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

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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 (NH₄⁺). To provide new insights into the mechanism of NH₄⁺ sensitivity in vtc1-1, root development, NH₄⁺ 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 NH₄⁺ 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 NH₄⁺ sensitivity in vtc1-1. Our data suggest that NH₄⁺ 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 (NH₄⁺), 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 G₁ 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.

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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 (NH₄⁺) and that the short-root phenotype in the mutant is independent of AA deficiency and the accumulation of H₂O₂. Therefore, we focused on investigating NH₄⁺ metabolism and hormone homeostasis in vtc1-1 to better understand root growth inhibition by NH₄⁺ in vtc1-1.

NH₄⁺ and nitrate are the major sources of nitrogen for plants. NH₄⁺ is the preferred nitrogen source with concentrations ranging from 20–200 μM in agricultural soils (Loque and von Wirén, 2004; Miller and Cramer, 2004). It is assimilated into essential amino acids. However, excess amounts of NH₄⁺ 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 NH₄⁺ toxicity in plants, several aspects of NH₄⁺ uptake, transport, metabolism, and interactions with plant hormones have been studied, particularly with respect to root development.

NH₄⁺ is directly taken up from the soil at the root surface and hairs of the rhizodermis and immediately transferred into the root symplast for NH₄⁺ assimilation by rhizodermis-localized glutamine synthetase (Ishiyama et al., 2004). Non-assimilated NH₄⁺ 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 NH₄⁺ in the endodermal apoplasm (Marschner, 1995).

Transport of NH₄⁺ 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). NH₄⁺ uptake may also occur through high- or low-affinity transport systems (Rawat et al., 1999). To minimize NH₄⁺ toxicity, NH₄⁺-sensitive species have been reported to actively pump out NH₄⁺ (Kronzucker et al., 2001).

Once inside the cell, NH₄⁺ is metabolized by glutamine synthetase (GS) into glutamine (Gln), which is further assimilated into glutamate (Glu) and 2-oxoglutarate into two molecules of glutamate. NH₄⁺ 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 NH₄⁺ nutrition. Plants with high GS activities are more tolerant to NH₄⁺ (Magalhaes et al., 1992; Glevarec et al., 2004). Arabidopsis GS isoforms have different kinetic properties and are differentially regulated by NH₄⁺.

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**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 (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.)

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D-Glucose-6-P

D-Fructose-6-P

D-Mannose-6-P

PMM

D-Mannose-1-P

VTC1/CYT1

GDP-d-Mannose

GDP-L-Galactose

VTC2/VTC5

L-Galactose-1-P

VTC4

L-Galactose

L-Galactono-1,4-lactone

L-Ascorbic acid

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By guest, on 27 July 2018
Glazebrook, 2002). 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; Schooerring et al., 2002).

Multiple studies suggest that nutrient signalling is intertwined with plant hormones (Feng and Barker, 1992; Cao et al., 1993; Barker, 1999b; 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, 1999a) 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% phytothene agar (Caisson Laboratories, Inc.) and were adjusted to a pH of 5.7 with KOH before autoclaving. None of the growth media contained NH$_4^+$-induced alterations in carbon and nitrogen metabolism. 

#### Seed-surface sterilization

Seeds were soaked for 1 min in 50% ethanol, followed by washing 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 microliters 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$_4$Cl standard curve and normalized to gram fresh weight of the sample. Glutamine content was determined using the t-asparagine, t-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$_4$NO$_3$. Extraction of IAA from plant tissue was performed using 80% methanol as previously described (Tanaka et al., 2001). Methylated IAA was quantified using the PhytoTest IAA kit following the manufacturer’s protocol (Agdia, Elkhart, IN).

Glutamine synthetase activity assay

Total glutamine synthetase activity was determined following a spectrophotometric assay (Mack, 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 microliters 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 trichloroacetic 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 sulfoximine (MSO, a GS inhibitor), sodium nitroprusside (SNP, an NO donor), 2-(4-carboxyphenyl)1,4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO, an NO inhibitor), indole-3-acetic acid (IAA), 1-aminocyclopropane-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 F1 progeny of these crosses were allowed to grow and self-fertile. The F2 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. F3 and F4 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′-TTGCTGGGAGGTCTCGT-TAGGATTG-3′ (NOS-F), 5′-CTCGTGTTTTGCGGATTGGT-TCTGGGGAGGTCTGGT-3′ (NOS-R), 5′-CACAAGAGTTCCTACGCAGTAG-3′ (TUB2-RT-F2), and 5′-TCACCTTCTTCTCATCCGCAGTT-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, H2O2 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 NH4+

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 NH4+ (i.e., no NH4NO3, but KNO3 still present) or when seedlings were grown in the absence of all nitrogen (–N, i.e., the medium contains neither NH4NO3 nor KNO3), root length was similar in the wild type and vtc1-1 (Fig. 3B). These data suggest that the presence of a high concentration of NH4+ in 1× MS has an inhibitory effect on root development in vtc1-1 mutants.
To further support that \textit{vtcl-1} is hypersensitive to NH$_4^+$, wild-type and \textit{vtcl-1} mutant plants were germinated on 1× MS medium lacking NH$_4^+$ (i.e., no NH$_4$NO$_3$, but KNO$_3$ present), increasing concentrations of NH$_4$Cl were added, and root length was determined. With increasing concentrations of NH$_4^+$, root elongation was strongly inhibited in \textit{vtcl-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$_4$Cl, 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 \textit{vtcl-1} is dose-dependent, an effect that has been reported previously in other plant species (Lasa \textit{et al.}, 2002; Dominguez-Valdivia \textit{et al.}, 2008). To ensure that the inhibitory effect on root

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**Fig. 2.** Physiological characterization of the wild type and \textit{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$_2$O$_2$ content in whole 7-d-old seedlings of the wild type and \textit{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 \textit{vtc} mutant seedlings grown on 1× MS. (E) Close-up of the \textit{vtc1-1} root developmental phenotype. Bar, 1 mm. (F) Primary root length of wild-type and \textit{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 \textit{vtc1-1}. (G) Primary root length in 7-d-old wild-type and \textit{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 \textit{t} test.
development in \textit{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 \textit{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 \textit{vtc1-1}, although root growth was slightly more inhibited in the \textit{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 \textit{vtc1-1} primary root development.

**NH$_4^+$ content is not altered in \textit{vtc1-1}, but glutamine synthetase activity and glutamine content are decreased**

To understand the mechanism of the NH$_4^+$ hypersensitivity of \textit{vtc1-1}, transcript levels of NH$_4^+$ transporters were examined, and NH$_4^+$ content and metabolism in the wild type and \textit{vtc1-1} were assessed. Wild type and \textit{vtc1-1} did not differ significantly in mRNA levels of the NH$_4^+$ transporters \textit{AMT1;1} and \textit{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 \textit{et al.}, 1984; Wang \textit{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 \textit{et al.}, 1992; King \textit{et al.}, 1993; Hirano \textit{et al.}, 2008). This inhibitor was used to test whether NH$_4^+$ metabolism is altered in \textit{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 \textit{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 \textit{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 increasing concentrations of ammonium chloride (NH$_4$Cl) 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.

\begin{figure}[h]
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\caption{Effect of various MS media compositions on primary root development in 7-d-old wild type and \textit{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$_4$Cl) 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.}
\end{figure}
activity was approximately 30% lower in vtcl-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 vtcl-1 (Fig. 4C). GS activity was only slightly decreased by MSO in the absence of NH4+ (Fig. 4G). The Gln content was approximately 40% lower in vtcl-1 compared to the wild type when plants were 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 NH4+ and was not significantly diminished in the presence of MSO (Fig. 4H). Finally, assimilation of NH4+ by GDH was similar in the wild type and vtcl-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 vtcl-1 in the presence of NH4+. In the absence of NH4+, vtcl-1 behaves like the wild type and is capable of readjusting NH4+ metabolism, presumably through deamination processes. This result seems at first perplexing, given the fact that the genetic defect is of course also present when vtcl-1 is grown on media lacking NH4+. Thus, our data suggest that the mutation in GMPase in combination with high concentrations of NH4+ causes the root developmental defect in vtcl-1.

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

To investigate whether the decreased GMPase activity in vtcl-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 vtcl-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 \( \text{NH}_4^+ \), 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 \( \mu \text{M} \), 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 \( \text{NH}_4^+ \) (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 \( \text{NH}_4^+ \).

**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, 1999a) are linked to NH4\(^+\)-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 NH4\(^+\) 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 NH4\(^+\).

Since IAA plays a fundamental role in root development, it was predicted that IAA content is altered in vtc1-1 in the presence of NH4\(^+\), but unchanged in the absence of NH4\(^+\). In fact, IAA content was decreased by approximately 40\% in vtc1-1 compared with the wild type when plants were grown on 1 \(\times\) MS. In the absence of NH4\(^+\), 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 NH4\(^+\). 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 \(\mu\)M and 5 \(\mu\)M 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 NH4\(^+\), 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 NH4\(^+\)

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 NH4\(^+\) 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 NH4\(^+\) (Fig. 2C, D, E). Therefore, it was investigated whether NO affects root development in the NH4\(^+\)-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 NH4\(^+\) and in the absence of NH4\(^+\) was, therefore, determined. As expected, the NO content was higher in vtc1-1 when grown on 1 \(\times\) MS, but decreased in the absence of NH4\(^+\) (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 NH4\(^+\). 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 NH4\(^+\). NOS mRNA levels were approximately 2-fold higher in vtc1-1 compared to the wild type in the presence of NH4\(^+\), whereas transcript levels were the same in both genotypes in the absence of NH4\(^+\) (Fig. 7D). These data correlate nicely with the NO content (Fig. 7C) and suggest that high concentrations of NH4\(^+\) 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 NH4\(^+\), 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
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 NH₄⁺ conditions in Arabidopsis. The vtc1-1 mutant, containing a point mutation in GMPase, exhibits hypersensitivity to NH₄⁺ (Fig. 3). The root growth inhibition in vtc1-1 cannot be explained by the AA deficiency or H₂O₂ 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 NH₄⁺ 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 NH₄⁺ (Figs 3B, C, 4E; see Supplementary Figs S2, S3, and S5C at JXB online). Third, increasing the concentration of NH₄⁺ 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. 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.
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 NH4+ stress (Fig. 3B, C; see Supplementary Fig. S5C at JXB online), indicating that the NH4+ 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-phosphate 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 NH4+ 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 NH4+ 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 NH4+ 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 NH4+ sensitivity in Arabidopsis. These results are in line with our data. However, our study provides new information.

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 NH4+ (1× MS) and in the absence of NH4+. 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 NH4+. 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.
suggested that N-glycosylation functions beyond protein folding under NH₄⁺ stress.

NH₄⁺ 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 NH₄⁺ transport and content may be altered in vtc1-1 under high NH₄⁺ conditions. NH₄⁺ 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 NH₄⁺ 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 NH₄⁺ concentrations greater than 1 mM, transport is passive (Ullrich et al., 1984; Wang et al., 1993). It is not clear whether NH₄⁺ uptake is altered in vtc1-1, because electrophysiological experiments investigating passive or active NH₄⁺ uptake were not conducted in this study. However, NH₄⁺ content was the same in the wild type and vtc1-1 when grown in the presence or absence of NH₄⁺ (Fig. 4B, F). Similar results were reported by Qin et al. (2008).

Once NH₄⁺ has entered the cell, GS catalyses the ATP-dependent condensation of NH₄⁺ and glutamate to yield glutamine, ADP, and inorganic phosphate. Plants with high GS activity have been reported to be more tolerant to NH₄⁺ (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 NH₄⁺ when compared to GS activity in the absence of NH₄⁺. This, however, was not the case in vtc1-1 (compare Fig. 4C and G). NH₄⁺ 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 NH₄⁺ in vtc1-1, because NH₄⁺ 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 NH₄⁺ is in excess, as GS activity and Gln content in vtc1-1 are similar to the wild type in the absence of NH₄⁺ (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 NH₄⁺ 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 NH₄⁺.

The role of hormones and NO in mediating NH₄⁺ 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 NH₄⁺ 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 NH₄⁺ (Fig. 6A). To inhibit root growth, NH₄⁺ must be blocking cell division and/or expansion. It is unlikely that NH₄⁺ 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 (Biliou et al., 2005). In fact, NH₄⁺ 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 NH₄⁺ treatment. This is supported by the fact that the IAA-resistant mutants aux1, aux1, and axr2 developed roots in the presence of 6 mM NH₄⁺ (Cao et al., 1993). Thus, the mutants are resistant to NH₄⁺ toxicity. Perhaps high levels of IAA have an inhibitory effect on root growth, as demonstrated by our IAA application experiments. Note, however, that the NH₄⁺ 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, NH₄⁺ may inhibit IAA distribution or promote IAA conjugation in vtc1-1, resulting in inhibited primary root growth and lateral root elongation (Fig. 2C, D; 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, 2005).
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-l 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-l (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-l is independent of IAA and instead mediated by NO, because NO content is high in vtc1-l when grown on excess NH₄⁺ (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-l, which is insensitive to ACC. The ethylene content in vtc1-l 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 NH₄⁺ 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 NH₄⁺ sensitivity in vtc1-l is linked to altered NH₄⁺ metabolism, IAA, ethylene, and/or NO signalling. Future experiments evaluating NH₄⁺ uptake, hormonal and cell cycle responses will aid in elucidating the mechanism of NH₄⁺ 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-l 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-l 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-l 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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