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RESEARCH PAPER

A mutation in GDP-mannose pyrophosphorylase causes conditional hypersensitivity to ammonium, resulting in *Arabidopsis* root growth inhibition, altered ammonium metabolism, and hormone homeostasis

Carina Barth*, Zachary A. Gouzd, Hilary P. Steele and Ryan M. Imperio

West Virginia University, Department of Biology, 5228 Life Sciences Building, 53 Campus Drive, PO Box 6057, Morgantown, WV 26506-6507, USA

* To whom correspondence should be addressed: E-mail: carina.barth@mail.wvu.edu

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Abstract

Ascorbic acid (AA) is an antioxidant fulfilling a multitude of cellular functions. Given its pivotal role in maintaining the rate of cell growth and division in the quiescent centre of the root, it was hypothesized that the AA-deficient *Arabidopsis thaliana* mutants *vtc1-1*, *vtc2-1*, *vtc3-1,* and *vtc4-1* have altered root growth. To test this hypothesis, root development was studied in the wild type and *vtc* mutants grown on Murashige and Skoog medium. It was discovered, however, that only the *vtc1-1* mutant has strongly retarded root growth, while the other *vtc* mutants exhibit a wild-type root phenotype. It is demonstrated that the short-root phenotype in *vtc1-1* is independent of AA deficiency and oxidative stress. Instead, *vtc1-1* is conditionally hypersensitive to ammonium (NH_4^+). To provide new insights into the mechanism of NH_4^+ sensitivity in *vtc1-1*, root development, NH_4^+ content, glutamine synthetase (GS) activity, glutamate dehydrogenase activity, and glutamine content were assessed in wild-type and *vtc1-1* mutant plants grown in the presence and absence of high NH_4^+ and the GS inhibitor MSO. Since *VTC1* encodes a GDP-mannose pyrophosphorylase, an enzyme generating GDP-mannose for AA biosynthesis and protein N-glycosylation, it was also tested whether protein N-glycosylation is affected in *vtc1-1*. Furthermore, since root development requires the action of a variety of hormones, it was investigated whether hormone homeostasis is linked to NH_4^+ sensitivity in *vtc1-1*. Our data suggest that NH_4^+ hypersensitivity in *vtc1-1* is caused by disturbed N-glycosylation and that it is associated with auxin and ethylene homeostasis and/or nitric oxide signalling.

Key words: Ammonium (NH_4^+), *Arabidopsis thaliana*, ascorbic acid (AA), auxin (IAA), GDP-mannose pyrophosphorylase (GMPase), glutamine synthetase (GS), N-glycosylation, root growth.

Introduction

Vitamin C (L-ascorbic acid, AA) is an essential antioxidant in plants and animals. It protects against oxidative stress caused by adverse environmental conditions and functions in various aspects of plant cell growth (Cordoba and Gonzalez-Reyes, 1994). Most notably, AA plays a pivotal role in maintaining the rate of cell growth and division in the quiescent centre of the root (Kerk and Feldman, 1995), in the transition from the G_1 to the S phase in the cell cycle. Actively dividing root meristem cells have high levels of AA and glutathione compared to cells in the quiescent zone. Exogenous application of AA and glutathione stimulates cell division and growth (Kerk and Feldman, 1995). Furthermore, redox activity (Sanchez-Fernandez *et al.*, 1997) and the formation of reactive oxygen species (Shin and Schachtman, 2004) are known to influence root elongation through cell proliferation in response to nutrient alterations.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; cPTIO, 2-(4-carboxyphenyl)4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; GDH, glutamate dehydrogenase; Gln, glutamine; MSO, methionine sulphoximine (MSO); MS, Murashige and Skoog; NO, nitric oxide; SNP, sodium nitroprusside.

To investigate the physiological role of AA in root development, advantage was taken of the availability of AA-deficient *Arabidopsis thaliana vtc* mutants. Five *vtc* mutant lines, *vtc1*, *vtc2*, *vtc3*, *vtc4*, and *vtc5* have been isolated so far (Conklin *et al.*, 2000; Dowdle *et al.*, 2007). Except for *vtc3*, whose genetic defect is still unknown, the *vtc* mutants were shown to contain mutations in genes encoding enzymes in the predominant L-galactose/D-mannose AA biosynthetic pathway (Fig. 1; Wheeler *et al.*, 1998). The *VTC1* gene encodes a GDP-mannose pyrophosphorylase (GMPase; Conklin *et al.*, 1999). *VTC2* and *VTC5* encode a GDP-L-galactose phosphorylase (Dowdle *et al.*, 2007), while *VTC2* also has GDP-L-galactose orthophosphate guanylyltransferase activity (Linster *et al.*, 2007, 2008; Laing *et al.*, 2007). *VTC4* encodes an L-galactose-1-phosphate phosphatase (Laing *et al.*, 2004; Conklin *et al.*, 2006). The *vtc* mutants contain between 25% and 50% of the wild-type leaf AA content (Conklin, 2001).

Given the pivotal function of AA in root development, we hypothesized that AA-deficient mutants have altered root growth. To test this hypothesis, the wild type and *vtc* mutants were grown on full-strength Murashige and Skoog (MS) medium and it was discovered that only the *vtc1-1* mutant has strongly retarded root growth, while the other *vtc* mutants exhibited a root developmental phenotype similar to the wild type. Thus, the goal of this study was to elucidate the mechanism through which root growth is inhibited in *vtc1-1*. Interestingly, our investigations demonstrate that *vtc1-1* is conditionally hypersensitive to ammonium (NH_4^+) and that the short-root phenotype in the mutant is independent of AA deficiency and the accumulation of H_2O_2. Therefore, we focused on investigating NH_4^+ metabolism and hormone homeostasis in *vtc1-1* to better understand root growth inhibition by NH_4^+ in *vtc1-1*.

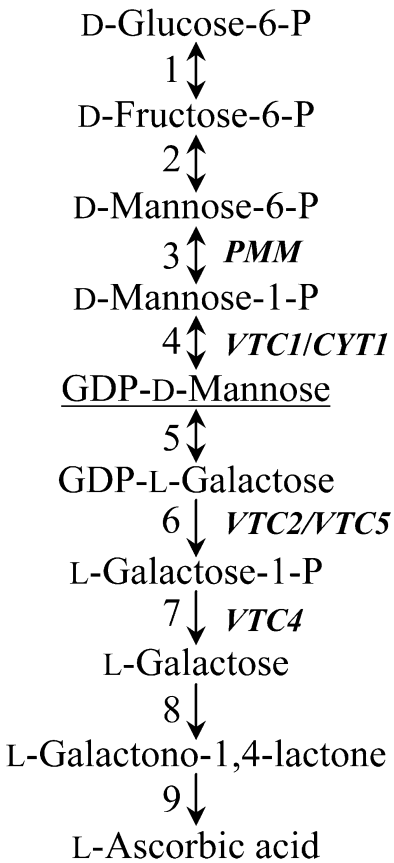

Fig. 1. Simplified representation of the D-mannose/L-galactose L-ascorbic acid biosynthetic pathway in higher plants. Enzymes are (1) phosphoglucose isomerase, (2) phosphomannose isomerase (3), phosphomannose mutase (*PMM*), (4) GDP-mannose pyrophosphorylase (*VTC1*), (5) GDP-mannose-3′,5′-epimerase, (6) GDP-L-galactose phosphorylase (*VTC2/VTC5*), (7) L-galactose-1-phosphate phosphatase (*VTC4*), (8) L-galactose dehydrogenase, (9) L-galactono-1,4-lactone dehydrogenase. (Adapted from Dowdle *et al.*, 2007.)

NH_4^+ and nitrate are the major sources of nitrogen for plants. NH_4^+ is the preferred nitrogen source with concentrations ranging from 20–200 μM in agricultural soils (Loque and von Wiren, 2004; Miller and Cramer, 2004). It is assimilated into essential amino acids. However, excess amounts of NH_4^+ may be toxic and inhibit root growth (Cao *et al.*, 1993; Britto and Kronzucker, 2002; Cruz *et al.*, 2006). In order to understand the mechanism of NH_4^+ toxicity in plants, several aspects of NH_4^+ uptake, transport, metabolism, and interactions with plant hormones have been studied, particularly with respect to root development.

NH_4^+ is directly taken up from the soil at the root surface and hairs of the rhizodermis and immediately transferred into the root symplast for NH_4^+ assimilation by rhizodermis-localized glutamine synthetase (Ishiyama *et al.*, 2004). Non-assimilated NH_4^+ might be stored in the vacuole (Miller *et al.*, 2001) or further transported via the symplast into the root stele for assimilation or loading into the xylem where it may accumulate in millimolar concentrations (Yuan *et al.*, 2007). The Casparian strip forms an apoplastic barrier, which favours a local accumulation of NH_4^+ in the endodermal apoplast (Marschner, 1995).

Transport of NH_4^+ is mediated by AMT-type transporters (Gazzarrini *et al.*, 1999; Ludewig *et al.*, 2001; Yuan *et al.*, 2007). When nitrogen content is high, *AMT* genes are transcriptionally down-regulated, whereas they are up-regulated under deficiency conditions (Rawat *et al.*, 1999). NH_4^+ uptake may also occur through high- or low-affinity transport systems (Rawat *et al.*, 1999). To minimize NH_4^+ toxicity, NH_4^+-sensitive species have been reported to actively pump out NH_4^+ (Kronzucker *et al.*, 2001).

Once inside the cell, NH_4^+ is metabolized by glutamine synthetase (GS) into glutamine (Gln), which is further metabolized by glutamate synthase (GOGAT) that converts Gln and 2-oxoglutarate into two molecules of glutamate. NH_4^+ may also be assimilated by glutamate dehydrogenase (GDH) that catalyses the formation of glutamate through reductive amination using NAD(P)H. Therefore, various studies have investigated the role of GS, GOGAT, and GDH in response to NH_4^+ nutrition. Plants with high GS activities are more tolerant to NH_4^+ (Magalhaes *et al.*, 1992; Glevarec *et al.*, 2004). *Arabidopsis* GS isoforms have different kinetic properties and are differentially regulated by NH_4^+

(Ishiyama *et al.*, 2004). The role of GDH in NH_4^+ metabolism is still a matter of debate, because GDH has low affinity to NH_4^+ (Harrison *et al.*, 2000). Its main role is thought to recycle glutamate (Robinson, 2001) and to return carbon in amino acids back into reactions of carbon metabolism and the tricarboxylic acid cycle (Miflin and Habash, 2002). In fact, proper C/N balance and sufficient carbohydrates in the roots have been suggested to play an important role in preventing NH_4^+ toxicity (Oliveira and Coruzzi, 1999; Schjoerring *et al.*, 2002).

Multiple studies suggest that nutrient signalling is intertwined with plant hormones (Feng and Barker, 1992; Cao *et al.*, 1993; Barker, 1999*b*; Rubio *et al.*, 2009). With regard to NH_4^+ nutrition, IAA (Cao *et al.*, 1993; Sattelmacher and Thoms, 1995), cytokinin (Cao *et al.*, 1993; Gerendas *et al.*, 1997), and ethylene (Feng and Barker, 1992; Barker, 1999*a*) have been shown to be linked to NH_4^+-induced alterations in growth and development. The work by Cao *et al.* (1993) not only demonstrated that NH_4^+ inhibition of *Arabidopsis* root growth can be reversed by auxin resistance mutations, but can also be rescued by the addition of potassium.

In the present study, evidence is provided that the genetic defect in *vtc1-1*, causing defective protein N-glycosylation, results in pleiotropic alterations in NH_4^+ metabolism, hormone homeostasis, and nitric oxide content.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana L. Heynh wild-type ecotype Columbia (Col-0) and the previously described *Arabidopsis* mutants *vtc1-1*, *vtc2-1*, *vtc3-1*, and *vtc4-1* (Conklin *et al.*, 2000; mutant seeds kindly provided by Dr Patricia Conklin) were grown in a growth chamber (Percival, Perry, IA). Surface-sterilized seeds (see below) were grown on full-strength (1×) Murashige and Skoog (MS) or variations of the MS medium (Caisson Laboratories, Inc., North Logan, UT). All growth media contained 1% phytoblend agar (Caisson Laboratories, Inc.) and were adjusted to a pH of 5.7 with KOH before autoclaving. None of the growth media contained sucrose. Seeds were germinated in 120×80 mm omni trays (Fisher Scientific, Pittsburgh, PA), which were filled with 30 ml of medium and sealed with parafilm. Trays were placed vertically for 7 d or 14 d under long-day (16/8 h light/dark) conditions. Light intensity was 160 μmol photons m^{-2} s^{-1} (fluorescent bulbs) and the temperature in the chamber was 23 °C day and night.

To assess root growth in the soil, wild-type and *vtc* mutant plants were grown on Metromix 360 soil (BFG Supplies, Burton, OH) in flats containing 32 inserts. Otherwise, growth conditions were the same as above.

Seed-surface sterilization

Seeds were soaked for 1 min in 50% ethanol, followed by washing the seeds in 50% bleach plus 0.01% sodium dodecyl sulphate for 8 min. Finally, seeds were rinsed six times with sterile water and stored in 0.1% sterile phytoblend agar for 2 d at 4 °C (Weigel and Glazebrook, 2002).

Root length measurements

Seven days or 14 d after germination, primary root length was measured using a ruler.

Ascorbic acid content assay

The AA content was determined using the AA oxidase assay as described previously (Conklin *et al.*, 1997).

Hydrogen peroxide content assay

Hydrogen peroxide production in whole seedlings of the wild type and *vtc* mutants (50–100 mg fresh tissue) was measured following a previously described protocol (Shin and Schachtman, 2004) using the Amplex red hydrogen peroxide/peroxidase assay kit (Invitrogen, Carlsbad, CA).

Nitric oxide content assay

Using liquid nitrogen, 20–50 mg of 7-d-old whole seedlings were ground in a Harbil paint shaker (Midwest Paint Equipment, Elwood, KS) and resuspended in 500 μl extraction buffer (0.1 mM $CaCl_2$, 10 mM KCl, and 10 mM MES at a pH of 5.6). Samples were centrifuged at 4 °C at 10 000 rpm for 10 min. The supernatant was collected and used for the GRIESS assay according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) with the following modifications: Equal volumes of Component A and Component B from the GRIESS reagent kit were mixed. To a 150 μl nitric oxide-containing sample, 130 μl of sterile water, and 20 μl of the GRIESS reagent (Component A and Component B) were added and mixed. The reaction mixture was incubated for 30 min at room temperature. Absorbance of each sample was recorded at 548 nm. The nitric oxide content was determined based on a standard curve of known sodium nitrite concentrations and normalized to the amount of fresh weight.

Ammonium and glutamine content assay

NH_4^+ content was determined in whole seedlings crushed in liquid nitrogen and tissue was extracted in 500 μl water or the extraction buffer used for the NO assay. Fifty microlitres of the supernatant were added to 50 μl of Nessler reagent. Absorbance was measured at 404 nm (Leleu and Vuylsteker, 2004). Concentration of NH_4^+ was determined using an NH_4Cl standard curve and normalized to gram fresh weight of the sample. Glutamine content was determined using the L-asparagine, L-glutamine, ammonia Rapid Kit from Megazyme (Megazyme International Ireland Ltd, Co. Wicklow, Ireland).

Indole-3-acetic acid content assay

The IAA content was determined in whole seedlings grown on 1× MS and 1× MS lacking NH_4NO_3. Extraction of IAA from plant tissue was performed using 80% methanol as previously described (Tanaka *et al.*, 2001). Methylated IAA was quantified using the Phytodetek IAA kit following the manufacturer's protocol (Agdia, Elkhart, IN).

Gutamine synthetase activity assay

Total glutamine synthetase activity was determined following a spectrophotometric assay (Mäck, 1995; Hirano *et al.*, 2008). In brief, 30–60 mg tissue was extracted using 500 μl of an imidazole buffer consisting of 50 mM imidazole-HCl, 0.5 mM EDTA, 1 mM DTT, 10 mM $MgSO_4$, and 1 mM phenylmethanesulphonyl fluoride. Fifty-five microlitres of sample were used in the enzyme reaction containing 20 μl of each of the following reagents: 80 mM imidazole-HCl, 8 mM $MgSO_4$, 160 mM hydroxylamine-HCl, 4 mM ATP, and 8 mM glutamate. The reaction was allowed to run for 15 min at 37 °C and was terminated after 15 min by adding 45 μl of a termination buffer (670 mM HCl, 88 mM $FeCl_3$, and 200 mM trichloracetic acid). A glutamine synthetase activity standard curve was prepared using the reaction end-product γ-glutamyl hydroxamate. Standards and samples were measured at both 498 nm and 540 nm with similar results.

Glutamate dehydrogenase activity assay

Enzyme activity was determined from the extract used for measuring GS activity. Aminating-GDH activity was assayed as previously described (Groat and Vance, 1981).

Exogenous application of pharmacological compounds

Growth media were supplemented with methione sulphoximine (MSO, a GS inhibitor), sodium nitroprusside (SNP, an NO donor), 2-(4-carboxyphenyl)4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO, an NO inhibitor), indole-3-acetic acid (IAA), 1-amino-cyclopropane-1-carboxylate (ACC, an ethylene precursor), tunicamycin (an inhibitor of N-glycosylation), D-mannose, GDP-D-mannose, or L-galactose. The final concentration of these compounds in the growth media is indicated in the figures.

Generation and identification of double mutants

The *vtc1-1* mutant was crossed with the salicylic acid (SA)-deficient mutants *pad4-1* and *eds5-1*, respectively (Weigel and Glazebrook, 2002). The F_1 progeny of these crosses were allowed to grow and self-fertilize. The F_2 progeny were screened for AA deficiency (Conklin *et al.*, 2000). Genomic DNA was extracted from AA-deficient individuals, PCR products spanning the two respective mutations were generated, purified, and analysed by sequencing to verify the presence of the mutations. F_3 and F_4 plants of homozygous double mutants were used for experimental analyses.

RNA isolation, cDNA synthesis, and NOS gene expression analysis

Total RNA was extracted from whole seedlings using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Five micrograms of total RNA were subjected to reverse transcription using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) and 10 pg of oligo dT primers. Two micrograms of cDNA were utilized for PCR using gene-specific primers for genes encoding nitric oxide synthase and *TUBULIN*, which served as an internal control, running 25 amplification cycles (linear range of amplification). PCR fragments were separated on 1% agarose gels containing ethidium bromide. Band intensities were quantified using ImageQuant 5.0 (Amersham Biosciences). Primer sequences were 5′-TCTGGGGAGGTCTCGT-TAGGATTG-3′ (NOS-F), 5′-CTCGTGTTTTGCGGATTGGT-TCA-3′ (NOS-R), 5′-CTCAAGAGGTTCTCAGCAGTA-3′ (TUB2-RT-F2), and 5′-TCACCTTCTTCATCCGCAGTT-3′ (TUB2-RT-R2).

Statistical analysis

Experiments were performed at least three times. Figures represent individual experiments. Data were expressed as mean values ±SE. *P* values were determined by Student's *t* test analysis.

Results

Among four different vtc *mutant lines, only* vtc1-1 *has retarded root growth*

The genetic defect in the *vtc* mutants causes AA-deficiency to a similar degree in roots and to varying degrees in shoots. All *vtc* mutants contained approximately 45% of the wild-type root AA content, whereas the AA content of shoots was approximately 30–50% of that of the wild type (Fig. 2A). At this early developmental stage, H_2O_2 content was similar in whole seedlings of the wild type and *vtc* mutants (Fig. 2B). Similar results were reported previously (Kotchoni *et al.*, 2009). Nevertheless, it was predicted that root growth would be inhibited in the *vtc* mutants given the function of AA in the root apical meristem (Kerk and Feldman, 1994). However, root growth on full-strength MS medium (without sucrose) was only affected in *vtc1-1*, while the *vtc2-1*, *vtc3-1*, and *vtc4-1* mutants exhibited a root developmental phenotype similar to the wild type. The *vtc1-1* mutants had four times shorter primary roots than the wild type and the other *vtc* mutants when plants were 7 d old (Fig. 2C). Additional developmental defects became apparent in 14-d-old seedlings. Whereas formation of lateral roots was normal in the *vtc2-1*, *vtc3-1*, and *vtc4-1* mutants compared to the wild type, *vtc1-1* mutants initiated lateral root primordia whose elongation was strongly retarded. Furthermore, *vtc1-1* mutants initiated adventitious roots at the hypocotyl–root transition zone, which were not observed in the other genotypes (Fig. 2D, E). When wild-type and *vtc1-1* mutant plants were germinated on 1× MS in darkness, root development was similar in both genotypes (Fig. 2F). Finally, root development was unaffected in *vtc1-1* when plants were grown on soil (Fig. 2G).

Collectively, these data suggest that (i) inhibition of root development in *vtc1-1* is not caused by AA-deficiency or oxidative stress; (ii) the root developmental defects in *vtc1-1* are due to one or more nutrients present in the full-strength MS medium; and (iii) the short-root phenotype in *vtc1-1* is light-dependent.

vtc1-1 *is hypersensitive to* NH_4^+

To determine whether the concentration of a nutrient in the MS medium could alter root growth in *vtc1-1*, wild-type and *vtc1-1* mutant plants were grown on increasing strength of MS medium and primary root length was measured. In the absence of any nutrients (i.e. seedlings grown on agar), primary root length was the same in the wild type and *vtc1-1*. With increasing concentrations of nutrients, root elongation was strongly inhibited in *vtc1-1* in a dose-dependent manner, whereas root growth was only slightly affected in the wild type and the other three *vtc* mutants (Fig. 3A; see Supplementary Fig. S1 at *JXB* online). This result confirms that one or more nutrients has or have an inhibitory effect on root development in *vtc1-1* when present at high concentrations.

To identify this/these nutrient/s, wild type and *vtc1-1* mutants were germinated on MS media lacking key nutrients. In the absence of phosphorous (P), roots of *vtc1-1* mutants were still approximately five times shorter than those of the wild type (Fig. 3B). A similar difference was observed in full-strength 1× MS medium (cf. Fig. 2C), suggesting that the *vtc1-1* short-root phenotype is independent of P nutrition. When seedlings were germinated in the absence of NH_4^+ (i.e., no NH_4NO_3, but KNO_3 still present) or when seedlings were grown in the absence of all nitrogen (–N, i.e., the medium contains neither NH_4NO_3 nor KNO_3), root length was similar in the wild type and *vtc1-1* (Fig. 3B). These data suggest that the presence of a high concentration of NH_4^+ in 1× MS has an inhibitory effect on root development in *vtc1-1* mutants.

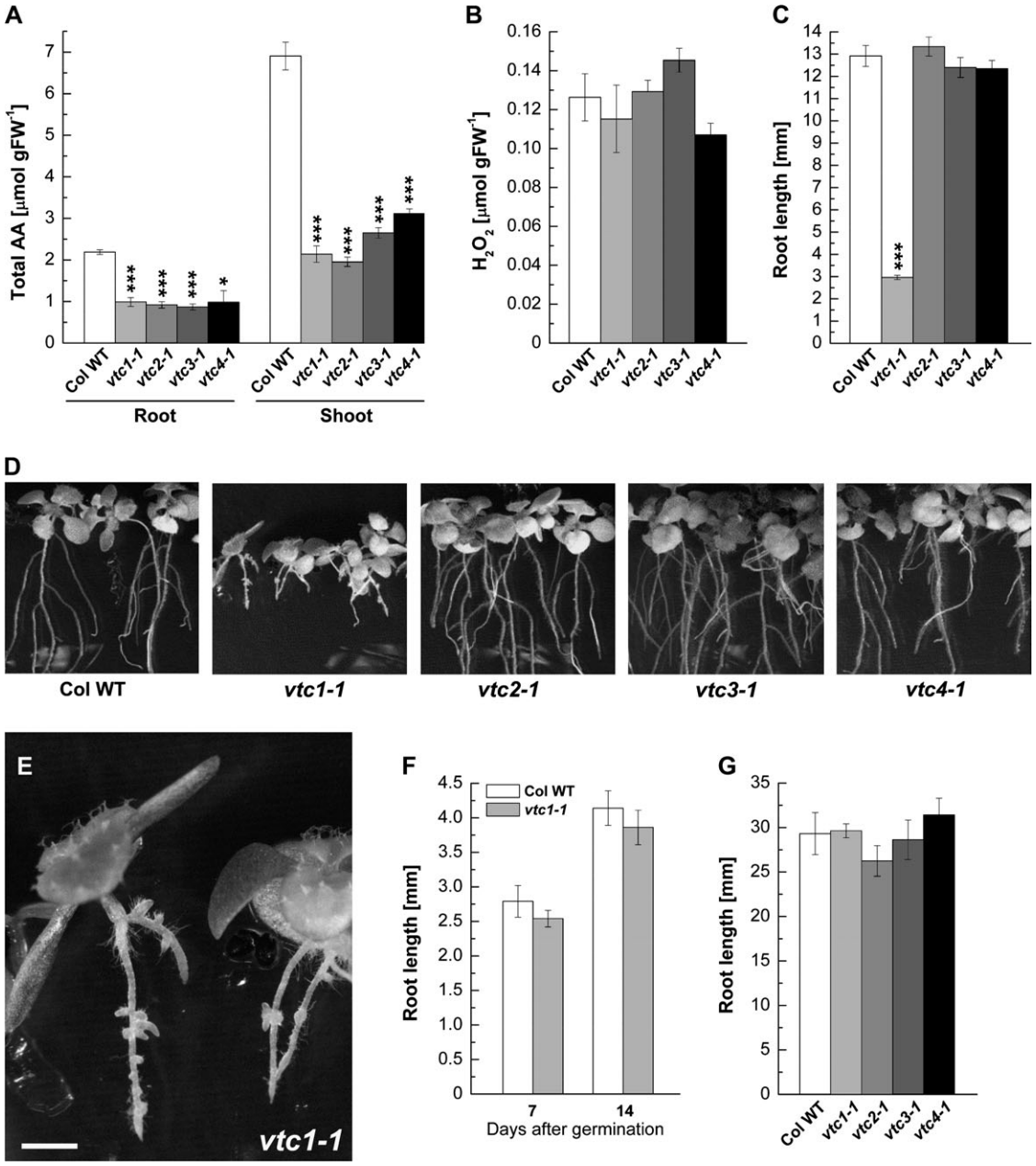

Fig. 2. Physiological characterization of the wild type and *vtc* mutants. (A) Ascorbic acid content in root and shoot tissue of 7-d-old seedlings grown on 1× MS. Mean values ±SE of three independent replicates are shown. (B) H_2O_2 content in whole 7-d-old seedlings of the wild type and *vtc* mutants. Results illustrate means ±SE of three independent replicates per genotype. (C) Primary root length in 7-d-old seedlings germinated on 1× MS. Data represent means ±SE of 53–70 replicates. (D) Phenotype of 14-d-old wild type and *vtc* mutant seedlings grown on 1× MS. (E) Close-up of the *vtc1-1* root developmental phenotype. Bar, 1 mm. (F) Primary root length of wild-type and *vtc1-1* mutant plants germinated on 1× MS medium in darkness. Data represent means ±SE of 21 replicates of the wild type and 24 replicates of *vtc1-1*. (G) Primary root length in 7-d-old wild-type and *vtc* mutant plants grown on soil. Means ±SE of 5–8 replicates are shown. Asterisks indicate significant differences between individual mutants and the wild type. **P* <0.05, ****P* <0.001, Student's *t* test.

To further support that *vtc1-1* is hypersensitive to NH_4^+, wild-type and *vtc1-1* mutant plants were germinated on 1× MS medium lacking NH_4^+ (i.e., no NH_4NO_3, but KNO_3 present), increasing concentrations of NH_4Cl were added, and root length was determined. With increasing concentrations of NH_4^+, root elongation was strongly inhibited in *vtc1-1*, and to a lesser degree in the wild type (Fig. 3C). Inhibition of primary root growth was similar in the presence of 20.6 mM NH_4Cl, the concentration of NH_4^+ that is present in the full-strength MS medium used here. These data indicate that the effect of NH_4^+ in *vtc1-1* is dose-dependent, an effect that has been reported previously in other plant species (Lasa *et al.*, 2002; Dominguez-Valdivia *et al.*, 2008). To ensure that the inhibitory effect on root

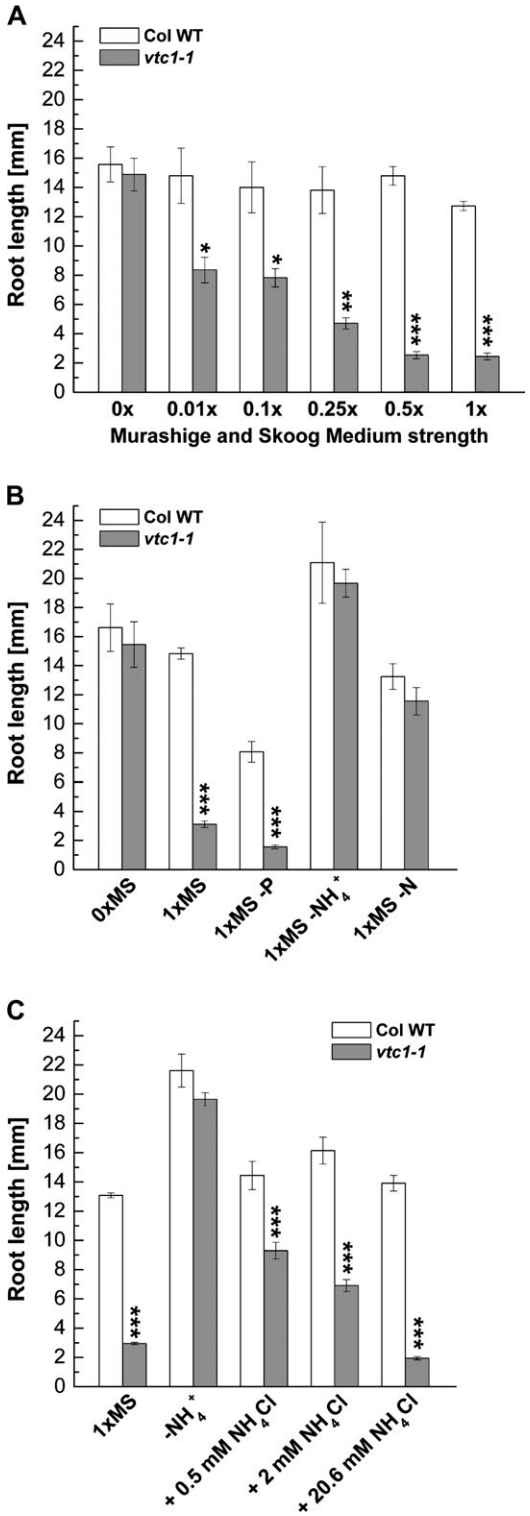

Fig. 3. Effect of various MS media compositions on primary root development in 7-d-old wild type and *vtc1-1*. (A) Primary root length when plants were grown on increasing strength of MS medium. Results illustrate means ±SE of 9–23 individual seedlings per genotype and treatment. (B) Primary root length of plants grown in the absence of phosphorous (–P), ammonium nitrate ($-NH_4^+$, with potassium nitrate still present) and in the absence of all nitrogen (–N, i.e. no potassium nitrate and no ammonium nitrate). Data represent means ±SE of 6–18 replicates. (C) Effect of increasing concentrations of ammonium chloride (NH_4Cl) on root growth in plants grown on 1× MS medium lacking ammonium nitrate ($-NH_4^+$), but potassium nitrate still present. Mean values ±SE of 44–102 individual seedlings per genotype and treatment are shown. Asterisks indicate significant differences between mutant and wild type. **P* <0.05, ***P* <0.01, ****P* <0.001, Student's *t* test.

development in *vtc1-1* was not caused by an increased concentration of Cl^- ions in the medium, a control experiment was performed in which wild-type and *vtc1-1* mutant seedlings were grown in the absence of NH_4^+ and in the presence of increasing KCl concentrations. As expected, root development was not inhibited in the presence of high amounts of KCl (see Supplementary Fig. S2 at *JXB* online).

Finally, the effect of high amounts of NH_4^+ added to 1× MS devoid of all N was investigated. Increasing concentrations of NH_4^+ have an inhibitory effect on root growth in both the wild type and *vtc1-1*, although root growth was slightly more inhibited in the *vtc1-1* mutant than in the wild type at higher concentrations (see Supplementary Fig. S3 at *JXB* online). Therefore, all experiments described in the following were carried out using media lacking NH_4^+, but containing KNO_3. This did not alter root development in the wild type, allowing for better comparisons with *vtc1-1* primary root development.

NH_4^+ content is not altered in vtc1-1*, but glutamine synthetase activity and glutamine content are decreased*

To understand the mechanism of the NH_4^+ hypersensitivity of *vtc1-1*, transcript levels of NH_4^+ transporters were examined, and NH_4^+ content and metabolism in the wild type and *vtc1-1* were assessed. Wild type and *vtc1-1* did not differ significantly in mRNA levels of the NH_4^+ transporters *AMT1;1* and *AMT2;1* when plants were grown in the presence or absence of NH_4^+ (data not shown). This was expected, because at higher concentrations (>1 mM), NH_4^+ transport is passive (Ullrich *et al.*, 1984; Wang *et al.*, 1993).

Once inside the cell, NH_4^+ is metabolized by GS and GDH. GS activity is inhibited by methionine sulphoximine (MSO), resulting in elevated NH_4^+ and reduced Gln levels (Lee *et al.*, 1992; King *et al.*, 1993; Hirano *et al.*, 2008). This inhibitor was used to test whether NH_4^+ metabolism is altered in *vtc1-1* compared to the wild type when plants were grown in the presence or absence of NH_4^+. As expected, in the presence of MSO, root growth was inhibited approximately 2-fold in the wild type, whereas root growth was not further impaired in *vtc1-1* (Fig. 4A). In the absence of NH_4^+, MSO equally inhibited root growth in both genotypes (Fig. 4E). Whole seedling NH_4^+ content was similar in the wild type and *vtc1-1* when seedlings were germinated on 1× MS. In the presence of MSO, NH_4^+ content doubled in both genotypes, as expected (Fig. 4B). A similar result was found when plants were grown in the absence of NH_4^+ (Fig. 4F). GS

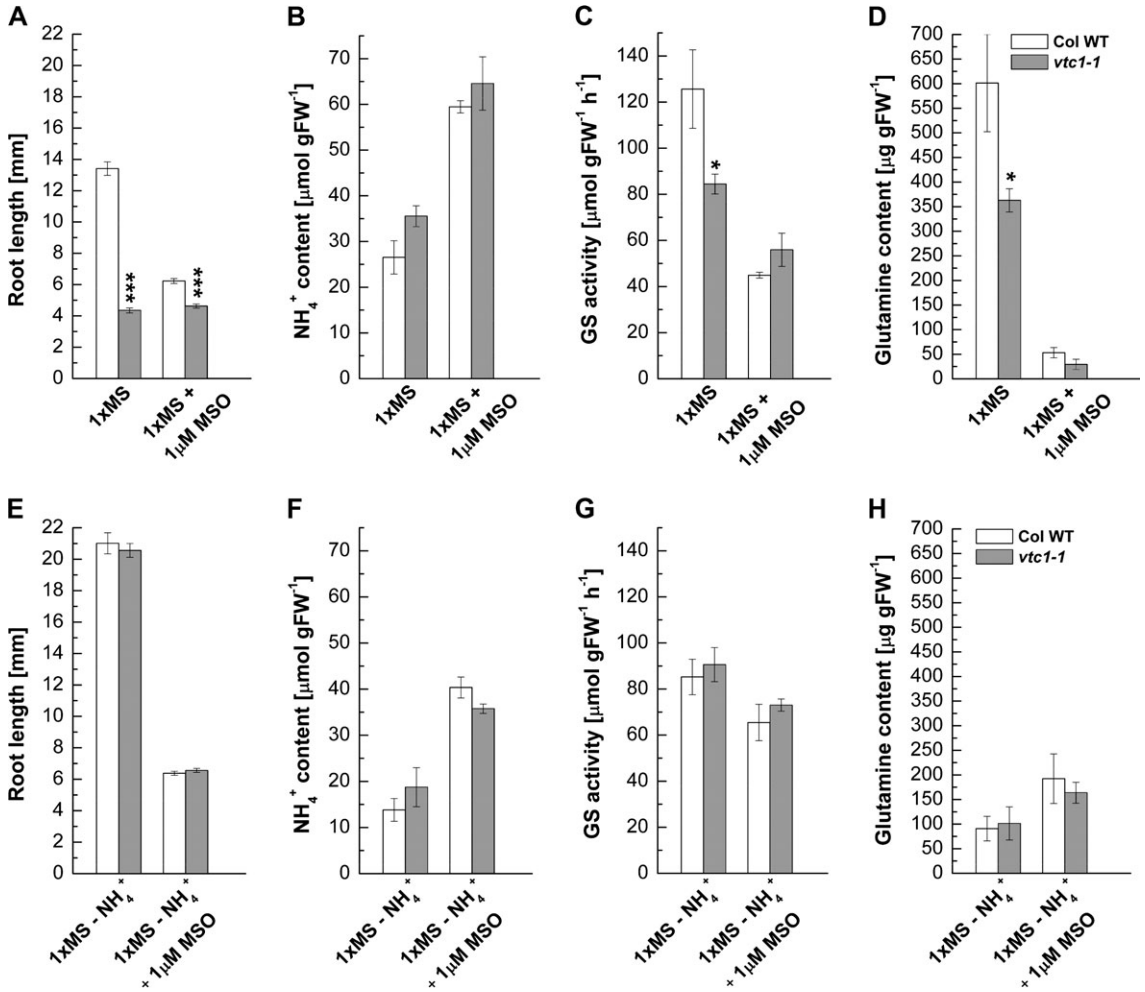

Fig. 4. Effect of methionine sulphoximine (MSO) on root development, ammonium (NH_4^+) content, glutamine synthetase (GS) activity, and glutamine content in whole 7-d-old seedlings of wild-type and *vtc1-1* mutant plants. Plants were germinated on 1× MS in the absence and presence of ammonium nitrate (–NH_4^+) and MSO, respectively. (A, E) Primary root length. Mean values ±SE of 147–196 independent replicates are shown. (B, F) Ammonium content g^{-1} fresh weight. Data represent means ±SE of three independent replicates. (C, G) Mean glutamine synthetase activity of three independent replicates ±SE. (D, H) Glutamine content g^{-1} fresh weight. Means ±SE of three independent replicates are shown. Asterisks indicate significant differences between mutant and wild type. *P <0.05, ***P <0.001, Student's t test.

activity was approximately 30% lower in *vtc1-1* compared to the wild type when grown on 1× MS. The GS inhibitor MSO decreased GS activity in the wild type approximately 3-fold, whereas the decrease in GS activity was less pronounced in *vtc1-1* (Fig. 4C). GS activity was only slightly decreased by MSO in the absence of NH_4^+ (Fig. 4G). The Gln content was approximately 40% lower in *vtc1-1* compared to the wild type when plants where grown on 1× MS. However, Gln content decreased dramatically in both genotypes in the presence of MSO (Fig. 4D). Gln content was significantly lower in both genotypes when grown in the absence of NH_4^+ and was not significantly diminished in the presence of MSO (Fig. 4H). Finally, assimilation of NH_4^+ by GDH was similar in the wild type and *vtc1-1* (see Supplementary Fig. S4 at *JXB* online). Note, however, that MSO also inhibited GDH activity (see Supplementary Fig. S4A at *JXB* online).

These data suggest that GS activity and Gln biosynthesis are negatively affected in *vtc1-1* in the presence of NH_4^+. In the absence of NH_4^+, *vtc1-1* behaves like the wild type and is capable of readjusting NH_4^+ metabolism, presumably through deamination processes. This result seems at first perplexing, given the fact that the genetic defect is of course also present when *vtc1-1* is grown on media lacking NH_4^+. Thus, our data suggest that the mutation in GMPase in combination with high concentrations of NH_4^+ causes the root developmental defect in *vtc1-1*.

Effect of tunicamycin, mannose, GDP-mannose, and galactose on root growth in vtc1-1

To investigate whether the decreased GMPase activity in *vtc1-1* (Conklin *et al.*, 1999), resulting in lower levels of GDP-mannose and thus in disturbed protein N-glycosylation (Lukowitz *et al.*, 2001), could be responsible for the root developmental defect in the mutant, wild-type and *vtc1-1* mutant plants were treated with tunicamycin. This antibiotic inhibits N-glycosylation (Elbein, 1988). If

decreased N-glycosylation causes root growth inhibition in *vtc1-1*, we predict that treatment of the wild type and the other *vtc* mutants, which are not affected in root growth in the presence of high NH_4^+, with tunicamycin will have an effect similar to that of the *vtc1-1* mutation on root growth. Since *vtc1-1* has a defect in the conversion of mannose to GDP-mannose, addition of GDP-mannose to the 1× MS growth medium should rescue the short-root phenotype in *vtc1-1*, while addition of mannose and galactose should not (Fig. 1).

Increasing concentrations of tunicamycin impair root elongation in the wild type, *vtc2-1*, *vtc3-1*, and *vtc4-1* mutants, but not in *vtc1-1*. At a concentration of 0.1 μM, these four genotypes mimicked the *vtc1-1* short-root phenotype (Fig. 5A; see Supplementary Fig. S5A at *JXB* online), suggesting that N-glycosylation is impaired in *vtc1-1*. Addition of mannose did not rescue the short-root phenotype in *vtc1-1*. Instead, high concentrations of mannose caused an inhibition in root elongation in the wild type, while root development in *vtc1-1* was unchanged (Fig. 5B). An inhibitory effect of mannose on root growth has been reported previously (Lukowitz *et al.*, 2001). Surprisingly, GDP-mannose did not rescue the *vtc1-1* root developmental phenotype (Fig. 5C), even at high concentrations. It is possible that GDP-mannose was not taken up or was unstable in the medium. As expected, galactose did not rescue the short-root phenotype in *vtc1-1*. Instead, it also had an inhibitory effect on root development (Fig. 5D). Similar results were found when ascorbic acid was applied to the growth medium (data not shown).

Our results suggest that root growth inhibition in *vtc1-1* is caused by a defect in N-glycosylation and not AA deficiency. Since defective N-glycosylation causes programmed cell death (Hauptmann and Lehle, 2008; Hoeberichts *et al.*, 2008), it was assessed whether cell death was altered in *vtc1-1*. Roots of *vtc1-1* grown on 1× MS exhibited enhanced cell death compared to the wild type (see Supplementary Fig. S5B at *JXB* online). However, only a few dead cells were detected when both genotypes were grown in the absence of NH_4^+ (see Supplementary Fig. S5C at *JXB* online). These results substantiate our findings that root growth inhibition in *vtc1-1* is caused by defective N-glycosylation but only in combination with NH_4^+.

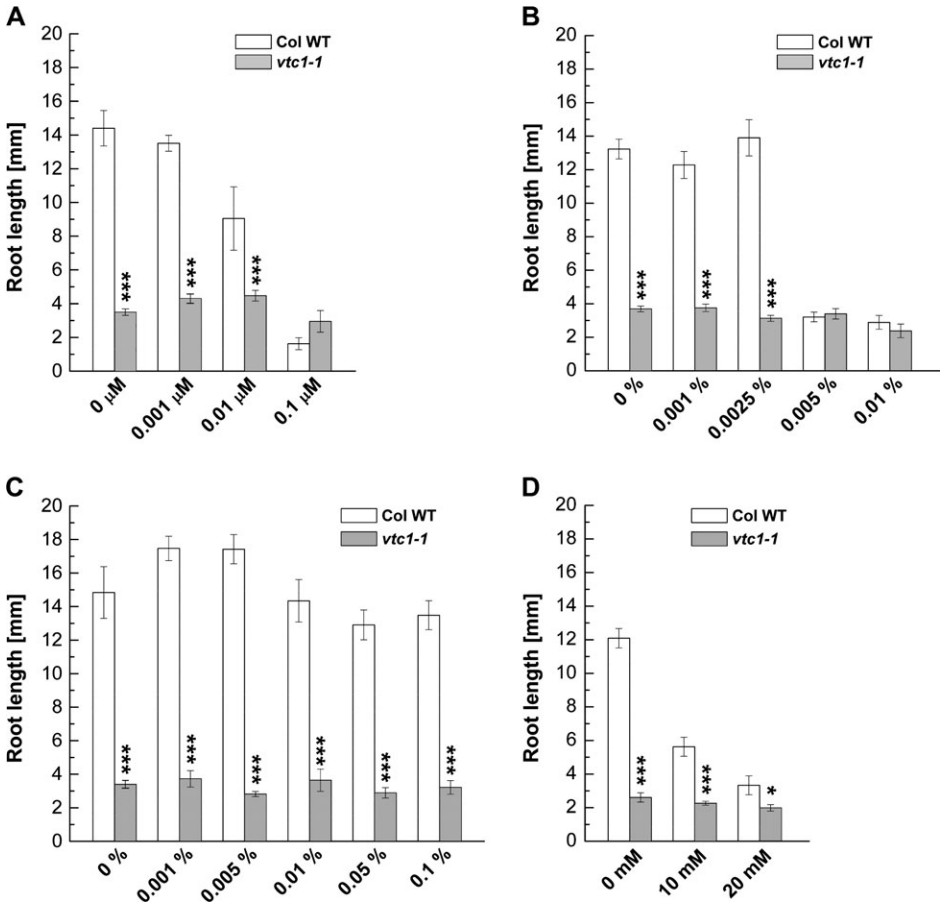

Fig. 5. Effect of the N-glycosylation inhibitor tunicamycin and ascorbic acid precursors on primary root growth in 7-d-old wild type and *vtc1-1* mutants grown on 1× MS. (A) Effect of increasing concentrations of tunicamycin. Data display 7–11 replicates per genotype and treatment. (B) Effect of increasing concentrations of D-mannose. Means ±SE of 9–16 individual replicates per genotype are shown. (C) Effect of increasing concentrations of GDP-D-mannose. Results represent means ±SE of 9–14 individual replicates per genotype and treatment. (D) Effect of L-galactose. Date illustrate means ±SE of 8–11 individual seedlings per genotype. Asterisks indicate significant differences between mutant and wild type. *P <0.05, ***P <0.001, Student's t test.

Effect of auxin, the ethylene precursor ACC, and salicylic acid on root growth in vtc1-1

The *vtc1-1* mutant exhibits pleiotropic phenotypes when grown on soil. These include alterations in the content of plant hormones, such as abscisic acid, salicylic acid (SA), and gibberellic acid (Pastori *et al.*, 2003; Barth *et al.*, 2004; Foyer *et al.*, 2007). Although not yet investigated, it is expected that ethylene content is affected as well, because AA serves as a co-factor in ethylene biosynthesis. While it is unknown whether IAA content is altered in *vtc1-1*, IAA (Cao *et al.*, 1993; Sattelmacher and Thoms, 1995) and ethylene (Feng and Barker, 1992; Barker, 1999*a*) are linked to NH_4^+-induced alterations in growth and development. Furthermore, SA is known either to inhibit (Manthe *et al.*, 1992) or to promote (Gutierrez-Coronado *et al.*, 1998) root growth, although a direct link between SA and NH_4^+ sensitivity has not yet been established. Nevertheless, investigating the role of SA in *vtc1-1* root development is of relevance, because *vtc1-1* contains constitutively elevated levels of SA (Barth *et al.*, 2004). Therefore, the effect of these three hormones was investigated to obtain clues on their role in *vtc1-1* root development in the presence of NH_4^+.

Since IAA plays a fundamental role in root development, it was predicted that IAA content is altered in *vtc1-1* in the presence of NH_4^+, but unchanged in the absence of NH_4^+. In fact, IAA content was decreased by approximately 40% in *vtc1-1* compared with the wild type when plants were grown on 1× MS. In the absence of NH_4^+, IAA content was similar in both genotypes (Fig. 6A). These data suggest that post-embryonic IAA biosynthesis and/or transport is affected in *vtc1-1* in response to NH_4^+. To test whether exogenous IAA could complement the *vtc1-1* short-root phenotype, IAA was added to the growth medium at 1 nM, at which root growth may be promoted, and at higher concentrations known to inhibit root elongation (Rahman *et al.*, 2007). The addition of IAA at 1 nM did not promote root elongation, neither in the wild type nor in *vtc1-1*. By contrast, in the presence of 1 μM and 5 μM IAA, primary root growth was strongly inhibited in the wild type and to a lesser extent in *vtc1-1* (Fig. 6B). However, IAA promoted the outgrowth and elongation of adventitious roots in the wild type and *vtc1-1* (data not shown).

Ethylene has been reported to influence primary root growth through IAA-dependent and -independent mechanisms (Ruzicka *et al.*, 2007; Thomann *et al.*, 2009). As illustrated in Fig. 6C, the ethylene precursor ACC had an inhibitory effect on root elongation in the wild type and the other *vtc* mutants (see Supplementary Fig. S6 at *JXB* online), but had no effect on *vtc1-1*. This suggests that *vtc1-1* is insensitive to ACC.

If high SA contributes to root growth inhibition in *vtc1-1* in the presence of high NH_4^+, it was predicted that double mutants of *vtc1-1* and the SA-deficient *pad4-1* and *eds5-1* mutants would have roots like the wild type. The *vtc1-1 eds5-1* and *vtc1-1 pad4-1* double mutants are SA-deficient, containing SA levels similar to the *pad4-1* and *eds5-1* single mutants (Mukherjee *et al.*, 2009). As shown in Fig. 6D, double mutants exhibited a root developmental phenotype similar to *vtc1-1* single mutants, suggesting that the *vtc1-1* short-root phenotype is independent of SA.

vtc1-1 *contains elevated levels of NO when grown on high* NH_4^+

Inhibition of root elongation by high nitrate concentrations was suggested to result from a reduction of nitric oxide synthase-dependent endogenous NO levels in maize root apical cells (Zhao *et al.*, 2007). However, a correlation between NH_4^+ nutrition and NO has not yet been established. Furthermore, NO is known to inhibit root elongation (He *et al.*, 2004) and to promote adventitious rooting (Pagnussat *et al.*, 2004), phenotypes that are present in *vtc1-1* when grown on high NH_4^+ (Fig. 2C, D, E). Therefore, it was investigated whether NO affects root development in the NH_4^+-sensitive *vtc1-1* mutant.

The response of the wild type and *vtc* mutants to exogenous NO was tested and the NO content was measured. With increasing concentrations of the NO donor, SNP, primary root growth was strongly inhibited in the wild type, *vtc2-1*, *vtc3-1*, and *vtc4-1* seedlings, whereas SNP had no significant effect on the already short roots in *vtc1-1* (Fig. 7A). This result suggests a high endogenous NO content in *vtc1-1* compared with the wild type and the other *vtc* mutants. The NO content in the wild type and *vtc1-1* in the presence of excess NH_4^+ and in the absence of NH_4^+ was, therefore, determined. As expected, the NO content was higher in *vtc1-1* when grown on 1× MS, but decreased in the absence of NH_4^+ (Fig. 7B). To test whether this is an NO-specific response, it was investigated whether root growth inhibition can be rescued by the addition of the specific NO scavenger cPTIO. The short-root phenotype is partially but significantly (P <0.001) recovered in *vtc1-1* in the presence of cPTIO (Fig. 7C), suggesting that NO contributes, in part, to root growth inhibition in *vtc1-1* in the presence of high NH_4^+. Finally, it was investigated whether NO is synthesized via nitric oxide synthase (*NOS*) by assessing *NOS* transcript levels in the wild type and *vtc1-1* in the presence and absence of NH_4^+. *NOS* mRNA levels were approximately 2-fold higher in *vtc1-1* compared to the wild type in the presence of NH_4^+, whereas transcript levels were the same in both genotypes in the absence of NH_4^+ (Fig. 7D). These data correlate nicely with the NO content (Fig. 7C) and suggest that high concentrations of NH_4^+ promote the formation of NO in *vtc1-1* and that NO contributes, in part, to the short-root phenotype in *vtc1-1*.

Discussion

vtc1-1 *is conditionally hypersensitive to* NH_4^+*, a response that is independent of AA deficiency and oxidative stress*

Since the isolation of the AA-deficient *Arabidopsis vtc* mutants (Conklin *et al.*, 2000; Dowdle *et al.*, 2007), multiple

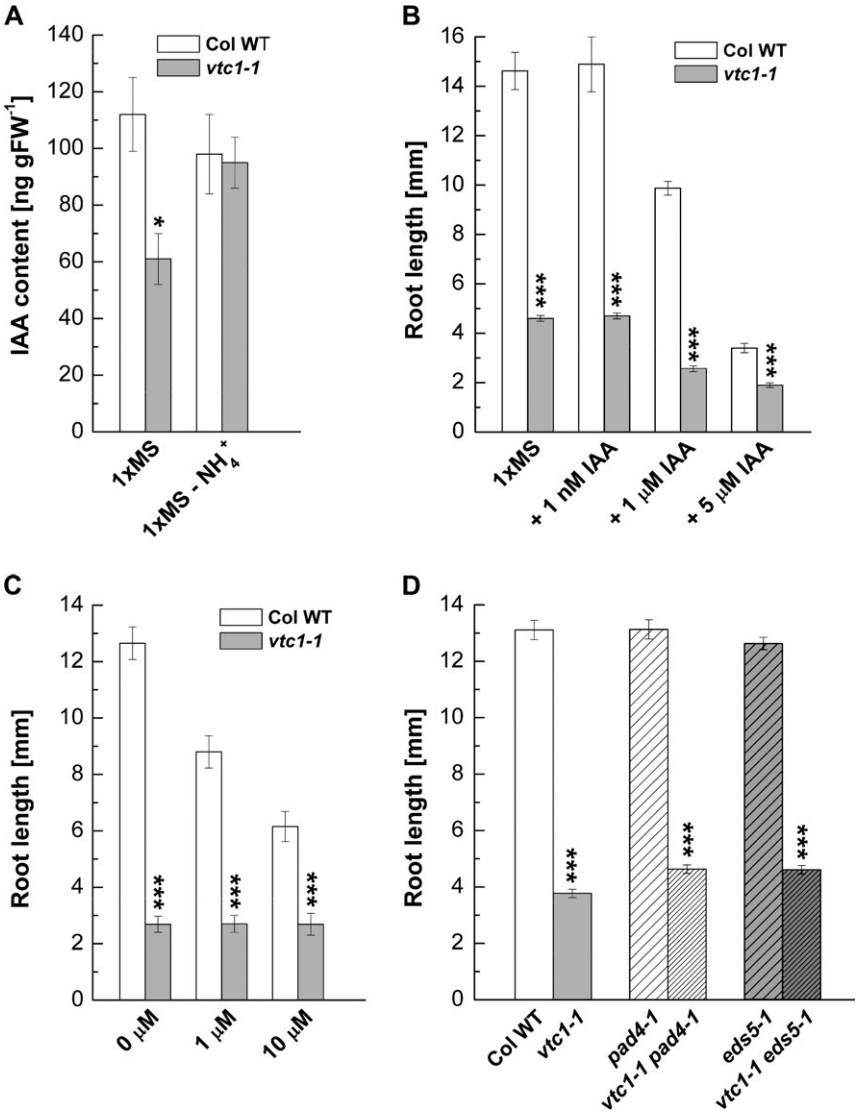

Fig. 6. Auxin (IAA, indole-3-acetic acid) content, and effect of IAA, the ethylene precursor ACC, and salicylic acid (SA) on primary root growth in 7-d-old wild-type and *vtc* mutant plants grown on 1× MS. (A) Total IAA content. Means ±SE of three individual replicates per genotype are shown. (B) Primary root length in the presence of IAA. Data show means ±SE of 12–35 individual seedlings. (C) Effect of increasing concentrations of ACC. Results represent means ±SE of 8–11 individual replicates per genotype and treatment. (D) Primary root growth in SA biosynthesis mutants and double mutants deficient in AA and SA. Data illustrate means ±SE of 54–108 individual seedlings per genotype. Asterisks indicate significant differences between individual mutants and the wild type. *P <0.05, ***P <0.001, Student's t test.

phenotypes of these mutants have been reported (Veljovic-Jovanovic *et al.*, 2001; Pastori *et al.*, 2003; Barth *et al.*, 2004; Pavet *et al.*, 2005; Kotchoni *et al.*, 2009). Evidence is provided here that functional GMPase, which generates GDP-mannose for AA biosynthesis and protein N-glycosylation, is essential for root growth under high NH_4^+ conditions in *Arabidopsis*. The *vtc1-1* mutant, containing a point mutation in GMPase, exhibits hypersensitivity to NH_4^+ (Fig. 3). The root growth inhibition in *vtc1-1* cannot be explained by the AA deficiency or H_2O_2 levels in the mutant (Fig. 2A, B). This is supported by the following facts: First, when grown on full-strength MS medium, root development is only inhibited in *vtc1-1*, whereas three additional AA-deficient mutants with defects in other AA biosynthetic genes have normal root growth (Fig. 2; see Supplementary Fig. S1 at *JXB* online). In support of these results, previous reports have demonstrated that NH_4^+ sensitivity is independent of antioxidant redox status and antioxidant enzymes (Dominguez-Valdivia *et al.*, 2008). Second, *vtc1-1* mutants develop roots similar to the wild type when grown in the absence of NH_4^+ (Figs 3B, C, 4E; see Supplementary Figs S2, S3, and S5C at *JXB* online). Third, increasing the concentration of NH_4^+ causes strong root growth inhibition in *vtc1-1* mutants, whereas root growth in the wild type (Fig. 3C) and the other *vtc* mutants is affected to a lesser extent. Together with the results of

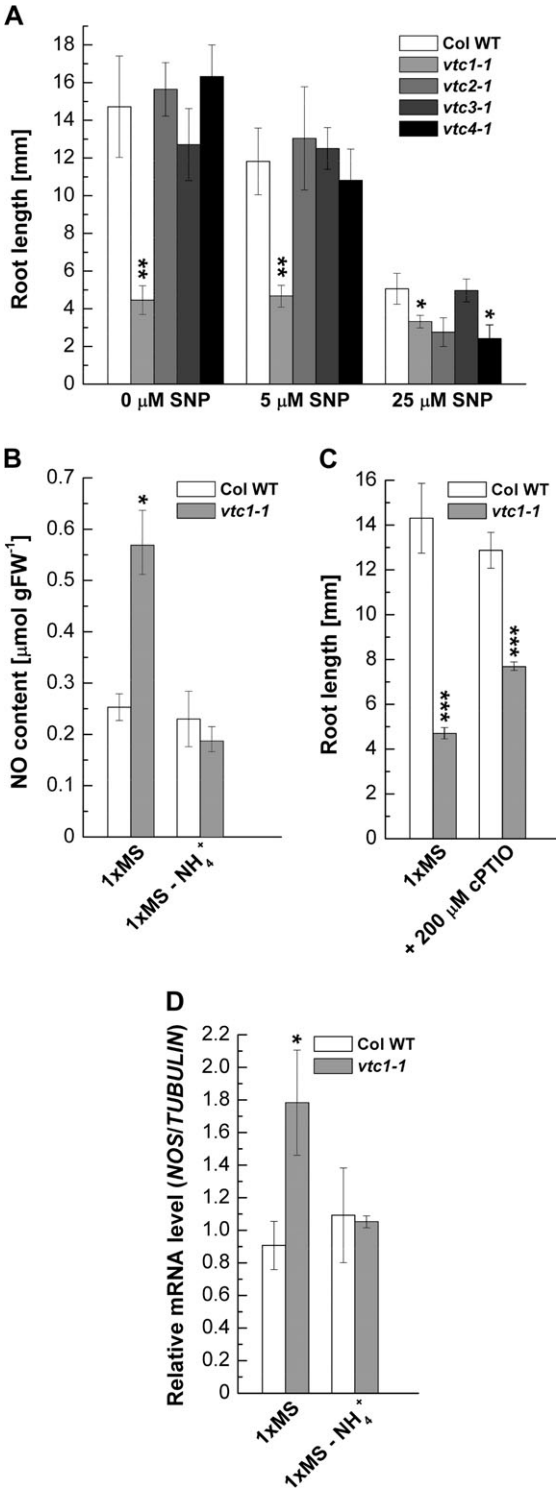

Fig. 7. The role of nitric oxide (NO) in primary root development of the wild type and *vtc* mutants. (A) Effect of increasing concentrations of the NO donor SNP on primary root growth in 7-d-old plants grown on 1× MS medium. Data represent means ±SE of 6–12 individual seedlings per genotype and treatment. (B) NO content in the wild type and *vtc1-1* in the presence of high NH_4^+ (1× MS) and in the absence of NH_4^+. Data represent means ±SE of three independent replicates. Asterisks indicate significant differences between the wild type and mutants. (C) Primary root growth in the presence of the specific NO scavenger cPTIO. Results show means ±SE of ten individual seedlings per genotype and treatment. (D) Relative *NOS* transcript levels in the presence and absence of NH_4^+. Results display means ±SE of four biological replicates of each genotype and treatment. **P* <0.05, ***P* <0.01, ****P* <0.001, Student's *t* test.

defective N-glycosylation (Fig. 5A; see Supplementary Fig. S5A at *JXB* online; Conklin *et al.*, 1999; Lukowitz *et al.*, 2001; Qin *et al.*, 2008) and enhanced cell death (see Supplementary Fig. S5B at *JXB* online), these results suggest that the defect in N-glycosylation is responsible for root growth inhibition in *vtc1-1*. However, the N-glycosylation defect alone does not impair root development, unless it is combined with NH_4^+ stress (Fig. 3B, C; see Supplementary Fig. S5C at *JXB* online), indicating that the NH_4^+ hypersensitivity phenotype of *vtc1-1* is conditional. Conditional mutants with defects in N-glycosylation and N-glycan maturation have been reported in *Arabidopsis* (Hoeberichts *et al.*, 2008; Kang *et al.*, 2008) and invertebrates (Sarkar *et al.*, 2006; Paschinger *et al.*, 2006; Koles *et al.*, 2007). Recently, Hoeberichts *et al.* (2008) reported that a mutation in phosphomannose mutase (PMM), which catalyses the interconversion of mannose 6-phospate and mannose 1-phosphate, causes conditional temperature sensitivity. PMM acts directly upstream of *VTC1* and also contributes to the formation of GDP-mannose and AA (Fig. 1). The authors provide evidence that *pmm* mutants exhibit cell death at restrictive temperatures due to a deficiency in protein glycosylation. Finally, GDP-mannose and protein glycosylation are also necessary for proper cell wall formation, as has been demonstrated for the *cyt1* mutant (Lukowitz *et al.*, 2001) and the glycosylation-deficient *cgl1* mutant, which is more sensitive to salt stress (Kang *et al.*, 2008). However, as to how cell wall formation is affected in *vtc1-1* during NH_4^+ stress has not yet been investigated. Taken together, these results and our data strongly suggest that mutants with defects in the synthesis of mannose or GDP-mannose are disrupted in N-glycosylation, triggering cell death. This also explains why *vtc2* and *vtc4* mutants are not affected in root development, because *VTC2* and *VTC4* genes act downstream of GDP-mannose (Fig. 1). The *VTC3* gene, which has not yet been identified, is most likely not involved in the formation of mannose or GDP-mannose.

Concurrently with our investigations, Qin and co-workers reported NH_4^+ sensitivity in the *Arabidopsis* mutant *hsn1*, which is allelic to *vtc1-1* (Qin *et al.*, 2008). In agreement with our results, the authors report that defective protein N-glycosylation in the roots, rather than decreased AA content, correlates with the hypersensitivity of the *hsn1* and *vtc1-1* mutants. The authors proposed that NH_4^+ inhibits GMPase activity and that defective protein N-glycosylation, the initiation of unfolded protein response, and cell death are downstream responses in the regulation of NH_4^+ sensitivity in *Arabidopsis*. These results are in line with our data. However, our study provides new information,

suggesting that N-glycosylation functions beyond protein folding under NH_4^+ stress.

NH_4^+ assimilation via glutamine synthetase is altered in vtc1-1

N-glycosylation constitutes a major post-translational modification. Thus, defects in N-glycosylation affect membrane and secreted proteins (Strasser *et al.*, 2004). Therefore, it was tested whether NH_4^+ transport and content may be altered in *vtc1-1* under high NH_4^+ conditions. NH_4^+ transport through AMT-type transporters, which are distantly related to Rhesus glycoproteins (Ludewig *et al.*, 2007), was not altered in *vtc1-1* mutants (data not shown). This was expected, because AMTs are not involved in transport at high NH_4^+ concentrations (used in our experiments) and because *AMT* genes are transcriptionally down-regulated when nitrogen content is high and up-regulated under deficiency conditions (Rawat *et al.*, 1999). Furthermore, at NH_4^+ concentrations greater than 1 mM, transport is passive (Ullrich *et al.*, 1984; Wang *et al.*, 1993). It is not clear whether NH_4^+ uptake is altered in *vtc1-1*, because electrophysiological experiments investigating passive or active NH_4^+ uptake were not conducted in this study. However, NH_4^+ content was the same in the wild type and *vtc1-1* when grown in the presence or absence of NH_4^+ (Fig. 4B, F). Similar results were reported by Qin *et al.* (2008).

Once NH_4^+ has entered the cell, GS catalyses the ATP-dependent condensation of NH_4^+ and glutamate to yield glutamine, ADP, and inorganic phosphate. Plants with high GS activity have been reported to be more tolerant to NH_4^+ (Magalhaes *et al.*, 1992; Glevarec *et al.*, 2004). Our data suggest that total GS activity is stimulated in the wild type in the presence of NH_4^+ when compared to GS activity in the absence of NH_4^+. This, however, was not the case in *vtc1-1* (compare Fig. 4C and G). NH_4^+ may have an inhibitory effect on GS activity (Ishiyama *et al.*, 2004). Furthermore, other metabolites, including nitrate, amino acids, and carbohydrates have been shown to influence GS activity (Oliveira and Coruzzi, 1999; Lancien *et al.*, 2000). We surmise that GS is not inhibited by NH_4^+ in *vtc1-1*, because NH_4^+ content is not elevated in the mutant. Thus, one may speculate that GS activity in *vtc1-1* may be affected due to altered carbon availability in this mutant when NH_4^+ is in excess, as GS activity and Gln content in *vtc1-1* are similar to the wild type in the absence of NH_4^+ (Fig. 4G, H). It is important to note that chloroplastic GS is induced by light, which is mediated by phytochrome (Oliveira and Coruzzi, 1999). It has been demonstrated that the conditional short-root phenotype in *vtc1-1* in the presence of high NH_4^+ is light-dependent (Fig. 2F). However, it appears that light exerts an indirect effect on GS expression that may depend on efficient photosynthetic activity to produce carbohydrates (Melo-Oliveira *et al.*, 1996). Thus, a more likely explanation for the low GS activity is that GS is not fully active due to the N-glycosylation deficiency in *vtc1-1*, which is enhanced by NH_4^+.

The role of hormones and NO in mediating NH_4^+ sensitivity in vtc1-1

Nutrient signalling involving different metabolites and hormones has been largely characterized in response to nutrient deficiencies or in response to nitrate but not NH_4^+ supply (Rubio *et al.*, 2009). Investigating the role of hormones in the inhibition of primary root growth in *vtc1-1* was important considering the fact that the mutant has altered levels of various hormones (Pastori *et al.*, 2003; Barth *et al.*, 2004; Foyer *et al.*, 2007).

While no evidence was found for the involvement of SA in root growth inhibition (Fig. 6D), lower IAA levels were observed in *vtc1-1* in the presence of NH_4^+ (Fig. 6A). To inhibit root growth, NH_4^+ must be blocking cell division and/or expansion. It is unlikely that NH_4^+ directly affects these processes. It is possible that the deficiency in N-glycosylation triggering the unfolded-protein response (Qin *et al.*, 2008) inhibits protein synthesis, causing cell-cycle arrest, as has been reported in mammals (Harding *et al.*, 1999; Brewer *et al.*, 1999). This response may be mediated by IAA, of which a high concentration in the root tip is required for correct cell division, cell elongation, and final cell size (Blilou *et al.*, 2005). In fact, NH_4^+ feeding led to a suppression of root IAA content (Kudoyarova *et al.*, 1997). Exogenous application of IAA did not rescue the short-root phenotype in *vtc1-1* (Fig. 6B). It is known that low concentrations of IAA may promote root growth, whereas higher concentrations inhibit root growth (Rahman *et al.*, 2007). Thus, our result is not surprising. The mutant responds to exogenous IAA, but not as dramatically as the wild type does. The *vtc1-1* mutant may have a defect in IAA distribution and/or conjugation. Note that only the amount of free IAA in seedlings has been measured. Since the *vtc1-1* phenotype can be mimicked in the wild type with high exogenous IAA, it is concluded that IAA synthesis, transport, and/or signalling are disrupted during NH_4^+ treatment. This is supported by the fact that the IAA-resistant mutants *aux1*, *axr1*, and *axr2* developed roots in the presence of 6 mM NH_4^+ (Cao *et al.*, 1993). Thus, the mutants are resistant to NH_4^+ toxicity. Perhaps high levels of IAA have an inhibitory effect on root growth, as demonstrated by our IAA application experiments. Note, however, that the NH_4^+ sensitivity phenotype reported by Cao *et al.* (1993) may be caused by a different mechanism, as the authors report almost complete root growth inhibition in the wild type, which can be rescued by the addition of potassium. Root IAA predominantly derives from shoot tissues before 10 d after germination (Ljung *et al.*, 2001; Bhalerao *et al.*, 2002), with IAA accumulating in the root tip between 1 d and 3 d after germination (Bhalerao *et al.*, 2002). Therefore, NH_4^+ may inhibit IAA distribution or promote IAA conjugation in *vtc1-1*, resulting in inhibited primary root growth and lateral root elongation (Fig. 2C, D, E; Blakely *et al.*, 1988; Celenza *et al.*, 1995; Casimiro *et al.*, 2001; Fukaki and Tasaka, 2009). Furthermore, outgrowth of lateral root primordia may be inhibited by the high ABA content in *vtc1-1* (Pastori *et al.* 2003; SO Kotchoni and C Barth,

unpublished results), as ABA negatively regulates the emergence of lateral root primordia (De Smet *et al.*, 2006).

While the outgrowth of lateral roots is suppressed, *vtc1-1* mutants form adventitious roots (Fig. 2D, E), a process that requires IAA (Hausman *et al.*, 1995; Guerrero *et al.*, 1999) and light (Sorin *et al.*, 2005). Since a lower content of free IAA was measured in *vtc1-1* (Fig. 6A), it is not clear whether IAA is involved in the formation of adventitious roots in the mutant. It is possible that adventitious root growth in *vtc1-1* is independent of IAA and instead mediated by NO, because NO content is high in *vtc1-1* when grown on excess NH_4^+ (Fig. 7B). This hypothesis is supported by data demonstrating that NO is involved in the adventitious rooting process in cucumber with NO acting downstream of IAA (Pagnussat *et al.*, 2004).

Finally, instead of NO or in addition to NO, ethylene may affect root development in *vtc1-1*, which is insensitive to ACC. The ethylene content in *vtc1-1* has not yet been measured. A high endogenous ethylene content may inhibit expansion of cells leaving the root meristem (Le *et al.*, 2001). It may also negatively impact lateral root formation by altering IAA transport (Negi *et al.*, 2008) and stimulate adventitious root development (Clark *et al.*, 1999). This would be in good agreement with our data.

In conclusion, we and a parallel study by Qin *et al.* (2008) identified GMPase as a genetic factor conferring conditional NH_4^+ hypersensitivity, resulting in root growth inhibition. This growth defect is not caused by AA deficiency or oxidative stress, but by a defect in N-glycosylation. Our data suggest that NH_4^+ sensitivity in *vtc1-1* is linked to altered NH_4^+ metabolism, IAA, ethylene, and/or NO signalling. Future experiments evaluating NH_4^+ uptake, hormonal and cell cycle responses will aid in elucidating the mechanism of NH_4^+ sensitivity.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Root developmental phenotype in the wild type and *vtc* mutants grown on increasing strength of MS medium.

Supplementary Fig. S2. Root developmental phenotype in 7-d-old wild-type and *vtc1-1* mutant plants grown on 1× MS in the absence of ammonium and increasing concentrations of potassium chloride.

Supplementary Fig. S3. Root developmental phenotype of 7-d-old wild-type and *vtc1-1* mutant plants grown on 1× MS in the absence of all nitrogen and increasing concentrations of ammonium chloride.

Supplementary Fig. S4. Glutamate dehydrogenase activity in whole 7-d-old seedlings of the wild type and *vtc1-1* grown on 1× MS in the presence and absence of ammonium and in the presence or absence of MSO, respectively.

Supplementary Fig. S5. Root developmental phenotype of 7-d-old wild-type and *vtc* mutant plants grown on 1× MS in the presence of increasing concentrations of tunicamycin and root cell death evaluation using Evan's blue staining.

Supplementary Fig. S6. Root developmental phenotype of 7-d-old wild-type and *vtc* mutant plants grown on 1× MS in the presence of increasing concentrations of the ethylene precursor ACC.

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