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Ammonium affects cell viability to inhibit root growth in *Arabidopsis*[*#]

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Abstract: Ammonium (NH_4^+) is an important form of nitrogen nutrient for most plants, yet is also a stressor for many of them. However, the primary events of NH_4^+ toxicity at the cellular level are still unclear. Here, we showed that NH_4^+ toxicity can induce the root cell death in a temporal pattern which primarily occurs in the cells of root maturation and elongation zones, and then spreads to the cells in the meristem and root cap. The results from the NH_4^+-hypersensitive mutant *hsn1* further confirmed our findings. Taken together, NH_4^+ toxicity inhibits primary root growth by inhibiting cell elongation and division and inducing root cell death.

Key words: Ammonium toxicity, Root, Cell viability, GDP-mannose pyrophosphorylase, *Arabidopsis*
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1 Introduction

Nitrogen (N) is an important nutrient to plants with two most important nitrogen forms being ammonium (NH_4^+) and nitrate (NO_3^-). Because NH_4^+ assimilation requires less energy than that of NO_3^- (Reisenauer, 1978), NH_4^+ is expected to be the preferred form of nutrient to plants. However, this ion has toxic effects on many plant species (Britto and Kronzucker, 2002). Two of the most dramatic symptoms of ammonium toxicity were the chlorosis of leaves and the overall suppression of growth (Britto and Kronzucker, 2002). Ammonium toxicity has also been linked to the damage to agricultural crops under certain ecological conditions (van Breemen and van Dijk, 1988). Several hypotheses to explain the possible mechanisms of NH_4^+ toxicity in plants have been proposed, including the acidification of external growth environment, displacement of important cations, such as K^+ and Mg^{2+}, or excessive energy being wasted due to the abundance of toxic NH_4^+ requiring removal from the cells (Britto and Kronzucker, 2002). None of these theories have yet been conclusively confirmed.

One characteristic of ammonium syndrome is the inhibition of primary root growth when plants are exposed to higher NH_4^+ concentrations (Britto and Kronzucker, 2002; Qin *et al*., 2008). Our previous study demonstrates that guanosine diphosphate (GDP)-mannose pyrophosphorylase (GMPase) is a genetic determinant of ammonium sensitivity in *Arabidopsis* (Qin *et al*., 2008). The root growth is severely inhibited in the mutant *hsn1* defective in GMPase grown in the growth medium with NH_4^+. Defective *N*-glycosylation of proteins, unfolded protein response, and cell death are likely important downstream molecular events involved in the NH_4^+-hypersensitive mutant. The root cellular response to

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NH_4^+, however, is still unclear. Understanding of the root cellular response to NH_4^+ may help us to elucidate the physiological and cellular mechanisms of the NH_4^+-mediated inhibition of root growth.

2 Materials and methods

2.1 Plant materials and growth conditions

The plant materials used in this work include wild type (WT) *Arabidopsis thaliana* and genetic mutant *hsn1* (Col-0 ecotype) (Qin *et al*., 2008). Seed germination and seedling growth were accomplished using modified Murashige and Skoog (MS) media, which consisted of $NO_3^- + NH_4^+$ medium (half-strength MS medium, with 10 mmol/L NH_4^+ and 20 mmol/L NO_3^- provided by KNO_3 and NH_4NO_3 as nitrogen sources), NH_4^+ medium (with 10 mmol/L NH_4^+ provided by $(NH_4)_2SO_4$ as sole nitrogen source), and NO_3^- medium (with 20 mmol/L NO_3^-, provided by KNO_3 as sole nitrogen source). The above media were all supplemented with 5 g/L sucrose and 0.5 g/L 2-morpholinoethane sulphonic acid (MES), adjusted to pH 5.7, and solidified with 10 g/L agar-agar (Fisons). The plates grew in a growth cabinet (Percival Scientific, Perry, IA) preset with a 16-h light/8-h dark photoperiod, 300 μmol/(m^2·s) light intensity, and a constant temperature of 20 °C.

To observe marker expression in the *hsn1* mutant, we examined F_3 seeds from a cross between *hsn1* and the marker lines.

2.2 Measurement of root growth

Seeds on plates were kept at 4 °C for 2 d and then were placed vertically in a growth cabinet kept at 20 °C under continuous illumination. Only seedlings that germinated at the same time were used for root growth measurements.

2.3 Histochemical analysis

The histochemical stain for β-glucuronidase (GUS) activity and stain for Starch granules were performed according to prevenient papers (Jefferson *et al*., 1987; Fukaki *et al*., 1998). The stained materials were cleared for 10 min in an 8:3:1 (v/v) mixture of chloral hydrate:water:glycerol (Willemsen *et al*., 1998) and photographed with a photomicroscope (Zeiss AxioCam HRC, Oberkochen, Germany). Starch granules in the columella root cap were visualized by staining with 1% (v/v) Lugol solution (Merck) for 3 min. The samples were rinsed with water, cleared with chloral hydrate, and imaged under a photomicroscope (Zeiss AxioCam HRC, Oberkochen, Germany).

For confocal scanning, roots were stained in 10 μg/ml propidium iodide (PI) solution for 5 min. The images were monitored by the LSM 510 (Carl Zeiss Co., Germany).

2.4 Cell viability assay

Cell viability was examined by detection of the green fluorescent dye fluorescein diacetate (FDA), of which fluorescence decreases as the dye leaks from dead cells (Bais *et al*., 2003). The roots of seedlings were mounted in 10 ml of 20 μmol/L FDA water solution for 10 min, washed with distilled water, and then imaged for no more than 5 min.

3 Results

3.1 Effect of NH_4^+ on root cell elongation and cell division

To characterize the effect of NH_4^+ on root development, we determined the root cell elongation and cell division of WT seedlings grown in the $NO_3^- + NH_4^+$ and NH_4^+ media over a 10-d period. When grown in the NH_4^+ medium, the WT seedlings displayed a typical NH_4^+ toxicity phenotype, which is distinct from that grown in the $NO_3^- + NH_4^+$ medium (Fig. 1a). To determine the effect of NH_4^+ on the root cell elongation, we measured the length of the newly formed mature cortical cells (the cells that are adjacent to the elongation zone). It showed that there was an inhibition of root cell elongation in WT grown in the NH_4^+ medium. The length of root mature cortical cells adjacent to the elongation zone of WT plants in the $NO_3^- + NH_4^+$ medium increased 46% (from 120 to 175 μm) 10 d after germination (DAG), while that of WT plants in the NH_4^+ medium decreased 55% after

10 d culture (from 108 μm at 1 DAG to 49 μm at 10 DAG) (Fig. 1b). As early as 3 DAG, an obvious inhibition of root cell elongation was observed in WT seedlings in the NH_4^+ medium. Up to 10 DAG, the length of mature cortical cells of WT seedlings in the NH_4^+ medium was 72% shorter than that in the $NO_3^- + NH_4^+$ medium (Fig. 1b). These results indicate that NH_4^+ toxicity affects root cell elongation. To confirm these results, we employed the NH_4^+-hypersensitive mutant *hsn1* for further characterization. It was reported that NH_4^+ can severely inhibit primary root of the *hsn1* mutant even when grown in the $NO_3^- + NH_4^+$ medium (Qin *et al*., 2008) (Fig. 1a). In the *hsn1* mutant, as early as 2 DAG, an obvious inhibition of root cell elongation was observed in the $NO_3^- + NH_4^+$ medium. Up to 10 DAG, the length of mature cortical cells of the *hsn1* mutant in the $NO_3^- + NH_4^+$ medium was 75% shorter than that of WT (Fig. 1b). These results suggest that NH_4^+ can inhibit root cell elongation and the *hsn1* mutant is much more sensitive to the inhibition.

To determine the effect of NH_4^+ on root cell division, we monitored the temporal expression of the cell cycle marker *CYCB1;1::GUS* in WT seedlings in both $NO_3^- + NH_4^+$ and NH_4^+ media. The expression of CYCB1;1 is a mark for dividing cells (Colon-Carmona *et al*., 1999). In the $NO_3^- + NH_4^+$ medium, a typical expression pattern of *CYCB1;1::GUS* in dividing cells at root meristem was observed (Fig. 1c). However, when grown in the NH_4^+ medium, the activity of *CYCB1;1::GUS* was obviously repressed at 3 DAG, and the activity was gradually reduced until nearly no dividing cell was detected at 10 DAG (Fig. 1c). It indicates that the NH_4^+ can inhibit root cell division in WT seedlings grown in the NH_4^+ medium. If this is true, we might expect that the root cell division would be inhibited in the *hsn1* mutant even if it was grown in the $NO_3^- + NH_4^+$ medium. Therefore, we introgressed *CYCB1;1::GUS* in the *hsn1* mutant background, and examined the GUS activity of the plants grown in the $NO_3^- + NH_4^+$ medium. Consistent with our hypothesis, the activity of *CYCB1;1::GUS* in *hsn1* mutant root was repressed at 3 DAG, and the activity was gradually reduced until nearly no cell division was detected at 10 DAG (Fig. 1c). Taken together, it indicated that NH_4^+ can inhibit root cell division at meristem.

Collectively, we showed that NH_4^+ can inhibit root cell elongation, and even repress root meristematic cell division.

3.2 Effect of NH_4^+ on organization and maintenance of the root meristem

To further determine the effect of NH_4^+ on organization and maintenance of the root meristem, we examined the expression patterns of some cell type-specific markers in WT seedlings grown in the $NO_3^- + NH_4^+$ and NH_4^+ media. The expressions of enhancer trap *J3612::GFP* (marks pericycle initials) and *SCRp::GFP* (marks the endodermis, the cortex/endodermal initial cells and the quiescent center (QC)) (Wysocka-Diller *et al*., 2000) showed a typical pattern in WT root grown in the $NO_3^- + NH_4^+$ medium (Fig. 2). In the NH_4^+ medium, *SCRp::GFP* showed a typical expression in WT. Ectopic expressions of *J3612::GFP* were observed in stele at 10 DAG (Fig. 2). Similar to that, in the NH_4^+-hypersensitive mutant *hsn1*, ectopic expressions of *J3612::GFP* and *SCRp::GFP* were all observed in stele of root at 10 DAG even in the $NO_3^- + NH_4^+$ medium (Fig. 2). These results indicate that the NH_4^+ can disorder the organization and maintenance of the root meristem, with defects either to specify or to maintain the root radial pattern and cell identity.

A failure in root meristem maintenance can be caused by the lack of QC activity. To determine whether NH_4^+ destroys QC activity, we examined the expression of QC marker, *QC25::GUS* (van den Berg *et al*., 1995) in seedlings in the $NO_3^- + NH_4^+$ and NH_4^+ media. The WT seedlings grown in the $NO_3^- + NH_4^+$ medium displayed the typical expression of the *QC25::GUS* in 2–3 QC cells during a 10-d period (Fig. 3a). When grown in the NH_4^+ medium, the WT seedlings displayed the typical expression of the *QC25::GUS* at 4 DAG, but no expression of *QC25::GUS* was detected in the root at 10 DAG

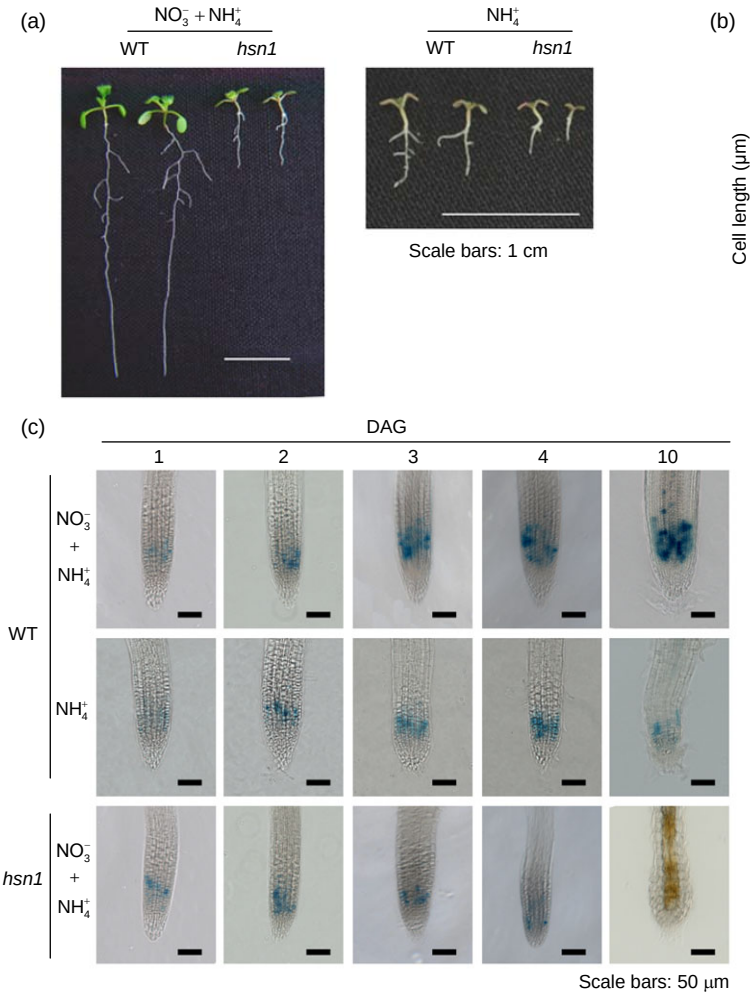

Fig. 1 Inhibitive effect of NH_4^+ on root cell elongation and cell division

(a) Comparisons of seedling growth of WT *Arabidopsis* and *hsn1* in the $NO_3^- + NH_4^+$ and NH_4^+ media at 10 d after germination (DAG); (b) Comparisons of mature cortical cells between WT *Arabidopsis* (Col-0) in the $NO_3^- + NH_4^+$ and NH_4^+ media, and *hsn1* mutant in the $NO_3^- + NH_4^+$ medium; (c) *CYCB1;1::GUS* marker expression in primary roots of WT in the $NO_3^- + NH_4^+$ and NH_4^+ media and *hsn1* mutant plants in the $NO_3^- + NH_4^+$ medium in a time course up to 10 d. These results have been repeated in three separate experiments

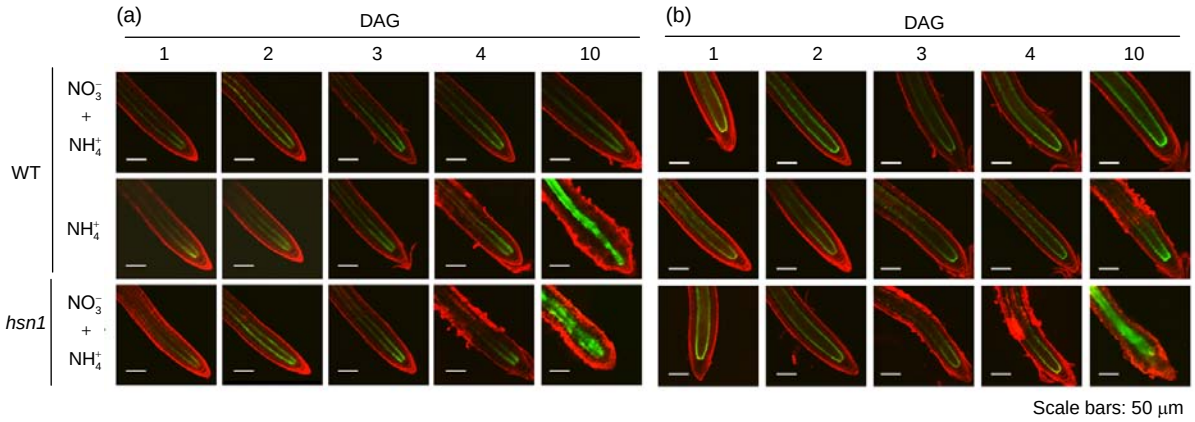

Fig. 2 Green fluorescent protein (GFP) expressions in primary roots of WT in the $NO_3^- + NH_4^+$ and NH_4^+ media and *hsn1* mutant plants in the $NO_3^- + NH_4^+$ medium in a time course up to 10 d carrying the enhancer traps *J3612::GFP* (a) and *SCRp::GFP* (b)

These results have been repeated in three separate experiments

(Fig. 3a). Further observation showed that QC cells were more distally positioned in WT seedlings in the NH_4^+ medium compared with seedlings in the $NO_3^- + NH_4^+$ medium at 4 DAG (Fig. 3a). Columella cells have starch granules, which can be stained by Lugol solution (Fukaki *et al*., 1998). The columella cells in WT seedlings in the $NO_3^- + NH_4^+$ and NH_4^+ media were examined using Lugol staining. Columella in WT seedlings in the $NO_3^- + NH_4^+$ medium showed a typical Lugol staining (Fig. 3b). In the NH_4^+ medium, Lugol staining signals of columella cells were decreased at 4 DAG (Fig. 3b). These results indicate that NH_4^+ can alter QC activity and root cap structure. To further confirm it, we examined the expression of *QC25::GUS* and Lugol staining in the *hsn1* mutant grown in the $NO_3^- + NH_4^+$ medium. At 10 DAG, no expression of *QC25::GUS* was detected in the *hsn1* mutant (Fig. 3a). Lugol staining signals of columella in the *hsn1* mutant in the $NO_3^- + NH_4^+$ medium were decreased at 4 DAG and finally disappeared at 10 DAG (Fig. 3b). These observations corroborate that NH_4^+ can alter QC activity and root cap structure, and then affect the organization and maintenance of the root meristem.

3.3 Disturbance of the root meristem organization and maintenance in the presence of NH_4^+ not likely related to auxin signaling, but cell death

It has been shown that the accumulation of auxin at the distal tip of root is required for the correct specification of cell division programs. Alteration of this auxin distal maximum or auxin transport produces defects in the cell fate and formation pattern (Sánchez-Calderón *et al*., 2005). To determine whether the NH_4^+ toxicity-mediated alteration in QC cell specification is related to changes of auxin, we visualized the auxin maximum in root tip using *DR5::GUS* report gene (Sabatini *et al*., 1999) and *IAA2::GUS* gene which expressed in the vasculature of the primary root and the root tip (Luschnig *et al*., 1998). The expression patterns of *DR5::GUS* and *IAA2::GUS* in WT seedlings grown in the $NO_3^- + NH_4^+$ medium were similar to the previous reports (Figs. 3c and 3d) (Luschnig *et al*., 1998; Sabatini *et al*., 1999). When grown in the NH_4^+ medium, the activities of *DR5::GUS* and *IAA2::GUS* were lessened at 4 DAG and not present at 10 DAG (Figs. 3c and 3d). Similar trends were also presented in NH_4^+-hypersensitive mutant *hsn1* even thought it was grown in the $NO_3^- + NH_4^+$ medium. To determine whether the activity or proper organization of the primary root meristem suffering from ammonium toxicities was due to lack of auxin, we tested the effects of exogenous application of auxin on WT grown in the NH_4^+ medium. As a result, it showed that the exogenous application of 3-indoleacetic acid (IAA) or 1-naphthylacetic acid (NAA) in the NH_4^+ medium could not alter the expression patterns of the markers (like *CYCB1;1::GUS*, *J3612::GFP*, *SCRp::GFP*, *QC25::GUS*, *DR5::GUS*, *IAA2::GUS*) and the staining pattern of starch granule in columella cells (data not shown). It suggested that the defects in meristem activities by NH_4^+ toxicity might not be due to the alteration of auxin signaling.

NH_4^+ was supposed to affect cell viability (Qin *et al*., 2008). We also employed fluorescein diacetate (FDA; Sigma, St. Louis, USA) to examine the root cell viability. This dye is retained by living cells but leaks from dead cells, rendering them nonfluorescent (Bais *et al*., 2003). When grown in the $NO_3^- + NH_4^+$ medium, the FDA fluorescence of WT primary root was relatively constant during 10 d (Fig. 4). While, in the NH_4^+ medium, the reductions of the FDA fluorescence in the mature zone and elongation zone were observed at 4 DAG, reflecting the loss of cell viability in these areas (Fig. 4). Subsequently, loss of cell viability in root meristem zone and root cap was detected at 6 and 8 DAG, respectively. Finally, the FDA fluorescence was lost in the whole root at 10 DAG (Fig. 4). A similar result was observed in the *hsn1* mutant grown in the $NO_3^- + NH_4^+$ medium, root cell viability in the different root zones was also lost in a temporal manner during the 10-d growth period (Fig. 4). These results suggested that loss of cell viability was also an important aspect of NH_4^+ toxicity inhibition of root growth.

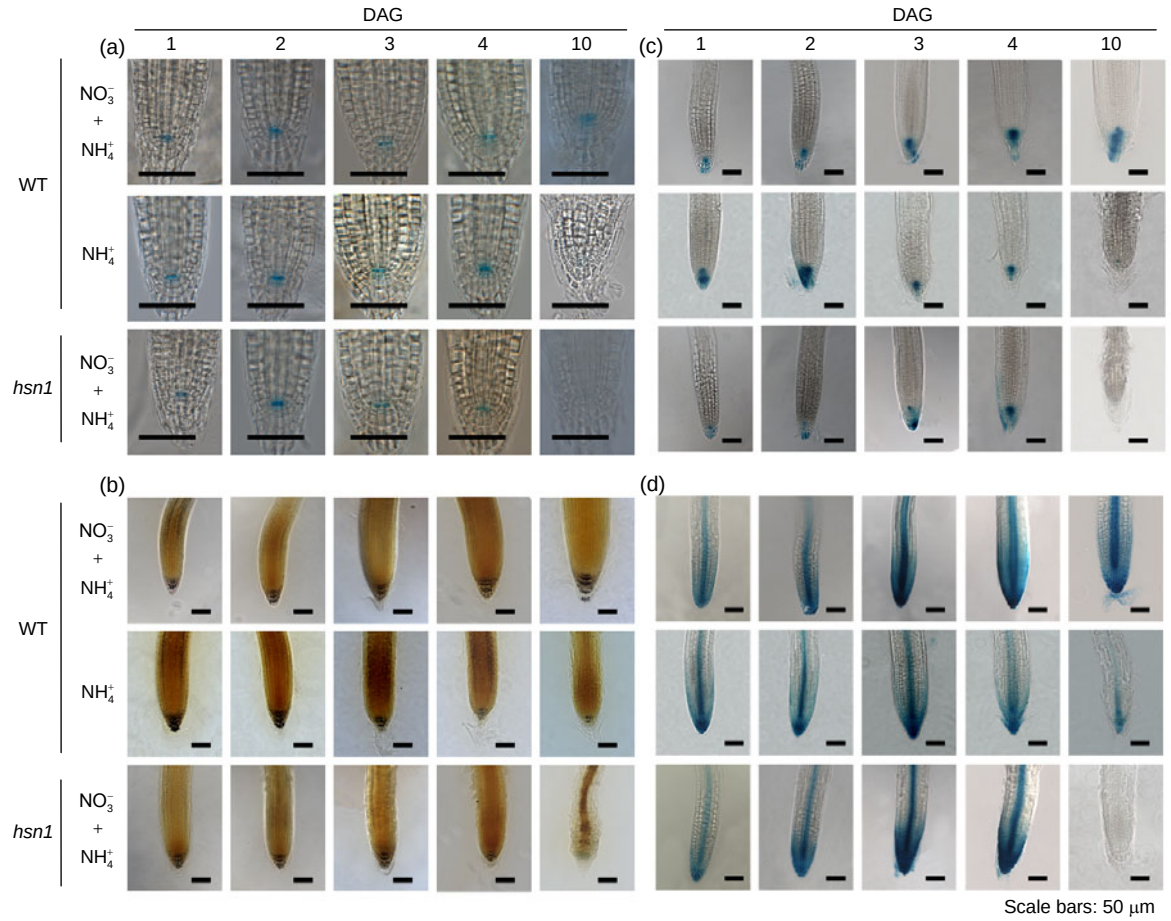

Fig. 3 *QC25::GUS* marker expression (a), staining of starch granule in columella cells (b), *DR5::GUS* marker expression (c), and *IAA2::GUS* marker expression (d) in primary roots of WT in the $NO_3^- + NH_4^+$ and NH_4^+ media and *hsn1* mutant plants in the $NO_3^- + NH_4^+$ medium in a time course up to 10 d

These results have been repeated in three separate experiments

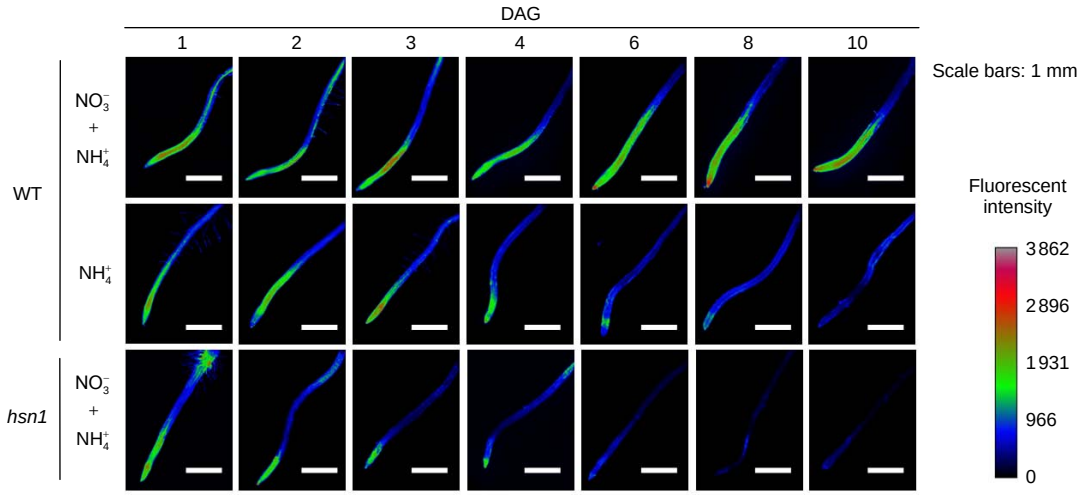

Fig. 4 Analyses of cell death in the roots of WT in the $NO_3^- + NH_4^+$ and NH_4^+ media and *hsn1* mutant plants in the $NO_3^- + NH_4^+$ medium in a time course up to 10 d

Cell death was analyzed by FDA staining. This dye is retained by living cells but leaks from dead cells, rendering them non-fluorescent. These results have been repeated in three separate experiments

4 Discussion

Although ammonium is one of the most important nutrients for plants, ammonium toxicity has been linked to the damage to agricultural crops, especially in areas with intensive agriculture. To understand the molecular and cellular mechanisms of plant response to NH_4^+ toxicity is required to improve crops with higher tolerance to NH_4^+ and consequently higher nitrogen use efficiency.

One character of ammonium syndrome is inhibition of root growth when plants are exposed to higher NH_4^+ concentration. High levels of NH_4^+ have toxic effects on plant cells (Marschner, 1995). Here, we demonstrate that the reduction in root length of plants induced by NH_4^+ toxicity is because of both reduction in the size of meristem zone and mature cell length. Furthermore, NH_4^+ can disturb the meristem patterning and maintenance in the root. Our data indicate that NH_4^+ affects seedlings from early in their development after its entry into root cells and the effect of NH_4^+ on root cells is through the progress from mature zone to root cap, which is supported by the observation of inhibition of cell length before 3 DAG, the repression of root meristem activity at 3 DAG, and the damage to the root cap after 4 DAG (Figs. 1a, 1b and 3b). Loss of cell viability in the different root zones determined by FDA staining further supports the temporal mode of response of root cells to NH_4^+ toxicity from mature zone to root cap (Fig. 4).

Most higher plants develop severe toxicity symptoms when grown on ammonium as the sole nitrogen source, and it can be alleviated by co-provision of nitrate (Britto and Kronzucker, 2002). WT seedlings had a toxic symptom when grown in the NH_4^+ medium, and had a normal phenotype when grown in the $NO_3^- + NH_4^+$ medium (Figs. 1–4). To further determine that the effects in WT grown in the NH_4^+ medium were actually due to the presence of NH_4^+, rather than a lack of NO_3^-, we examined root growth/marker line expressions of WT and *hsn1* when grown in the NO_3^- medium (using NO_3^- as the sole nitrogen source). When WT and *hsn1* seedlings were grown in the NO_3^- medium, their root growth/marker line expressions were similar as WT seedlings grown in the $NO_3^- + NH_4^+$ medium (Figs. S1–S4). The repression of WT seedlings in the NH_4^+ medium was due to ammonium toxicity, and the inhibition of cell elongation in the *hsn1* mutant in the $NO_3^- + NH_4^+$ medium was actually due to the NH_4^+, and not a general phenotype of the mutant.

Plant hormones, such as auxin, control most of the characteristics of root systems, including primary root growth. It is suggested that root responses to nutrients may originate from hormonal signals that are triggered by specific nutrient pathways. In this study, we found that the reduction of primary root length in NH_4^+ toxicity was also caused by a reduction in auxin levels in the meristem, as visualized by the expressions of *DR5::GUS* and *IAA2::GUS* (Figs. 3c and 3d). However, the exogenous application of IAA or NAA in the NH_4^+ medium could not alter the expression patterns of the markers (like *CYCB1;1::GUS*, *J3612::GFP*, *SCRp::GFP*, *QC25::GUS*, *DR5::GUS*, *IAA2::GUS*) and the staining patterns of starch granules in columella cells as described above. To determine whether the defects in meristem activities in NH_4^+ toxicity were due to the alteration of auxin signaling or not, further experiments should be performed.

Recent information showed that nutrient-specific signal transduction pathways exist in the root development. Some genes that involved in the nitrate- and phosphate-signaling pathways have been identified (Zhang and Forde, 1998; Rubio *et al*., 2001; Miura *et al*., 2005). GMPase, the only component discovered recently, is involved in hypersensitive response to ammonium (Qin *et al*., 2008). The insights gained in this work may offer clues to aid in the understanding of the mechanism of NH_4^+ toxicity among different plant families and species. Unraveling the mechanisms of nutrient-induced regulation of root architecture will enable us to increase the crop yield by manipulating the root architecture in field with unevenly distributed nutrients.

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List of electronic supplementary materials

Fig. S1 *CYCB1;1::GUS* marker expression in primary roots of WT and *hsn1* mutant plants in NO_3^- medium in a time course up to 10 d. These results have been repeated in three separate experiments

Fig. S2 GFP expression in primary roots of WT and *hsn1* mutant plants in NO_3^- medium in a time course up to 10 d carrying the enhancer traps *J3612::GFP* (a) and *SCRp::GFP* (b). These results have been repeated in three separate experiments

Fig. S3 *QC25::GUS* marker expression (a), staining of starch granule in columella cells (b), *DR5::GUS* marker expression (c), and *IAA2::GUS* marker expression (d) in primary roots of WT and *hsn1* mutant plants in NO_3^- medium in a time course up to 10 d. These results have been repeated in three separate experiments

Fig. S4 Analysis of cell death in the roots of WT and *hsn1* mutant plants in NO_3^- medium in a time course up to 10 d. These results have been repeated in three separate experiments