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Root growth inhibition by NH_4^+ in *Arabidopsis* is mediated by the root tip and is linked to NH_4^+ efflux and GMPase activity

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ABSTRACT

Root growth in higher plants is sensitive to excess ammonium (NH_4^+). Our study shows that contact of NH_4^+ with the primary root tip is both necessary and sufficient to the development of arrested root growth under NH_4^+ nutrition in *Arabidopsis*. We show that cell elongation and not cell division is the principal target in the NH_4^+ inhibition of primary root growth. Mutant and expression analyses using *DR5:GUS* revealed that the growth inhibition is furthermore independent of auxin and ethylene signalling. NH_4^+ fluxes along the primary root, measured using the Scanning Ion-selective Electrode Technique, revealed a significant stimulation of NH_4^+ efflux at the elongation zone following treatment with elevated NH_4^+, coincident with the inhibition of root elongation. Stimulation of NH_4^+ efflux and inhibition of cell expansion were significantly more pronounced in the NH_4^+-hypersensitive mutant *vtc1-1*, deficient in the enzyme GDP-mannose pyrophosphorylase (GMPase). We conclude that both restricted transmembrane NH_4^+ fluxes and proper functioning of GMPase in roots are critical to minimizing the severity of the NH_4^+ toxicity response in *Arabidopsis*.

Key-words: abiotic stress; ammonium toxicity; cell elongation; flux; N-glycosylation; root system.

INTRODUCTION

Ammonium (NH_4^+), a major nitrogen source, is not only essential for living cells but also a ubiquitous intermediate in plant metabolism (Glass *et al*. 1997). However, when present in excess, NH_4^+ is notorious for its toxic effects in most higher plants (Givan 1979; Britto & Kronzucker 2002; Krupa 2003). In addition to NH_4^+ being the predominant source of inorganic N in some of earth's most extensive natural ecosystems, such as the boreal forest and arctic tundra (Kronzucker, Siddiqi & Glass 1997; Britto & Kronzucker 2002), application of large quantities of nitrogen fertilizer, irrational fertilization patterns, and deposition of atmospheric NH_3/NH_4^+ (Pearson & Stewart 1993; Krupa 2003) have led to the accumulation of excess NH_4^+ in many agricultural and natural soils normally governed by nitrate. In some soils, high NH_4^+ concentrations of up to 40 mM are achieved (Wolt 1994; Glass, Britto, Kaiser *et al*. 2002; Kronzucker *et al*. 2003). Recently, excess NH_4^+ has been proposed as a factor in the loss of plant species richness in a variety of ecosystems (Kronzucker *et al*. 2003; Krupa 2003; Stevens *et al*. 2004; Clark & Tilman 2008).

For terrestrial plants, the root system is critical to nutrient and water uptake from soil and displays considerable plasticity in response to developmental and environmental signals. Plants equipped with taproot systems produce laterals along the length of the primary root (Deak & Malamy 2005); hence, the developing root system in these plants depends on a well-developed primary root. The primary root originates from a rudimentary embryonic root, and most growth and differentiation is post-embryonic (Deak & Malamy 2005). During post-embryonic development, primary root growth depends on two fundamental developmental processes: cell division in the meristematic zone of the root apex, and elongation of cells in the elongation zone behind the root meristem (Scheres, Benfey & Dolan 2002). During primary root growth, environmental signals in the soil are sensed, and remarkable developmental plasticity to diverse environmental conditions is typically seen, which includes an integration of root- and shoot-derived signals, such as those involved in the regulation of nitrate (Scheible *et al*. 1997), potassium (Szczerba, Britto & Kronzucker 2009), phosphate (Delhaize & Randall 1995; Williamson *et al*. 2001) and sucrose acquisition (MacGregor *et al*. 2008). However, the growth of the primary root is often reduced in stressful soil environments, such as those deficient in phosphate (Williamson *et al*. 2001; Svistoonoff *et al*. 2007) or L-glutamate (Walch-Liu *et al*. 2006; Walch-Liu & Forde 2008), or those afflicted by excess aluminium (Ryan, DiTomaso & Kochian 1993; Jones & Kochian 1995). A stunted root system is also a significant symptom of NH_4^+ toxicity (Britto & Kronzucker 2002; Balkos, Britto & Kronzucker 2010). This is readily observed in seedlings grown in solution culture with NH_4^+ as the sole nitrogen source, but not with nitrate, even in cases where shoot growth may be

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similar (Kudoyarova, Farkhutdinov & Veselov 1997). However, the physiological and molecular processes underlying root growth inhibition by high NH_4^+ are only partially understood.

Previous studies have explored several important physiological links in the development of NH_4^+ toxicity, such as rhizosphere acidification, nutrient imbalance, damage to the photosynthesis system and carbohydrate limitation (Gerendas *et al*. 1997; Britto & Kronzucker 2002). Moreover, NH_4^+-mediated inhibition of primary root development have been suggested to be linked to auxin transport or signalling pathways when plants were reared in low-K^+ media (Cao, Glass & Crawford 1993), and ethylene evolution has been observed in seedlings that accumulate excessive NH_4^+ in shoots (Barker 1999; You & Barker 2004).

Recently, two novel, and seemingly independent, mechanisms of NH_4^+ toxicity have been proposed. Firstly, elevated unidirectional NH_4^+ fluxes at the root plasma membrane have been linked to root and shoot biomass suppression in NH_4^+-sensitive species supplied with elevated levels of NH_4^+ (Britto *et al*. 2001). The rates of NH_4^+ transport across the root (and shoot) plasma membranes have been shown to be significantly less pronounced in genotypes that are relatively tolerant of high external NH_4^+ (Britto *et al*. 2001, 2002; Kronzucker *et al*. 2003; Britto & Kronzucker 2006), and they have also, more recently, been shown to be sensitive to environmental factors such as the availability of potassium (Szczerba *et al*. 2008); when fluxes are reduced by such factors, biomass can be greatly enhanced (Szczerba *et al*. 2008; Balkos *et al*. 2010).

Secondly, the enzyme GDP-mannose pyrophosphorylase (GMPase) has been shown to be linked to NH_4^+ sensitivity of *Arabidopsis* roots (Qin *et al*. 2008; Barth *et al*. 2010). In this second model, decreased GMPase activity, and the consequent defective N-glycosylation of proteins, are assumed to be important downstream molecular events involved in the inhibition of root growth induced by NH_4^+ (Qin *et al*. 2008). N-glycosylation is essential for correct protein folding, proper targeting in protein secretion (Lerouge *et al*. 1998), triggering of the protein unfolding response, cellulose synthesis, and cell wall stability and cell viability in *Arabidopsis* (Lukowitz *et al*. 2001; Koiwa, Li, McCully *et al*. 2003; Hoeberichts, Vaeck, Kiddle *et al*. 2008; Kang, Frank, Kang *et al*. 2008). GDP-mannose is important for the correct N-glycosylation of proteins and the biosynthesis of ascorbic acid in *Arabidopsis* (Conklin *et al*. 1999; Lukowitz *et al*. 2001). *cty1* is a null mutant of GMPase, a key gene in GDP-mannose synthesis, which results in embryonic lethality (Nickle & Meinke 1998; Lukowitz *et al*. 2001). *vtc1* and *hsn1* are weak alleles of GMPase, displaying a phenotype similar to that of the wild type under normal conditions (Conklin *et al*. 1999; Qin *et al*. 2008), but showing seriously inhibited primary root growth in high-NH_4^+ conditions (Qin *et al*. 2008; Barth *et al*. 2010). The embryonic lethality in *cty1* and the NH_4^+-induced inhibition of root growth in *vtc1* and *hsn1* result in deficiencies of N-glycosylation, but decreases in ascorbic acid in the mutant are not related to NH_4^+ sensitivity (Lukowitz *et al*. 2001; Qin *et al*. 2008; Barth *et al*. 2010). Whether this N-glycosylation of proteins interacts with the transport of NH_4^+ across the plasma membrane in plants is still unknown.

The previously described physiological and molecular processes related to NH_4^+ toxicity do not clarify the inhibitory effect of high NH_4^+ on root elongation, but provide important clues for further evaluation. In this study, we investigate the properties and mechanisms of the inhibition of root growth by root-supplied NH_4^+ in *Arabidopsis* using a segregated culture system. We address the following research questions: (1) Is NH_4^+ contact with the root tip necessary and sufficient for the development of root growth inhibition under elevated NH_4^+? (2) Is the principal growth inhibition target cell elongation or division? (3) Is NH_4^+-dependent growth inhibition caused by NH_4^+ itself or does it depend on NH_4^+ metabolism? (4) Is the inhibition of primary root growth independent of ethylene and auxin signalling? (5) Is the inhibition of primary root growth linked to elevated NH_4^+ efflux along the root? and (6) Are root growth inhibition and NH_4^+ efflux stimulation accentuated in the NH_4^+-hypersensitive mutant *vtc 1-1*, deficient in the enzyme GDP mannose pyrophosphorylase?

MATERIALS AND METHODS

Plant material and growth conditions

Seedlings of the following lines were used in this study: *Arabidopsis thaliana* ecotype *Columbia-0* (*Col-0*); the mutants *aux1-7* (Pickett, Wilson & Estelle 1990), *eir1-1* (Roman *et al*. 1995), *ein2-1* and *etr1-3* (Guzman & Ecker 1990) and *vtc1-1* (Conklin *et al*. 1999) in the *Col-0* background (obtained from the Arabidopsis Biological Resource Centre of Ohio State University); and the transgenic lines *DR5::GUS* (Ulmasov *et al*. 1997), *CycB1::GUS* (Colon-Carmona *et al*. 1999), and *QC25::GUS* (Sabatini *et al*. 2003). The seeds were surface-sterilized and cold-treated at 4 °C for 48 h before being sown on normal growth medium. The medium, modified from Cao *et al*. (1993), contained 2 mM KH_2PO_4, 5 mM $NaNO_3$, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM Fe-EDTA, 50 μM H_3BO_3, 12 μM $MnSO_4$, 1 μM $ZnC1_2$, 1 μM $CuSO_4$, 0.2 μM Na_2MoO_4, 1% sucrose, 0.5 g/L MES and 0.8% agar (adjusted to pH 5.7 with 1 M NaOH). The plates were placed vertically in a growth chamber at 23 ± 1 °C under a light intensity of 100 μmol photons m^{-2} s^{-1}, with a photoperiod of 16 h light and 8 h dark. For uniform-nutrient treatments, the seedlings were germinated for 4 to 6 d and subsequently transferred to agar plates containing the appropriate treatment.

Whole root and localized root supply of NH_4^+ to the primary root

The model for the whole root and localized supply of NH_4^+ to only the root tip or the mature zone of the primary root is shown in Fig. 2a and is briefly described here: segmented agar plates (13 × 13 cm) were separated into upper and

bottom parts with a 3-mm air gap (Zhang & Forde 1998) using movable glass strips 3 mm in width. Normal growth medium (control medium) was poured into the upper part, and control medium with various concentrations of $(NH_4)_2SO_4$ added to it was poured into the bottom part. 'Root-supplied NH_4^+' plants have the whole root in contact with the bottom medium after seedling transfer. Plates on which only the primary root tip of the seedlings (~2 mm) was in contact with the bottom of the medium are called 'root-tip supplied NH_4^+' plants. In order to perform localized applications of nutrients to the mature zone of the primary root, but not to the root tip and shoot, two movable glass strips were used, and the segmented plates were separated into three parts: upper, middle and bottom. The medium containing NH_4^+ was only poured into the middle part of plates; other regions of the plate received no added NH_4^+. For root or root tip supply of NH_4^+, 5-day-old seedlings (with a primary root length of about 2 cm) were transferred and treated for 5 d prior to analysis. For the mature root zone treated with NH_4^+, 6-day-old seedlings (with primary roots long enough to cross the middle agar region) were transferred, and the treatment length was also 5 d.

It is important to note that in agar media, unlike in soil or hydroponic culture, diffusion limitation for nutrients necessitates the application of larger-than-normal concentrations of nutrients, including those of the toxicant NH_4^+ (see also Barth *et al*. 2010). We have previously shown (Li & Shi 2007) that 5–15 mM NH_4^+ are not sufficient to induce toxicity symptoms in this system. To achieve growth suppression and tissue NH_4^+ contents (Barth *et al*. 2010) similar to those seen in hydroponic NH_4^+ toxicity studies (e.g. 10 mM: Britto *et al*. 2001; Szczerba *et al*. 2008; Balkos *et al*. 2010), 20–60 mM NH_4^+ must, at minimum, be applied. In select cases, where hypotheses demanded it, even higher concentrations (up to 120 mM) were applied.

Measurement of root length

Roots on the agar surface were sampled. The lengths of individual primary roots of individual seedlings were measured either directly with a ruler or with Image J software (National Institutes of Health; http://rsb.info.nih.gov/ij) from digital images captured with a Canon G7 camera. Primary root elongation was defined as the length of the root parts newly grown after treatment; whereas the primary root length was set as the length of the whole primary root. All experiments for phenotypic analysis were performed at least twice and the data represent one independent experiment.

Microscopic analysis of cell development in roots

Histochemical analysis of GUS reporter enzyme activity was performed according to the procedure (Weigel & Glazebrook 2002). Starch granules in the columella root cap were visualized with Lugol staining as previously described (Willemsen *et al*. 1998).

Roots were mounted directly in double-distilled water to avoid cell shrinkage (Beemster & Baskin 1998). Measurements were made of the length from the root cap to the first root hair, the meristem length from the QC to the first elongated cell, and the final cell length as indicated by the cortex cell length at the mature zone on newly grown parts of the primary root. All images were obtained using an Olympus BX51 microscope equipped with differential interference contrast (DIC) optics and an Olympus DP71 camera. The images shown are representative of at least eight individual plants for each treatment, and the experiments were repeated at least twice. The final cell length was assessed from at least 150 cells from more than six individual seedlings. The length of a single cell or tissue in the primary root was analysed with the software Image-Pro Express version 5.1 (Media Cybernetics Inc., Bethesda, MD, USA).

Measurement of net NH_4^+ flux with the SIET system

Net fluxes of NH_4^+ were measured non-invasively using SIET (scanning ion-selective electrode technique, SIET system BIO-003A; Younger USA Science and Technology Corp.; Applicable Electronics Inc.; Science Wares Inc., Falmouth, MA, USA). The principle of this method and the instrument are detailed in Sun, Chen, Dai *et al*. (2009). Measurements were performed at room temperature (24–26 °C). After treatment for 1 d on medium containing, or lacking, 60 mM NH_4^+, the roots of seedlings were immediately equilibrated in measuring solution for 20–30 min. The equilibrated seedlings were then transferred to the measuring chamber, a small plastic dish (3-cm diameter) containing 2 to 3 mL of fresh measuring solution. When the root became immobilized at the bottom of the dish, the microelectrode was vibrated in the measuring solution between two positions, 5 μm and 35 μm from the root surface, along an axis perpendicular to the root. The background was recorded by vibrating the electrode in measuring solution not containing roots. The glass microelectrodes with 2–4 μm aperture were made and silanized by Xuyue Science and Technology Co., Ltd. 100 mM NH_4Cl was added as backfilling solution, followed by 20 μM of a commercially available ionophore cocktail for measuring NH_4^+ (NH_4^+-selective liquid ion-exchange cocktail #09879, Fluka Chemicals, Buchs, Switzerland) in front of the microelectrode. Prior to the flux measurements, the ion-selective electrodes were calibrated using NH_4^+ concentrations of 0.2, 2.0 and 1.2 mM. During the entire measurement process, the shoot was not in contact with the measuring solution. The net fluxes of NH_4^+ at the meristem, elongation zone and mature zone were measured individually. Each plant was measured once. The final flux values at each zone were the means of more than five individual plants from each treatment. The measuring solution was composed of 0.2 mM $CaCl_2$, 0.1 mM KCl, 0.1 mM $NaNO_3$, 0.6 mM $(NH_4)_2SO_4$ and 0.5 g/L MES (pH 5.7 adjusted with 1 M NaOH). All

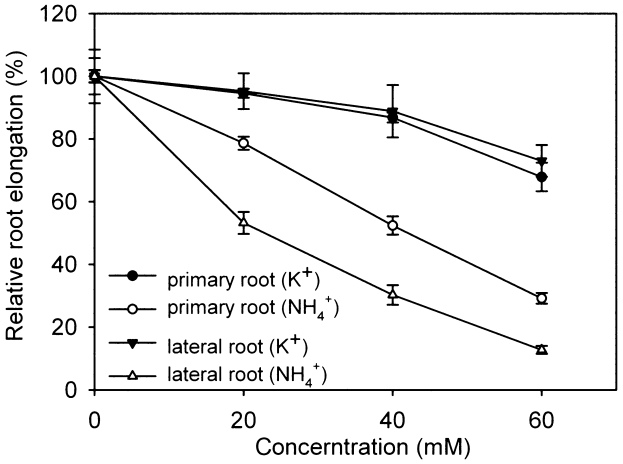

Figure 1. The inhibitory effect on the elongation of primary and lateral roots in *Arabidopsis* when roots are supplied with NH_4^+ ($(NH_4)_2SO_4$) or K^+ (K_2SO_4). 100% corresponds to a primary root length of 5.7 ± 0.21 cm and a lateral root length (total lengths per plant) of 12.0 ± 0.80 cm in the root-supplied NH_4^+ treatment, and a primary root length of 5.57 ± 0.13 cm and a lateral root length of 12.78 ± 0.74 cm in root-supplied K^+ treatment. The values represent the mean ± SE of more than 12 individual plants.

measurements of net NH_4^+ fluxes were carried out at Xuyue Science and Technology Co., Ltd (Beijing, China).

Statistical and graphical analyses

For all experiments, the data were statistically analysed using SPSS version 13.0 (SPSS, Chicago, IL). One-way analysis of variance with a Duncan post hoc test was used for testing differences in primary root length, lateral root length and net NH_4^+ fluxes after NH_4^+ treatment. For analysis of NH_4^+ responses in mutants, we used univariate analysis with a Tukey B post hoc test. Other analyses involved a *t*-test. Graphs were produced using Sigma Plot 10.0. All graphs and images were arranged using Adobe Photoshop 7.0.

RESULTS

Root-tip contact with NH_4^+ is crucial for inhibition of root growth under root-supplied NH_4^+

In the agar medium used in our study, root-supplied NH_4^+ from 20 to 60 mM significantly inhibited ($P < 0.05$) the elongation of both primary and lateral roots (Fig. 1), but not the formation of lateral roots (Fig. S1a). By contrast, concentrations of 40 to 60 mM suppressed shoot growth only slightly, while 20 mM did not at all (Fig. S1b). Figure 1 shows that, with an equivalently high concentration of K_2SO_4, the reduction in the length of both primary and lateral roots was significantly ($P < 0.05$) less than with $(NH_4)_2SO_4$, and was maximally 20% at 60 mM.

To understand the inhibitory nature of root-supplied NH_4^+ on root elongation, we tested the growth response of primary roots to a localized supply of NH_4^+ (Fig. 2a; lateral roots were affected to a similar extent – data not shown), and found that the inhibitory effect was identical whether NH_4^+ was applied to the whole root, or only the tip (Fig. 2b). In a further experiment, little growth inhibition occurred when the root body was in contact with NH_4^+ but the root tip did not touch the NH_4^+-containing medium, even when NH_4^+ concentration was raised to 120 mM (Fig. 2b). In addition, methionine sulfoximine (MSO), a compound known to block NH_4^+ assimilation by inhibiting the activity of glutamine synthetase (GS), was added to eliminate the effect of downstream metabolic products of NH_4^+ assimilation (Kronzucker, Siddiqi & Glass 1995). However, MSO application slightly augmented, and did not rescue, primary root growth repression (Fig. S2), supporting the idea that NH_4^+ itself triggers the inhibition of primary roots, independent of processes involved in NH_4^+ metabolism, although inhibitory effects of MSO itself cannot be discounted.

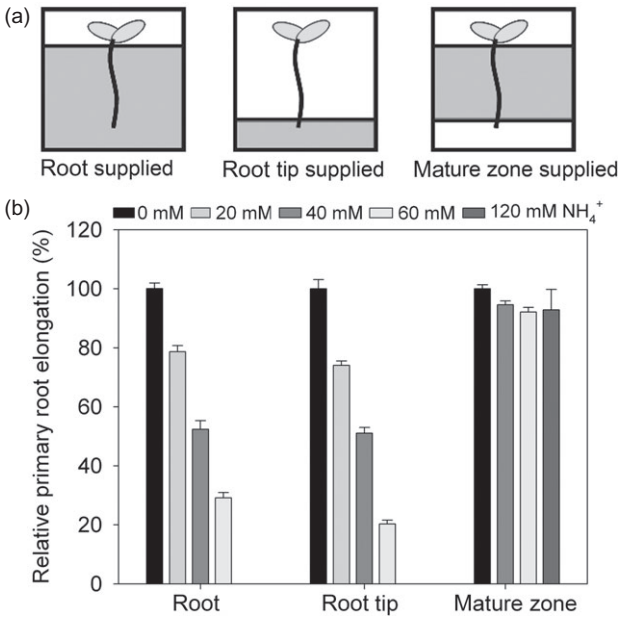

Figure 2. The inhibitory effect on primary root growth by exposure of different parts of the root to NH_4^+. (a) Schematic diagram of experimental setup for applying whole-root and localized NH_4^+ treatments, including the root tip, to *Arabidopsis* roots. The white sections indicate the basal growth medium and the gray areas indicate the NH_4^+ enriched medium. (b) Effect on the primary root elongation of applying serial concentrations of NH_4^+ to different parts of the primary root. 100% corresponds to 5.8 ± 0.21 cm in root-supplied NH_4^+ treatment, 5.6 ± 0.14 cm in root tips supplied with NH_4^+ treatment, and 6.3 ± 0.16 cm in mature zones supplied with NH_4^+ treatment. The values represent the mean ± SE of more than 12 individual plants, except for mature zones supplied with 120 mM NH_4^+ treatment (five seedlings).

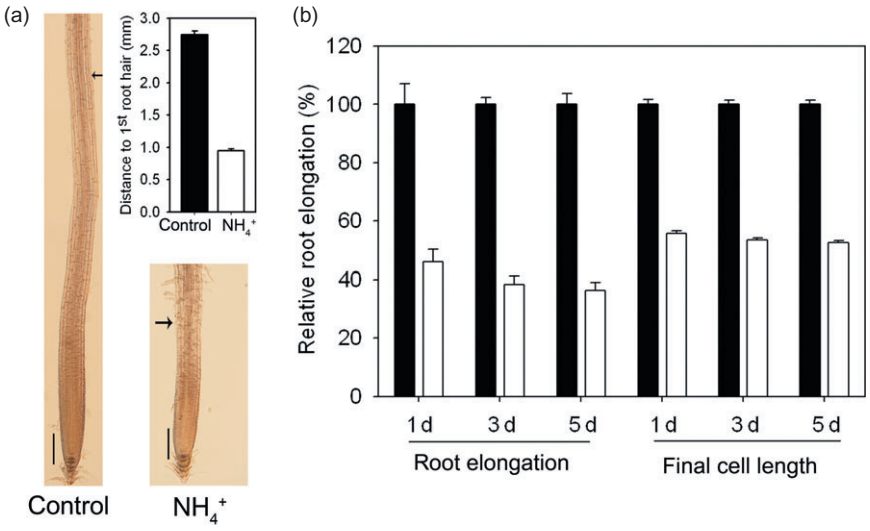

Figure 3. Effect of root-supplied NH_4^+ on final cell length in *Col-0*. (a) Distance from the root apex to the first root hair of the primary root. Photographs show representative seedlings treated for 3 d. Black arrows indicate the position of the first root hair. The quantification of samples is shown in the inset ($P < 0.05$, *t*-test). (b) Comparison of the temporal effect of root-supplied NH_4^+ on root elongation and the final cell length in primary roots with the root-supplied NH_4^+ treatment for 1, 3 or 5 d ($P < 0.05$, *t*-test). A hundred percent corresponds to primary root elongation 0.8 ± 0.06 cm, 1.3 ± 0.05 cm and 1.1 ± 0.12 cm, and the final cell length of 185.3 ± 3.01 μm, 194.0 ± 2.86 μm, and 231.8 ± 3.54 μm, as quantified at 1, 3, and 5 d, respectively. Closed bars, control; open bars, root-supplied NH_4^+ (60 mM). Values represent the mean ± SE of more than seven individual plants, or more than 150 cells from no fewer than six independent plants. Scale bars = 50 μm.

Root-supplied NH_4^+ affects cell elongation and cell division in primary roots

In high-magnification images of NH_4^+-inhibited seedlings, the distance from the root apex to the emergence of the first root hair was reduced to 34.5% of that in controls (Fig. 3a). In particular, the elongation zone was markedly shorter in NH_4^+-inhibited seedlings (Fig. 3a), indicating either a reduced capacity for cell expansion in the elongation zone and/or reduced cell division in the meristem. To address these two possibilities, we firstly examined the length of differentiated cells just above the elongation zone, representing root cell expansion (Aida *et al*. 2004). The average length of differentiated cells in seedlings subjected to NH_4^+ treatment was 55.7% of that in control roots at 1 d after transfer, and 53.0% at 3 and 5 DAT (days after transfer; Fig. 3b). The response in the final cell length was rapid and followed the arrest pattern of the primary root (Fig. 3b). It can be assumed that the length of the primary root, which, due to the short length of the immature zone (about 2-3 mm), is mainly governed by the mature zone, is roughly equal to the average mature cell length multiplied by the number of mature cells. On the first day after treatment (DAT), individual cell length was inhibited by 44.3%, compared with the 54% inhibition of total root growth, i.e. individual cell length in the elongation zone accounted for some 82% of the total root growth inhibition. These results demonstrate that the NH_4^+-mediated inhibition of primary root growth is mostly due to a repression of cell elongation.

To test whether the number of cells in the primary root could also be affected, we measured the capacity for cell division, including meristem length and meristematic activity, in the primary root apex. Meristem length of inhibited seedlings was reduced by 19.2% ($P < 0.05$) on the first day, reaching 40.5% inhibition at 5 DAT (Fig. 4a). We further used expression of the *CycB1::GUS* reporter to monitor cell cycle activity, specifically the G_2-to-M transition during the cell cycle (Doerner *et al*. 1996), within the root meristem in response to root-supplied NH_4^+. Even though root-supplied NH_4^+ had no significant effect on the intensity of expression of *CycB1::GUS* in the root meristem compared to control (Fig. 4b), measurements of the extent of the GUS-stained region of the primary root tip indicate that the mitotically active zone displayed a reduction of 21.3% ($P < 0.05$) during the first day, and reaching 28.2% inhibition by 5 DAT (Fig. 4a). These results indicate that meristematic cell division and/or meristem cell size are reduced significantly, and more severely with longer treatment, but to a much lesser extent than cell elongation.

Root-supplied NH_4^+ alters the distal organizers of pattern and polarity of the primary root tip

To assess the effect of root-supplied NH_4^+ on the stability of the quiescent centre (QC), we examined the expression of *QC25*, an established QC marker (Sabatini *et al*. 2003). The expression of *QC25::GUS* was not ectopic in the early response, but almost disappeared by 5 DAT (Fig. 5a). Secondly, Lugol staining was used to monitor the deposition of starch granules in differentiated columella cells. Root-supplied NH_4^+ did not affect starch granule formation or differentiation (Fig. 5b). Furthermore, to visualize

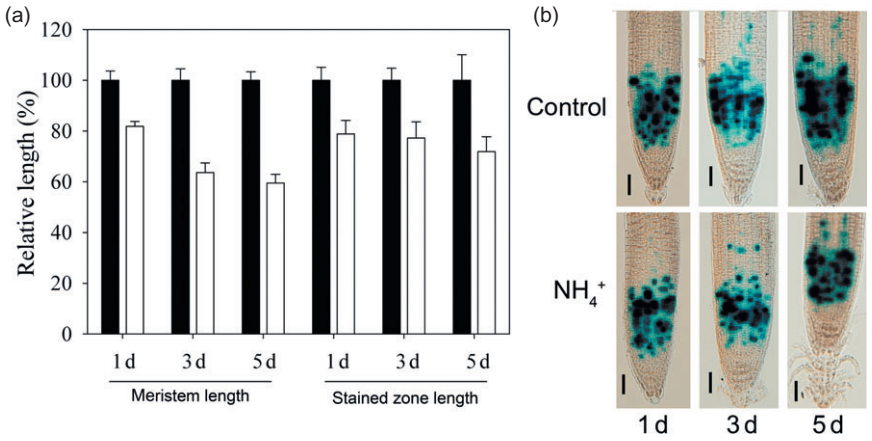

Figure 4. Effect of root-supplied NH_4^+ on the capacity for cell division in the primary root meristem. (a) Length of the meristem and the stained zone in *CycB1::GUS* ($P < 0.05$, *t*-test). 100% corresponds to meristem length of 305.9 ± 11.31 μm, 333.0 ± 15.02 μm and 321.1 ± 10.71 μm, and lengths of the GUS-stained zone of 203.6 ± 11.74 μm, 185.8 ± 8.88 μm and 189.0 ± 19.10 μm, as quantified at 1, 3 and 5 d, respectively. Closed bars, control; open bars, root-supplied NH_4^+ (60 mM). The values represent the mean ± SE of more than seven individual plants. (b) Expression of the *CycB1::GUS* reporter in the meristem of the primary root. One representative sample for each experiment is shown. Scale bars = 50 μm.

responses to active auxins at cellular resolution (Sabatini, Beis, Wolkenfelt *et al*. 1999), we used the expression of *DR5::GUS* and short-term staining to visualize the auxin response maximum at the root tip. Compared to control, expression in columella initial cells with NH_4^+ treatment still displayed an auxin maximum, although there was lower expression in the root cap, especially at 3 DAT (Fig. 5c). Shrinkage of the root cap was observed in three experiments with root-supplied NH_4^+ for prolonged periods. These results indicate that a change in the distal patterning at the primary root apex might result from relatively long-term root applications of NH_4^+.

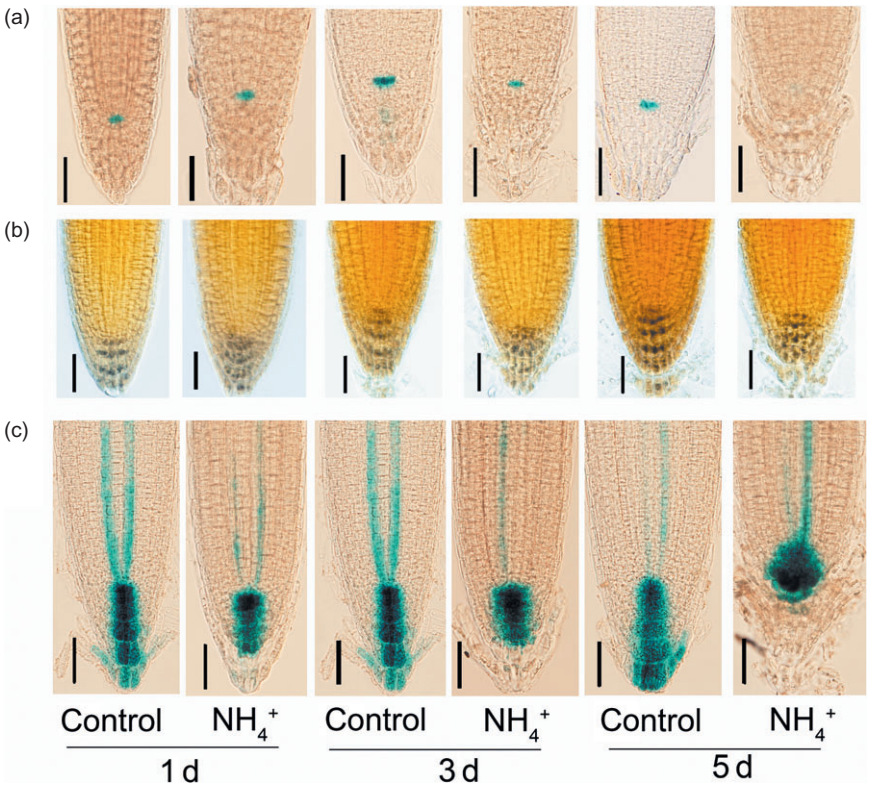

Figure 5. Effect of root-supplied NH_4^+ on the distal organizer pattern and polarity of primary root tips in *Arabidopsis*. (a) Effect of root-supplied NH_4^+ on the expression of *QC25::GUS*, after staining for 16 h (b) Lugol staining (indicating columella differentiation), and (c) Expression of the auxin reporter *DR5::GUS*, after staining for 4 h (indicating the auxin gradient maximum and distribution) in the primary root tip of *Arabidopsis* seedlings. One representative sample for each experiment is shown. Scale bars = 50 μm.

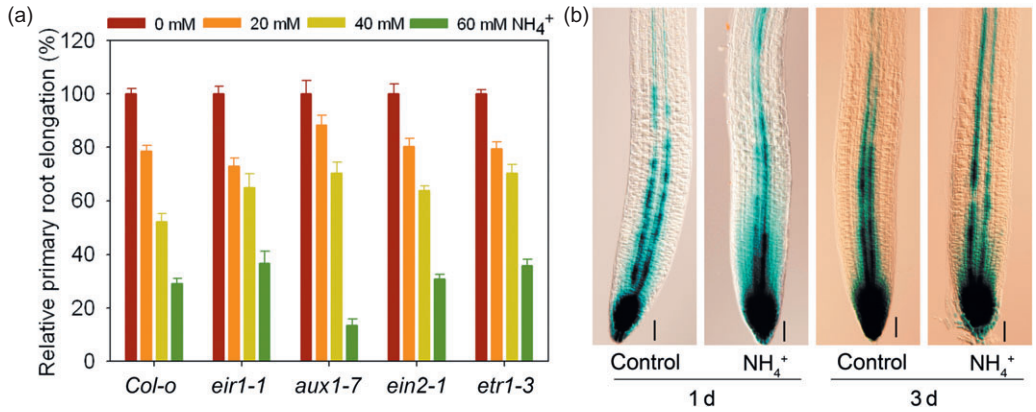

Figure 6. Dependence of root growth inhibition by root-supplied NH_4^+ on auxin and ethylene signals. (a) Primary root elongation in auxin transporter mutants (*eir1-1*, *aux1-7*) and ethylene-resistant mutants (*ein2-1*, *etr1-3*) under root-supplied NH_4^+, in contrast to the wild type (*Col-0*) ($P = 0.21$, univariate). 100% corresponds to primary root elongation of 5.8 ± 0.21 cm in *Col-0*, 4.2 ± 0.12 cm in *eir1-1*, 4.1 ± 0.20 cm in *aux1-7*, 6.0 ± 0.23 cm in *ein2-1* and 4.75 ± 0.08 cm in *etr1-3*. Closed bars, control; open bars, root-supplied NH_4^+ (60 mM). The values represent the mean ± SE of more than 12 individual plants. (b) Effect of root-supplied NH_4^+ on the expression of *DR5::GUS* in the primary root apex, stained for 16 h. One representative sample for each experiment is shown. Scale bars = 50 μm.

The primary root growth of auxin-transport mutants and an ethylene-insensitive mutant displays no resistance to inhibition by root-supplied NH_4^+

An increase in auxin transport in roots has been speculated to occur with high NH_4^+ nutrition (Sattelmacher & Thoms 1995; Gerendas *et al.* 1997). If the local accumulation of auxin at the elongation zone participates in the NH_4^+-mediated inhibition of root length, the root growth of the *eir1-1* and *aux1-7* mutants, defective in basipetal auxin transport in roots (Ruzicka *et al.* 2007; Stepanova *et al.* 2007), should be resistant to high NH_4^+. However, the primary root growth in both of these mutants was not NH_4^+-resistant compared to the wild type; moreover, the primary root elongation of *eir1-1* was more sensitive to NH_4^+ than that of *Col-0* at 20 mM NH_4^+, and, with root-supplied NH_4^+ at 60 mM, the primary root growth of *aux1-7* was also more sensitive (Fig. 6a). These results suggest that root-supplied NH_4^+ triggers inhibition of root elongation independent of the auxin-mediated repression of cell expansion in the root elongation zone.

We also used the well-established spatial expression auxin-response reporter *DR5::GUS* (Ulmasov *et al.* 1997) to examine root-supplied NH_4^+ for better monitoring of local auxin distribution in roots. In control roots (without NH_4^+), the expression of *DR5::GUS*, after 16 h of staining, was restricted to cells of the columella, QC, and provascular tissue (Fig. 6b). As expected, the expression pattern of *DR5::GUS* was not distinctly altered, nor was *DR5::GUS* staining in the elongation zone markedly enhanced in roots subjected to NH_4^+ treatment for 1 or 3 d (Fig. 6b). This experiment further strengthened the results of the two auxin-transport mutants treated with root-supplied NH_4^+.

We further investigated the root growth of the ethylene-insensitive mutants *ein2-1* and *etr1-3* (Ruzicka *et al.* 2007) in response to root-supplied NH_4^+ to test whether NH_4^+ inhibition of root elongation is also independent of other ethylene pathways. The relative ratio of primary root elongation in *ein2-1* and *etr1-3* at different NH_4^+ concentrations indicated a similar sensitivity to *Col-0* (Fig. 6a). Therefore, these results do not support the participation of ethylene in this NH_4^+-induced inhibition of root elongation.

Exposure of root tips to NH_4^+ impacts the NH_4^+ flux at the elongation zone of primary roots

Excessive fluxes of NH_4^+ at the root plasma membrane have been previously linked to the development of the NH_4^+ toxicity syndrome (Britto *et al.* 2001). As described above, NH_4^+-inhibited root growth requires the root tip to come into contact with external NH_4^+. It was therefore important to ask whether NH_4^+ fluxes at the root apex, or in the elongation zone, may be changed by direct NH_4^+ contact. We investigated the temporal net NH_4^+ fluxes in these zones in roots of 6-day-old seedlings (including treatment with NH_4^+ for 1 d), using high-resolution SIET. In seedlings in which the whole root was pre-treated with NH_4^+, the pattern of NH_4^+ fluxes in roots was different than in control seedlings not pre-treated with NH_4^+ (i.e. seedlings in which fluxes were measured upon first exposure to NH_4^+). NH_4^+ efflux at the surface of the meristem was stimulated to 203 pmol cm^{-2} s^{-1} after whole-root treatment with NH_4^+ (Fig. 7a, c), whereas net NH_4^+ influx in the elongation zone switched to sustained NH_4^+ efflux, assuming a value of 183 pmol cm^{-2} s^{-1} (Fig. 7b, c). When NH_4^+ was not supplied to the root tip, NH_4^+ efflux at the meristem increased to 156 pmol cm^{-2} s^{-1}, similar to whole roots treated with NH_4^+. However, there remained an NH_4^+ influx of 8.0 pmol cm^{-2} s^{-1} in the elongation zone, similar to the

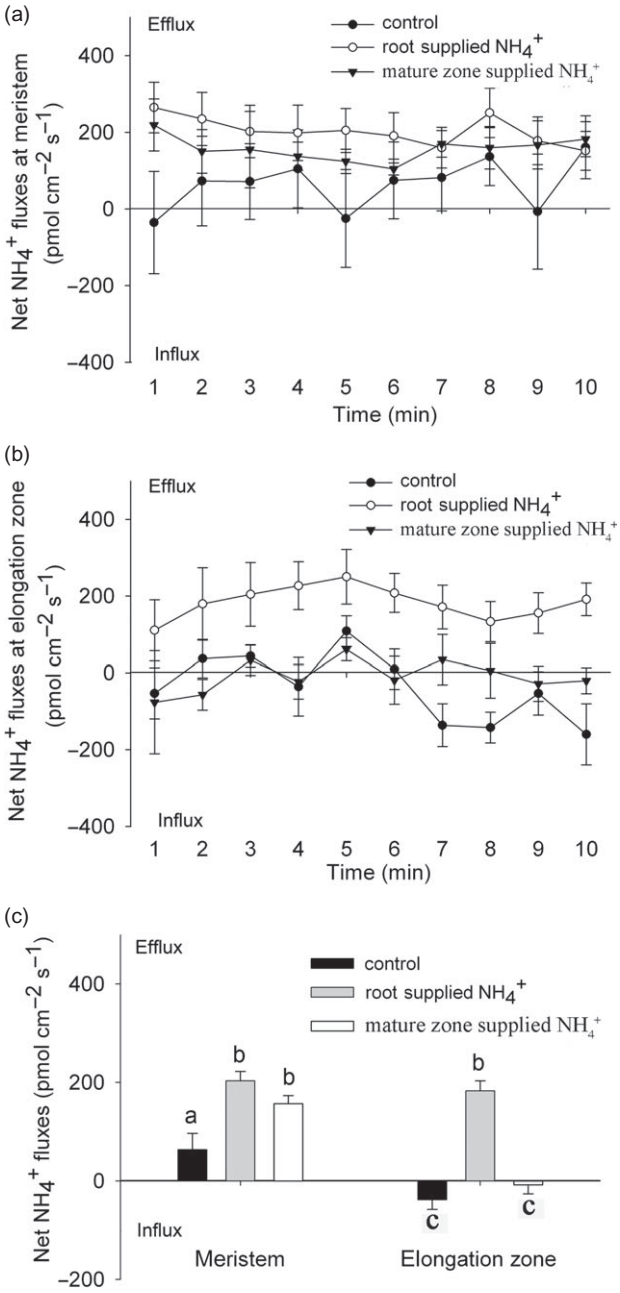

Figure 7. Influence of root supply of NH_4^+ on net plasma membrane NH_4^+ fluxes at (a) the meristem, and (b) the elongation zone of primary root tips in *Col-0*. (c) Mean values of NH_4^+ fluxes from (a) and (b). Each point represents the mean ± SE of more than five individual plants. Significant differences between treatments are indicated with different letters ($P < 0.05$, one-way ANOVA).

control, and in stark contrast to treatment of the whole root NH_4^+ (Fig. 7). In the mature zone of primary roots, an NH_4^+ influx (73.6 pmol cm^{-2} s^{-1}) was measured following NH_4^+ treatment, compared to 39.0 pmol cm^{-2} s^{-1} in controls (Fig. S3). These results show that NH_4^+-induced efflux in the root elongation zone coincides with its inhibitory effect on cell length and primary root elongation.

NH_4^+ efflux at the elongation zone is enhanced in primary roots of the mutant *vtc1-1*

Recently, it was reported that a mutation in the *AtVTC1* gene, which encodes a GDP-mannose pyrophosphorylase (GMPase) critical to cell wall formation (Lukowitz *et al.* 2001), confers hypersensitivity to NH_4^+, especially in primary root growth (Qin *et al.* 2008). To determine whether GMPase affects NH_4^+ flux in the root apex, we monitored the growth of the primary root and the accompanying NH_4^+ flux at the meristem and elongation zone as affected by root-supplied NH_4^+ in the *vtc1-1* mutant (Barth *et al.* 2010). Under our experimental conditions, growth of the primary root of *vtc1-1* was indeed more sensitive to NH_4^+ than that of *Col-0* (Fig. 8a). In addition, as in the wild type, the root tip was necessary and sufficient to sense NH_4^+ and bring about the inhibition of primary root growth in the *vtc1-1* mutant (Fig. 8b). NH_4^+ fluxes in the elongation zone, although not the meristem, differed between wild-type *Col-0* and *vtc1-1* (compare Figs 7 and 8d–f). While under (NH_4^+-free) control conditions, the average meristem zone flux values were similar in the mutant and wild type ($P = 0.78$, *t*-test), the mean NH_4^+ efflux of 107.6 pmol cm^{-2} s^{-1} in the elongation zone of *vtc1-1* contrasted strongly with the NH_4^+ influx in this zone in *Col-0*. In the case of root-supplied NH_4^+, the increase in the NH_4^+ efflux (75%) in the meristematic zone in *vtc1-1* was slightly higher than in *Col-0* (69%), and the net NH_4^+ efflux in the elongation zone was 335 pmol cm^{-2} s^{-1}, or 1.8-fold that in the wild type. To test whether this higher absolute value of net NH_4^+ efflux gives rise to the shorter meristem length and final root cell size, we measured the final cell length and meristem length of the primary root in *vtc1-1* plants. Roots of *vtc1-1* supplied with 60 mM NH_4^+ for 1 d showed a dramatic drop of 56.8% in final cell length, compared with a decrease of 44.3% in *Col-0*, whereas meristem length was only reduced by 20.6% in *vtc1-1*, similar (19.2%) to the wild type (Fig. 8c). These data confirm an association of NH_4^+ efflux at the elongation zone with the NH_4^+-mediated inhibition of primary root elongation, and indicate that GMPase directly or indirectly regulates the process of NH_4^+ efflux in roots.

DISCUSSION

Excess NH_4^+ arrests primary root elongation and is mediated through the root tip

In this work, we show that NH_4^+ supplied to roots in *Arabidopsis* can regulate cell division, elongation and the distal organizer pattern in the root tip. There is no obvious systemic inhibition of root growth by NH_4^+. Instead, NH_4^+-induced root growth inhibition requires the root tip to be in direct contact with external NH_4^+. Where the root tip is in direct contact with NH_4^+, cell elongation is immediately and dramatically suppressed, with cell division being reduced to a much lesser extent, whereas the effect on the distal organizer pattern in the root tip takes longer to develop. From these observations, we conclude that root-supplied NH_4^+

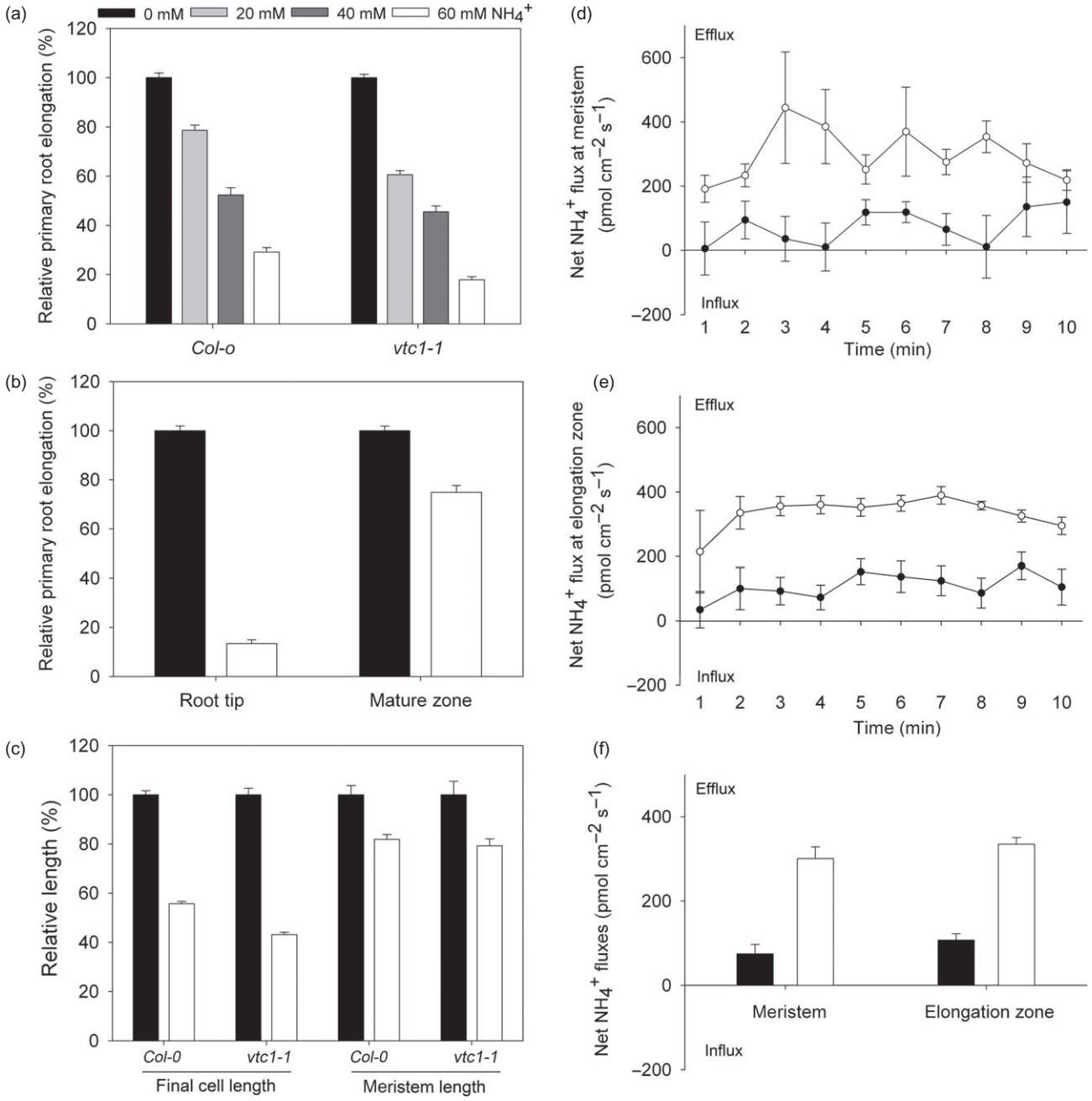

Figure 8. Primary root elongation, final cell length, meristem length and net NH_4^+ fluxes in the NH_4^+-hypersensitive mutant *vtc1-1* as affected by root-supplied NH_4^+. (a) Primary root elongation ($P < 0.05$, univariate). 100% corresponds to primary root elongation of 5.8 ± 0.21 cm for *Col-0* and 4.3 ± 0.12 cm for *vtc1-1*. More than 12 independent seedlings were analysed. (b) Effect of root-supplied NH_4^+ on primary root growth in *vtc1-1*. A hundred percent corresponds to primary root elongation of 4.48 ± 0.09 cm in root tips supplied with NH_4^+ treatment and 5.0 ± 0.09 cm in mature zones supplied with NH_4^+ treatment. More than 12 independent seedlings were analysed. (c) Final cell length in *vtc1-1* compared with *Col-0* after treatment for 1 d ($P < 0.05$ for the final cell length, $P = 0.45$ for the meristem length). A hundred percent corresponds to the final cell length of 185.3 ± 3.01 μm in *Col-0* and 184.9 ± 4.89 μm in *vtc1-1*, and length of meristem of 305.9 ± 11.31 μm in *Col-0* and 278.6 ± 15.28 μm in *vtc1-1*. More than seven independent seedlings and 150 cells were analysed. Net NH_4^+ fluxes of *vtc1-1* (d) at the meristem and (e) at the elongation zone, Mean values of fluxes in (d) and (e) are shown in (f). More than five independent seedlings were analysed for fluxes experiments. Closed symbols, control; open symbols, treatment with NH_4^+ (60 mM). The values represent the mean ± SE.

arrests root growth primarily *via* decreasing cell elongation and mainly results from the direct effects of external NH_4^+ upon the root tip. Surprisingly, our mutant analyses indicate that the inhibitory effect of root-supplied NH_4^+ is independent of auxin and ethylene signalling, which have been previously implicated as important cues for NH_4^+ toxicity in plants (Cao *et al*. 1993; Britto & Kronzucker 2002; Barth *et al*. 2010). Neither the auxin transport mutants *eir1-1* and *aux1-7* nor the ethylene-resistant lines *ein2-1* and *etr1-3* showed resistance to the root growth inhibition brought about by NH_4^+. Furthermore, our results clearly demonstrate that inhibition of cell elongation is associated with NH_4^+ efflux in the root elongation zone. Excessive NH_4^+ efflux from cells has previously been shown to constitute an important mechanism of NH_4^+ toxicity in plants (Britto *et al*. 2001; Kronzucker *et al*. 2001, 2003; Szczerba *et al*. 2008). Moreover, we demonstrate that GMPase alleviates the inhibition of cell elongation and the increased NH_4^+ efflux in the elongation zone induced by root-supplied NH_4^+. GMPase has been argued to determine the sensitivity to NH_4^+ by affecting the N-glycosylation of proteins in *Arabidopsis* (Qin *et al*. 2008; Barth *et al*. 2010).

It is instructive to compare the suppression of root growth by NH_4^+ demonstrated here with the effects reported for nitrate and glutamate. High external concentrations of all three nitrogen compounds appear to inhibit root growth, but each *via* a different mechanism. Firstly, high nitrate mainly suppresses lateral, but not primary, root growth in *Arabidopsis* (Zhang *et al*. 1999). High L-glutamate has a strong inhibitory effect on primary root growth, followed by a later effect on lateral root elongation in *Arabidopsis* (Walch-Liu *et al*. 2006). Our results demonstrate that high NH_4^+ synchronously affects the length of the primary and the lateral root. Secondly, although the growth-promoting effect of nitrate is sensed in the root tip (Zhang & Forde 1998; Forde & Walch-Liu 2009), the inhibitory effect exerted on root growth by high nitrate is more a consequence of systemic changes in nitrogen metabolism (Zhang *et al*. 1999). Plants perceive the inhibitory effect of external NH_4^+ and L-glutamate *via* the root tip, but lateral roots acquire glutamate sensitivity later in their development (Walch-Liu *et al*. 2006). Thirdly, high nitrate suppresses lateral root growth *via* an ABA-mediated pathway, as demonstrated by an analysis of *abi4* and *aba3* mutants (Signora *et al*. 2001), and L-glutamate may interact with auxin transport, as seen in the partial resistance of root growth in the *aux1-7* mutant to L-glutamate (Walch-Liu *et al*. 2006). Our results suggest that the NH_4^+-induced inhibition of the primary root is independent of auxin transport, and that the growth of lateral roots displays no resistance to root-supplied NH_4^+ in *abi4* or *aba3* (data not shown). Hence, different nitrogen compounds have distinct effects on root growth *via* a suite of distinct regulatory pathways.

It is also important to note that there are interrelationships during root system development among nitrogen compounds. Nitrate is known to act as a signal antagonizing the inhibitory effect of glutamate on the primary root tip (Walch-Liu & Forde 2008; Forde & Walch-Liu 2009). Likewise, toxicity is observed in many species when NH_4^+ is provided alone, and can be alleviated by co-provision of nitrate (Kronzucker *et al*. 1999; Roosta & Schjoerring 2008). This effect may also suggest a role for nitrate as a signal rather than merely a nutrient (Stitt & Krapp 1999; Tischner 2000; Wang *et al*. 2004). Therefore, great care should be taken in selecting the nitrogen source in growth media. In our study, moderate nitrate is included as an additional nitrogen source in the growth medium (Li & Shi 2007). This method is established for elucidating the mechanisms of NH_4^+ toxicity in *Arabidopsis* (Qin *et al*. 2008; see also Rawat *et al*. 1999).

In addition to the effects of NH_4^+ and L-glutamate, inhibitory effects by other environmental stresses, such as low phosphate (Svistoonoff *et al*. 2007) or aluminium (Ryan *et al*. 1993; Jones & Kochian 1995) on root elongation, are also sensed by the root tip. Similarly, positive environmental signals, such as gravity, water (Hawes *et al*. 2003; Eapen *et al*. 2004) and nitrate (Walch-Liu & Forde 2008), are perceived by the root tip. In some cases, receptors for environmental signals have been reported; examples of this include NRT1.1 and ANR1 in nitrate signalling, with both being strongly expressed in the root tip (Guo *et al*. 2001; Remans *et al*. 2006; Ho *et al*. 2009). LPR1, which senses low Pi, is expressed especially strongly in the root cap (Svistoonoff *et al*. 2007). Therefore, the root tip, especially the root cap, may be a vital area for sensing environmental signals. Thus, identifying the key gene(s) that sense(s) NH_4^+ stress will be a crucial direction for future research.

Enhanced NH_4^+ efflux at the elongation zone is coupled with inhibition of primary root growth

It is widely accepted that cells export toxic or excess ions to avoid toxicity or maintain ion balance, but this process can carry a substantial energy burden and, thus, have significant implications for plant performance, particularly under conditions in which growth is energy-limited; this is particularly well-documented in the cases of the ions Na^+ and NH_4^+ (Britto *et al*. 2001; Britto & Kronzucker 2006, 2009). Both ions have toxic effects on photosynthesis and the supply of energy to roots, and energetic consequences of futile cycling may thus have a great impact on plant survival; by contrast, high K^+ supply, which does not compromise photosynthesis or the supply of energy to the roots, even though its transport can be energetically costly, does not appear to be detrimental to growth to any similar extent (Britto & Kronzucker 2006; Szczerba, Britto & Kronzucker 2006). Here, we demonstrate that high NH_4^+ fluxes (in particular efflux) in the elongation zone of the primary root are strongly negatively correlated with cell elongation and primary root growth under conditions of root-supplied NH_4^+.

Our results show increasing NH_4^+ efflux in the elongation zone and meristem, contrasted by NH_4^+ influx in the mature zone when roots were supplied with NH_4^+. These results suggest that growing cells, such as in the elongation zone and meristem, are the most sensitive to NH_4^+, whereas cells of the mature zone may be more tolerant of NH_4^+.

Consequently, growing cells may rapidly send signals to stimulate NH_4^+ efflux when high NH_4^+ is sensed, in contrast to mature cells in the basal part. The different characteristics of growing cells and mature cells may be important. Cells that are not completely differentiated, especially in the meristem, are characterized by a small vacuole, low volume, low water content and low fresh weight, but a high level of protein (Luxova 1988). Thus, this part of the root might be more sensitive to NH_4^+ and export it even at low concentrations. By contrast, because mature cells have large vacuoles that can occupy more than 90% of the differentiated plant cell and possess a great capacity for NH_4^+ storage, the bulk of the NH_4^+ taken up may be located in the vacuole (Loque & von Wiren 2004). In analogy to Na^+ (Apse & Blumwald 2007), the vacuolar sequestration of the toxicant ion may be a method to avoid NH_4^+ toxicity in plants (Wang *et al*. 1993; Miller *et al*. 2001; Loque & von Wiren 2004).

GMPase provides a defence against excess NH_4^+ efflux in primary roots

The strong relationship of elevated NH_4^+ efflux and cell arrest of the primary root apex in the conditional NH_4^+-sensitive *vtc1-1* mutant further supports the idea that NH_4^+ efflux at the elongation zone is mechanistically linked to the inhibition of cell elongation and primary root growth. However, elevated NH_4^+ efflux in *vtc1-1* plants occurred not only at the surface of the elongation and meristematic zones, at a significantly higher level than in the wild type, but also in the mature zone of the primary root (NH_4^+ influx was seen in the mature zone of the wild type) with root-supplied NH_4^+. The more systemic trends in root NH_4^+ fluxes in *vtc1-1* plants suggest that enhanced NH_4^+ efflux in cells can be prevented by properly functioning GMPase in the wild type. Interestingly, enhanced NH_4^+ efflux is not necessarily tied to enhanced NH_4^+ accumulation, as tissue NH_4^+ content in the GMPase mutants *hsn1-1* and *vtc1-1* is not significantly different from that in the wild type (Qin *et al*. 2008; Barth *et al*. 2010). In addition to the greatly enhanced NH_4^+ efflux at the surface of all primary roots in *vtc1-1* following NH_4^+ treatment, the elongation zone displayed NH_4^+ efflux even in seedlings grown in medium containing nitrate as the sole nitrogen source, which supports the suggestion that cells are more sensitive to NH_4^+ in *vtc1-1* plants in general. It has been previously shown that the sensitivity of cells to excess NH_4^+ in mutants with a partial loss of GMPase function (Qin *et al*. 2008; Barth *et al*. 2010), and reduced cellulose biosynthesis, cell wall stability and cell viability seen in the null mutant of GMPase (*cty1-2*), are mainly the result of a deficiency in N-glycosylation (Lukowitz *et al*. 2001). Therefore, the NH_4^+ flux in roots may be regulated by the N-glycosylation of proteins. In addition to the GMPase gene, mutants with defects in the *STT3a* or *CGL1* genes, which play an important role in N-glycosylation, also show growth inhibition, especially in the growing root tip under salt stress (Koiwa *et al*. 2003; Kang *et al*. 2008). Furthermore, a deficiency in N-glycosylation resulting in insufficient cellulose biosynthesis and cell wall formation due to aberrant modification of the plasma membrane glycoprotein KORRIGAN 1/RADIALLY SWOLLEN 2 (KOR1/RSW2) has been shown to lead to a dramatic inhibition of root growth in *stt3a* and *cgl1* plants under salt stress (Kang *et al*. 2008). Taken together, our results support the suggestion that the processes of correct N-glycosylation of proteins plays an important role in guarding against the detrimental effects of ionic stresses such as those associated with Na^+ and NH_4^+. Unlike in previous studies (Qin *et al*. 2008; Barth *et al*. 2010), however, our present study shows that, rather than being in conflict with one another, the N-glycosylation hypothesis of NH_4^+ toxicity and that of futile NH_4^+ cycling (Britto *et al*. 2001; Kronzucker *et al*. 2003) are, in fact, both correct, and may well be causally related.

The inhibitory effects of root-supplied NH_4^+ on root growth are unlikely to be mediated by auxin and ethylene pathways

A previous report showed that the auxin-resistant mutant *aux1-7* could reverse the NH_4^+-induced inhibition of *Arabidopsis* primary root growth in low-K^+ medium during germination (Cao *et al*. 1993). Other researchers have also proposed that the auxin signal is involved in NH_4^+ toxicity leading to growth retardation. In a recent study on the conditional NH_4^+-hypersensitive mutant *vtc1-1* (Barth *et al*. 2010), lower free auxin levels than wild type were reported in the presence (but not in the absence) of NH_4^+, also suggesting a potential role of auxin signalling in the growth inhibition on NH_4^+ media. In our study, root-supplied NH_4^+ only slightly disturbed the auxin maximum at the root cap, but did not change the pattern of auxin responses in the elongation zone. Furthermore, primary root elongation in the *aux1-7* and *eir1-1* mutants displayed the same sensitivity to root-supplied NH_4^+ as the wild type. There are two explanations for this. Firstly, Cao *et al*. found that the NH_4^+-induced inhibition of primary root elongation at the germination stage occurred in medium containing low K^+, so the process may be also influenced by low K^+. Secondly, more importantly, NH_4^+ can not only enter the plant through the roots but also directly *via* shoots in whole-medium culture conditions. Therefore, the inhibition of primary root growth may have been a synthetic effect of NH_4^+ induced by both root-supplied and shoot-supplied NH_4^+ in the study by Cao *et al*. In fact, we found that the growth of the primary root in *aux1-7* plants is partially resistant to shoot-supplied NH_4^+ as compared to wild type (data not shown). Hence, the mechanisms of inhibition induced by root-supplied and shoot-supplied NH_4^+ may be independent. A slightly altered tissue content of free auxin has been documented in *vtc1-1* mutants under NH_4^+ exposure in the study by Barth *et al*. (2010), but, as the authors point out, more general pleiotropic effects of the mutation with respect to hormone homeostasis have been observed (Pastori *et al*. 2003; Foyer, Kiddle & Verrier 2007). Further to the above, in our study, the growth of the primary root in ethylene-insensitive mutants was also similar

to that in the wild type treated with NH_4^+, suggesting that root-supplied NH_4^+ inhibits primary root elongation independent of ethylene pathways, although this does not exclude the possibility that NH_4^+ affects ethylene synthesis in treated seedlings, especially in shoots, because this was not examined in this study.

In conclusion, our results show that NH_4^+ contact with the root tip is both necessary and sufficient to the sensing of NH_4^+ stress in the root system of *Arabidopsis*, and to the ensuing suppression of primary root growth, and that the effect is predominantly upon cell elongation not division. Furthermore, we show that this inhibition is independent of auxin- and ethylene-mediated pathways, and is coupled with NH_4^+ efflux in the elongation zone, which in turn appears to be regulated by GMPase. Our findings not only demonstrate the processes of the arrest of root elongation by root-supplied NH_4^+, but also connect the latter two important mechanisms of NH_4^+ toxicity, that of futile NH_4^+ cycling at the plasma membrane of root cells (Britto *et al*. 2001; Kronzucker *et al*. 2003) and that of impaired N-glycosylation of proteins through disruptions of GMPase (Qin *et al*. 2008; Barth *et al*. 2010). We hope that the working model gained in this study may offer clues to understanding the growth response of the primary root in response to other abiotic stresses.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:
This is given as Eqn 3 in materials and methods

Figure S1. Effects on the number of lateral roots (a) and the fresh weight of shoots (b) in *Arabidopsis* when roots are supplied with $NH_4 + ((NH_4)_2SO_4)$ or $K + (K_2SO_4)$. Values represent means ± SE in (a), $n \geq 12$ individual plants. The shoot fresh weight was determined as the sum of twelve individual seedlings because of low individual seedling weights.

Figure S2. MSO supply to roots cannot alleviate the suppression of primary root growth by root-supplied NH_4 + (60 mM) in *Col-0*. Significant difference between treatments is indicated by different letters ($P < 0.05$ one-way ANOVA). 100% correspond to 2.1 ± 0.09 cm of primary root elongation. Values represent means ± SE, $n = 12$–17 individual plants.

Figure S3. The net NH_4 + fluxes at mature zone in the primary roots *Col-0* (a) and *vtc1-1* (b). The mean values of fluxes in (a) and (b) within the measuring periods are shown in (c). Significant differences between treatments are indicated by different letters ($P < 0.05$, one-way ANOVA). Closed symbols, control; open symbols, root supplied 60 mM NH_4+. Values represent mean ± SE, $n = 5$–7 individual plants.

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