed, which are regulated by AMOS1/EGY1-dependent plastid retrograde signaling and the ethylene–auxin pathway, respectively. Under combined RSA and SSA conditions, lateral roots are similar to those seen when SSA conditions occur alone. DPMS1 and AMOS2 appear to participate in several signaling pathways based on the phenotypes observed in overexpression and loss-of-function mutant studies [40,43], although this warrants more detailed examination. The greater hypersensitivity of the shoot to SSA than to RSA implies that the shoot may be more vulnerable to NH_4^+ than the roots and, therefore, compartmentalization of NH_4^+ in roots could serve as a cost-effective strategy to avoid NH_4^+ toxicity in the more sensitive shoot. This hypothesis may explain why some plant species are hypersensitive and even extinct in regions of high NH_4^+/NH_3 deposition. It also provides a new foundation for the rational application of NH_4^+-release fertilizers, with the goal of optimizing nitrogen-use efficiency and reducing the risk to increasingly fragile ecological systems. Abbreviations: AMOS1/2, ammonium overly sensitive 1/2; AMT1.3, ammonium transporter 1.3; AUX1, auxin-resistant 1; DPMS1, dolichol phosphate mannose synthase 1; GSA1, gravitropism-sensitive-to-ammonium 1; PIN2, auxin efflux carrier PIN-FORMED 2; VTC1, vitamin C defective 1.

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].

Acknowledgments

This work was supported by the National Science Foundation of China (grant no. 31200189 and 30771285), the Chinese Academy Sciences Innovation Program (CAS ISSASIP1103), and the Natural Sciences and Engineering Research Council of Canada (NSERC, Discovery Grant 217277-2009).

References

1 Gerendas, J. *et al.* (1997) Physiological and biochemical processes related to ammonium toxicity in higher plants. *Z. Pflanzenernähr. Bodenkd.* 160, 239–251
2 Wiesler, F. (1997) Agronomical and physiological aspects of ammonium and nitrate nutrition of plants. *Z. Pflanzenernähr. Bodenkd.* 160, 227–238

3 Britto, D.T. and Kronzucker, H.J. (2002) NH_4^+ toxicity in higher plants: a critical review. *J. Plant Physiol.* 159, 567–584
4 Van Dijk, H.F.G. and Roelofs, J.G.M. (1988) Effects of excessive ammonium deposition on the nutritional status and condition of pine needles. *Physiol. Plant.* 73, 494–501
5 Alber, J.D. *et al.* (1989) Nitrogen saturation in northern forest ecosystems. *BioScience* 39, 378–386
6 Pearson, J. and Stewart, G.R. (1993) The deposition of atmospheric ammonia and its effects on plants. *New Phytol.* 125, 283–305
7 Krupa, S.V. (2003) Effects of atmospheric ammonia (NH_3) on terrestrial vegetation: a review. *Environ. Pollut.* 124, 179–221
8 Stevens, C.J. *et al.* (2004) Impact of nitrogen deposition on the species richness of grasslands. *Science* 303, 1876–1879
9 Stevens, C.J. *et al.* (2006) Loss of forb diversity in relation to nitrogen deposition in the UK: regional trends and potential controls. *Global Change Biol.* 12, 1823–1833
10 Camargo, J.A. and Alonso, A. (2006) Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. *Environ. Int.* 32, 831–849
11 Clark, C.M. and Tilman, D. (2008) Loss of plant species after chronic low-level nitrogen deposition to prairie grasslands. *Nature* 451, 712–715
12 Dupre, C. *et al.* (2010) Changes in species richness and composition in European acidic grasslands over the past 70 years: the contribution of cumulative atmospheric nitrogen deposition. *Global Change Biol.* 16, 344–357
13 Bobbink, R. *et al.* (2010) Global assessment of nitrogen deposition effects on terrestrial plant diversity: a synthesis. *Ecol. Appl.* 20, 30–59
14 Liu, X. *et al.* (2013) Enhanced nitrogen deposition over China. *Nature* 494, 459–462
15 von Wiren, N. *et al.* (2000) The molecular physiology of ammonium uptake and retrieval. *Curr. Opin. Plant Biol.* 3, 254–261
16 von Wiren, N. and Merrick, M. (2004) Regulation and function of ammonium carriers in bacteria, fungi and plants. *Top. Curr. Genet.* 9, 95–120
17 Hess, D.C. *et al.* (2006) Ammonium toxicity and potassium limitation in yeast. *PLoS Biol.* 4, e351
18 Norenberg, M.D. *et al.* (2009) Signaling factors in the mechanism of ammonia neurotoxicity. *Metab. Brain Dis.* 24, 103–117
19 Balkos, D.T. *et al.* (2010) Optimization of ammonium acquisition and metabolism by potassium in rice (*Oryza sativa* L. cv. IR-72). *Plant Cell Environ.* 33, 23–34
20 Britto, D.T. and Kronzucker, H.J. (2013) Ecological significance and complexity of N-source preference in plants. *Ann. Bot.* 112, 957–963
21 Walch-Liu, P. *et al.* (2000) Rapid effects of nitrogen form on leaf morphogenesis in tobacco. *J. Exp. Bot.* 51, 227–237
22 Britto, D.T. and Kronzucker, H.J. (2005) Nitrogen acquisition, PEP carboxylase, and cellular pH homeostasis: new views on old paradigms. *Plant Cell Environ.* 28, 1396–1409
23 Li, B. and Shi, W. (2007) Effects of elevated NH_4^+ on *Arabidopsis* seedlings different in accessions. *Acta Pedol. Sin.* 44, 508–515
24 Qin, C. *et al.* (2008) GDP-mannose pyrophosphorylase is a genetic determinant of ammonium sensitivity in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 18308–18313
25 Li, B. *et al.* (2011) Shoot-supplied ammonium targets the root auxin influx carrier AUX1 and inhibits lateral root emergence in *Arabidopsis*. *Plant Cell Environ.* 34, 933–946
26 van den Berg, L.J.L. *et al.* (2005) Decline of acid-sensitive plant species in heathland can be attributed to ammonium toxicity in combination with low pH. *New Phytol.* 166, 551–564
27 Britto, D.T. *et al.* (2001) Futile transmembrane NH_4^+ cycling: a cellular hypothesis to explain ammonium toxicity in plants. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4255–4258
28 Szczerba, M.W. *et al.* (2008) Alleviation of rapid, futile ammonium cycling at the root plasma membrane by potassium reveals K^+-sensitive and -insensitive components of NH_4^+ transport. *J. Exp. Bot.* 59, 303–313
29 Lasa, B. *et al.* (2001) The sensitivity to ammonium nutrition is related to nitrogen accumulation. *Sci. Hort.* 91, 143–152
30 Barker, A.V. (1999) Foliar ammonium accumulation as an index of stress in plants. *Commun. Soil Sci. Plant Anal.* 30, 167–174
31 Holm, L.M. *et al.* (2005) NH_3 and NH_4^+ permeability in aquaporin-expressing *Xenopus* oocytes. *Pflügers Arch.* 450, 415–428
32 ten Hoopen, F. *et al.* (2010) Competition between uptake of ammonium and potassium in barley and *Arabidopsis* roots: molecular mechanisms and physiological consequences. *J. Exp. Bot.* 61, 2303–2315
33 Loque, D. *et al.* (2007) A cytosolic trans-activation domain essential for ammonium uptake. *Nature* 446, 195–198
34 Loque, D. *et al.* (2009) Pore mutations in ammonium transporter AMT1 with increased electrogenic ammonium transport activity. *J. Biol. Chem.* 284, 24988–24995
35 Lanquar, V. *et al.* (2009) Feedback inhibition of ammonium uptake by a phospho-dependent allosteric mechanism in *Arabidopsis*. *Plant Cell* 21, 3610–3622
36 Yuan, L. *et al.* (2013) Allosteric regulation of transport activity by heterotrimerization of *Arabidopsis* ammonium transporter complexes in vivo. *Plant Cell* 25, 974–984
37 Rogato, A. *et al.* (2010) Characterization of a developmental root response caused by external ammonium supply in *Lotus japonicus*. *Plant Physiol.* 154, 784–795
38 Li, Q. *et al.* (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
39 Barth, C. *et al.* (2010) A mutation in GDP-mannose pyrophosphorylase causes conditional hypersensitivity to ammonium, resulting in *Arabidopsis* root growth inhibition, altered ammonium metabolism, and hormone homeostasis. *J. Exp. Bot.* 61, 379–394
40 Jadid, N. *et al.* (2011) DOLICHOL PHOSPHATE MANNOSE SYNTHASE1 mediates the biogenesis of isoprenyl-linked glycans and influences development, stress response, and ammonium hypersensitivity in *Arabidopsis*. *Plant Cell* 23, 1985–2005
41 Kempinski, C.F. *et al.* (2011) Toward the mechanism of NH_4^+ sensitivity mediated by *Arabidopsis* GDP-mannose pyrophosphorylase. *Plant Cell Environ.* 34, 847–858
42 Li, B. *et al.* (2011) Roles of abscisic acid and auxin in shoot-supplied ammonium inhibition of root system development. *Plant Signal. Behav.* 6, 1451–1453
43 Li, G. *et al.* (2012) Isolation and characterization of a novel ammonium overly sensitive mutant, *amos2*, in *Arabidopsis thaliana*. *Planta* 235, 239–252
44 Zou, N. *et al.* (2012) Ammonium-induced loss of root gravitropism is related to auxin distribution and TRH1 function, and is uncoupled from the inhibition of root elongation in *Arabidopsis*. *J. Exp. Bot.* 63, 3777–3788
45 Li, B. *et al.* (2012) *Arabidopsis* plastid metalloprotease AMOS1/EGY1 integrates with ABA signaling to regulate global gene expression in response to ammonium stress. *Plant Physiol.* 160, 2040–2051
46 Li, B. *et al.* (2013) Molecular components of stress-responsive plastid retrograde signaling networks and their involvement in ammonium stress. *Plant Signal. Behav.* 8, e23107
47 Li, G. *et al.* (2013) Ammonium-induced shoot ethylene production is associated with the inhibition of lateral root formation in *Arabidopsis*. *J. Exp. Bot.* 64, 1413–1425
48 Liu, Y. *et al.* (2013) Ammonium inhibits primary root growth by reducing the length of meristem and elongation zone and decreasing elemental expansion rate in the root apex in *Arabidopsis thaliana*. *PLoS ONE* 8, e61031
49 Zou, N. *et al.* (2013) GSA-1/ARG1 protects root gravitropism in *Arabidopsis* under ammonium stress. *New Phytol.* 200, 97–111
50 Beemster, G.T. *et al.* (2003) Cell cycle: the key to plant growth control? *Trends Plant Sci.* 8, 154–158
51 Petricka, J.J. *et al.* (2012) Control of *Arabidopsis* root development. *Annu. Rev. Plant Biol.* 63, 563–590
52 Overvoorde, P. *et al.* (2010) Auxin control of root development. *Cold Spring Harb. Perspect. Biol.* 2, a001537
53 Cao, Y. *et al.* (1993) Ammonium inhibition of *Arabidopsis* root growth can be reversed by potassium and by auxin resistance mutations *aux1*, *axr1*, and *axr2*. *Plant Physiol.* 102, 983–989
54 Löpez-Bucio, J. *et al.* (2003) The role of nutrient availability in regulating root architecture. *Curr. Opin. Plant Biol.* 6, 280–287
55 Peret, B. *et al.* (2011) Root developmental adaptation to phosphate starvation: better safe than sorry. *Trends Plant Sci.* 16, 442–450

56 Conklin, P.L. *et al.* (1999) Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4198–4203
57 Kronzucker, H.J. *et al.* (2001) Ammonium toxicity and the real cost of transport. *Trends Plant Sci.* 6, 335–337
58 Britto, D.T. *et al.* (2002) Subcellular NH_4^+ flux analysis in leaf segments of wheat (*Triticum aestivum*). *New Phytol.* 155, 373–380
59 Masakapalli, S.K. *et al.* (2013) The metabolic flux phenotype of heterotrophic *Arabidopsis* cells reveals a complex response to changes in nitrogen supply. *Plant J.* 74, 569–582
60 Hoeberichts, F.A. *et al.* (2008) A temperature-sensitive mutation in the *Arabidopsis thaliana* phosphomannomutase gene disrupts protein glycosylation and triggers cell death. *J. Biol. Chem.* 283, 5708–5718
61 Maruta, T. *et al.* (2008) *Arabidopsis* phosphomannose isomerase 1, but not phosphomannose isomerase 2, is essential for ascorbic acid biosynthesis. *J. Biol. Chem.* 283, 28842–28851
62 Kempinski, C.F. *et al.* (2013) The novel *Arabidopsis thaliana svt2* suppressor of the ascorbic acid-deficient mutant *vtc1-1* exhibits phenotypic and genotypic instability. *F1000Research* 2, 6
63 Sedbrook, J.C. *et al.* (1999) ARG1 (altered response to gravity) encodes a DnaJ-like protein that potentially interacts with the cytoskeleton. *Proc. Natl. Acad. Sci. U.S.A.* 96, 1140–1145
64 Boonsirichai, K. *et al.* (2003) ALTERED RESPONSE TO GRAVITY is a peripheral membrane protein that modulates gravity-induced cytoplasmic alkalinization and lateral auxin transport in plant statocytes. *Plant Cell* 15, 2612–2625
65 Harrison, B.R. and Masson, P.H. (2008) ARL2, ARG1 and PIN3 define a gravity signal transduction pathway in root statocytes. *Plant J.* 53, 380–392
66 Baluška, F. *et al.* (2010) Root apex transition zone: a signalling-response nexus in the root. *Trends Plant Sci.* 15, 402–408
67 Jasik, J. *et al.* (2013) PIN2 turnover in *Arabidopsis* root epidermal cells explored by the photoconvertible protein dendra2. *PLoS ONE* 8, e61403
68 Sun, F. *et al.* (2008) Salt modulates gravity signaling pathway to regulate growth direction of primary roots in *Arabidopsis*. *Plant Physiol.* 146, 178–188
69 Li, Q. *et al.* (2011) Response and mechanism of *Arabidopsis* lateral root to excessive ammonium stress. *Soils* 43, 374–381
70 Lima, J.E. *et al.* (2010) Ammonium triggers lateral root branching in *Arabidopsis* in an AMMONIUM TRANSPORTER1;3-dependent manner. *Plant Cell* 22, 3621–3633
71 Signora, L. *et al.* (2001) ABA plays a central role in mediating the regulatory effects of nitrate on root branching in *Arabidopsis*. *Plant J.* 28, 655–662
72 De Smet, I. *et al.* (2003) An abscisic acid-sensitive checkpoint in lateral root development in *Arabidopsis*. *Plant J.* 33, 543–555
73 MacGregor, D.R. *et al.* (2008) Root system architecture in *Arabidopsis* grown in culture is regulated by sucrose uptake in the aerial tissues. *Plant Cell* 20, 2643–2660
74 Laskowski, M. *et al.* (2008) Root system architecture from coupling cell shape to auxin transport. *PLoS Biol.* 6, e307
75 Roosta, R.H. *et al.* (2009) Responses of cucumber plant to NH_4^+ and NO_3^- nutrition: the relative addition rate technique vs. cultivation at constant. *Sci. Hort.* 121, 397–403
76 Li, B. *et al.* (2011) The differing responses of two *Arabidopsis* ecotypes to ammonium are modulated by the photoperiod regime. *Acta Physiol. Plant.* 33, 325–334
77 Chen, G. *et al.* (2005) EGY1 encodes a membrane-associated and ATP-independent metalloprotease that is required for chloroplast development. *Plant J.* 41, 364–375
78 Szczerba, M.W. *et al.* (2008) NH_4^+-stimulated and -inhibited components of K^+ transport in rice (*Oryza sativa L.*). *J. Exp. Bot.* 59, 3415–3423
79 Engelsberger, W.R. and Schulze, W.X. (2012) Nitrate and ammonium lead to distinct global dynamic phosphorylation patterns when resupplied to nitrogen-starved *Arabidopsis* seedlings. *Plant J.* 69, 978–995
80 Patterson, K. *et al.* (2010) Distinct signalling pathways and transcriptome response signatures differentiate ammonium- and nitrate-supplied plants. *Plant Cell Environ.* 33, 1486–1501
81 Ariz, I. *et al.* (2011) Depletion of the heaviest stable N isotope is associated with NH_4^+/NH_3 toxicity in NH_4^+-fed plants. *BMC Plant Biol.* 11, 83
82 Moeller, I.S. *et al.* (2009) Shoot Na^+ exclusion and increased salinity tolerance engineered by cell type-specific alteration of Na^+ transport in *Arabidopsis*. *Plant Cell* 21, 2163–2178
83 Chen, G. *et al.* (2013) Nitrogen use efficiency (NUE) in rice links to NH_4^+ toxicity and futile NH_4^+ cycling in roots. *Plant Soil* 369, 351–363
84 Poulton, P.R. *et al.* (1990) Evaluation of the benefit of substituting foliar urea for soil-applied nitrogen for winter wheat. *Aspects Appl. Biol.* 25, 301–308
85 Gooding, M.J. and Davies, W.P. (1992) Foliar urea fertilization of cereals: a review. *Nutr. Cycl. Agroecosyst.* 32, 209–222
86 Phillips, S.B. and Mullins, G.L. (2004) Foliar burn and wheat grain yield responses following topdress-applied nitrogen and sulfur fertilizers. *J. Plant Nutr.* 27, 921–930