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RESEARCH PAPER

Ammonium-induced loss of root gravitropism is related to auxin distribution and TRH1 function, and is uncoupled from the inhibition of root elongation in *Arabidopsis*

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Abstract

Root gravitropism is affected by many environmental stresses, including salinity, drought, and nutrient deficiency. One significant environmental stress, excess ammonium (NH_4^+), is well documented to inhibit root elongation and lateral root formation, yet little is known about its effects on the direction of root growth. We show here that inhibition of root elongation upon elevation of external NH_4^+ is accompanied by a loss in root gravitropism (agravitropism) in *Arabidopsis*. Addition of potassium (K^+) to the treatment medium partially rescued the inhibition of root elongation by high NH_4^+ but did not improve gravitropic root curvature. Expression analysis of the auxin-responsive reporter gene *DR5::GUS* revealed that NH_4^+ treatment delayed the development of gravity-induced auxin gradients across the root cap but extended their duration once initiated. Moreover, the β-glucuronidase (GUS) signal intensity in root tip cells was significantly reduced under high NH_4^+ treatment over time. The potassium carrier mutant *trh1* displayed different patterns of root gravitropism and *DR5::GUS* signal intensity in root apex cells compared with the wild type in response to NH_4^+. Together, the results demonstrate that the effects of NH_4^+ on root gravitropism are related to delayed lateral auxin redistribution and the TRH1 pathway, and are largely independent of inhibitory effects on root elongation.

Key words: Auxin, gravitropism, K^+, NH_4^+ stress, root elongation, TRH1.

Introduction

Root gravitropism is the process that dictates the growth of roots along a specific angle relative to gravity, also known as the gravitational set point angle (GSPA) (Blancaflor and Masson, 2003). Gravitropism is a major determinant in the distribution of root systems in soil and thus influences critical activities such as anchorage and uptake of water and nutrients from the soil (Forde and Lorenzo, 2001; Perrin *et al.*, 2005). Upon gravistimulation, the gravitropic response of roots has been conceptually separated into four phases: gravity perception, signal transduction, signal transmission, and curvature response (Perrin *et al.*, 2005). Gravity sensing occurs mainly in the root cap and through the sedimentation of amyloplasts within the columella cells (Chen *et al.*, 2002; Masson *et al.*, 2009). Although the receptors and molecular mechanisms that sense amyloplast motion are still unknown, there have been observations suggesting that cytosolic ions such as Ca^{2+} and the rapid changes of cytoplasmic pH within columella cells may participate in gravity signal transduction in roots (Scott and Allen, 1999; Fasano *et al.*, 2001, 2002; Hou *et al.*, 2004; Monshausen *et al.*, 2011). Gravity perception and signal transduction promote the formation of a lateral auxin gradient across the stimulated organs, leading to differential cell expansion on opposite flanks of elongation zone tissues, responsible for tip curvature (Swarup *et al.*, 2005; Masson *et al.*, 2009). However, the root cap and elongation zone, the locations of stimulus perception and growth response, respectively, are spatially separated. Therefore, the root gravitropic response requires the transmission of a gravitational signal, which occurs via a lateral auxin gradient, toward the elongation zone (Chen *et al.*, 2002; Swarup *et al.*, 2005). In *Arabidopsis* roots, auxin influx carriers of the AUX1/LAX family, the PIN family of auxin efflux facilitators, ABC transporters, and the putative potassium transporter TRH1 all participate in a complex network that mediates polar auxin transport and regulates morphogenesis and growth of roots (Perrin *et al.*, 2005).

In addition to gravity, an omnipresent environmental signal, root growth direction, is also affected by various environmental stresses, such as nutrient status, water availability, gradients in temperature, salinity, and mechanical impedance (Bonser *et al.*, 1996; Liao *et al.*, 2001; Forde and Lorenzo, 2001; Fasano *et al.*, 2002; Takahashi *et al.*, 2003; Vicente-Agullo *et al.*, 2004; Sun *et al.*, 2008; Shibasaki *et al.*, 2009). In all these cases, a complex mix of signals must be sensed by roots and integrated into an appropriate developmental response in order to overcome the signal from gravity and reorient root growth to navigate past barriers or toward favourable conditions (Fasano *et al.*, 2002). For example, the availability of phosphorus can regulate the root configuration of leguminous plants by altering the growth angle of the basal roots so as to better take up phosphorus from soil (Bonser *et al.*, 1996; Liao *et al.*, 2001). Likewise, reductions in external potassium trigger agravitropic root growth so that roots can grow away from potassium-impoverished regions, which may well represent a mechanism by which plants respond to mineral deficiencies in general (Vicente-Agullo *et al.*, 2004). In addition, moisture gradients or water stress cause immediate degradation of amyloplasts in columella cells of the root cap, so as to reduce the response of roots to gravity and allow them to exhibit hydrotropism (Takahashi *et al.*, 2003). These studies show that plants have evolved highly adaptive regulatory mechanisms in the control of root-directional growth and are capable of perceiving and responding to a variety of external stimuli so as to maintain optimal development.

Ammonium (NH_4^+), an important source of nitrogen for many species (Kronzucker *et al.*, 1997), is frequently present in soil environments in excessive quantities and leads to growth retardation (Britto and Kronzucker, 2002). This is especially so when NH_4^+ is supplied as the sole nitrogen source or in combination with a low availability of potassium (K^+) (Kronzucker *et al.*, 2003; Qin *et al.*, 2008; Balkos *et al.*, 2010; ten Hoopen *et al.*, 2010). In recent years, significant advances have been made in the study of the mechanisms of ammonium toxicity (Gerendás *et al.*, 1997; Britto and Kronzucker, 2002; Qin *et al.*, 2008; Li *et al.*, 2010; Kempinski *et al.*, 2011; Li *et al.*, 2011*a*, *b*). However, very little is known about the root gravitropic response to excess ammonium.

Root tips act as sensors for different stimuli such as gravity and moisture gradients, and can integrate multiple tropic responses essential for root navigation (Takahashi *et al.*, 2009). Laser ablation of defined columella cell layers in the cap of *Arabidopsis* primary roots inhibited root curvature (Blancaflor *et al.*, 1998). Previous research found an alteration in the distal organizer pattern of the primary root tip in *Arabidopsis* with NH_4^+ treatment, although this was not the ion's primary inhibitory effect on root elongation. Cell elongation was the major target in the suppression of primary root growth by NH_4^+ (Li *et al.*, 2010). Whether NH_4^+ may affect other processes that require the participation of root tips, such as root gravitropism, is still unknown. As explained above, a differential cellular elongation on opposite flanks of the elongation zone is responsible for gravitropic curvature. This study was intended to explore whether NH_4^+ influences root gravitropism in *Arabidopsis* and its relationship with the retardation of root growth. In addition, it was interesting to test whether exogenous K^+ can alleviate root growth inhibition and/or effects on gravitropism. Furthermore, it was determined whether influences of NH_4^+ on gravitropism arise from changes in auxin redistribution and auxin signal intensity in root tips

Materials and methods

Plant material and growth conditions

Columbia-0 ecotype *Arabidopsis thaliana* (Col-0), the *Arabidopsis* mutant *trh1* (SALK_086060), and the transgenic *Arabidopsis DR5::GUS* (β-glucuronidase; Ulmasov *et al.*, 1997) in the Col-0 background were used. *trh1* plants carrying the *DR5::GUS* construct were derived from crosses between *trh1* and *DR5::GUS*-transformed plants, and homozygous plants for both *trh1* and the *DR5::GUS* insertion were used.

After being surface sterilized and cold treated at 4 °C for 2 d, the seeds were sown on *Arabidopsis* normal growth medium. The composition of the normal growth medium was as described by Li *et al.* (2010): 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 $ZnCl_2$, 1 μM $CuSO_4$, 0.2 μM Na_2MoO_4, 1% (w/v) sucrose, 0.5 g l^{-1} MES, and 0.8% (w/v) agar (adjusted to pH 5.7 with 1 M NaOH).

The culture 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 16 h light/8 h dark cycle. Five-day-old seedlings germinated on normal growth medium, with relatively straight root tips and ~1.5 cm in length, were selected for gravity stimulation experiments.

Ammonium treatment and gravity stimulation

The ammonium treatment medium consisted of normal growth medium supplemented by varying concentrations of $(NH_4)_2SO_4$. Ion effects were analysed by using NH_4Cl with the same NH_4^+ concentration, Na_2SO_4 and K_2SO_4 with the same SO_4^{2-} concentration, and KNO_3 with the same N concentration as substitutes for $(NH_4)_2SO_4$. Exogenous K^+ experiments were performed by adding KNO_3 ($NaNO_3$) or KCl (NaCl) of appropriate concentrations to the 30 mM $(NH_4)_2SO_4$ medium. It is important to note that in agar media, unlike in soil or hydroponic culture, diffusion limitation for nutrients necessitates the application of higher than normal concentrations of nutrients, including those of the toxicant NH_4^+ (Li *et al.*, 2010; Barth *et al.*, 2010; Li *et al.*, 2011*a*). To achieve growth suppressions and tissue NH_4^+ contents (Barth *et al.*,

2010) similar to those seen in hydroponic NH_4^+ toxicity studies (e.g. 10 mM: Szczerba *et al.*, 2008; Balkos *et al.*, 2010), 20–60 mM NH_4^+ must, at minimum, be applied.

For agar plate cultivation, 5-day-old seedlings of similar size were transferred to new agar plates containing the appropriate treatment. The roots were placed vertically, with the initial positions of root tips recorded, reoriented by rotating the plates by 90 °, and placed vertically for gravitropic response measurement under ammonium stress in a cultivation chamber at time zero. Digital images of seedlings were captured with a Canon G7 at the specified time after gravistimulation. ImageJ software was used for the measurement of root elongation and bending angles as described by Sun *et al.* (2008). Root elongation refers to the length of root growth after transfer to treatment medium, while root length refers to the entire length of the root. The gravitropic angle is the angle of the root tip with respect to the gravity vector.

Microscopic observations of DR5::GUS

Five-day-old seedlings of similar root lengths were selected and transferred to ammonium treatment medium for gravitropic analysis over a 3–72 h time span. Histochemical analyses of *DR5::GUS* gene enzyme activity were carried out according to Weigel and Glazebrook (2002). Images were obtained using an Olympus BX51 optical microscope equipped with differential interference contrast (DIC) for observation and an Olympus DP71 system for photographing. The images shown are representative of at least 10 plants for each treatment, and the experiments were repeated at least twice.

Data analyses

Data were statistically analysed with SPSS version 13.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) with an LSD (least significance difference) test was used for the analysis of differences in root growth and gravitropism following ammonium treatments. Sigma Plot 13.0 was used for generation of graphs and Photoshop for photocomposition.

Results

Influence of ammonium on Arabidopsis *root growth and gravitropism*

With an increase in ammonium concentration, growth of *Arabidopsis* roots was inhibited and their orientation of growth was changed (Fig. 1A–D). The effects of ammonium on root growth and root gravitropic angle were quantified (Fig. 1E). The results showed that all of the $(NH_4)_2SO_4$ treatments impeded root growth. Primary root lengths of 5-day-old seedlings germinated on treatment medium with 5, 10, 20, and 30 mM $(NH_4)_2SO_4$ were shortened to 81.10, 68.66, 39.25, and 13.24%, respectively (Table 1). Unlike the concentration-dependent inhibition of root growth, root gravitropic angles first decreased and then increased with the elevation of ammonium concentration. Root gravitropic angles of 5-day-old seedlings on media with 5 mM and 10 mM $(NH_4)_2SO_4$ were 4.53 ° and 5.87 °, respectively, much smaller relative to the control (10.45 °), and closer to the gravity vector. However, with an additional increase of $(NH_4)_2SO_4$ in the culture medium, *Arabidopsis* roots gradually deviated from the direction of gravity and lost gravitropism. The loss of gravitropism manifested in horizontal growth, curling, and occasionally upward growth of roots. This latter condition was observed in 30 mM $(NH_4)_2SO_4$ treatments (Fig. 1D). Given the influence of root length on the gravitropic angle during growth, comparisons were performed of changes in the angle between plants of similar root lengths. For similar root lengths, the average gravitropic angle of 30 mM $(NH_4)_2SO_4$-treated plants (5-day-old seedlings) was much greater than that of 2-day-old control plants (Fig. 1C, D; Table 1). These results imply that intermediate levels of $(NH_4)_2SO_4$ can promote positive gravitropism while excess levels can cause roots to deviate from gravity; that is, lead to agravitropism. Moreover, the influence of excess NH_4^+ on the gravitropic angle may not be caused by the inhibitory effect of ammonium on root growth.

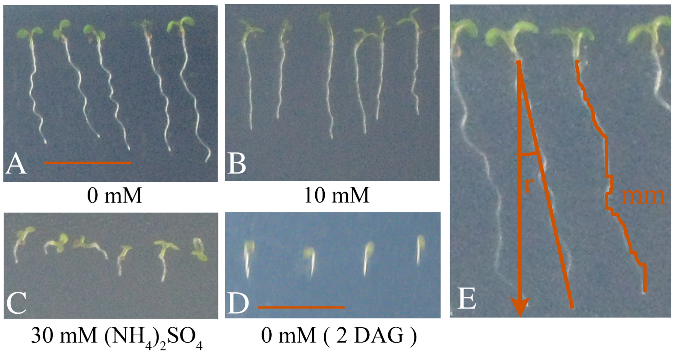

Fig. 1. Regulation of root growth direction and induction of agravitropism by ammonium in *Arabidopsis*. (A–C) Col-0 grown on medium with different concentrations of $(NH_4)_2SO_4$ (0, 10, and 30 mM) for 5 d. Scale bar=1 cm. (D) Col-0 grown on normal growth medium [0 mM $(NH_4)_2SO_4$] for 2 d. Scale bar=1 cm. (E) Schematic diagram shows measurement of the primary root length (in mm) and gravitropic angle (r). (This figure is available in colour at *JXB* online.)

Table 1. Effect of different concentrations of $(NH_4)_2SO_4$ on root growth and gravitropic angle

$(NH_4)_2SO_4$ (mM)	**Days after germination**	**Root length (mm)**	**Gravitropic angle (°)**
0	5	15.57±0.25 a	10.45±0.43 b
5	5	12.63±0.24 b	4.53±0.27 d
10	5	10.69±0.15 c	5.87±0.34 d
20	5	6.11±0.22 d	9.53±0.73 bc
30	5	2.06±0.09 e	24.29±3.34 a
0	2	2.31±0.10 e	6.99±0.54 cd

Values of root length and angle are means ± SE ($n > 60$) from two independent experiments. Letters after the SE indicate whether the different treatments have a significant influence ($P < 0.05$) by one-way ANOVA followed by least significance difference (LSD) post-hoc tests.

To better understand the influence of ammonium on root growth and gravitropism, an additional experiment was carried out to study the effect of $(NH_4)_2SO_4$ on the root gravitropic response (Fig. 2A). After transfer of seedlings to ammonium medium, initiation of root elongation and gravitropic bending were both highly delayed relative to controls. For this reason, data were collected from the sixth

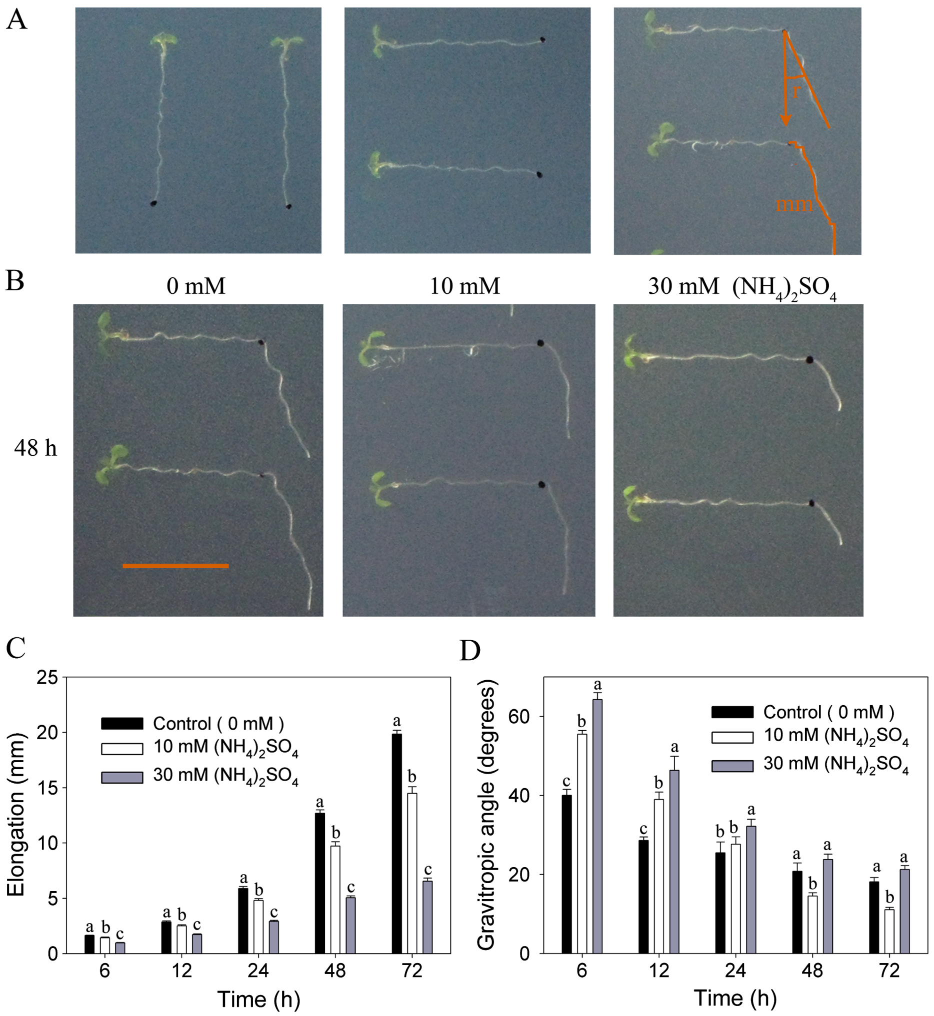

Fig. 2. Time–concentration dynamics of root elongation and gravitropic curvature in *Arabidopsis* upon ammonium exposure. (A) Diagram of the experimental set-up for the study of the root gravitropic response: seedlings were cultivated on normal growth medium for 5 d, transferred onto medium with varying concentrations of $(NH_4)_2SO_4$ (0, 10, and 30 mM), root tips were marked, and culture plates were rotated by 90 ° to initiate gravistimulation at time zero, and photographed at the indicated time intervals following treatments. Root elongation (in mm) refers to the new length established after transfer onto treatment medium, and the gravitropic angle is defined as the angle of the root tip with respect to the gravity vector (r). (B) Ammonium-induced alteration of gravitropic root growth on medium with various concentrations of $(NH_4)_2SO_4$. Pictures were taken 48 h after initiation of treatment. Scale bar=1 cm. (C and D) Time course of root elongation (C) and gravitropic response (D). Data are from three independent experiments with 12–16 seedlings per experiment, and bars represent the standard error (SE). Letters above the bars indicate whether the different treatments have a significant influence ($P < 0.05$) by ANOVA followed by LSD. (This figure is available in colour at *JXB* online.)

hour of treatment imposition. The results (Fig. 2B, C) showed that inhibition brought about by ammonium increased with both ammonium dose and time. For example, average root elongation on the third day was reduced to 73.0% and 33.0%, at 10 mM $(NH_4)_2SO_4$ and 30 mM $(NH_4)_2SO_4$, respectively. On the other hand, ammonium affected root gravitropism in two ways: in the short term (<12 h), $(NH_4)_2SO_4$ at all concentrations inhibited root gravitropism; but with extended time (24 h and beyond) the gravitropic angle became increasingly smaller. The ammonium-treated roots continued to bend after control roots had attained their final gravitropic angle of ~20 °. Consequently, 10 mM $(NH_4)_2SO_4$ increased the maximum curvature of treated roots (displaying a smaller gravitropic angle) in contrast to controls after 48/72 h of gravistimulation, which might be a result of the postponement of the gravitropic bending period (Fig. 2D). Although 30 mM $(NH_4)_2SO_4$ also postponed the bending period, the bending capability of plants at this ammonium level was much lower relative to those in the 10 mM $(NH_4)_2SO_4$ treatment, and, moreover, the gravitropic angles between the two treatments diverged increasingly over time (Fig. 2B, D).

Further analyses revealed that elongation of roots with 30 mM $(NH_4)_2SO_4$ treatment at 12 h was not significantly different from that of control at 6 h ($P > 0.05$); corresponding gravitropic angles were 46.4 ° and 40.0 ° ($P < 0.05$). For seedlings under 10 mM $(NH_4)_2SO_4$ treatment, the root gravitropic angle was not distinguishable from that of the control at 24 h, but root elongation was significantly smaller than in controls. These findings demonstrated that the influence of ammonium on the gravitropic angle was not directly linked to its inhibition of root elongation.

Specificity of the influence of ammonium on root elongation and gravitropic response

As ammonium application in the present study was in the form of $(NH_4)_2SO_4$, it is possible that effects on root elongation and gravitropism may have occurred through the actions of either the NH_4^+ or SO_4^{2-} ions, or due the fact that highly concentrated levels were typically applied. To address these possibilities, NH_4Cl, with the same concentration of NH_4^+, Na_2SO_4 and K_2SO_4, with the same concentration of SO_4^{2-}, and KNO_3, with the same concentration of N, were substituted for $(NH_4)_2SO_4$ in the treatment medium. Because the gravity of roots in control seedling was also affected by light (compared with Figs 2 and 3, also in Supplementary Table S1 available at *JXB* online), to exclude interference by light, experiments were carried out in the dark. It was observed that NH_4Cl had very similar effects on the root gravitropic angle to $(NH_4)_2SO_4$, while Na_2SO_4 had very little effect. After 6 h of gravistimulation, K_2SO_4 and KNO_3 reduced the root gravitropic angle, but to a much smaller extent relative to $(NH_4)_2SO_4$. Thus, it was clear that the influence of ammonium on root gravitropic angle was largely caused by NH_4^+, and was not duplicated upon exposure to SO_4^{2-}, Cl^-, Na^+, N, or high ionic concentration *per se*. Moreover, based on the data (compared with Figs 2 and 3, and Supplementary Table S1), the gravitropic angle under NH_4^+ stress seemed to be independent of light, but this aspect will need further experiments for confirmation. Nevertheless, in the early stage, K_2SO_4 and KNO_3 showed some influence on root gravitropism, probably through the influence of K^+, but the alteration was far less than that caused by NH_4^+ of the same concentration.

The inhibition intensity of root growth by these substances was as follows: $(NH_4)_2SO_4 \geqslant NH_4Cl > Na_2SO_4 > K_2SO_4 \geqslant KNO_3$, and elongation of roots was reduced to 39.8, 48.5, 73.6, 88.4, and 93.8%, respectively, after 3 d treatments with each of these salts (Fig. 3B). Although $(NH_4)_2SO_4$ caused inhibition primarily via NH_4^+, SO_4^{2-} at high concentration also showed some effect on root growth, which was demonstrated by the fact that inhibition by 30 mM $(NH_4)_2SO_4$ was significantly greater than that by 60 mM NH_4Cl ($P < 0.05$) based on the 3 d treatment.

Influence of exogenous K^+ on ammonium-induced root elongation and gravitropic response

Because of their similarity in ionic radius and electric charge, K^+ and NH_4^+ compete with each other for absorption, and excess NH_4^+ can inhibit the absorption and accumulation of K^+ (Szczerba, 2008; ten Hoopen *et al.*, 2010). Research has shown that low K^+ not only inhibits root growth of *Arabidopsis* but also weakens its gravitropic response (Vicente-Agullo *et al.*, 2004). This raised the question of whether the influence of ammonium on *Arabidopsis* roots in this study was caused by a K^+ deficiency induced by highly concentrated NH_4^+. The results (Fig. 4A) showed that addition of KNO_3 at different concentrations to 30 mM $(NH_4)_2SO_4$ treatment media significantly reversed the inhibition of root elongation, recovering the root elongation from 27.3% to 36.6–43.4% following 3 d treatments. However, KNO_3 did not affect rescue of the root gravitropic response, and the addition of 20 mM KNO_3, in fact, slightly enhanced the disruption in gravitropism, following gravistimulation treatments for ⩾12 h (Fig. 4B). In order to determine further the influence of exogenous KNO_3 on root growth and gravitropism and the relationship between KNO_3 and the role of the K^+ ion in this, experiments were carried out with 20 mM $NaNO_3$, 20 mM KCl, or 20 mM NaCl substituted for 20 mM KNO_3. KCl addition relieved ammonium's inhibition of root elongation, while $NaNO_3$ and NaCl accentuated the inhibition (Fig. 4C). In terms of the gravitropic response,

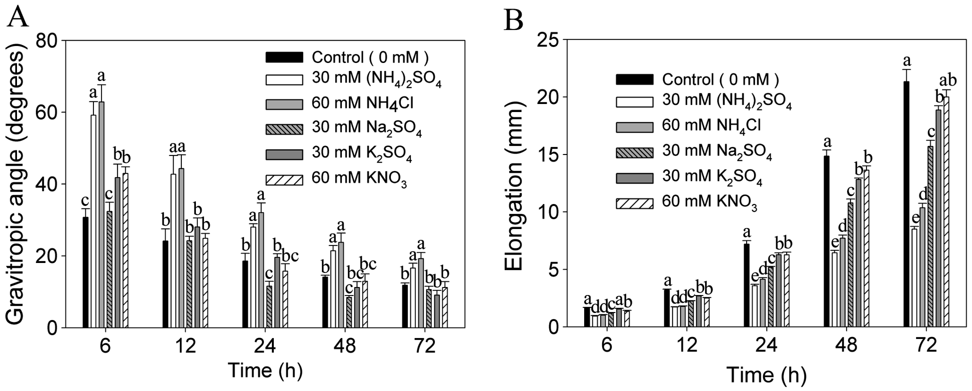

Fig. 3. Growth and gravitropic response of Col-0 on $(NH_4)_2SO_4$, NH_4Cl, K_2SO_4, KNO_3, and Na_2SO_4 in darkness. (A) Influence of an equal concentration of various ions on the gravitropic response. The effect of ammonium on root gravitropism was generated by NH_4^+ rather than by SO_4^{2-}, Cl^-, Na^+, K^+, or NO_3^-. (B) Influence of various salts on root growth. Data are from four independent experiments with 12 seedlings per experiment, and bars represent the SE. Letters above the bars indicate whether the different treatments have a significant influence ($P < 0.05$) by ANOVA followed by LSD.

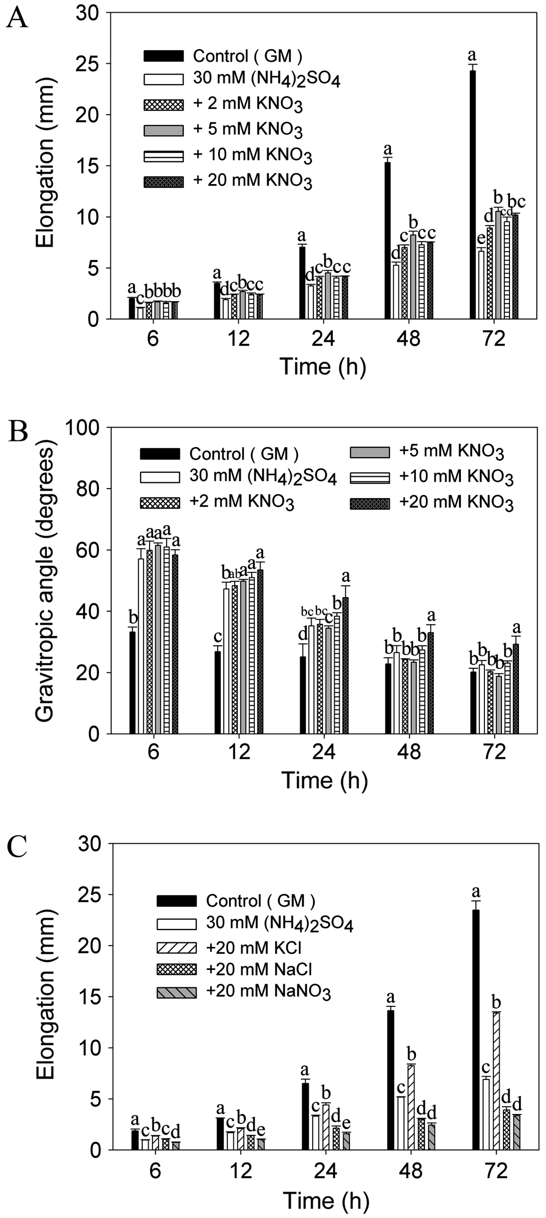

Fig. 4. K^+ partially restores the ammonium-induced inhibition of root elongation but does not relieve its influence on gravitropism. Five-day-old seedlings were transferred onto treatment medium with 30 mM $(NH_4)_2SO_4$ containing additional concentrations of KNO_3. (A) All concentrations of KNO_3 alleviated the inhibition of ammonium-induced inhibition of root growth, and at all time points ($P < 0.05$). (B) KNO_3 failed to rescue (indeed accentuates) the effect of ammonium on root gravitropic curvature. (C) The K^+ ion was responsible for the KNO_3 alleviation of ammonium-induced inhibition of root elongation. Data are from four independent experiments with 12 seedlings per experiment, and bars represent the SE. Letters above the bars indicate whether the different treatments have a significant influence ($P < 0.05$) by ANOVA followed by LSD.

neither KCl nor NaCl reduced the effects of ammonium, while $NaNO_3$ accentuated agravitropism (data not shown). That exogenously supplied K^+ partially relieved ammonium's inhibition of root elongation but not the gravitropic response underscores that the influence of ammonium on gravitropism was not due to K^+ deficiency. This was consistent with research on bean by Bonser *et al.* (1996), who found that, with the exception of Pi, the deficiencies of minerals such as N, K, S, Ca, or Mg had no effect on root angle.

Influence of ammonium on DR5::GUS *expression in the root tip*

In *Arabidopsis* roots, the expression of *DR5::GUS*, an auxin-responsive promoter (Ulmasov *et al.*, 1997), has been used to infer the development of a lateral auxin gradient during the gravitropic process (Hou *et al.*, 2004). Histochemical methods were used to test the influence of ammonium on auxin signals in root tips during the gravitropic response. The results showed that in vertically grown roots (0 h), *DR5::GUS* was expressed mainly in the quiescent centre, columella initial cells, and columella cells of the root cap. In control roots, after 3 h of gravistimulation, 69% of plants (29/42) activated the expression of *DR5::GUS* on the lower side of the root tips, and extending basipetally along the lateral root caps and epidermal cells to the meristematic zone. Thereafter, the lateral auxin gradient was gradually weakened with the passage of time. At 24 h, it disappeared in 80% (20/25) of plants, and the expression of *DR5::GUS* returned to pre-treatment states (Fig. 5). In contrast, $(NH_4)_2SO_4$ treatment delayed the lateral redistribution of auxin in root tips. After 3 h of gravistimulation, only 23.7% (9/38) of the roots displayed asymmetric expression of *DR5::GUS* upon treatment with 30 mM $(NH_4)_2SO_4$. At 6 h, the asymmetric auxin gradient was observed in 68.4% (26/38) of roots treated with $(NH_4)_2SO_4$. Furthermore, after 24 h of gravistimulation, it was still observed in 56.7% (17/30) of roots, while disappearing at 72 h. Moreover, in contrast to controls and roots treated with 10 mM $(NH_4)_2SO_4$, the expression of *DR5::GUS* in apical cells of roots treated with 30 mM $(NH_4)_2SO_4$ decreased over time (>1 d), but auxin signals in stele cells increased (Fig. 5). Expression of *DR5::GUS* was also observed in stele cells of controls, with results fluctuating slightly.

TRH1 participates in ammonium inhibition of root gravitropism and auxin signals in root tip cells

The potassium transporter TRH1 (AtKT3/AtKUP4), which is strongly expressed in the root cap, is required for auxin transport in *Arabidopsis* roots (Rigas *et al.*, 2001; Vicente-Agullo *et al.*, 2004). Disruption of this gene in the *trh1* (tiny root hair 1) mutant not only blocks the translocation of auxin in the root cap but also weakens root gravitropism (Vicente-Agullo *et al.*, 2004). In order to examine whether the alteration of root gravitropism and auxin signalling in root caps was related to TRH1, we tested the gravitropic response of the *trh1* mutant under high ammonium exposure. If $(NH_4)_2SO_4$ inhibits *Arabidopsis* root gravitropism via TRH1-mediated auxin transport in the root cap, the gravitropism of *trh1* is expected to show at least partial resistance to ammonium stress relative to Col-0. The results

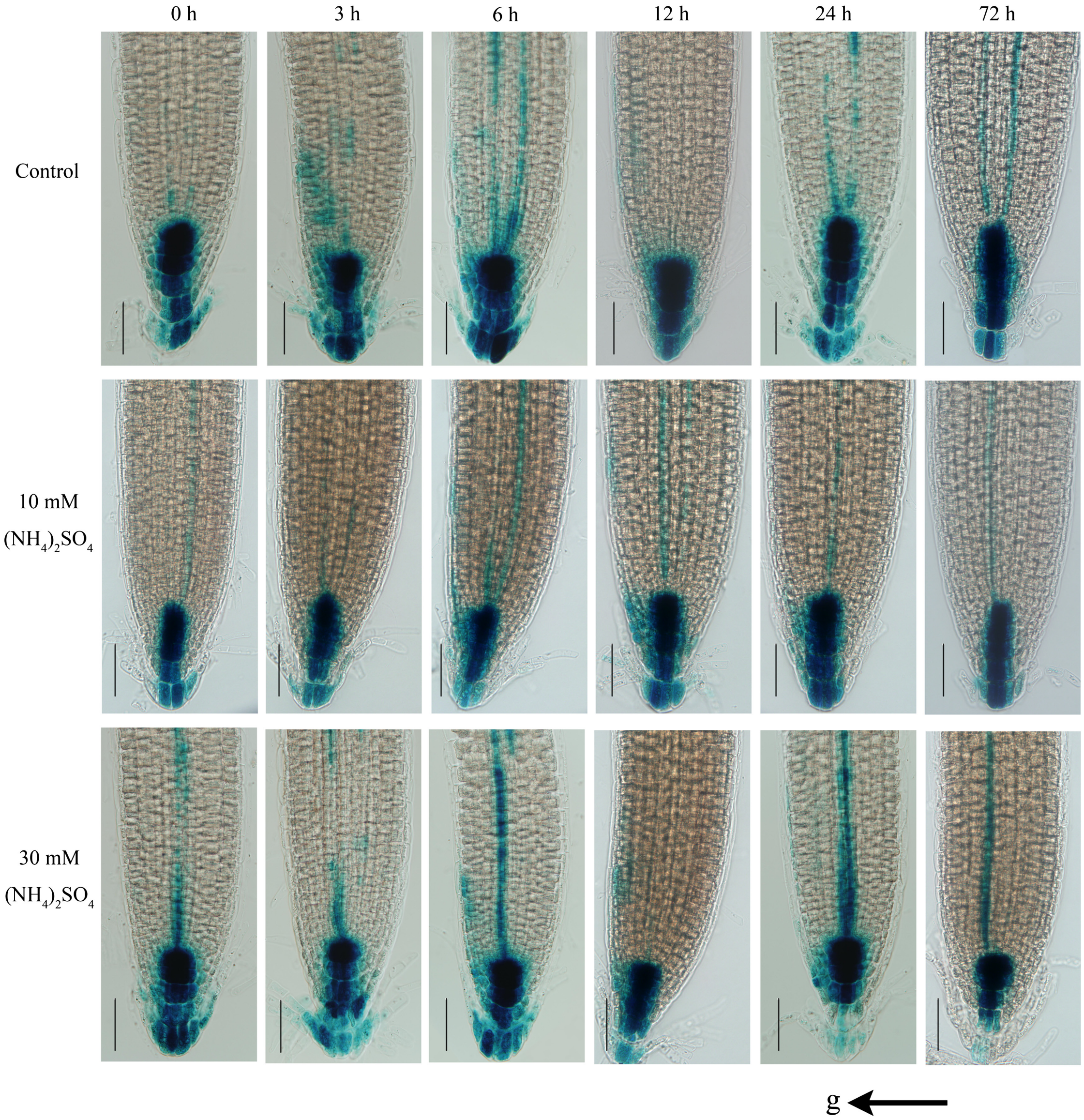

Fig. 5. Influence of ammonium on *DR5::GUS* expression in the root tip during the gravitropic response. GUS staining for 4 h following gravistimulation on treatment medium with the indicated concentrations of $(NH_4)_2SO_4$. The arrow indicates the direction of the gravity vector. One representative image for each experiment is shown. Scale bar=50 μm. (This figure is available in colour at *JXB* online.)

showed that, in the early stages (6 h), the gravitropic curvature of the *trh1* mutant root was less than that of Col-0, but, with time (48/72 h), *trh1* mutants displayed similar gravitropism to the wild type under ammonium (Fig. 6). In controls, *trh1* showed clearly weakened gravitropism compared with the wild type. These data indicate that TRH1 participates in the ammonium-induced gravity response in roots. Nevertheless, the reduction of root elongation in *trh1* by ammonium was similar to that observed in Col-0. Under a 3 d treatment with 10 mM and 30 mM $(NH_4)_2SO_4$ separately, root elongation of *trh1* and Col-0 was 75.74% and 67.81%, and 30.42% and 29.93% of their controls, respectively.

With the *DR5::GUS* construct in the *trh1* mutant, the influence of ammonium on the expression of *DR5::GUS* in *trh1* root tips was analysed in the context of the gravitropic response. No significant differences were found in auxin signals between *trh1* and Col-0 under control conditions (Fig. 7A, B). After 1 d and 3 d of treatments under 30 mM $(NH_4)_2SO_4$, auxin signals decreased noticeably in root tip cells but increased in central tissues of Col-0. Similar to Col-0, ammonium treatment caused auxin accumulation in stele cells of *trh1* roots. However, the auxin signal intensity in the root apex cells did not decrease over time (Fig. 7B). These findings indicate that TRH1 regulates the reduction of auxin signal intensity in root tip cells under ammonium exposure.

Discussion

The relationship of ammonium to root gravitropism and elongation

Root gravitropism responses always depend on root elongation. However, it is not known whether these two

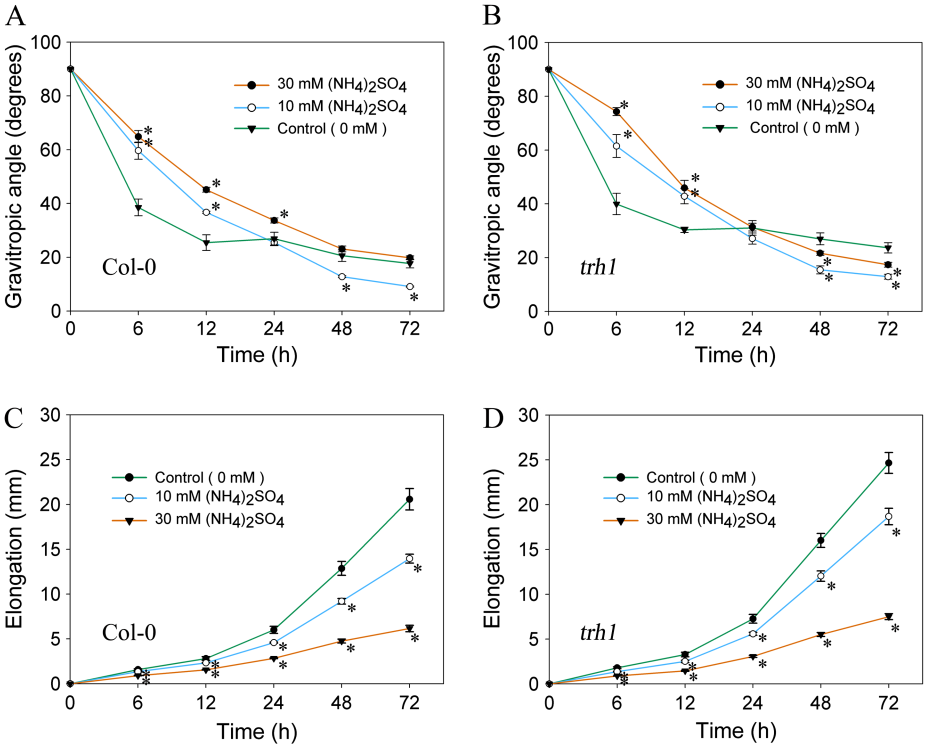

Fig. 6. Influence of ammonium on root growth and gravitropism in Col-0 and *trh1*. (A and B) Root gravitropic response to $(NH_4)_2SO_4$ exposure as a function of time and concentration in Col-0 (A) and *trh1* (B). (C and D) Time course of root elongation in Col-0 (C) and *trh1* (D) under ammonium treatment. Data are from four independent experiments with 10–12 seedlings per experiment, and bars represent the SE. *indicates significant differences (P <0 .05) compared with the controls. (This figure is available in colour at *JXB* online.)

biological processes are always synchronized. This study provides compelling evidence that responses of root gravitropism and elongation to ammonium stress are regulated independently. Firstly, with the rise of ammonium concentration, root length shortened while gravitropism initially strengthened and only later weakened (Table 1). Secondly, when a root length similar to that of the control was attained, roots under 30 mM $(NH_4)_2SO_4$ treatment deviated from the gravity vector to a greater degree. Thirdly, the dynamic results obtained with variations in time and concentration also showed that ammonium's influence on the gravitropic bending angle was not a secondary effect of its inhibition of root elongation (Fig. 2; see also Fig. 3). Furthermore, added KNO_3 relieved NH_4^+-induced inhibition of root elongation by partial restoration of root length, but failed to rescue the gravitropic angle (Fig. 4). These results suggest that root gravitropism and elongation in response to ammonium stress are regulated by two at least partially distinct pathways.

As plants have evolved an elaborate and sophisticated set of growth responses to environment cues (Cassab, 2008), it is hypothesized that the alteration of root gravitropism with increased ammonium supply may be a key process by which the plant acclimates to a changed soil condition. When at a low concentration, NH_4^+ is an important N fertilizer, especially when nitrate is also present in solution (Kronzucker *et al.*, 1999). Therefore, under the experimental conditions in this study (2 mM K^+, 5 mM NO_3^-), and concentrations of $(NH_4)_2SO_4$ <10 mM, ammonium may be an important source of N and, under such conditions, the gravitropic angle becoming smaller might be due to the fertilizer tropism of roots. In fact, when supplied via the roots, 10 mM $(NH_4)_2SO_4$ had no inhibitory effect on the aerial parts of *Arabidopsis*; moreover, it increased the number of lateral roots (Li *et al.*, 2010). However, excess ammonium increased the angle of the root tip from the gravity vector, and this may be a mechanism of risk aversion by the root system. That the NH_4^+ inhibition of root growth was partially relieved by exogenous K^+ can probably be explained as follows: supplied K^+ can effectively alleviate the futile ammonium cycling at the plasma membrane and reduce the ion's entry and accumulation (Nielsen and Schjoerring, 1998; Szczerba *et al.*, 2008; Balkos *et al.*, 2010; ten Hoopen *et al.*, 2010).

Effect of ammonium on root gravitropism relates to the redistribution of auxin in root tips

The asymmetric distribution of the plant hormone auxin in root tips has long been regarded as an important factor in the regulation of root gravitropism (Ottenschläger *et al.*, 2003). The present results showed that in the early stages of the root gravitropic response, ammonium treatment delayed the gravity-induced development of asymmetric *DR5::GUS* expression across the root caps, but, once the asymmetry was established, ammonium further prolonged

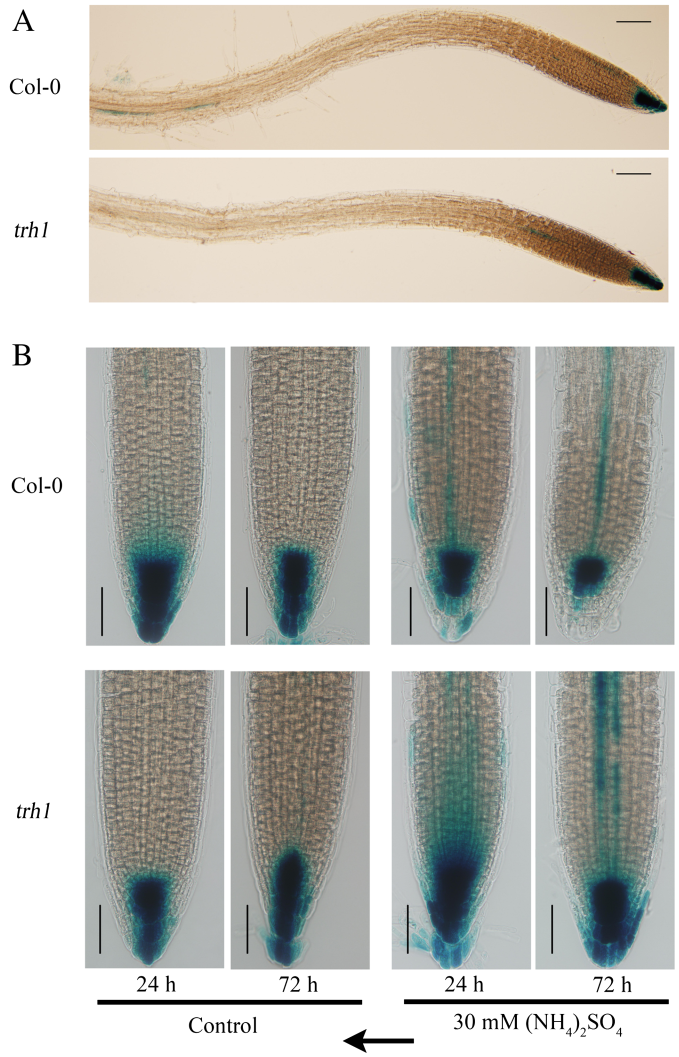

Fig. 7. Influence of ammonium on *DR5::GUS* expression in root tips of Col-0 and *trh1* during the gravitropic response. (A) Expression of the auxin reporter *DR5::GUS* in Col-0 and *trh1* seedlings germinated on normal growth medium for 5 d. (B) Effect of 30 mM $(NH_4)_2SO_4$ on the expression of *DR5::GUS* in Col-0 and *trh1* seedlings following 24 h and 72 h of gravistimulation. GUS staining was conducted for 4 h. One representative image for each experiment is shown. Scale bar=100 µm in A and 50 µm in B. The arrow indicates the direction of the gravity vector. (This figure is available in colour at *JXB* online.)

its expression on the lower side (Fig. 5). This was consistent with the observation that 10 mM $(NH_4)_2SO_4$ treatment prolonged the period of gravitropic response of the treated root and consequently increased its maximum curvature (Figs 2D, 6A). The continuous asymmetric auxin signal as a consequence of ammonium exposure is clearly related to the increased root gravitropic curvature, in particular at 10 mM $(NH_4)_2SO_4$. From these observations, it can be concluded that the weakening of root gravitropism by ammonium in *Arabidopsis* relates to the redistribution of auxin in root tips. A close relationship between gravity-induced cytoplasmic alkalization in columella cells and lateral auxin redistribution in root caps has been demonstrated (Fasano *et al.*, 2001; Boonsirichai *et al.*, 2003; Wolverton *et al.*, 2011). Ammonium absorption, especially under high external supply conditions, can induce alkalization of the cytoplasm in root cells (Britto and Kronzucker, 2005; Monshausen *et al.*, 2011). Whether the persistent auxin gradient is related to the alkalization of cytoplasm in root tip cells under ammonium treatments needs further study.

In addition to delaying and prolonging the formation of a lateral auxin gradient, 30 mM $(NH_4)_2SO_4$ treatment in this experiment notably decreased the auxin signal in apical cells of root tips (Fig. 5). With time further prolonged (5 d after treatment), the auxin distribution in the root cap was altered, with the maximum shifted upward encompassing the quiescent centre and vascular initials (Li *et al.*, 2010). However, the gravitropism of seedling roots in 30 mM $(NH_4)_2SO_4$ treatments at the later stage was recovered to control levels; that is, the reduction of the auxin response in the root apex should not be the primary reason for the ammonium-induced loss of root gravitropism.

TRH1 is related to root gravity response and auxin signals in the root apex under ammonium treatment

It has been shown that the potassium transporter TRH1 affects the gravity response of *Arabidopsis* roots and is involved in auxin transport in the root apex (Vicente-Agullo *et al.*, 2004). In this study, it was also observed that the mutation of TRH1 was associated with weakened gravitropism compared with the wild type. Under 30 mM $(NH_4)_2SO_4$, the *trh1* mutant showed more weakened gravitropism than the wild type at the early stage, and recovered gravitropism in later stages. Thus, it was clear that TRH1, while not sufficient to explain the phenomenon, was nevertheless associated with the weakening of root gravitropism by excess ammonium in *Arabidopsis*. Due to the fact that external potassium failed to recover the root gravity response under ammonium in the wild type, the data suggest the involvement of TRH1-mediated auxin transport rather than potassium transport in the ammonium-induced weakening of the gravitropism response. However, it is not known at this time whether the potassium carrier TRH1 transports auxin directly or is involved indirectly, through the generation of ionic and electric gradients across the plasma membrane that favour auxin efflux via other auxin transporters, as suggested by Vicente-Agullo *et al* (2004). This warrants further investigation.

In addition, it was interesting to find that the auxin signal in apical cells in the *trh1* mutant did not significantly change in response to ammonium (Fig. 7). This result demonstrates that TRH1-mediated auxin translocation is likely to be involved in this process. However, this study did not confirm that the TRH1-mediated auxin response in the root apex is related to the ammonium-induced loss of gravitropism, because the reduction of *DR5::GUS* expression in the root apex was not always coupled with loss of root gravitropism.

In plants, auxin is synthesized mainly in young shoot tissues, and transported through the central tissues to the roots. There, auxin is transported acropetally toward the tip and adds to a pool of locally synthesized auxin, forming an auxin-maximal centre that overlaps with the quiescent centre and upper cap, and is then redistributed toward the flanks (lateral cap) and transported basipetally through the lateral root cap and epidermal cells toward the elongation zone (Massion *et al.*, 2009). Research has shown that auxin transport is sufficient to generate a maximum as well as a gradient to guide root growth (Grieneisen *et al.*, 2007). Therefore, for the reduction of auxin signal intensity within the root apex under excess ammonium treatment, it is probable that NH_4^+ inhibits acropetal translocation of auxin towards the root tip, such as the functions of the auxin influx carrier AUX1, but has no effect on the auxin translocation away from the root cap (Li *et al.*, 2011*a,b*), so that the auxin signal within apex cells is reduced. Yet, in the *trh1* mutant, the disruption of TRH1-mediated auxin export resulted in an accumulation of auxin in the root cap (Vicente-Agullo *et al.*, 2004; this study), which can compensate for the reduction of acropetal auxin transport. This is consistent with the observation that there was an increased auxin signal in stele tissues in *trh1* and Col-0 roots under ammonium treatment (Fig. 7), indicating that the auxin whose transport was inhibited accumulated in the stele, and activated the expression of *DR5::GUS*. However, it is also plausible that the translocation of auxin through the root cap by TRH1 was promoted and thus the auxin signal accumulation in the root cap of Col-0 was reduced under excess ammonium. Results have shown that ammonium accumulation can increase ethylene evolution from leaf tissues (Barker, 1999), while ethylene can promote transport-dependent auxin distribution (Ruzicka *et al.*, 2007). In addition, under excess ammonium, cell membranes in root tips can be induced to depolarize rapidly, and thereafter efflux of K^+ will increase (Higinbotham *et al.*, 1964; Wang *et al.*, 1994; Nocito *et al.*, 2002; Coskun *et al.*, 2010). As the molecular mechanism of TRH1-dependent auxin transport is not yet clear, whether ammonium regulates TRH1 activity via ethylene and/or ion and electrochemical potential gradients to promote TRH1 or other carriers mediating auxin translocation warrants future study.

Interestingly, it was found that the oscillating growth pattern for roots was inhibited at elevated $(NH_4)_2SO_4$, implying that ammonium did act on the auxin transport systems, because the oscillating growth was disturbed in mutants of auxin transporters such as *aux1* and *agr1* (alellic with *eir1/pin2/wav6*) or NPA (naphthylphthalamic acid)-treated Col-0 roots (Rashotte *et al.*, 2000; Migliaccio and Piconese, 2001), all of which related to polar auxin transport. Evidence has been obtained that the ammonium-induced reduction of the auxin signal in root tips may not be due to the lack of auxin content (Qin *et al.*, 2011). Nevertheless, the possibility that the alteration of the auxin maximum was a consequence of changes in metabolism and synthesis of auxin within the root apex under ammonium stress cannot be excluded, because mutants relevant to auxin synthesis, transport, and response have been shown to affect gravitropism (Masson *et al.*, 2009). Nor can it be excluded that there may be other auxin carriers in addition to TRH1 taking part in the ammonium-induced agravitropic response and auxin distribution.

In summary, although root gravitropism and root growth were both greatly influenced by excessive ammonium, they seemed to be under the influence of independent mechanisms. The latter was partially relieved by exogenously supplied K^+, while the former was related to the alteration of auxin redistribution in the root apex. The data suggest an involvement of TRH1 in the ammonium-induced loss of root gravitropism in *Arabidopsis*. In addition, it was interesting to observe that the reduction of auxin signal intensity within the root apex caused by prolonged ammonium exposure was eliminated by the mutation in TRH1.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Comparison of the effects of NH_4^+ on root gravitropism, affected by light and dark

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