Regulation of Ammonia Homeostasis by the Ammonium Transporter AmtA in Dictyostelium discoideum

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Ammonia has been shown to function as a morphogen at multiple steps during the development of the cellular slime mold Dictyostelium discoideum; however, it is largely unknown how intracellular ammonia levels are controlled. In the Dictyostelium genome, there are five genes that encode putative ammonium transporters: amtA, amtB, amtC, rghA, and rghB. Here, we show that AmtA regulates ammonia homeostasis during growth and development. We found that cells lacking amtA had increased levels of ammonia/ammonium, whereas their extracellular ammonia/ammonium levels were highly decreased. These results suggest that AmtA mediates the excretion of ammonium. In support of a role for AmtA in ammonia homeostasis, AmtA mRNA is expressed throughout the life cycle, and its expression level increases during development. Importantly, AmtA-mediated ammonia homeostasis is critical for many developmental processes. amtA+ cells are more sensitive to NH4Cl than wild-type cells in inhibition of chemotaxis toward cyclic AMP and of formation of multicellular aggregates. Furthermore, even in the absence of exogenously added ammonia, we found that amtA+ cells produced many small fruiting bodies and that the viability and germination of amtA+ spores were dramatically compromised. Taken together, our data clearly demonstrate that AmtA regulates ammonia homeostasis and plays important roles in multiple developmental processes in Dictyostelium.

The cellular slime mold Dictyostelium discoideum has a unique life cycle consisting of a unicellular growth phase and a multicellular developmental phase. When food sources such as bacteria are available, Dictyostelium amoeboid cells proliferate by cytokinesis. Starvation triggers cells to undergo developmental processes, during which up to 10^9 cells display chemotaxis toward cyclic AMP (cAMP) and form multicellular aggregates. On top of the aggregates, a small projection is formed, and this process is called tip formation. Cells located at the anterior part of aggregates differentiate into prestalk cells, precursors of stalk cells, while the rest of the aggregates become prespore cells, precursors of spores. The tipped aggregates form elongated multicellular structures called slugs. Slugs migrate and eventually culminate to form a fruiting body consisting of a mass of spores supported by a stalk (25, 44, 47).

A number of diffusible molecules regulate the development of Dictyostelium, including cAMP, differentiation-inducing factor, adenosine, and ammonia (5, 47, 74, 78). Ammonia has been shown to affect many developmental events in Dictyostelium. For example, in the presence of ammonia, the production and secretion of cAMP are inhibited, resulting in the impairment of chemotaxis toward cAMP and subsequent tip formation during early development (23, 61, 77). At later stages of development, ammonia acts against differentiation-inducing factor, suppresses differentiation into prestalk cells, and promotes differentiation into prespore cells (6, 26, 64, 72). In addition, ammonia plays an important role in the choice between the formation of a migrating slug and culmination. High concentrations of ammonia keep slugs migrating and block the initiation of culmination (60). The exhaustion of the ammonia supply triggers culmination at least in part by activating protein kinase A (PKA) through the DhkC signaling pathway (30, 61, 63). In fruiting bodies, extremely high concentrations of ammonium phosphate in sori maintain spore dormancy through the activation of the sporulation-specific adenyl cyclase ACG (8).

Ammonia is produced by protein catabolism, and ammonia levels rise during development, when most energy is generated by the degradation of protein and RNA (27, 60, 71, 76). It has been suggested that glutamine synthetases, which incorporate ammonia into glutamine, control intracellular levels of ammonia. The expression of glutamine synthetases is developmentally regulated, and their activity becomes elevated during the culmination stage (12–14, 24), suggesting a role of glutamine...
synthetases in culmination. Indeed, the pharmacological inhibition of glutamine synthetase blocks culmination during development (14). In addition, there are five genes, \textit{amtA}, \textit{amtB}, \textit{amtC}, \textit{rhgA}, and \textit{rhgB}, which belong to the evolutionarily conserved family of ammonium transporter/methylammonium permease/rhesus protein (Amt/Mep/Rh) in the \textit{Dictyostelium} genome (15). Previous studies have shown that developmental phenotypes in cells lacking AmtC can be rescued by deleting the AmtA protein (62). These studies suggest that AmtC and AmtA antagonistically regulate developmental processes and that AmtA and AmtC function in either ammonium transport or ammonium sensing (20, 37, 62). In this study, we show that AmtA regulates intracellular ammonium/ammonia levels during growth and development and is critical for the morphogenesis of multicellular aggregates and normal spore formation.

**MATERIALS AND METHODS**

**Strains, culture, and development.** The \textit{D. discoideum} strain Ax2 (40, 73) was used for wild-type cells in this study. For sequencing, strain Ax4 (40) was used. Cells were cultured at 22°C either in HL-5 medium (73) or on A medium agar plates (0.5% glucose, 0.05% yeast extract, 16.5 mM KH$_2$PO$_4$, 2.0 mM MgSO$_4$, 1.5% agar) associated with LPS and housed in 6-cm-diameter petri dishes at 22°C. When NH$_4$Cl was added, ammonia buffer A (20 mM KCl, 2.5 mM MgCl$_2$, 4.0 mM K$_2$HPO$_4$, pH 6.4) and placed on Geneticin (Calbiochem). Peptone and yeast extract were obtained from Difco Ltd. containing LPS and housed in 6-cm-diameter petri dishes at 22°C. When NH$_4$Cl was added, ammonia buffer A (20 mM KCl, 2.5 mM MgCl$_2$, 4.0 mM KH$_2$PO$_4$, pH 7.3) was used instead of LPS buffer. Developmental processes were observed with an Olympus SZX12 microscope. Stalk lengths were determined using the NIH Image program (developed at the National Institutes of Health [http://rsb.info.nih.gov/nih-image/]).

**Nucleic acid analyses.** Genomic DNA was extracted as described previously (33, 35). Total RNA was isolated using TRIzol reagent (Invitrogen) by following the manufacturer’s instructions. Poly(A)-containing RNA was purified using Oligotex-dT30 Super (JSR). RNA from prespore and prestalk cells was extracted following the manufacturer’s instructions. Whole-cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted on polyvinylidene difluoride membranes. Blots were immunostained with monoclonal antiactin antibody (C4; Chemicon) or monoclonal antiphosphotyrosine antibody clone PY20 (ICN), as previously described (81).

**RESULTS**

**AmtA controls ammonia homeostasis during growth and development.** The \textit{Dictyostelium} genome contains five genes, \textit{amtA}, \textit{amtB}, \textit{amtC}, \textit{rhgA}, and \textit{rhgB}, which belong to the Amt/Mep/Rh family. A phylogenetic analysis showed that these five genes are divided into three groups: Amt alpha, Amt beta, and Amt gamma. These studies suggest that AmtC and AmtA antagonistically regulate developmental processes and that AmtA and AmtC function in either ammonium transport or ammonium sensing (20, 37, 62). These studies suggest that AmtC and AmtA antagonistically regulate developmental processes and that AmtA and AmtC function in either ammonium transport or ammonium sensing (20, 37, 62). In this study, we show that AmtA regulates intracellular ammonium/ammonia levels during growth and development and is critical for the morphogenesis of multicellular aggregates and normal spore formation.
We observed 5.24 pmol ammonia/ammonium during growth and development using AmtA. 6.2- and 4.6-kb fragments were observed in cells disrupted for the AmtA gene. On the other hand, 5.1- and 3.5-kb DNA fragments generated by HincII digestion were observed in Southern blot analysis (Fig. 1B and C). Wild-type cells showed no phenotypes were caused by the loss of the AmtA gene by homologous recombination. The disruption was confirmed by Southern blotting, using the probe shown in panel B. Genomic DNA isolated from wild-type and AmtA− cells was analyzed after digestion with HincII or BamHI and EcoRV (B−V).

FIG. 1. Disruption of the AmtA gene. (A) Phylogenetic analysis of ammonium transporters in D. discoideum (Dd), M. thermautotrophicum (Mt), E. coli (Ec), S. cerevisiae (Sc), C. reinhardtii (Cr), C. elegans (Ce), D. melanogaster (Dm), X. laevis (Xl), H. sapiens (Hs), and A. thaliana (At). The scale indicates the number of substitutions per site. (B) Disruption of AmtA. A blastidicin resistance marker (bsr cassette) replaced the second intron of the AmtA gene by homologous recombination. Exons are indicated by white boxes. B, Hc, and V indicate BamHI, HincII, and EcoRV restriction sites, respectively. (C) The AmtA disruption was confirmed by Southern blotting, using the probe shown in panel B. Genomic DNA isolated from wild-type and AmtA− cells was analyzed after digestion with HincII or BamHI and EcoRV (B−V).

increased in wild-type cells during development. Although AmtA− cells also showed a gradual decrease in ammonia/ammonium levels, intracellular levels of ammonia/ammonium in AmtA− cells were much higher than those seen in wild-type cells at both the aggregation stage (12 h after starvation) and culmination stage (20 h). Increases in intracellular ammonia/ammonium levels could result from either overproduction of ammonia/ammonium or defects in its transport out of cells. Supporting the latter possibility, we found that extracellular levels of ammonia/ammonium were lower in AmtA− cells (Fig. 2B). We confirmed that these phenotypes were caused by the loss of the AmtA gene by expressing wild-type AmtA in AmtA− cells. All the phenotypes were significantly rescued by the expression of AmtA. These results clearly demonstrate that AmtA is required for normal ammonia homeostasis during growth and development.

Consistent with a role for AmtA in ammonia homeostasis, we found that AmtA mRNA (1.7 kb) is expressed during both growth and development using Northern blot analysis (Fig. 3A). The expression level increased continuously during development. This is in contrast with previous studies using reverse transcription-PCR that showed that AmtA mRNA levels do not change during development (20). The apparent difference may result from different methods used to measure mRNA levels. Nevertheless, the previous study and our current study clearly demonstrate that AmtA is expressed during development. Furthermore, we confirmed the localization of AmtA mRNA in prespore cells in slugs (20), using Northern blotting (Fig. 3B).

We confirmed the results of previous studies (62) showing that AmtA− cells normally grow both in shaking culture and on bacterial plates and that the time courses of development in wild-type and AmtA− cells are similar, differentiating into fruiting bodies in 24 h upon starvation (data not shown). Wild-type and AmtA− cells show similar sensitivities to ammonia in culmination (Fig. 3C). In Fig. 3C, cells were developed in the absence of NH4Cl, and then different amounts of NH4Cl were added to pads for further development. In addition, AmtA− cells produced more aggregates and fruiting bodies than wild-
In wild-type cells, the average density of aggregates was 593 aggregates/cm². In contrast, \textit{amtA} \textit{H}11002 cells produced 1,128 aggregates/cm². The resulting fruiting bodies of \textit{amtA} \textit{H}11002 cells were smaller than those of wild-type cells. The average stalk length in \textit{amtA} mutants (0.77 mm) was almost half that in the wild-type (1.63 mm). The expression of wild-type \textit{AmtA} in \textit{amtA} \textit{H}11002 cells significantly suppressed those \textit{amtA} phenotypes. These results are consistent with the previous observation that \textit{AmtA} plays a critical role in the number and size of aggregates and fruiting bodies (62). We also found that \textit{amtA} \textit{H}11002 cells show smaller aggregation territory sizes and frequent group breakup (Fig. 3G). Since glucose has been suggested to increase aggregation size, we measured intracellular levels of glucose (34, 22). We found that glucose levels in \textit{amtA} \textit{H}11002 cells were higher than those in wild-type cells (Fig. 3H). In contrast, protein amounts in wild-type and \textit{amtA} cells were indistinguishable (data not shown). These results suggest that the decreases of aggregation sizes in \textit{amtA} \textit{H}11002 cells are affected mainly by ammonia, not glucose. The results of ammonia homeostasis in early morphogenesis. It has been shown that \textit{NH}_4\text{Cl} inhibits multiple steps in early developmental processes, including the aggregation and tip formation of aggregates (10, 23, 60). Since \textit{amtA} \textit{H}11002 cells contain higher levels of ammonia/ammonium in early development than wild-type cells, we reasoned that mutant cells may be more sensitive to ammonia than wild-type cells. To test this idea, we induced development in \textit{amtA} \textit{H}11002 and wild-type cells in the presence of ammonia. Cells were incubated throughout development on filter membranes containing 0, 30, or 50 mM \textit{NH}_4\text{Cl}. As shown in Fig. 4A, in wild-type cells,
ammonia inhibited aggregation and tip formation in a concentration-dependent manner. In the absence of NH₄Cl, wild-type cells developed normally and formed fruiting bodies in 24 h after starvation. However, in the presence of 30 mM NH₄Cl, wild-type cells were able to form only aggregates with pointed ends (tip formation) and did not differentiate further into fruiting bodies. In 50 mM NH₄Cl, wild-type cells aggregated without tip formation. This result is not simply due to a delay in development. When we examined cells for prolonged periods of time (3 days), the cells still did not form fruiting bodies. In contrast, the mutants did not show tip formation of aggregates in 30 mM NH₄Cl, although amtA⁻ cells formed fruiting bodies in the absence of NH₄Cl. Furthermore, 50 mM NH₄Cl completely blocked aggregation in amtA⁻ cells. These results show that the loss of AmtA makes cells hypersensitive to ammonia in aggregation and tip formation during development.

amtA⁻ cells are defective in chemotaxis in the presence of ammonia. The inhibitory effect of ammonia on the aggregation of amtA⁻ cells suggests that ammonia suppresses chemotaxis toward cAMP in mutant cells. To test this possibility, we examined chemotaxis toward cAMP using a spot assay (Fig. 4B). Cells were spotted on the center of plates which contained 10 μM cAMP in the presence or absence of NH₄Cl. Since there were no nutrients available on the plate, cells initiated development processes immediately after being spotted. Cells secrete phosphodiesterase and degrade extracellular cAMP, generating a difference in cAMP concentrations around the spot, with higher concentrations outside and lower concentrations inside. Along the cAMP gradient, cells move outward from the spotted area. If cells are defective in chemotaxis, they will stay inside the spotted areas. When wild-type and amtA⁻ cells were spotted on plates containing 10 μM cAMP in the absence of NH₄Cl, both wild-type and mutant cells developed normally. At the periphery of the spotted area, we found that many cells moved outward (Fig. 4B). This outward movement is chemotactic migration toward cAMP, since cells move out only when cAMP is present. On plates lacking cAMP, cells developed normally, but they stayed in the spotted area and did not move outward. However, when we added 30 mM NH₄Cl to the plates, wild-type and amtA⁻ cells showed distinct chemotactic behaviors. In the presence of 30 mM NH₄Cl, wild-type cells were still able to develop, form aggregates, and move outward toward cAMP. In contrast, when amtA⁻ cells were spotted, the mutant cells developed normally and formed aggregates, but they failed to move out of the spotted area. Our data suggest that cells lacking AmtA become more sensitive to NH₄Cl during chemotaxis toward cAMP.

AmtA is required for normal spore formation. In Dictyostelium, the spore formation process is affected by ammonia through the regulation of cAMP-dependent PKA (30, 80). In addition, extra spore solution contains high concentrations of ammonium phosphate, which has been suggested to maintain the dormancy of spores (8, 9, 70). To determine whether AmtA is involved in ammonium homeostasis in spore and extra spore solution in sori, we collected sori, separated spores from extra spore solution by centrifugation, and measured levels of ammonium/ammonium. As shown in Fig. 5A, the levels of ammonium/ammonium increased twofold in amtA⁻ spores. Wild-type spores contained 1.50 fmol of ammonium/ammonium per cell, whereas amtA⁻ spores contained 2.69 fmol of ammonium/ammonium per cell. In addition, ammonium levels in extra spore solution decreased twofold in the mutant cells (Fig. 5B). Therefore, our data indicate that AmtA is required for normal ammonium homeostasis in spores. To probe the role of AmtA in spores, we examined the morphology, viability, and germination rates of spores. We observed spore morphology using phase-contrast microscopy and checked spore viability using PI, which stains only dead spores (7, 50, 52). We found that the viability of spores lacking AmtA is compromised as the spores age. Immediately after the completion of fruiting body formation, both wild-type and mutant spores showed oval shapes and very few spores were stained by PI. However, as spores aged, amtA⁻ spores appeared to be darker under the phase-contrast microscope (Fig. 5C and D). The majority of the dark spores were stained by PI, indicating that they were dead. Twelve days after fruiting body formation, 79.0% of amtA mutant spores were stained by PI, whereas only 4.4% of wild-type spores were PI positive. Furthermore, we found that germination rates were decreased in amtA⁻ spores (Fig. 5E). Probably as a result of their compromised viability, amtA⁻ spores are also defective in normal germination. Immediately after spore formation, ~95% of wild-type spores germinated, whereas only 67% of amtA mutant spores germinated. Eight days after the completion of fruiting body formation, wild-type cells maintained a germination rate similar to that seen at day 0. In contrast, the germination rate of amtA⁻ spores decreased as they aged. We found that only 20% of amtA⁻ spores had germinated at 8 days. It has been shown that actins are organized into thick bundles in spores and are phosphorylated on tyrosine residues during the formation and maturation of spores (38, 59). Supporting the idea that amtA⁻ cells are defective in normal spore formation, we found that tyrosine-phosphorylated actin levels decreased in amtA⁻ spores (Fig. 5F). Therefore, our data indicate that AmtA is required for the formation and maintenance of viable spores.

**DISCUSSION**

A large number of studies have shown that ammonia regulates multiple processes during the development of Dictyostelium (45, 47, 78–80). However, the molecular mechanisms underlying ammonia homeostasis are poorly understood. Previous studies have suggested that AmtA functions in ammonia transport or ammonia sensing (20, 62). In this paper, supporting a role for AmtA in ammonium transport, we have shown that AmtA is required for normal ammonia homeostasis in Dictyostelium during growth and development. Our data strongly suggest that AmtA is involved in the excretion of ammonia/ammonium from cells. Supporting our conclusion, cells lacking AmtA accumulate ammonia/ammonium. The impairment of ammonium efflux results in a reduction in extra-cellular levels of ammonia/ammonium. Furthermore, intrasporic levels of ammonia/ammonium are also highly increased in amtA mutants. Since the extracellular levels of ammonia/ammonium are slightly higher than the intracellular levels in Dictyostelium, AmtA might actively transport ammonium out of cells. Although our data suggest that AmtA is an important ammonium transporter in development, they do not rule out...
the possibility that AmtA also functions in ammonia sensing, as previously proposed (62).

Previous studies have shown that AmtA is important for the morphogenesis of fruiting bodies and for resistance to high concentrations of ammonia in culmination (62). Our study confirmed the previous findings and further identifies three additional developmental processes which involve AmtA-mediated ammonia homeostasis, including chemotaxis, tip formation, and spore formation. At the beginning of development, single cells undergo chemotaxis toward aggregation centers, which release the chemoattractant cAMP, leading to the formation of multicellular aggregates. We found that AmtA affects the sensitivity to ammonia in inhibition of chemotactic migration toward cAMP. \textit{amtA} \textsuperscript{-} cells fail to undergo chemotaxis.

FIG. 5. AmtA is required for ammonia homeostasis, viability, and the germination of spores. Ammonia levels in spores (A) and spore mass solution (B). Three days after fruiting body formation, spore masses were collected and spores and sorus media were separated by centrifugation. The ammonia levels of each fraction were determined by an ammonia electrode. Values are normalized to 10\textsuperscript{8} spores in 10 \(\mu\)l sori. (C) Spores were collected 0 and 12 days after fruiting body formation was completed and stained with PI. Spores were observed by phase-contrast and fluorescence microscopy. Bar, 50 \(\mu\)m. (D) Quantitation of PI-positive spores. Spores were collected at the indicated periods of time after fruiting body formation and stained with PI. Spores that were stained with PI were counted. (E) Spores were collected at the indicated time points after the completion of fruiting body formation and examined for germination, as described in Materials and Methods. (F) Changes of tyrosine phosphorylation of actin with aging. Total proteins were prepared from 1-, 2-, 6-, and 10-day-old spores and immunostained with antiactin antibody and antiphosphotyrosine antibody.
taxis in the presence of NH$_4$Cl. Since the concentrations of NH$_4$Cl that block chemotaxis do not inhibit the normal development of $amtA^-$ cells, it is unlikely that the chemotaxis phenotypes result simply from developmental defects. In addition, in our experiments, we examined chemotaxis toward exogenously added cAMP. Therefore, the observed chemotaxis defect is not due to the inhibition of the production and secretion of cAMP by ammonia. Rather, we suggest that ammonia directly affects signaling pathways for chemotaxis. Supporting our hypothesis, it has been shown that ammonia affects intracellular pH and thereby inhibits chemotaxis (10, 19, 69). It is possible that intracellular pH is regulated at least partially by AmtA during chemotaxis. Since the morphogenesis of aggregates involves cAMP signaling (66), the impaired chemotaxis may result in defects in tip formation in aggregates in $amtA^-$ cells. Furthermore, at the final stage of development, $amtA^-$ cells formed much smaller fruiting bodies than those of wild-type cells, even in the absence of exogenously added ammonia. Many smaller fruiting bodies may result from incomplete chemotaxis, which could produce many smaller aggregates. Alternatively, it is also possible that ammonia homeostasis directly controls the size of the fruiting bodies by affecting the differentiation of spore and stalk cells.

One of our most interesting observations is that AmtA regulates ammonia levels in spores. We found that $amtA^-$ spores in fruiting bodies contained higher levels of ammonia/ammonium but that extracellular levels were decreased. In addition, $amtA^-$ spores were severely defective in viability, and these phenotypes became more severe as spores aged. It has been shown that ammonia in sori is critical for the formation and maintenance of spores (8, 9, 30, 70, 80). Thus, our studies demonstrate that AmtA is a critical regulator for ammonia homeostasis during spore formation. It is likely that the reduced viability of $amtA^-$ spores leads to their defect in germination. Unlike with cells lacking the histidine kinase dhkB, which are defective in spore dormancy and prematurely germinate in sori (82), we did not observe premature germination in $amtA^-$ spores; it is unlikely that $amtA^-$ cells are unable to inhibit premature germination in sori, but rather they seem to be defective in formation of fully resistant spores. It has been suggested that the organization of the actin cytoskeleton is important for the formation and stabilization of spores (38, 58, 59). During spore formation, a large fraction of actin molecules are phosphorylated on tyrosine residues and organized into thick bundles. This process is induced under PKA activity and later mediated by the MADS box transcription factor SrfA (16–18). We found that the level of tyrosine phosphorylation of actin remains low in $amtA^-$ spores, suggesting that ammonia homeostasis might regulate spore formation and stabilization by affecting the actin organization mediated by PKA and SrfA.

Our current study and previous study have shown that the disruption of AmtA does not cause the severe defects in the culmination step of aggregates on its own (62). Wild-type and $amtA^-$ cells showed similar sensitivities to exogenously added ammonia in culmination, suggesting that other ammonium transporters regulate ammonia levels at the initiation of culmination. Supporting this idea, previous studies showed that AmtC was important for the induction of culmination and that cells lacking AmtC failed to culminate and remained as slugs (20, 37). In contrast to AmtA, AmtC is proposed to function as a sensor which monitors ammonia levels and activates intracellular signaling. Consistent with this idea, AmtC is preferentially expressed at the tip region of slugs, where the culmination signal may be generated (20). Interestingly, the disruption of $amtA$ in the $amtC$ null strain restored the ability of cells to differentiate to undergo the culmination stage (62). In this study, we showed that $amtA$ functions as an ammonium transporter, although it is possible that $amtA$ controls the slug/culmination transition as an ammonia sensor. To determine whether AmtC functions as an ammonium sensor or transporter, it would be important to determine whether intracellular levels of ammonia are also altered in $amtC^-$ cells.

Members of the Amt/Mep/Rh family are found in all domains of life and involved in a variety of biological processes. In microorganisms, ammonia is a nutrient, and most Amt/Mep/Rh proteins participate in the uptake of ammonium into cells (11, 31, 36, 42, 49). In contrast, in animals, ammonia is a waste product resulting from amino acid catabolism (29). Rh proteins have been suggested to excrete ammonia to maintain intracellular homeostasis (3, 4, 32, 48, 55, 75), although several recent studies suggest that the substrate of Rh proteins is CO$_2$, not ammonia (41, 54). Furthermore, yeast Mep2 ammonium permease has been shown to function as a sensor of ammonia, generating a signal to regulate pseudohyphal differentiation (21, 46). Mep2 may lack the ability to transport ammonium across membranes (46). In contrast to most organisms, which carry only one type of ammonium transporter among the three subfamilies (Amt alpha, Amt beta, and Rh groups), Dictyostelium contains five ammonium transporters that belong to all three subfamilies. It would be tempting to speculate that Dictyostelium uses the different functions of ammonium transporters, such as ammonium uptake, efflux, and sensing, to control its unique development.

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