Review: Mechanisms of ammonium toxicity and the quest for tolerance

Raquel Esteban[a*], Idoia Ariz[b], Cristina Cruz[b], Jose Fernando Moran[a*]

[a]*Institute of Agrobiotechnology, IdAB-CSIC-UPNA-Government of* Navarre, *Avda. de Pamplona 123; E-31192 Mutilva, Navarre, Spain*

[b] *Faculdade de Ciências, Centro Ecologia Evolução e Alterações Ambientais, Universidade de Lisboa, 1749-016 Lisboa, Portugal*

**Author for correspondence: Jose Fernando Moran*

Phone: (+34) 948168000; Fax: (+34) 948 232191; e-mail: jose.moran@unavarra.es

Running title: ammonium toxicity and tolerance

Abstract

Ammonium sensitivity of plants is a worldwide problem, constraining crop production. Prolonged application of ammonium as the sole nitrogen source may result in physiological and morphological disorders that lead to decreased plant growth and toxicity. The main causes of ammonium toxicity/tolerance described until now include high ammonium assimilation by plants and/or low sensitivity to external pH acidification. The various ammonium transport-related components, especially the non-electrogenic influx of NH_3 (related to the depletion of ^{15}N) and the electrogenic influx of NH_4^+, may contribute to ammonium accumulation, and therefore to NH_3 toxicity. However, this accumulation may be influenced by increasing K^+ concentration in the root medium. Recently, new insights have been provided by "omics" studies, leading to a suggested involvement of GDP mannose-pyrophosphorylase in the response pathways of NH_4^+ stress. In this review, we highlight the cross-talk signaling between nitrate, auxins and NO, and the importance of the connection of the plants' urea cycle to metabolism of polyamines. Overall, the tolerance and amelioration of ammonium toxicity are outlined to improve the yield of ammonium-grown plants. This review identifies future directions of research, focusing on the putative importance of aquaporins in ammonium influx, and on genes involved in ammonium sensitivity and tolerance.

Keywords: Ammonium, counterbalance, gas, sensitivity, tolerance, urea.

Abbreviation: Aquaporins (AQP), Ammonium Transporters (AMT), Carbamoyl phosphate synthase (CPS), Non-Specific Cation Channel (NSCC), Nitrogen Use Efficiency (NUE), Oxygen-Evolving Complex (OEC), Reactive Oxygen Species (ROS)

1. Nitrogen: A global issue with socio-economic and environmental consequences

We live in a world surrounded by nitrogen (N_2): 78% of the Earth's atmosphere is N_2. However, to be available to plants, N_2 must first be converted into reactive nitrogen, a term which encompasses all N forms that are not involved in C-N bonding and elemental N_2, and applies to species including NH_3 to NO_3^-, covering the valence spectrum from -3 to + 5 (excluding 0, the valence of N_2). In nature, the conversion of N_2 into reactive nitrogen is mediated by biological N_2 fixation and by atmospheric lightning. However, the fixation of N_2 through natural processes is insufficient to ensure the food production necessary to maintain the current and future human population. Agriculture requires the intensive use of N fertilizers: nitrate (NO_3^-), ammonium (NH_4^+) and/or urea (CH_4N_2O). Due to crop breeding for high yields irrespective of the amounts of fertilizer required, most current crops exhibit very low nutrient (including N) use efficiency. The intensive application of NO_3^-- and NH_4^+-based fertilizers causes environmental problems, including the eutrophication of water reservoirs, aquifer contamination, and atmospheric pollution; these problems are recognized as a serious worldwide issue of public and economic concern. A recent economic analysis indicated that excess reactive nitrogen in the environment and its contributions to climate change and biodiversity loss cost the European Union between 70 and 320 billion € per year [1]. The scientific proposal "20:20 for 2020 goal" addressed global intergovernmental cooperation to improve N Use Efficiency (NUE), and was intended to provide environmental and health benefits worth approximately 160 (50–370) billion € per year [1]. The aim of the "20:20 for 2020 goal" is to improve NUE by 20%, thus reducing the use of N by 20 million t/year by 2020 [1]. Understanding the mechanisms underlying N use by plants and improving its use efficiency is therefore of social, economic, agricultural and ecological importance.

Soil NO^-_3 and NH_4^+ concentrations are usually unpredictable and change with time and location. The abundance of NH_4^+ in ecosystems is determined by many factors, including the chemical nature of the soil, pH, temperature, the accumulation of organic compounds, oxygenation, light, and CO_2 [2–4]. Soils with low pH and anoxic conditions (such as those found in wetlands including fens, bogs, saltmarshes, mangroves and rice paddies) exhibit higher ammonification than nitrification rates [2,5] and so are rich in NH_4^+. In many soils of natural and semi-natural ecosystems, NH_4^+ is the predominant N source, presenting a mean concentration of 2 mM in boreal and temperate forest soils [6]. Plant growth in these ecosystems is mainly supported by direct (via roots) or indirect (via mycorrhizae) NH_4^+ uptake. At low concentrations (<3 mM), NH_4^+ is typically the N source preferred by plants, but above a certain threshold, NH_4^+ becomes toxic [7]. This threshold depends on plant species and on variety (in crops) (as examples, see [8–11]). Environmental factors such as temperature, soil pH, CO_2 concentration and light intensity can affect the threshold for NH_4^+ toxicity (e.g., [3,4,7,12]; Fig. 1). Crops, such as potato or sugar beet, are generally more sensitive to NH_4^+ than their respective wild relatives (reviewed in [2]). However, some crops, such as rice, blueberries and onions, are adapted to high NH_4^+ concentrations [2] and rarely reach the threshold for NH_4^+ toxicity. In the case of trees, higher sensitivity to ammonium is often found in early-successional trees, such as poplars or douglas-fir, rather than in late-successional conifers such as spruce species [2]. NH_4^+ sensitivity is not unique to terrestrial plants, but has also been observed in animals, (including mammals) [13], algae [14], cyanobacteria [14,15] and yeast [16], among others.

Although understanding of the causes of NH_4^+ sensitivity/tolerance has greatly improved during the last two decades, the plant traits that are responsible for plant NH_4^+ sensitivity or

tolerance remain unclear. This review highlights key factors that determine plant tolerance or sensitivity to NH_4^+ nutrition, especially in crops, and draws comparisons with nitrate where useful. The review also summarizes and integrates recent research addressing: (i) the main causes of NH_4^+ toxicity in plants and (ii) how these causes can be targeted to achieve plant tolerance to NH_4^+ nutrition.

2. Ammonium toxicity in plants: From classical to recent hypothesis

NH_4^+ toxicity in plants is not a new topic; the detrimental visual effects of NH_4^+ nutrition on roots, leaves and plant growth were first reported more than a century ago (e.g., [17]). Since then, much work has been conducted to elucidate the mechanisms underlying the toxicity of NH_4^+ nutrition.

2.1 Classical hypotheses of plant ammonium toxicity

NH_4^+ toxicity symptoms include: reduced plant growth, changes in root architecture, decreases in the root/shoot ratio, and leaf chlorosis, among others [7,18]. These phenotypic symptoms reflect the integrated effect of NH_4^+ excess, which causes the following: inhibition of cations (K^+, Mg^{2+} or Ca^{2+}) uptake and consequent changes in plant ion balance; intra-cellular alkalinization and extracellular acidification; the inhibition of root respiration and stimulation of photorespiration; interference with photosynthetic activity; the altered expression/activity of NH_4^+ assimilating enzymes; the disruption of hormonal homeostasis; increased oxidative stress; and high energy cost to maintain low levels of cytosolic NH_4^+content (e.g., [7,9,12,18–20] and references cited therein) (Fig. 1).

To avoid toxicity, plants need to maintain a fine balance between the uptake, production, and consumption of NH_4^+ [18]. This leads to the question of the nature of plants'

first response to high levels of NH_4^+. In NH_4^+-sensitive plants, such as *Arabidopsis* sp., shoots tend to be the most sensitive part of the plant to NH_4^+ nutrition [21]. However, roots constitute the first NH_4^+ sensor, and the initial signals of NH_4^+ toxicity appear at root level with a severe modification of the root system architecture; commonly observed modifications include: shorter primary root systems [e.g.,[8,23]]; the inhibition of root elongation, embracing primary and lateral roots [23–25]; the stimulation of lateral root branching [24,26] with changes in the insertion of lateral roots in the main root [25]; and a loss of gravitropism [27]. It is necessary to understand whether these effects are part of a cascade-type response or merely the summation of several simultaneous plant responses to the presence of NH_4^+ (or to one product of its metabolism). Studies of *Lotus japonicus* using split-root systems showed that root-specific NH_4^+-induced responses are mediated by NH_4^+ transporters [24,26] and are more related to the perception of NH_4^+ than to its assimilation. A member of the *L. japonicus* AMT1-type transporter family (LjAMT1;3 gene) appears a good candidate for the agent responsible for promotion of the characteristic NH_4^+-root phenotype for the following reasons: its transcription is induced in the same range of NH_4^+ concentrations that promotes the appearance of the NH_4^+-root phenotype; and its overexpression is sufficient for phenocopying the short-root phenotype into transgenic plants [24]. The function of AMT1;3 as a mediator of the short-root phenotype induced by NH_4^+ nutrition was also observed in *Arabidopsis* [26]. Together, these observations suggest that NH_4^+ is locally sensed, and that it is possible that the NH_4^+-sensing machinery employs regulatory modules similar to those that have been suggested to play a role in nitrate sensing.

Exogenous NH_4^+ (in the root medium) only reaches plant leaves after saturating the storage capacity of the root system. In leaves, the toxic effects described are controversial and

132 are mainly related to the effect of NH_4^+ on photosynthesis and its capacity to generate
133 oxidative stress. NH_4^+ (1 mM) was described as uncoupling photophosphorylation in isolated
134 spinach (*Spinacia oleracea*) thylakoids in the late 1950s [28]. Forty years later, the uncoupling
135 effect of NH_4^+ was ruled out, based on a study performed on bean (*Phaseolus vulgaris*) leaves,
136 which showed that NH_4^+ supply (2 mM) does not affect the operation of photosynthetic
137 protein complexes [29] and therefore does not affect leaf CO_2 assimilation capacity [30].
138 However, studies of *Synechocystis* (cyanobacteria) cells showed that NH_4^+ (5 mM) can affect
139 photosynthesis through the binding of NH_3 to two sites in the oxygen-evolving complex (OEC)
140 of photosystem II [15]. Secondary binding occurs in the outer shell of the amino acids of the
141 OEC, and is competitive with chloride. In the primary binding site, NH_3 coordinates with Mn in
142 the OEC upon formation of the S_2 state. The binding of NH_3 does not completely block O_2
143 evolution, indicating that water molecules can still bind and react at the OEC in the presence
144 of NH_3 [31]. The interaction of NH_3 with the OEC might explain the effect of NH_4^+ (1 mM), but
145 not of NO_3^- or urea, on chlorophyll fluorescence transients in *Medicago truncatula*, results
146 that showed that NH_4^+ affected the energy conservation of photons absorbed by photosystem
147 II, involving the reduction of intersystem electron acceptors [25]. However, these effects of
148 NH_4^+ on photosynthesis are not universal and appear to depend on species (variety) and NH_4^+
149 concentration. For instance, the functional impairment associated with NH_4^+ nutrition (5 mM)
150 in *A. thaliana* was mainly associated with redox reactions that occur outside the chloroplast,
151 because this NH_4^+ nutrition did not impair photosynthetic capacity but increased the leaf
152 levels of mitochondrial reactive oxygen species (ROS) [32]. This finding is consistent with
153 several studies showing that NH_4^+ nutrition leads to increased lipid peroxidation, enhanced
154 glutathione-ascorbate cycle enzyme activity, or changes in the redox state (most of the

reductant is oxidized), thus implicating greater ROS production [29,33]. This oxidative stress and stimulation of anti-oxidative metabolism appears to be characteristic of the more sensitive plant species, since NH_4^+ nutrition does not induce oxidative stress in legumes, which are known to be relatively tolerant to ammonium [8,25].

Since there is no agreement on the main cause of NH_4^+ toxicity, agreement also lacks on which key traits confer plant tolerance to NH_4^+ nutrition. These traits might include high activity of the mitochondrial alternative oxidase pathway [9]; high NH_4^+ assimilation and the maintenance of low levels of NH_4^+ in leaves, as it has been observed for pea (*Pisum sativum*) and *A. thaliana* ecotypes [9,11]; and/or low sensitivity to external pH acidification [34]. The latter traits might be related to the acidification of the root environment due to the activation of ATPases, which pump out H^+, thus causing medium acidification (as it is commonly observed under NH_4^+ nutrition). Taken together, the above considerations indicate that the ability of plants to tolerate high NH_4^+ concentrations depends on several main drivers: (i) root C metabolism and the ability to maintain high respiration rates [9,12]; (ii) tolerance of acidification of the root zone [35]; and (iii) the capacity to restrict NH_4^+ accumulation inside tissues [11]. However, the NH_4^+ tolerance puzzle could only be solved after the NH_4^+ futile cycle [36] had been described.

2.2. NH_3 gas uptake as the basis of ammonium toxicity (sensitivity)

When first proposed as a mechanism to explain plant NH_4^+ toxicity, the futile transmembrane NH_4^+ cycle hypothesis [36] linked the futile cell cycling of NH_4^+ uptake followed by NH_4^+ extrusion from the cytoplasm to an excessive energy consumption (associated with NH_4^+ extrusion) that caused plants to slow or cease growth. However, later,

177 based on the use of the short-lived radioisotope ^{13}N, the same team realized that the increase
178 in root NH_4^+ uptake (due to an increase in pH) was not accompanied by an increase in root O_2
179 consumption, and that the intensification of NH_4^+ influx, at external concentrations greater
180 than 2 mM, was not proportional to the observed membrane depolarization, suggesting that
181 most of the observed influx was not electrogenic. This implies that NH_3, not NH_4^+, is the main
182 N species transported across the cell membrane [37]. These observations did not support the
183 original NH_4^+ futile cycle hypothesis [36] and led to the development of a revised hypothesis
184 [37]. This result excludes the notion that excessive energy consumption is associated with
185 NH_4^+ efflux (as was proven incorrect) and reintroduced the status of NH_4^+ accumulation in the
186 cell and nutrient imbalance (lower K^+, Ca^{2+} and Mg^{2+} concentrations) caused by NH_4^+ nutrition
187 as the main causes of NH_4^+ toxicity.

188 Evidence supporting the involvement of NH_3 transport across the plasma membrane
189 also comes from the N natural isotopic signature ($\delta^{15}N$) and discrimination ($\Delta^{15}N$) of NH_4^+-fed
190 plants, which tend to be enriched in ^{14}N (depleted in ^{15}N) in comparison with plants fed with
191 NO_3^- [38]. This observation can only be explained if at least part of the NH_4^+ entering the plant
192 is deprotonated before transport across the membrane (Fig. 2); this explanation is based on
193 the fact that the lighter isotope (^{14}N) is more easily deprotonated than the heavier one (^{15}N)
194 [38]. Furthermore, the depletion in the heavier isotope ^{15}N is correlated with plant tolerance
195 to NH_4^+ across a large number of species and plant functional types, a finding that provides
196 strong evidence concerning the physiological implications of NH_3 futile cycling [38] (Fig. 3).
197 This correlation is also supported by later data on two model species, *A. thaliana* and *M.*
198 *truncatula* (unpublished results), and two crops: tomato (*Solanum lycopersicum*) [4] and
199 wheat (*Triticum aestivum*) [3] (Fig. 3). Interestingly, legume species such as lupin (*Lupinus* sp.),

clover (*Tripholium* sp.) and pea (*P. sativum*) are among the most tolerant species, the exception being the legume *M. truncatula*. This lower tolerance of *M. truncatula* might be related to its susceptibility to the hydroponic growth system (authors´ personal observation).

The hypothesis that NH_3 can enter cells as ammonia is not new and was previously demonstrated to occur in bacterial [39], fungal [40], animal [13] and plant cells [40] through the involvement of transporters of the MEP/AMT/Rh protein family. The MEP/AMT/Rh proteins transporting NH_3 most likely recruit NH_4^+ from the extracellular medium, deprotonate NH_4^+ to NH_3 in a highly hydrophobic area of the transporter facing the outer layer of the plasmalemma, and then transport NH_3 across the membrane [39]. However, MEP/AMT/Rh proteins are mainly described as high-affinity transport systems with a high affinity for low NH_4^+ concentrations [41]. Therefore, transport of NH_4^+ across these proteins is not expected to contribute to NH_4^+ toxicity. Considering the physico-chemical similarities between the molecular structure of NH_3 and H_2O, aquaporins (AQP) appear good candidates for performing the NH_3 transport that is associated with NH4+ toxicity at high NH4+ concentrations [18,41]. Only recently, a high-resolution structure of the ammonia-permeable AQP AtTIP2;1 from A. thaliana (TIP subfamily) showed surprising features associated with the substrate selectivity filter, including a conserved arginine in a new orientation that is stabilized by interactions to a histidine. Which determines ammonia specificity. An additional histidine in a different part of AtTIP2;1 reinforces the position of the arginine and interacts directly with the substrate in the channel. It is possible that a water-filled side pore, next to the substrate-binding histidine, participates in deprotonating ammonium [43]. Further studies are necessary in order to understand if these are peculiar characteristics of this AQP or if they are common to other AQPs. In fact, various non-orthodox AQP are able to transport NH_3; some wheat AQP restored

223 NH_4^+ transport and acidified the external medium, thereby functionally complementing a
224 yeast mutant that was deficient in NH_4^+ transport [42]. In barley, inhibitors (albeit nonspecific)
225 of AQP such as Hg_2^+, H_2O_2 and propionic acid suppressed NH_4^+ uptake, further implicating AQP
226 in NH_3 transport [37]. This finding implies that plants with distinct sensitivities to NH_4^+
227 nutrition should have AQP that differ in their capacity to transport NH_3 and in their relative
228 expression and/or regulation. In fact, it has been recently crystallized an AQP of the tonoplast
229 intrinsic proteins subfamily, which are known to be able to transport NH_3 and water [43]. In
230 this study, new insights into the substrate selectivity and the conceivable via for NH_4^+
231 deprotonation are given for the ammonia-permeable AQP *AtTIP2;1* from *A. thaliana.* Since
232 yeast cells contain only 2 non-orthodox AQP, which can also transport other molecules
233 different from water, experiments involving the heterologous expression of plant plasma
234 membrane AQP in yeast mutants without ammonium permeases (MEPs) are expected to shed
235 light on the potential of plant AQPs to transport NH_3. The great number of natural accessions
236 of *A. thaliana* and crop plants with distinct tolerances of NH_4^+, and the vast number of mutants
237 available, are important tools that could be used to identify quantitative trait loci and genes
238 involved in plant NH_4^+ sensitivity/tolerance (Fig. 5).

239 In addition to entering the cell as NH_3 (as recently proposed), inorganic reduced N
240 might also enter the cell as NH_4^+ (as has long been assumed). In fact, multiple channel types,
241 including non-selective cation and K^+-specific channels, might mediate the entrance of NH_4^+
242 into root cells [37,44–46]; the main reason for this is that K^+ and NH_4^+ ions are of very similar
243 size and surface charge density; thus, these ions might compete and pass through protein
244 channels that can transport cations (non-specific cation channels - NSCC). The proportion of
245 the inorganic reduced N that is entering the cell as NH_4^+ may depend on the NH_4^+/ K^+ present

246 in the root medium and on the electrical potential difference between the cytoplasm and the
247 root medium.

248 The effect of NH_4^+ nutrition on reducing cation (K^+, Ca^{2+} and Mg^{2+}) concentrations in
249 plant tissue is recognized as a major cause of NH_4^+ toxicity [7,12]. However, it is also known
250 that the toxicity of NH_4^+ nutrition can be alleviated by increasing K^+ concentrations in the root
251 medium [47]. K^+ can decrease NH_4^+ toxicity: i) by increasing the incorporation of NH_4^+ into
252 organic N-compounds by activating enzymes such as glutamine synthetase, glutamate
253 dehydrogenase and others [11,19]; and/or ii) by inhibiting the acquisition of NH_4^+ by low
254 affinity transporters [44,47]. Even in cases where NH_3 is presumed to be the chemical species
255 that is transported into the cell, K^+ concentrations in the root medium can interfere with that
256 transport through the effect of K^+ on regulating the activity of AQP in particular and on the
257 water balance of the plant [46].

258 Together, these findings indicate the existence of two NH_4^+-transport-related
259 components that operate at high NH_4^+ concentrations and contribute to NH_4^+ accumulation in
260 the cell with toxic effects: i) the non-electrogenic flux of NH_3 across cell membranes, which is
261 putatively mediated by AQP [37] and is related to the depletion of ^{15}N in plant material in
262 relation to the NH_4^+source (Fig. 2) and ii) the electrogenic influx of NH_4^+, which is putatively
263 mediated by non-specific cation channels. Both components of this NH_3/NH_4^+ transport can
264 be fine-tuned by K^+ concentrations in the medium either: (i) through the regulation of protein
265 synthesis and function; or (ii) by direct competition for the transporter, which would explain
266 the general benefit of high K^+ concentrations in regard to plant NH_4^+ toxicity.

267 **3. New insights provided by "omics" studies**

Most “omics” studies to date have addressed rapidly responding gene expression under NH_4^+ nutrition in various plants, including sensitive species such as *A. thaliana* and more tolerant species such as rice (*Oryza sativa*). The transcriptomic studies in a short term study with *A. thaliana* roots showed that NH_4^+ nutrition induced a rapid and sustained upregulation of GDH enzyme as well as the regulation of a group of genes common to biotic stress and plant defense [33]. While the GDH induction may be related to its anaplerotic activity implicated in the changes in the C/N balance [19], the pathogenesis-related response may reflect the extracellular acidification caused by NH_4^+/NH_3 uptake [33].

Another important gene involved in NH_4^+ nutrition is the GDP mannose-pyrophosphorylase (GMPase; [23,48]), which produces GDP-mannose from GDP-mannose 1-phosphate and is involved in ascorbic acid biosynthesis, cell wall synthesis, glycosylphospahtidyl anchoring and protein N-glycosylation in plants. GMPase is also related to the response pathways of NH_4^+ stress, since GMPase deficiency leads to hypersensitivity to NH_4^+. Initial experiments linked this hypersensitivity to defective N-glycosylation of proteins in roots [48]. Strong correlations between the extents of decreased GMPase activity, the degrees of defective N-protein glycosylation in the roots, and the different magnitudes of growth retardation exhibited by wild type and defective GMPase mutant seedlings in the presence of NH_4^+ were also shown. In contrast, the enzyme GDP mannose-pyrophosphohydrolase, which catalyzes the reverse reaction to produce GDP mannose 1-phosphate, induced the growth of *A. thaliana* plants under NH_4^+ nutrition, which is attributed to the modulation of the protein N-glycosylation in the roots [49]. Furthermore, the GMPase NH_4^+-hypersensitive mutant exhibited a more pronounced stimulation of NH_4^+ efflux and inhibition of cell expansion, indicating that the proper functioning of GMPase in roots is critical

291 to minimizing the severity of the NH_4^+ toxicity response in *Arabidopsis*, and suggesting a role
292 for the enzyme in the regulation of the transmembrane NH_4^+ futile cycling [21]. Also recently,
293 a metabolic flux study with *A. thaliana cells* grown hetetrophically on Murashige and Skoog
294 medium showed that introduction of ammonium into the culture medium revealed higher cell
295 maintenance costs which also may well support the futile NH_4^+ cycle hypothesis [50].

296 "Omics" studies on the long-term response to NH_4^+ (18 days) were conducted using *A.*
297 *thaliana* [51] and showed changes in the expression of genes that are involved in
298 photosynthesis, mitochondrial and cell wall metabolism, and auxin-mediated growth. No
299 changes were found in the expression of glycosylation-associated genes [51] or in genes that
300 are directly related to ATP hydrolysis, which would be expected if NH_4^+ toxicity were related
301 to ATP loss via excessive H^+ pumping [51].

302 The involvement of oxidative metabolism [33] in response to NH_4^+ nutrition has been
303 confirmed by "Omics" studies. Heme-heme oxygenase 1, a novel antioxidant regulatory
304 enzyme that has recently been detected in plants, is involved in the improvement of plant
305 tolerance to NH_4^+ in both NH_4^+ tolerant (rice) and sensitive species (*A. thaliana*) [52]. Higher
306 NH_4^+ tolerance was achieved both by the activation of antioxidant defense signaling and by
307 the modulation of ROS homeostasis, thereby neutralizing excess oxygen species that are
308 produced in response to NH_4^+ excess [52].

309 It was also found that genes involved in cell wall loosening (polygalacturonases,
310 expansins and endo-transglycosylases) are down-regulated in NH_4^+-fed plants [33].
311 Interestingly, pathogen susceptibility is also associated with cell wall loosening and the ROS
312 response [53]. Furthermore, the NH_4^+- grown tobacco plants that were exposed to virulent
313 *Pseudomonas syringae* strains exhibited reduced NO production and a lower induction of the

hypersensitive response, which is associated with a decrease in resistance to the pathogen when compared with NO_3^-- grown plants [54]. In addition, NH_4^+ nutrition induced a decrease in biomarkers for the salicylic acid-mediated response and a shift from polyamine biosynthesis under nitrate nutrition to a 4-aminobutyric accumulation under NH_4^+ nutrition. Therefore, the question arises as to how the defence-associated signaling pathway of NH_4^+ nutrition is compromised during the pathogenic response.

4. Toward an improved knowledge of the signaling of NH_4^+ tolerance

4.1. Nitrate, auxins and NO

In the scenario of NH_4^+ sensitivity and tolerance, various factors are involved in the cross-talk signaling that determines plant growth and development, including NO_3^-, which alleviates NH_4^+ toxicity, even when present at very low concentrations (see [7] for a review). Curiously, the alleviation of NH_4^+ toxicity by NO_3^- is not associated with lower tissue NH_4^+ accumulation; or with organic anion and inorganic cation depletion (symptoms that are associated with NH_4^+ toxicity) in *A. thaliana* shoots [55]. It has been demonstrated that both the NO_3^- transporter NRT1.1 and NO_3^- itself participate in the regulation of endogenous auxin uptake in root cells. Auxin is known to stimulate lateral root development, although the sensing mechanism is not fully understood [56]. The expression of the NRT1.1 transporter gene is induced by NO_3^-, therefore, this signaling pathway can be disrupted in plants that lack NO_3^- in the root medium (for example, plants that are grown using NH_4^+ or urea as the only N-source), as has been shown to be true for NH_4^+- and urea-fed *Medicago truncatula* plants, which exhibit shortened (for NH_4^+) and less-branched roots (for urea) [25]. The role of auxin signaling in NH_4^+-grown primary roots has been studied in an *A. thaliana* line containing an

auxin responsive reporter, DR5:GUS-reporter. This line exhibited an acute decrease in the reporter response to auxins under NH_4^+-nutrition, but the growth of its roots was only partially rescued with exogenous auxin [51]. In contrast, NRT1.1-defective mutants exhibited higher tolerance of high concentrations of NH_4^+, and nitrate application did not enhance this tolerance of NH_4^+ of NRT1.1-defective mutants; it was therefore concluded that a NO_3^--independent function of NRT1.1 exists. These researchers noted the regulatory role of some genes that are relevant to the aliphatic glucosinolate-biosynthetic pathway; these genes were induced in the NRT1.1 mutant during ammonium nutrition [57]. Therefore, increased knowledge of the NO_3^-- dependent and –independent signaling mechanisms could provide new lines of research for managing ammonium stress.

NO_3^- efflux channels such as SLAH3 are strongly selective for nitrate over chloride and also play important roles in the nitrate-mediated alleviation of stress [58]. SLAH3-defective mutants are hypersensitive to high levels of NH_4^+ and low pH in the absence of NO_3^-. Recently, our group studied *M. truncatula* plants that were grown under axenic conditions in the total absence of NO_3^- [25], an approach that excludes microorganisms that could produce phytohormones or NO_3^-. Roots (both main and lateral) grew more slowly on NH_4^+- and urea-grown plants than on NO_3^--grown plants, and the total length of the plant was positively correlated with auxin content, although no recovery was detected when auxin was applied externally to NH_4^+- and urea-grown plants. These results are consistent with the roles proposed for NO_3^- and NRT1.1, which do not regulate the initiation of lateral root primordia but slow their development in the absence of NO_3^- [25,56]. Together, these results suggest that NO_3^- generates root signals that determine NH_4^+ tolerance, and highlight the need to understand the signaling mechanisms in NH_4^+-grown plants grown with nano- or micro-molar

concentrations of NO_3^-. Conversely, the complementary action of NH_4^+ and NO_3^- on lateral root development has raised the question as to whether NH_4^+ invokes a similar regulatory sensing machinery to NO_3^- [59] .

It has been proposed that NO, which may originate from NO_3^- via nitrate reductase, might play a central role in signaling, by acting downstream of auxin. In fact, two *A. thaliana* plants with mutations in the arginase isoenzymes ARGAH1 or ARGAH2 (encoding arginine amidohydrolase-1 and -2, respectively), which exhibited a higher accumulation and efflux of NO, also promoted the formation of lateral and adventitious roots. Furthermore, these mutants responded to the auxin analog naphthaleneacetic acid by increasing their NO contents. Additionally, exogenous exposure of the *A. thaliana* arginase mutant plants to naphthaleneacetic acid resulted in a doubling of the number of lateral roots, thus confirming the importance of auxin-mediated regulation. Consistently, both mutants presented increased auxin signaling in the root tips, emerging lateral roots, and hypocotyls. Currently, the origin of NO in plants that are grown with NH_4^+ or urea as the sole N source remains unknown; such knowledge might be essential to understand the nitrate and auxin interaction. However, several enzymatic assays have detected increased NO production from reduced N-containing molecules in plants, although no specific enzyme has been associated with such catalysis. Conversely, NO can also lead to the formation of NO_3^- through its interaction with hemoglobins [60], and this might be relevant for understanding the NO_3^- signaling effect.

4.2 The “urea cycle” in plants

Plants lack an ornithine-urea cycle homologous to that of metazoans for the removal of N from amino acid catabolism (Fig. 4). The key enzyme of the animal urea cycle, carbamoyl

383 phosphate synthase (CPS)-type I, is absent from green algae and plants; however, this enzyme
384 is present in some lower photosynthetic eukaryotic phyla, such as Rhodophyta and
385 Stramenopila [61]. Thus, in diatoms, this type of urea cycle serves as a distribution and
386 repackaging hub for inorganic C and N, and contributes significantly to the metabolic response
387 of diatoms to episodic N availability. In contrast, plants appear to have a functional CPS-type
388 II that uses glutamine as the NH_3 source for synthesis of carbamoyl-P [62]. Consequently,
389 plants cannot synthesize urea directly from NH_4^+, which would reduce the internal NH_4^+
390 content during stress. Unfortunately, we lack data on transgenic plants that are endowed with
391 a CPS-type I gene, and the effects thus produced on NUE and stress tolerance during NH_4^+
392 nutrition.

393 The plant urea cycle includes a biosynthetic phase that produces arginine, which is
394 followed by a catabolic phase in which arginine can be degraded either by arginase to produce
395 urea and ornithine, or by serving as a substrate for arginine decarboxylase, producing
396 agmatine (Fig. 4). Ornithine can condensate with a carbamoyl phosphate molecule in a
397 reaction catalyzed by ornithine transcarbamylase to produce citrulline and other
398 intermediates in the biosynthetic phase. Either agmatine or ornithine can subsequently
399 originate putrescine, spermidine and other polyamines. Several oxidases participate in the
400 pathway leading to polyamines; for example, the Cu-binding diamino oxidase and FAD-binding
401 polyamino oxidases, which produce H_2O_2 (Fig. 4). The importance of the urea cycle in plants is
402 not as clear as it is in diatoms [63], and the biosynthetic phase may be
403 temporally/developmentally distinct from the catabolic phase. However, it may be a
404 regulatory control point in N metabolism, since this cycle connects N with C metabolism:
405 arginine is the amino acid with the lowest C/N ratio, it can be used to store N, and it has also

been considered a signal molecule. As mentioned above, arginine has been proposed as an intermediate in NO synthesis, and arginase-defective mutants exhibit greater NO production during root development than those with the wild type enzyme [64]. However, when applied exogenously, the polyamines spermidine and spermine, but not putrescine or arginine, enhanced NO production in *A. thaliana* plants [65]. Additionally, another research group observed that the exogenous application of spermidine increased NO biosynthesis in control plants but not in two knock-out mutants of the Cu-amino oxidase-1 and -2 isoenzymes; moreover, the exogenous application of ABA decreased the production of H_2O_2 and the sensitivity to regulatory processes that are controlled by ABA, such as germination and post-germination growth [65]. We speculate that an excess of ammonium that originates either from polyamine metabolism (Fig. 4) or from NH_4^+-nutrition can act as a feedback inhibitor of polyamine deamination, further impairing polyamine metabolism. Additionally, metabolic studies showed that arginine was accumulated under stress by ammonium nutrition in pea and in *Arabidopsis* plants, possibly indicating impairment of polyamine synthesis [19,59]. Interestingly, high irradiance, which improved tolerance to ammonium in pea plants, also reduced the relative arginine content; this arginine was apparently transformed into putrescine under high light intensity, thus improving tolerance to ammonium stress and improving the plants' energetic condition [19].

5. Ammonium toxicity can be counterbalanced: the tolerance response

A final objective of understanding ammonium toxicity is to identify which plant traits confer ammonium tolerance. Several environmental factors can counterbalance ammonium toxicity in the laboratory (Fig. 1), although these are sometimes difficult to apply in the field.

These factors include: higher light intensity [3,12,19]; increased CO_2 concentration [4]; the exogenous supply of organic or inorganic carbon [66], which partially reverses C-starvation symptoms associated with ammonium nutrition; increased concentrations of K^+ that eventually inhibiting the entrance of NH_4^+, as shown for rice (a very tolerant plant, which exhibited even greater tolerance of ammonium at higher K^+ concentrations [44,47]); and complementation with nitrate at low doses, which has long been practiced in agriculture. In crop soils, the usual fertilization of ammonium together with nitrate, either in the form of NH_3NO_3 or using the remainder of previous agricultural manures, may counterbalance the effect of NH_4^+, as the NO_3^- uptake alkalinizes the rhizosphere [2] and exerts the positive regulatory effect mentioned in the previous section. In addition to the previous biological reasons supporting the alleviation of ammonium toxicity by NO_3^-, there is evidence that a nitrate efflux channel is required for plant growth under low nitrate/high ammonium nutrition [35]. It was observed that a shift in the pH of the medium from 5.7 to 7 mitigated the toxic effect of ammonium on *A. thaliana* plants due to the effect of SLAH3 on ammonium toxicity that is mediated by pH regulation [35]. Accordingly, SLAH3 orthologs represent promising targets for crop enhancement [35]. Together, these findings suggest that controlling the pH of the growth solution is a key factor in retarding the appearance of ammonium toxicity symptoms.

Hormones such as ABA or auxins have also been linked to the alleviation of ammonium nutrition toxicity [21]. It has been proposed that under NH_4^+ nutrition, the membrane of the chloroplasts of ammonium-sensitive mutants (based on the chlorotic phenotype) receive the stress signal, thus triggering AMOS1/EGY1-dependent retrograde signaling and recruiting downstream ABA signaling to regulate NH_4^+ responses, since the hormone is a downstream

452 messenger [21]. The metabolic imbalance derived from ammonium nutrition of *A. thaliana* is
453 partially overcome by elevated auxin levels [51].
454
455 **6. Prospects and conclusions: networking the farm with the laboratory**
456 Recent findings in the laboratory regarding ammonium tolerance might determine
457 future field actions under the increasing use of slow-release NH_4^+ fertilizers. However, the
458 extrapolation of laboratory results to the field is complicated by the fact that multiple factors
459 affect the assimilation of nutrients (including ammonium) by plants. Nevertheless, all data
460 indicate that the use of nitro-ammoniacal [56] or ammonium/K^+ fertilizers is recommended to
461 improve ammonium tolerance and reduce N fertilizer application. The diffusion of NH_3 within
462 plant cells and the natural N isotopic signature might have important agricultural,
463 environmental and technical applications (e.g., as a novel indicator for ammonium tolerance,
464 fertilizer management, or measuring pH balance in aquatic environments).
465 Moreover, the use of *A. thaliana*, for which accessions with very distinct ammonium
466 tolerances are available, is aiding progress in understanding ammonium toxicity, although this
467 information has to be combined with information obtained from wild plants that are
468 characteristic of ammonium-rich locations, and from wild relatives of crops.
469 Very important advances in our knowledge of ammonium toxicity and its amelioration
470 have recently occurred. However, further research is necessary to elucidate how ammonium
471 is transported out of the cell. Studies that focus on changes and modulation of the extra- and
472 intracellular pH, which might influence subcellular ammonia gas concentrations in the cytosol,
473 vacuoles and other organelles, are warranted (Fig. 5).
474

Acknowledgments

We greatly acknowledge all the researchers involved in “ammonium”, and we apologize to all researchers whose works were not cited. The authors acknowledge the support of research grants AGL2014-52396-P from the Spanish Ministry of Competitiveness and Innovation (MICINN) and PTDC/BIA-BEC/099323/2008 and PTDC/BIA-ECS/122214/2010 from the Portuguese Fundação para a Ciência e Tecnologia (FCT). RE received a JAE-Doc-2011-046 fellow from the Spanish CSIC, co-financed by the European Social Fund. IA was supported by a postdoctoral Fellowship from the FCT (SFRH/BPD/904 36/2012). We thank Steve Houghton for editing the English text and Beatriz Royo for technical assistance during the growing of the plants shown in figure 3.

References

[1] M.A. Sutton, Too much of a good thing?, Nature. 472 (2011) 159–161.

[2] D.T. Britto, H.J. Kronzucker, Ecological significance and complexity of N-source preference in plants, Ann. Bot. 112 (2013) 957–963.

[3] I. Setién, et al., High irradiance improves ammonium tolerance in wheat plants by increasing N assimilation., J. Plant Physiol. 170 (2013) 758–71.

[4] I. Vega-Mas, et al., CO_2 enrichment modulates ammonium nutrition in tomato adjusting carbon and nitrogen metabolism to stomatal conductance, Plant Sci. 241 (2015) 32–44.

[5] T. Nakamura, N. Motoka, Ecophysiological mechanisms characterising fen and bog species: focus on variations in nitrogen uptake traits under different soil-water pH, Oecologia. 168 (2012) 913–921.

[6] R.J. Bijlsma, H. Lambers, S.A.L.M. Kooijman, A dynamic whole-plant model of integrated metabolism of nitrogen and carbon 1. Comparative ecological implications of

498 ammonium-nitrate interactions, Plant Soil. (2000) 49–69.
499 [7] D.T. Britto, H.J. Kronzucker, NH_4^+ toxicity in higher plants : a critical review, J. Plant
500 Physiol. 159 (2002) 567–584.
501 [8] M.D. Domínguez-Valdivia, et al., Nitrogen nutrition and antioxidant metabolism in
502 ammonium-tolerant and -sensitive plants, Physiol. Plant. 132 (2008) 359–369.
503 [9] C. Cruz, et al., Intra-specific variation in pea responses to ammonium nutrition leads to
504 different degrees of tolerance, Environ. Exp. Bot. 70 (2011) 233–243.
505 [10] B. Li, W. Shi, Y. Su, The differing responses of two *Arabidopsis* ecotypes to ammonium
506 are modulated by the photoperiod regime, Acta Physiol. Plant. (2010) 1–10.
507 [11] A. Sarasketa, M.B. González-Moro, C. González-Murua, D. Marino, Exploring
508 ammonium tolerance in a large panel of *Arabidopsis thaliana* natural accessions., J. Exp.
509 Bot. 65 (2014) 6023–33.
510 [12] I. Ariz, et al., High irradiance increases NH_4^+ tolerance in *Pisum sativum*: Higher carbon
511 and energy availability improve ion balance but not N assimilation, J. Plant Physiol. 168
512 (2011) 1009–1015.
513 [13] S. Biver, et al., A role for Rhesus factor Rhcg in renal ammonium excretion and male
514 fertility., Nature. 456 (2008) 339–343.
515 [14] G.Z. Dai, J.L. Shang, B.S. Qiu, Ammonia may play an important role in the succession of
516 cyanobacterial blooms and the distribution of common algal species in shallow
517 freshwater lakes, Glob. Chang. Biol. 18 (2012) 1571–1581.
518 [15] M. Drath, et al., Ammonia triggers photodamage of photosystem II in the
519 cyanobacterium *Synechocystis* sp. Strain PCC 6803, Plant Physiol. 147 (2008) 206–215.
520 [16] D.C. Hess, W. Lu, J.D. Rabinowitz, D. Botstein, Ammonium toxicity and potassium

521 limitation in yeast, PLoS Biol. 4 (2006) e351.
522 [17] C. Darwin, The action of carbonate of ammonia on the roots of certain plants, Linn. Soc.
523 Lond. Bot. 19 (1882) 239–261.
524 [18] A. Bittsánszky, K. Pilinszky, G. Gyulai, T. Komives, Overcoming ammonium toxicity, Plant
525 Sci. 231 (2015) 184–190.
526 [19] I. Ariz, et al., Changes in the C/N balance caused by increasing external ammonium
527 concentrations are driven by carbon and energy availabilities during ammonium
528 nutrition in pea plants: The key roles of asparagine synthetase and anaplerotic
529 enzymes, Physiol. Plant. 148 (2013) 522–537.
530 [20] C. Cruz, H. Lips, M.A. Martins-Loução, Nitrogen use efficiency by a slow-growing species
531 as affected by CO_2 levels, root temperature, N source and availability., J. Plant Physiol.
532 160 (2003) 1421–8.
533 [21] B. Li, G. Li, H.J. Kronzucker, F. Baluška, W. Shi, Ammonium stress in *Arabidopsis*:
534 Signaling, genetic loci, and physiological targets, Trends Plant Sci. 19 (2014) 107–114.
535 [22] G. Li, et al., Isolation and characterization of a novel ammonium overly sensitive
536 mutant, amos2, in *Arabidopsis thaliana*, Planta. 235 (2012) 239–252.
537 [23] Qi. Li, B.-H. Li, H.J. Kronzucker, W.-M. Shi, Root growth inhibition by NH_4^+ in *Arabidopsis*
538 is mediated by the root tip and is linked to NH_4^+ efflux and GMPase activity, Plant. Cell
539 Environ. (2010) 1529–1542.
540 [24] A. Rogato, et al., Characterization of a developmental root response caused by external
541 ammonium supply in *Lotus japonicus*, Plant Physiol. 154 (2010) 784–795.
542 [25] R. Esteban, et al., Both free indole-3-acetic acid and the phtosynthetic efficiency play a
543 relevant role in the response of *Medicago truncatula* to urea and ammonium nutrion

544 under axenic conditions, Front. Plant Sci. in press (2016).
545 [26] J.E. Lima, S. Kojima, H. Takahashi, N. von Wirén, Ammonium triggers lateral root
546 branching in *Arabidopsis* in an AMMONIUM TRANSPORTER1;3-dependent manner.,
547 Plant Cell. 22 (2010) 3621–33.
548 [27] N. Zou, B. Li, G. Dong, H.J. Kronzucker, W. Shi, Ammonium-induced loss of root
549 gravitropism is related to auxin distribution and TRH1 function, and is uncoupled from
550 inhibition of root elongation in *Arabidopsis*, J. Exp. Bot. 63 (2012) 3777–3788.
551 [28] D.W. Krogmann, a T. Jagendorf, M. Avron, Uncouplers of spinach chloroplast
552 photosynthetic phosphorylation., Plant Physiol. 34 (1959) 272–277.
553 [29] Z. Zhu, et al., Different tolerance to light stress in NO_3^-- and NH_4^+-grown *Phaseolus*
554 *vulgaris* L., Plant Biol. 2 (2000) 558–570.
555 [30] H. Brück, S. Guo, Influence of N form on growth photosynthesis of *Phaseolus vulgaris* L.
556 plants, J. Plant Nutr. Soil Sci. 169 (2006) 849–856.
557 [31] M. Askerka, D.J. Vinyard, G.W. Brudvig, V.S. Batista, NH_3 binding to the S_2 state of the
558 O_2 -evolving complex of Photosystem II: Analogue to H_2O binding during the $S_2 \rightarrow S_3$
559 transition, Biochemistry. 54 (2015) 5783–5786.
560 [32] A. Podgórska, et al., Long-term ammonium nutrition of *Arabidopsis* increases the
561 extrachloroplastic NAD(P)H/$NAD(P)^+$ ratio and mitochondrial reactive oxygen species
562 level in leaves but does not impair photosynthetic capacity, Plant. Cell Environ. (2013)
563 2034–2045.
564 [33] K. Patterson, et al., Distinct signalling pathways and transcriptome response signatures
565 differentiate ammonium- and nitrate-supplied plants, Plant. Cell Environ. (2010) 1486–
566 1501.

567 [34] Y. Zhu, et al., Adaptation of plasma membrane H^+-ATPase of rice roots to low pH as
568 related to ammonium nutrition, Plant, Cell Environ. 32 (2009) 1428–1440.
569 [35] X. Zheng, K. He, T. Kleist, F. Chen, S. Luan, Anion channel $SLAH_3$ functions in nitrate-
570 dependent alleviation of ammonium toxicity in *Arabidopsis*, Plant, Cell Environ. 38
571 (2015) 474–486. doi:10.1111/pce.12389.
572 [36] D.T. Britto, M.Y. Siddiqi, A.D. Glass, H.J. Kronzucker, Futile transmembrane $NH_4(^+)$
573 cycling: a cellular hypothesis to explain ammonium toxicity in plants, Proc. Natl. Acad.
574 Sci. USA. 98 (2001) 4255–4258.
575 [37] D. Coskun, D.T. Britto, M. Li, A. Becker, H.J. Kronzucker, Rapid ammonia gas transport
576 accounts for futile transmembrane cycling under NH_3/NH_4^+ toxicity in plant roots, Plant
577 Physiol. 163 (2013) 1859–1867.
578 [38] I. Ariz, et al., Depletion of the heaviest stable N isotope is associated with NH_4^+/NH_3
579 toxicity in NH_4^+-fed plants., BMC Plant Biol. 11 (2011) 83.
580 [39] S. Khademi, et al., Mechanism of ammonia transport by Amt/MEP/Rh: Structure of
581 AmtB at 1.35 A, Science 305 (2004) 1587–1594.
582 [40] M. Guether, et al., A mycorrhizal-specific ammonium transporter from *Lotus japonicus*
583 acquires nitrogen released by arbuscular mycorrhizal fungi, Plant Physiol. 150 (2009)
584 73–83.
585 [41] O. Pantoja, High affinity ammonium transporters: molecular mechanism of action,
586 Front. Plant Sci. 3 (2012) 1–10.
587 [42] A. Bertl, R. Kaldenhoff, Function of a separate NH_3-pore in Aquaporin TIP2;2 from
588 wheat, FEBS Lett. 581 (2007) 5413–5417.
589 [43] A. Kirscht, et al., Crystal structure of an ammonia-permeable aquaporin. PLoS Biol

590 14(2016) e1002411. doi:10.1371/journal.pbio.1002411
591 [44] K.D. Balkos, D.T. Britto, H.J. Kronzucker, Optimization of ammonium acquisition and
592 metabolism by potassium in rice (*Oryza sativa* L. cv. IR-72), Plant, Cell Environ. 33 (2010)
593 23–34.
594 [45] S. Scherzer, et al., The *Dionaea muscipula* ammonium channel DmAMT1 provides NH_4^+
595 uptake associated with Venus flytrap's prey digestion., Curr. Biol. 23 (2013) 1649–57.
596 [46] D. Coskun, D.T. Britto, H.J. Kronzucker, The nitrogen-potassium intersection:
597 Membranes, metabolism, and mechanism, Plant. Cell Environ. (2015) doi:
598 10.1111/pce.12671.
599 [47] M.W. Szczerba, D.T. Britto, K.D. Balkos, H.J. Kronzucker, Alleviation of rapid, futile
600 ammonium cycling at the plasma membrane by potassium reveals K^+-sensitive and -
601 insensitive components of NH_4^+ transport., J. Exp. Bot. 59 (2008) 303–13.
602 [48] C. Qin, et al., GDP-mannose pyrophosphorylase is a genetic determinant of ammonium
603 sensitivity in *Arabidopsis thaliana*., Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 18308–13.
604 [49] H. Tanaka, et al., Identification and characterization of *Arabidopsis* AtNUDX9 as a GDP-
605 d-mannose pyrophosphohydrolase: its involvement in root growth inhibition in
606 response to ammonium., J. Exp. Bot. 66 (2015) erv281–.
607 [50] S.K. Masakapalli, F.M. Bryant, N.J. Kruger, R.G. Ratcliffe, The metabolic flux phenotype
608 of heterotrophic *Arabidopsis* cells reveals a flexible balance between the cytosolic and
609 plastidic contributions to carbohydrate oxidation in response to phosphate limitation,
610 Plant J. 78 (2014) 964–977.
611 [51] H. Yang, et al., Auxin-modulated root growth inhibition in *Arabidopsis thaliana*
612 seedlings with ammonium as the sole nitrogen source, Funct. Plant Biol. 42 (2014) 239–

251.

[52] Y. Xie, et al., Heme-heme oxygenase 1 system is involved in ammonium tolerance by regulating antioxidant defence in *Oryza sativa*., Plant. Cell Environ. (2014) 129–143.

[53] X. Ding, et al., Activation of the indole-3-acetic acid-amido synthetase GH3-8 suppresses expansin expression and promotes salicylate- and jasmonate-independent basal immunity in rice, Plant Cell Online. 20 (2008) 228–240.

[54] K.J. Gupta, Y. Brotman, S. Segu, T. Zeier, J. Zeier, S.T. Persijn, et al., The form of nitrogen nutrition affects resistance against *Pseudomonas syringae* pv. *phaseolicola* in tobacco, J. Exp. Bot. 64 (2013) 553–568.

[55] T. Hachiya, C.K. Watanabe, M. Fujimoto, T. Ishikawa, K. Takahara, M. Kawai-Yamada, et al., Nitrate addition alleviates ammonium toxicity without lessening ammonium accumulation, organic acid depletion and inorganic cation depletion in *Arabidopsis thaliana* shoots, Plant Cell Physiol. 53 (2012) 577–591.

[56] G. Krouk, et al., Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants, Dev. Cell. 18 (2010) 927–937.

[57] T. Hachiya, Y. Mizokami, K. Miyata, D. Tholen, C.K. Watanabe, K. Noguchi, Evidence for a nitrate-independent function of the nitrate sensor NRT1.1 in *Arabidopsis thaliana*, J. Plant Res. 124 (2011) 425–430.

[58] D. Geiger, et al., Stomatal closure by fast abscisic acid signaling is mediated by the guard cell anion channel SLAH3 and the receptor RCAR1., Sci. Signal. 4 (2011) ra32.

[59] L. Yuan, et al., Allosteric regulation of transport activity by heterotrimerization of *Arabidopsis* ammonium transporter complexes *in vivo*., Plant Cell. 25 (2013) 974–84.

[60] A.U. Igamberdiev, R.D. Hill, Nitrate, NO and haemoglobin in plant adaptation to

hypoxia: An alternative to classic fermentation pathways, J. Exp. Bot. 55 (2004) 2473–2482.

[61] A.E. Allen, et al., Evolution and metabolic significance of the urea cycle in photosynthetic diatoms, Nature. 473 (2011) 203–207.

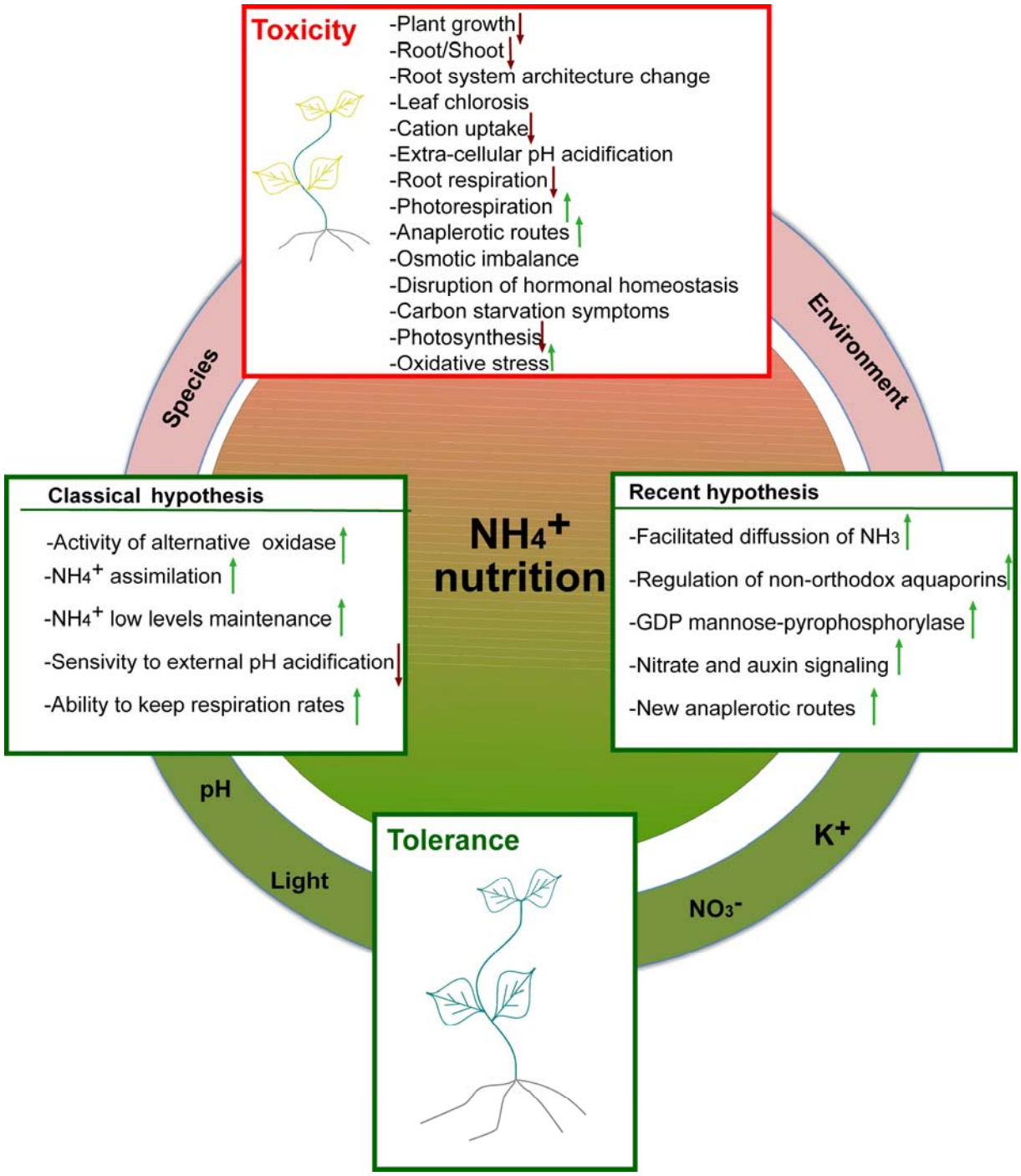
[62] Z. Zhou, A.E. Metcalf, C.J. Lovatt, B.C. Hyman, Alfalfa (*Medicago sativa*) carbamoylphosphate synthetase gene structure records the deep lineage of plants., Gene. 243 (2000) 105–14.

[63] P.M. Glibert, F.P. Wilkerson, R.C. Dugdale, J.A. Raven, C.L. Dupont, P.R. Leavitt, et al., Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions, Limnol. Oceanogr. (2015) 165–197.

[64] T. Flores, et al., Arginase-negative mutants of *Arabidopsis* exhibit increased nitric oxide signaling in root development, Plant Physiol. 147 (2008) 1936–1946.

[65] R. Wimalasekera, C. Villar, T. Begum, G.F.E. Scherer, COPPER AMINE OXIDASE1 (CuAO1) of *Arabidopsis thaliana* contributes to abscisic acid-and polyamine-induced nitric oxide biosynthesis and abscisic acid signal transduction, Mol. Plant. 4 (2011) 663–678.

[66] H.R. Roosta, J.K. Schjoerring, Effects of ammonium toxicity on nitrogen metabolism and elemental profile of cucumber plants, J. Plant Nutr. 30 (2007) 1933–1951.



657

658 **Fig. 1.** Ammonium toxicity (sensitivity) and tolerance in plants from classical to recent
659 hypothesis. The red box shows the main toxicity symptoms of NH_4^+ nutrition in plants, which
660 depend on several environmental factors and the species (varieties) used. The green boxes
661 show both the classical and recent theories of ammonium tolerance. Factors such as neutral
662 pH, light, NO_3^- and K^+ have been described as able to counterbalance ammonium toxicity and
663 therefore increase tolerance.
664

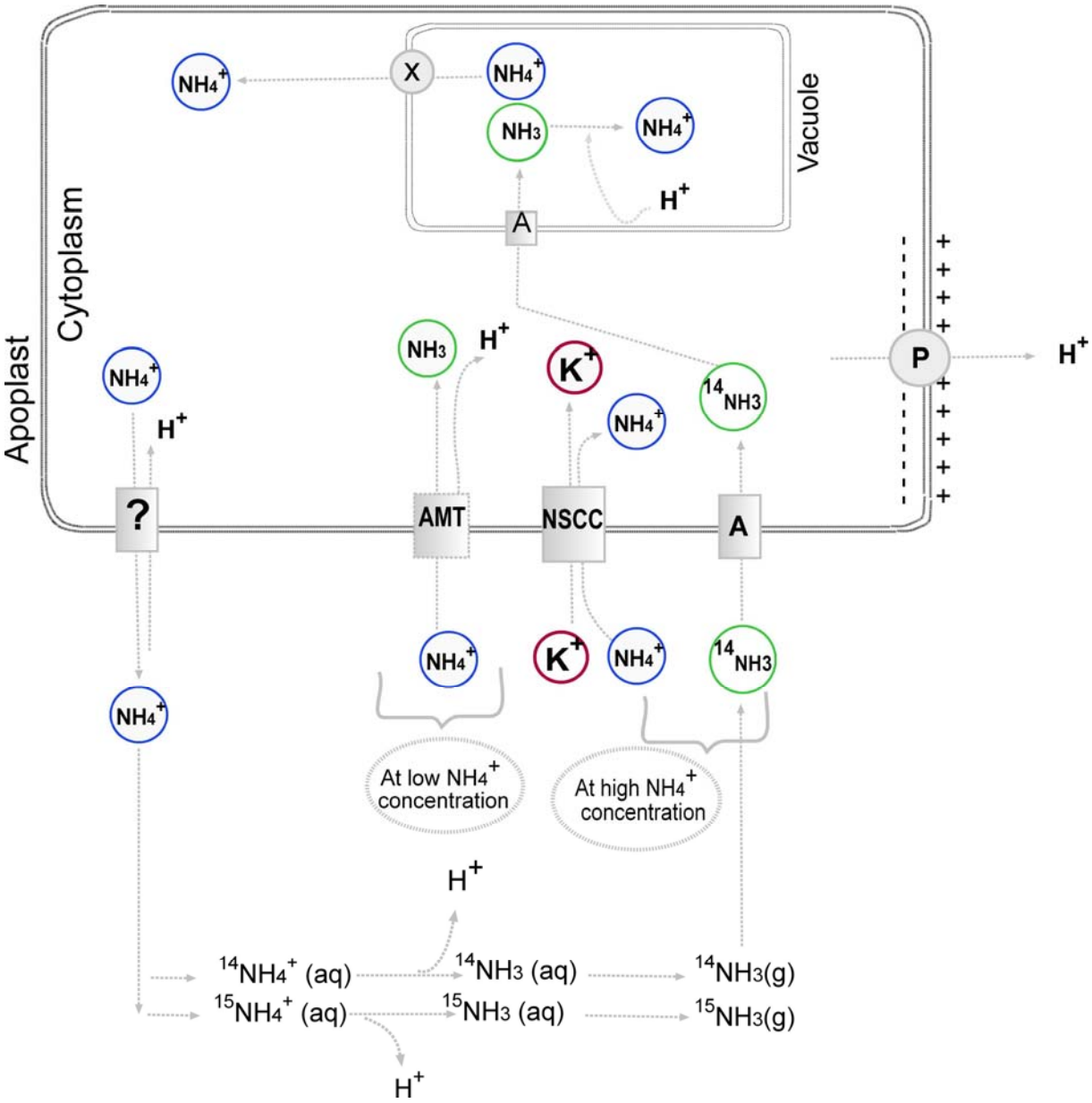
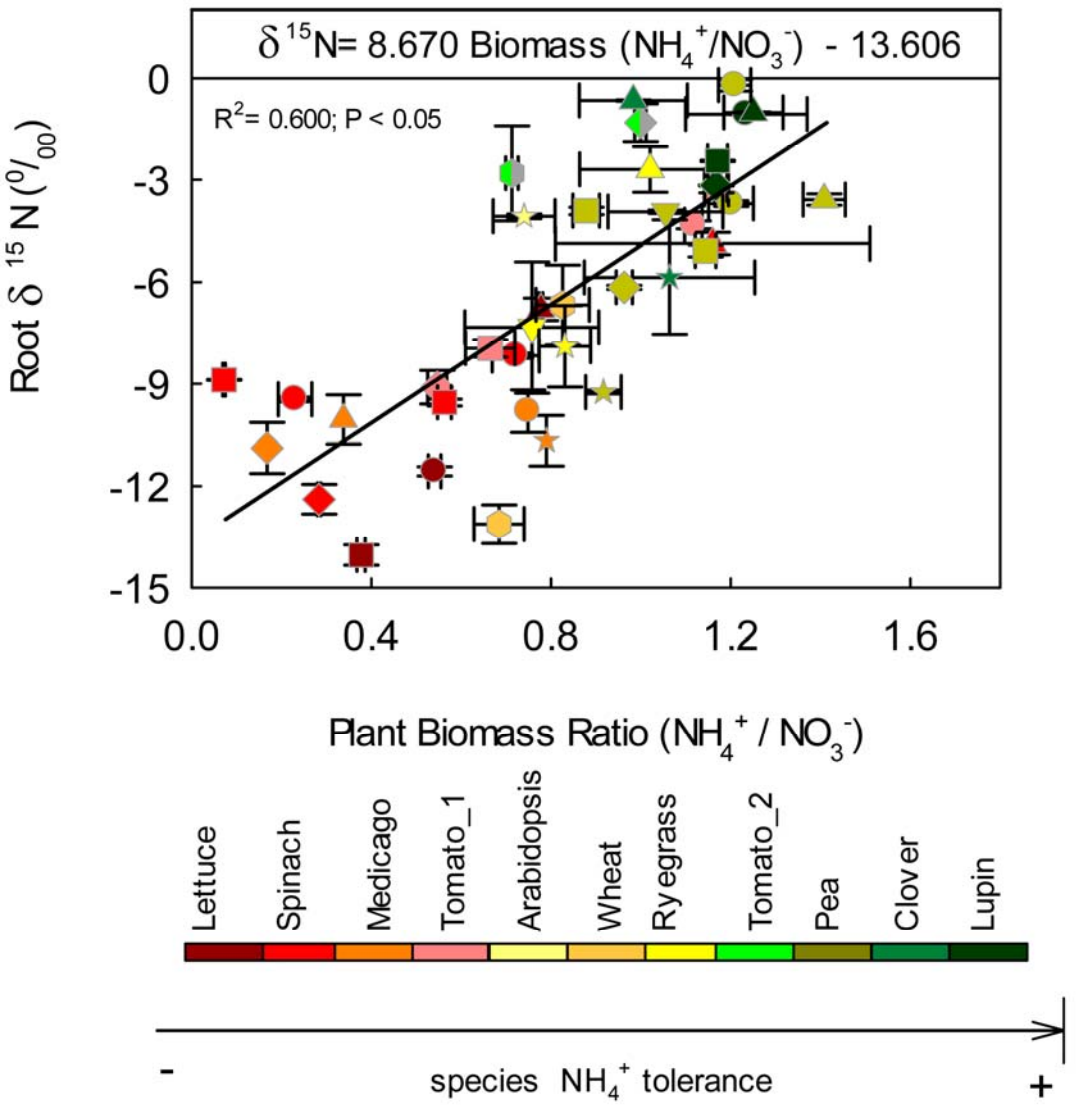
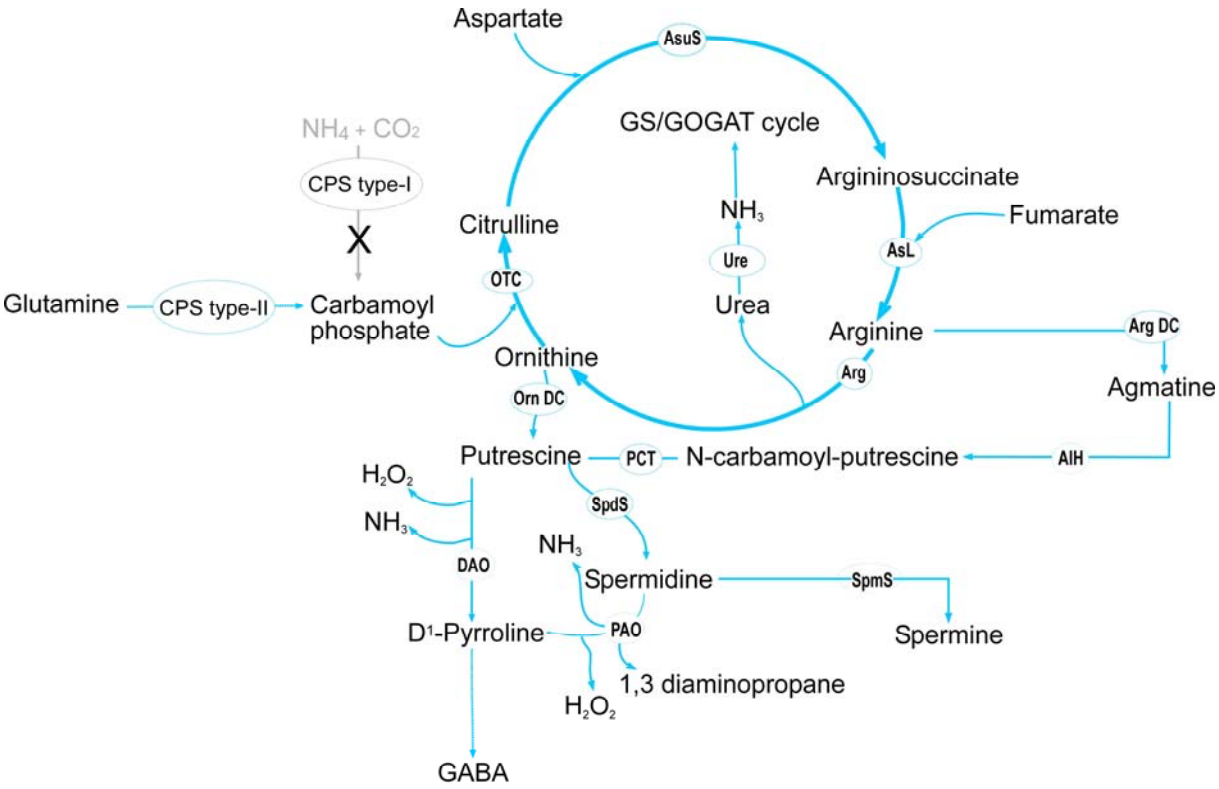


Fig. 2. Ammonium uptake by the plant cell, which contribute to NH_4^+ accumulation in the cell with toxic effects. Ammonium uptake is believed to be achieved by the diffusion of non-electrogenic flux of NH_3 across the plasma membrane, which might be facilitated by AQP. Once NH_3 enters the cell, it is re-protonated to form NH_4^+ (at neutral pH) in the cytosol, which causes a transient alkalinisation of the cytosol. This non-electrogenic flux is related to the depletion of ^{15}N in plant tissues in relation to the NH_4^+ source. The light N-isotope (^{14}N) is thermodynamically favoured over the heavy isotope (^{15}N). In other words, NH_3 (gas) is enriched in ^{14}N by about 5‰ relative to NH_3 (aqueous), while the latter is enriched in ^{14}N by about 20‰ relative to NH_4^+ (aqueous). Additionally, ammonium, (as NH_4^+), can be transported through high- affinity AMT transporters and, to a lesser extent, through K^+ channels. Passive transport of NH_3 from the cytoplasm to the vacuole can also be mediated by vacuolar AQP. Within the vacuole, the NH_3 is also protonated to form NH_4^+. The molecule that transports ammonium from the vacuole to the cytoplasm is unknown. P, proton pump; A, aquaporin; NSCC: non-specific cation channels; AMP, ammonium transporters.



681

682 **Fig. 3.** Correlation of the biomass yield ratio of plants grown hydroponically with NH_4^+ or NO_3^-
683 (this ratio is used as an indicator of plant tolerance/sensitivity to ammonium) with the root N
684 isotopic signature (δ15N, ‰) of NH_4^+-grown plants (updated in ref. [38]). Model species, such
685 as *A. thaliana* and *M. truncatula*, have been included in the figure. Additionally, new points
686 have been include for crops such as tomato [4] and wheat [3]. The N concentrations
687 represented are as follows: 0.5-1 mM (upward triangle), 1.5-2 mM (circle), 2.5 mM (downward
688 triangle), 3 mM (square), 4-5 mM (star), 6 mM (diamond), 7.5 mM (semi-filled diamond), 10
689 mM (hexagon) and 15 mM (semi-filled hexagon). The K^+ concentration was within the same
690 range (4-5 mM) for all the treatments and species. δ^{15} N data representing the $(NH_4)_2SO_4$ used
691 in NH_4^+-fed plants were obtained at +0.029, +0.5 and +2.31‰, and all three values fall within
692 the area indicated (upper part of the graph). Symbols represent the average values of at least
693 3 replicates ± SE. Further information is provided in [38].
694

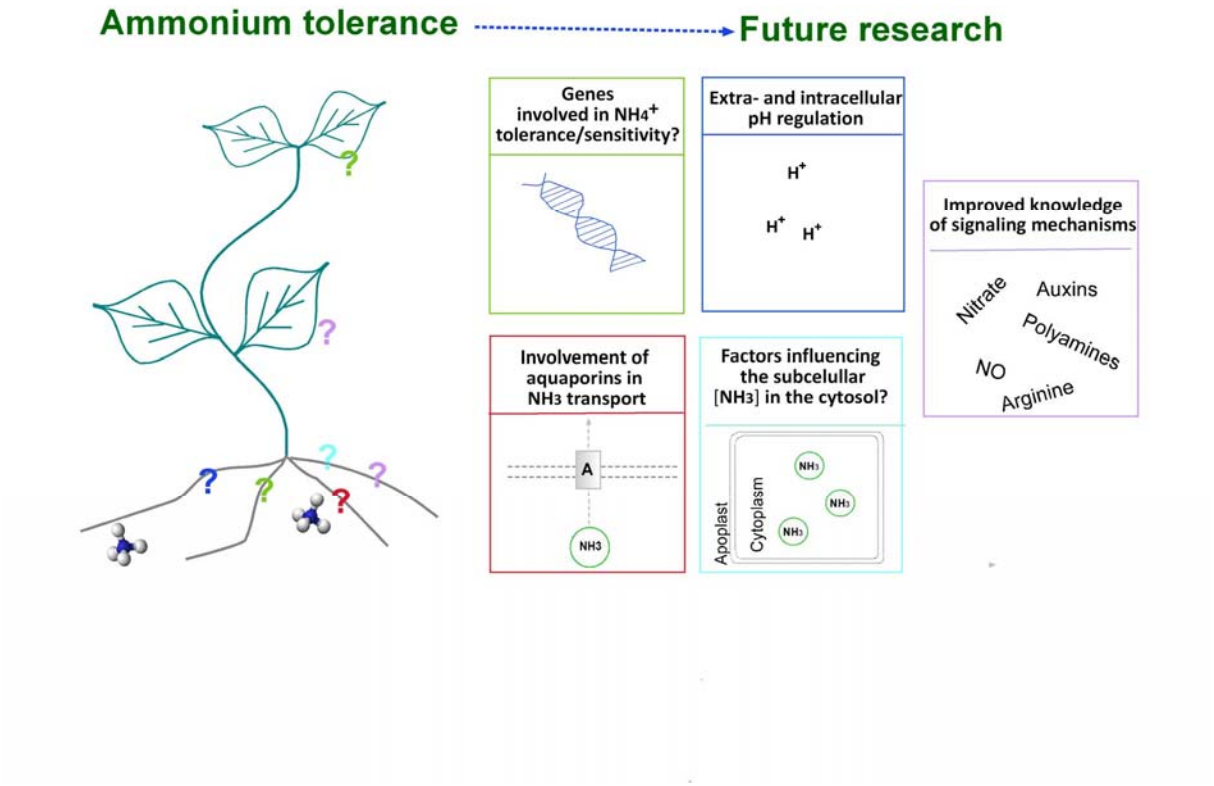


695

696 **Fig. 4.** The urea cycle in plants, which is linked to the polyamines synthesis. Carbamoyl
697 phosphate is synthesized from glutamine, but not from NH_4^+ in plants. Arginine and ornithine
698 can be used as the precursors of polyamines synthesis. Inter-conversions of polyamines give
699 rise to NH_4^+ and H_2O_2 molecules that result from oxidases catalysis. The enzymes are
700 represented inside a circle as: AIH, agmatine imidohydrolase; AsL, argininosuccinate lyase;
701 ARG, arginase, Arg DC; arginine decarboxylase; AsuS, argininosuccinate synthase; DAO,
702 diamine oxidase; Orn DC, ornithine decarboxylase; OTC, transcarbamylase; PAO, polyamine
703 oxidase; PCT, putrescine carbamoyl transferase; SpdS, spermidine synthase; SpmS, spermine
704 synthase; Ure, urease.

705

706



707

708 **Fig. 5.** Major challenges for future research on plants' ammonium tolerance as proposed in
709 this review. This figure identifies future directions of research as: the possible importance of
710 AQP in ammonium influx; and the identification of genes/loci involved in ammonium
711 sensitivity/tolerance. The colours of the boxes are related to the plant tissues whose study is
712 suggested, corresponding to locations of question marks on the figure.

713