GDP-mannose pyrophosphorylase is a genetic determinant of ammonium sensitivity in *Arabidopsis thaliana*

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Higher plant species differ widely in their growth responses to ammonium (NH_4^+). However, the molecular genetic mechanisms underlying NH_4^+ sensitivity in plants remain unknown. Here, we report that mutations in the *Arabidopsis* gene encoding GDP-mannose pyrophosphorylase (GMPase) essential for synthesizing GDP-mannose confer hypersensitivity to NH_4^+. The in planta activities of WT and mutant GMPases all were inhibited by NH_4^+, but the magnitude of the inhibition was significantly larger in the mutant. Despite the involvement of GDP-mannose in both L-ascorbic acid (AsA) and N-glycoprotein biosynthesis, defective protein glycosylation in the roots, rather than decreased AsA content, was linked to the hypersensitivity of GMPase mutants to NH_4^+. We conclude that NH_4^+ inhibits GMPase activity and that the level of GMPase activity regulates *Arabidopsis* sensitivity to NH_4^+. Further analysis showed that defective N-glycosylation of proteins, unfolded protein response, and cell death in the roots are likely important downstream molecular events involved in the growth inhibition of *Arabidopsis* by NH_4^+.

glycosylation | NH_4^+ toxicity | unfolded protein response | L-ascorbic acid

Ammonium (NH_4^+) is an essential ion in living cells. NH_4^+ and nitrate (NO_3^-) form the major sources of nitrogen nutrition for plants and microorganisms. Furthermore, NH_4^+ is also an indispensable intermediate in the biosynthesis of essential cellular components. However, NH_4^+ is toxic to cellular organisms when present in excess amounts (1). In fact, NH_4^+ sensitivity is a widespread phenomenon in animals, plants, and fungi, although the levels of sensitivity differ considerably among different species (1). Plants, being unable to escape from harmful environments, are especially prone to NH_4^+-induced growth inhibition. Owing to the application of large quantities of nitrogen fertilizers in intensive agriculture, high levels of NH_4^+ accumulation are becoming more common in many natural and agricultural soils (2). Consequently, NH_4^+ toxicity has been linked to plant species extinction and decline of forest under certain ecological conditions in recent years (3).

The molecular mechanisms underlying plant sensitivity to this ion are still unclear. Past studies have revealed important physiological changes (for example, acidification of external growth environment, disturbance in the acid/base balance, or excessive energy consumption in pumping the toxic level of NH_4^+ out of cells) accompanying NH_4^+ uptake and toxicity symptoms (1, 2). However, the genetic determinant of NH_4^+ sensitivity in plants remains unknown. Interestingly, recent biochemical investigations in animal cells have shown that NH_4^+ sensitivity is linked with reduced efficiencies in protein glycosylation and the correct processing and secretion of glycoproteins (4–6). But, again, the genetic trigger for these changes has not been determined. From the fact that different plant families, or different species in the same plant family, can differ considerably in their growth response to NH_4^+ (7), we deduce that NH_4^+ sensitivity is genetically controlled. Identification of a genetic determinant should provide valuable clues for understanding the molecular mechanisms of NH_4^+ sensitivity. To this end, we have taken a forward genetics approach by identifying an *Arabidopsis thaliana* mutant showing enhanced NH_4^+ sensitivity than WT control and have characterized the hypersensitive response of *hsn1* and its allelic mutant *vtc1* to NH_4^+.

We found that *hsn1* was the result of a point mutation in the gene encoding GDP-mannose pyrophosphorylase (GMPase; EC 2.7.7.22), which has been established to be essential for synthesizing the vital cellular metabolite GDP-mannose in *Arabidopsis* (8). We provide molecular genetic and biochemical evidence on the inhibition of GMPase by NH_4^+ and describe the consequential downstream molecular events likely to be important for the inhibition of *Arabidopsis* growth by NH_4^+. The relevance of our finding on further studies of the mechanisms underlying NH_4^+ sensitivity in higher plants is discussed.

Results

Morphological and Physiological Characterization of *hsn1* Mutant. Previous studies have shown that Brassicaceae is one of the NH_4^+-sensitive plant families (1) and that the growth of the Col-0 ecotype of *Arabidopsis*, which is a member of Brassicaceae, is inhibited by physiological concentrations of NH_4^+ (9). When cultured on defined media with both NO_3^- and NH_4^+ as nitrogen sources, the growth of *Arabidopsis*, especially the elongation of its roots, is increasingly inhibited by rising concentrations of NH_4^+ (9). Based on these findings, we screened 20,000 ethylmethanesulfonate-mutagenized M_2 seedlings on a half-strength Murashige–Skoog (MS) medium (with 20 mM NO_3^- and 10 mM NH_4^+ as nitrogen sources, hereafter referred as $NO_3^- + NH_4^+$ medium), and isolated a mutant exhibiting hypersensitivity to NH_4^+ (named as *hsn1*, hypersensitive to NH_4^+ 1). The *hsn1* mutant showed dramatically reduced growth of both aerial and root organs in the media containing NH_4^+ irrespective of the presence or absence of NO_3^- as an additional nitrogen source (Fig. 1). Moreover, the level of growth retardation of *hsn1*, especially that of its root, increased substantially with rising NH_4^+ concentrations (Fig. 1 *A* and *B*), demonstrating that the growth of *hsn1* (particularly its root) is more sensitive to NH_4^+ than that of WT *Arabidopsis*. A typical comparison of WT and *hsn1*

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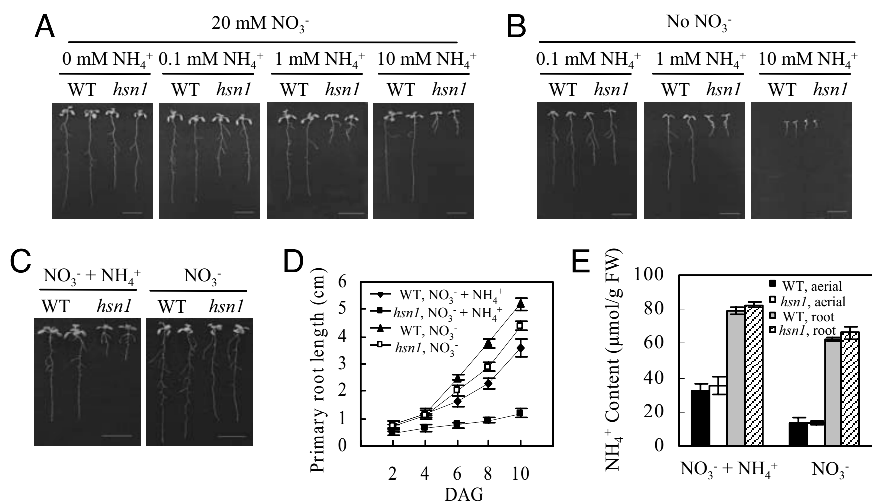

Fig. 1. Phenotypes of *hsn1* mutant. (*A* and *B*) Comparisons of seedling growth of WT *Arabidopsis* and *hsn1* on modified MS media at 10 days after germination (DAG). In *A*, the nitrogen source included 20 mM NO_3^- with increasing concentrations of NH_4^+ (from 0 to 10 mM). In *B*, the nitrogen source was provided solely by ascending concentrations of NH_4^+ (from 0.1 to 10 mM). (Bars: 1 cm.) (*C*) A typical example of WT and *hsn1* seedlings cultured on $NO_3^- + NH_4^+$ or NO_3^- medium. (Bars: 1 cm.) (*D*) Comparisons of postgerminative root growth between WT *Arabidopsis* and *hsn1* on $NO_3^- + NH_4^+$ and NO_3^- medium. Statistically significant differences ($P < 0.05$) were found between the 4 data points at 8 or 10 DAG. (*E*) NH_4^+ contents (expressed as μmol/g of fresh weight) in the aerial and root tissues of WT *Arabidopsis* and *hsn1* seedlings (10 DAG) grown on $NO_3^- + NH_4^+$ or NO_3^- medium. Significant differences ($P < 0.01$) were found between the NH_4^+ contents of aerial and root tissues for both WT *Arabidopsis* and *hsn1* grown on either $NO_3^- + NH_4^+$ or NO_3^- medium.

seedlings germinated on $NO_3^- + NH_4^+$ medium or a modified 1/2 MS medium with NO_3^- as the sole nitrogen source (henceforth named as NO_3^- medium) is shown in Fig. 1*C*. Quantitative analysis confirmed that the primary root of *hsn1* was markedly shorter than that of WT control on both $NO_3^- + NH_4^+$ and NO_3^- medium, with the difference in primary root length between the 2 genotypes being much larger on $NO_3^- + NH_4^+$ medium (Fig. 1*D*). Consistent with previous findings (9), the primary root length of WT *Arabidopsis* seedlings was also significantly reduced on the medium containing NH_4^+ (i.e., $NO_3^- + NH_4^+$ medium; Fig. 1 *C* and *D*) compared with that attained on the medium with NO_3^- as the only nitrogen source (i.e., NO_3^- medium; Fig. 1 *C* and *D*).

The NH_4^+ content did not differ significantly between WT control and *hsn1* in both aerial and root tissues on either $NO_3^- + NH_4^+$ or NO_3^- medium (Fig. 1*E*). For both WT *Arabidopsis* and *hsn1* and on either $NO_3^- + NH_4^+$ or NO_3^- medium, the NH_4^+ content in the roots was significantly higher than that in the shoots (Fig. 1*E*), indicating that NH_4^+ uptake and translocation processes did not differ significantly between WT control and *hsn1*. The NH_4^+ contents in the aerial and root tissues of WT and *hsn1* seedlings all were significantly higher on $NO_3^- + NH_4^+$ medium compared with their corresponding values on NO_3^- medium (Fig. 1*E*), reflecting a higher level of uptake and assimilation of the NH_4^+ present in the former medium by both genotypes.

Molecular and Biochemical Analysis of *hsn1* and Its Allelic Mutant. The *hsn1* allele was isolated by using a positional cloning strategy and found to be caused by a point mutation in the gene (*At2g39770*) encoding GMPase (Fig. 2*A*). In addition to *At2g39770*, the *Arabidopsis* genome contains 2 more predicted genes (*At3g55590*, *At4g30570*) encoding putative GMPase (www.Arabidopsis.org). However, the 2 additional genes have been found not to complement the functional deficiency of *At2g39770* (refs. 8 and 10). The mutation in *hsn1* replaced a WT glycine residue (at position 11 of GMPase) by serine. The *hsn1* phenotype on $NO_3^- + NH_4^+$ medium was fully complemented by transgenic expression of WT GMPase in the mutant background (Fig. 2*B*), confirming that a defective GMPase is responsible for the enhanced NH_4^+ sensitivity of *hsn1*.

GMPase is a highly conserved protein in eukaryotic organisms, with nearly 60% identity among *Arabidopsis*, human, and yeast orthologs and >85% identity between the orthologs from dicot and monocot plant species (data not shown). GMPase is necessary for synthesizing GDP-mannose, which is indispensable for proper N-glycosylation of proteins in eukaryotic cells (11). In higher plants, GDP-mannose is also an important intermediate for the biosynthesis of ascorbic acid (AsA) through the Smirnoff–Wheeler pathway (12). Previous studies have identified 2 types of mutations affecting the function of *Arabidopsis* GMPase. The *vtc1* allele, causing a P22S change in GMPase protein (Fig. 2*A*), decreases GMPase activity and AsA content in *Arabidopsis* (8). Compared with WT controls, *vtc1* seedlings are more susceptible to oxidation stress and their root length is constitutively shorter when grown with either single (NO_3^-) or dual (NO_3^- and NH_4^+) nitrogen sources (8, 13). Two *cyt* alleles (*cyt1–1*, *cyt1–2*; Fig. 2*A*), with a P89L substitution in the middle and an alteration of the C-terminal amino acid sequence of GMPase protein, respectively, display severe defects in N-glycosylation of proteins and are thus embryo-lethal (10). As anticipated, the root elongation of *vtc1* was strongly inhibited on $NO_3^- + NH_4^+$ medium (Fig. 2*C Left*). Moreover, the degree of the inhibition displayed by *vtc1* was more severe than that by *hsn1* (Fig. 2*C Left*). Consistent with our earlier data (Fig. 1 *C* and *D*) and those published (13), the root lengths of *hsn1* and *vtc1* seedlings were both shorter than that of WT controls on NO_3^- medium (Fig. 2*C Right*).

The mutations in *hsn1* and *vtc1* did not decrease the transcript and protein accumulation levels of GMPase on either $NO_3^- + NH_4^+$ or NO_3^- medium [supporting information (SI) Fig. S1]. However, the in planta GMPase activity levels of *hsn1* and *vtc1* all were significantly lower than those of WT control in both the aerial and root tissues and on either $NO_3^- + NH_4^+$ or NO_3^- medium, although the decrease of GMPase activity in *hsn1* was less severe than that in *vtc1* (Fig. 2*D*). Further inspection of the data in Fig. 2*D* revealed 2 additional GMPase activity responses common to WT, *hsn1*, and *vtc1* seedlings. First, in either the aerial or root tissues, GMPase activity levels in the 3 genotypes all were significantly lower when grown on $NO_3^- + NH_4^+$ medium compared with the corresponding values when NO_3^- medium

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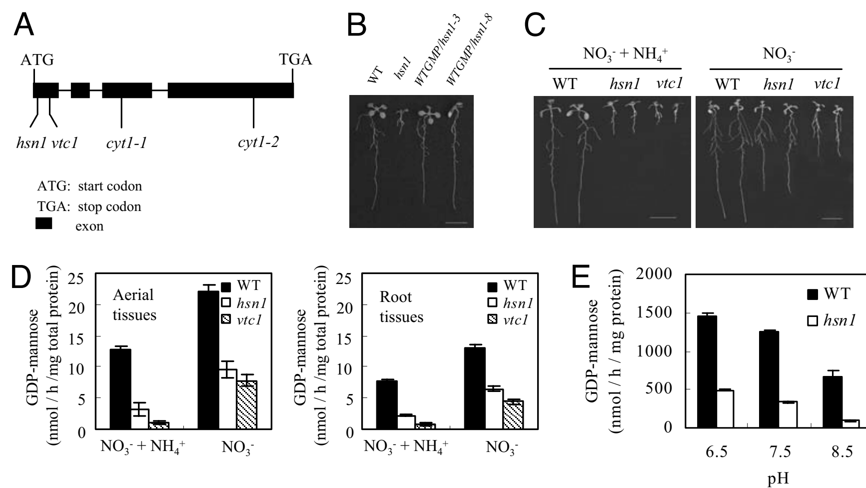

Fig. 2. Molecular characterization of *hsn1*. (*A*) A diagram illustrating the genomic coding sequence of *Arabidopsis* GMPase and its mutant alleles *hsn1*, *vtc1*, *cyt1–1*, and *cyt1–2*. (*B*) In the complementation experiments ($n = 3$), the NH_4^+-induced growth inhibition of *hsn1* on NO_3^- + NH_4^+ medium was fully reversed by the expression of WT GMPase coding sequence in independent transgenic lines (*WTGMP/hsn1–3*, *WTGMP/hsn1–8*). (Bar: 1 cm.) (*C*) The *vtc1* allele exhibited a similar tendency of growth inhibition as *hsn1* on either NO_3^- + NH_4^+ (*Left*) or NO_3^- medium (*Right*), although the inhibition was much stronger for *vtc1* than for *hsn1*. (Bars: 1 cm.) (*D*) Comparisons of in planta GMPase activity levels in the aerial and root tissues of 10 DAG seedlings of WT *Arabidopsis* and 2 GMPase mutants (*hsn1*, *vtc1*) cultured on NO_3^- + NH_4^+ or NO_3^- medium. GMPase activity was determined by monitoring the production of GDP-mannose (nmol/h per mg of total protein). Significant differences ($P < 0.05$) were found in GMPase activity levels among the 3 genotypes by either tissues types (aerial vs. root) or culture media (NO_3^- + NH_4^+ vs. NO_3^-). (*E*) Comparisons of the activities of recombinant WT GMPase and hsn1 enzymes under 3 pH regimes. Enzyme activity was determined as in *D* except that the purified recombinant proteins of WT GMPase and hsn1 were used for the assays. Significant differences ($P < 0.01$) were found in the activity levels between WT GMPase and hsn1 enzymes at all pH conditions and among the 3 activity levels exhibited by WT GMPase or hsn1.

used for the culture (Fig. 2*D*). Second, GMPase activity levels in the roots of the 3 genotypes were generally lower than those in the shoots irrespective of NO_3^- + NH_4^+ or NO_3^- medium used for the growth (Fig. 2*D*).

In addition to the experiments above, we investigated whether GMPase activity may be affected by alkaline pH, because one of the earliest cellular responses to NH_4^+ uptake is the alkalinization of the cytosol (reviewed in ref. 14). To this end, we compared the activities of recombinant WT GMPase and hsn1 proteins under 3 different pH regimes. At pH 7.5, which is within the range of cytosolic pH in unstressed plant cells (14), hsn1 enzyme activity was <30% of that WT GMPase (Fig. 2*E*). At pH 8.5, the activity of WT GMPase was reduced by >45% and that of hsn1 was reduced by >70% compared with the corresponding values at pH 7.5 (Fig. 2*E*). At pH 6.5, the activity of WT GMPase was enhanced by ≈15% and that of hsn1 was enhanced by ≈32% relative to the corresponding values at pH 7.5 (Fig. 2*E*). The data in Fig. 2*E* prompted us to test whether raising the rhizosphere pH would affect the in planta activity of GMPase in WT *Arabidopsis* and GMPase mutants. For this purpose, seedlings germinated on NO_3^- medium (pH 5.7) were transferred onto 3 different media, NO_3^- + NH_4^+ (pH 5.7), NO_3^- (pH 5.7), and NO_3^- (pH 6.7) and allowed to grow for 24 h, followed by measuring in planta GMPase activity. In agreement with the data depicted in Fig. 2*D*, GMPase activity levels in the shoots and roots were generally and significantly higher on NO_3^- medium (pH 5.7) than on NO_3^- + NH_4^+ medium (pH 5.7) (Fig. S2). However, raising the pH of NO_3^- medium from 5.7 to 6.7 caused considerable decreases of GMPase activity levels in both the shoots and roots and for either WT *Arabidopsis* or GMPase mutants (*hsn1*, *vtc1*) (Fig. S2). By contrast, GMPase protein level remained relatively stable in the shoots and roots of the 3 genotypes (Fig. S2).

Analysis of AsA Content and Protein N-Glycosylation in WT and Mutant Seedlings in Relation to NH_4^+ Sensitivity. To find out whether decreased AsA content or defective N-glycosylation of proteins may be linked to the enhanced NH_4^+ sensitivity of *hsn1* and *vtc1*, we first determined the total AsA content of *hsn1* and compared the growth of WT *Arabidopsis*, *hsn1*, *vtc1*, and a knockout mutant (*vtc4-KO*) of the *Arabidopsis VTC4* gene (15). *VTC4* encodes L-Gal-1-P phosphatase devoted to AsA biosynthesis, and the total AsA content of *vtc4-KO* is <50% of that of WT *Arabidopsis* (15). As expected, the total AsA content in the aerial and root tissues of *hsn1* was generally and substantially lower than those of WT control (Fig. S3). In contrast to the clear differences in the root length among WT, *hsn1*, and *vtc1* seedlings on NO_3^- + NH_4^+ or NO_3^- medium, the growth of *vtc4-KO* seedlings was indistinguishable from that of WT controls on either of the 2 media (Fig. 3*A*). Quantitative measurements confirmed that the root length of *vtc4-KO* was highly similar to that of WT *Arabidopsis* and significantly longer than the one displayed by either *hsn1* or *vtc1* (Fig. 3*B*). Like WT control, the root length of *vtc4-KO* was much longer on NO_3^- medium than that on NO_3^- + NH_4^+ medium (Fig. 3).

Two approaches were undertaken to investigate whether defective N-glycosylation of proteins may occur in *hsn1* and *vtc1* mutants on the medium containing NH_4^+. First, a peroxidase-conjugated Con A reagent, which binds to branches of oligomannose chains on N-glycoproteins (16), was used to compare the glycoproteins between WT and GMPase mutant (*hsn1* and *vtc1*) tissues. This experiment revealed that the level of N-glycoproteins detected by Con A was drastically lower in the root tissues of *hsn1* and *vtc1* relative to that in WT *Arabidopsis* roots when grown on NO_3^- + NH_4^+ medium (Fig. 3*C Right*). However, no apparent reduction in the level of Con A-binding N-glycoproteins was observed in the root tissues of the 3 genotypes cultured on NO_3^- medium (Fig. 3*C Right*) or in the aerial tissues of the 3 genotypes irrespective of NO_3^- + NH_4^+ or NO_3^- medium used (Fig. 3*C Left*).

Second, the glycosylation status of protein disulfide isomerase (PDI), which is an abundant protein of the endoplasmic reticulum (ER) and posttranslationally modified with N-linked glycans under normal growth conditions (17), was examined by immunoblotting. PDI was resolved as a single protein band in the aerial tissues of WT control, *hsn1*, and *vtc1* grown on either NO_3^- + NH_4^+ or NO_3^- medium (Fig. 3*C Left*). A single PDI band

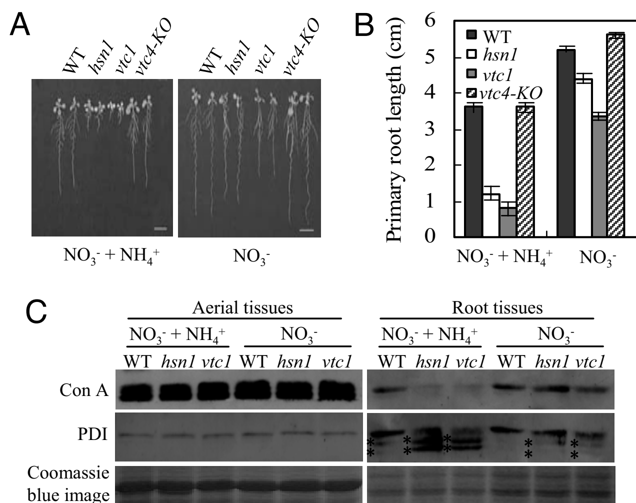

Fig. 3. Analysis of AsA and protein N-glycosylation in WT and mutant seedlings. (*A*) Comparisons of seedling growth of WT *Arabidopsis*, *hsn1*, *vtc1*, and *vtc4-KO* at 10 DAG. (Bars: 1 cm.) (*B*) Quantitative analysis of the root elongation of WT *Arabidopsis*, *hsn1*, *vtc1*, and *vtc4-KO* seedlings (10 DAG) cultured on NO_3^- + NH_4^+ or NO_3^- medium. Significant differences ($P < 0.05$ at least) were found between WT *Arabidopsis* and GMPase mutants (*hsn1*, *vtc1*) or among *vtc4-KO* and the 2 mutants but not between WT *Arabidopsis* and *vtc4-KO*. (*C*) Detection of defective N-glycosylation of proteins in WT and GMPase mutants. N-glycosylation of proteins was evaluated by using a Con A-peroxidase reagent (*Top*) or an antibody specific for PDI (*Middle*). The Coomassie blue-stained gel images (*Bottom*) illustrate equal loading of total protein samples during SDS/PAGE. Asterisks mark the underglycosylated PDI forms.

was also detected in the roots of WT *Arabidopsis* cultured on NO_3^- medium (Fig. 3*C Right*). By contrast, in the roots cultured on NO_3^- + NH_4^+ medium, 2 additional PDI bands, showing faster electrophoretic mobility and representing underglycosylated PDI forms (marked by asterisks in Fig. 3*C Right*), were detected for *hsn1* and *vtc1*. Interestingly, the 2 underglycosylated PDI forms were also present, although in much smaller amounts, in the roots of WT *Arabidopsis* cultured on NO_3^- + NH_4^+ medium (Fig. 3*C Right*) or in the roots of *hsn1* and *vtc1* grown on NO_3^- medium (Fig. 3*C Right*).

Comparisons of Unfolded Protein Response (UPR) and Cell Death in the Roots of WT and Mutant Seedlings in the Presence of NH_4^+. It is well known that N-glycosylation is an important form of posttranslational modification of proteins in eukaryotic cells (18). Consequently, the NH_4^+-induced decreases of GMPase activity and availability of GDP-mannose in WT and GMPase mutants may debilitate the processing, targeting, and functionality of many glycoproteins functioning in diverse cellular processes. Because delayed glycoprotein processing has been shown to trigger UPR in animal and yeast cells (19, 20), we tested whether UPR may occur in WT and GMPase mutants (*hsn1* as a representative) in the presence of NH_4^+. This experiment was accomplished by monitoring the expression of BiP, an isoform of the HSP70 chaperone that is rapidly induced by N-glycosylation defects in the ER (21, 22). The biomarker BiP–β-glucuronidase (GUS) (21), which can be reliably followed by histochemical staining, was introduced into WT and *hsn1* backgrounds. The marker-tagged lines were grown on a series of modified MS media with a constant level of NO_3^- (20 mM) but rising concentrations of NH_4^+. Compared with WT roots, in *hsn1* roots, BiP–GUS expression (indicated by the blue signals in Fig. 4*A*) was observed at much lower NH_4^+ concentration (0.1 mM) and was substantially stronger at higher NH_4^+ concentrations (1–10 mM). Furthermore, at 10 mM NH_4^+, BiP–GUS expression was induced in the entire root tip region of *hsn1* (Fig. 4*A*), whereas in WT *Arabidopsis* BiP–GUS signals were absent from the root apical meristematic region (Fig. 4*A*). Corresponding to the difference in BiP–GUS expression patterns, cell death, as revealed by the color precipitates after staining with Evans blue, was much more extensive in *hsn1* roots than in WT controls in the presence of 10 mM NH_4^+ (in NO_3^- + NH_4^+ medium; Fig. 4*B*). A lower level of cell death, indicated by fewer Evans blue signals, was also observed in *hsn1* roots in the absence of NH_4^+ (i.e., on NO_3^- medium; Fig. 4*B*).

Discussion

In this work, a forward genetic analysis of NH_4^+ sensitivity was conducted with *Arabidopsis* as the model. We found that mutations in the gene encoding *Arabidopsis* GMPase, essential for synthesizing GDP-mannose, conferred hypersensitivity to NH_4^+ present in the growth medium, which provided a well-defined genetic system to study not only the mechanism underlying the function of GMPase, but also the downstream molecular events, in NH_4^+ sensitivity of *Arabidopsis*.

Our results reveal that strong correlations exist among the extents of decreased GMPase activity, the degrees of defective N-protein glycosylation in the roots, and the different magnitudes of growth retardation exhibited by WT, *hsn1*, and *vtc1* seedlings in the presence of NH_4^+. From these correlations, we suggest that *Arabidopsis* GMPase activity is sensitive to inhibition by physiological concentrations of NH_4^+, which is further aggravated by mutations in GMPase protein (as in *hsn1* and *vtc1* seedlings). Consequently, the activity level of GMPase regulates *Arabidopsis* sensitivity to NH_4^+. From the findings that the in vitro GMPase activity was decreased by alkaline pH (Fig. 2*E*) and in planta GMPase activity was reduced by raising the pH of NO_3^- medium from 5.7 to 6.7 (Fig. S2), we hypothesize that cytosolic alkalinization associated with NH_4^+ uptake may play a

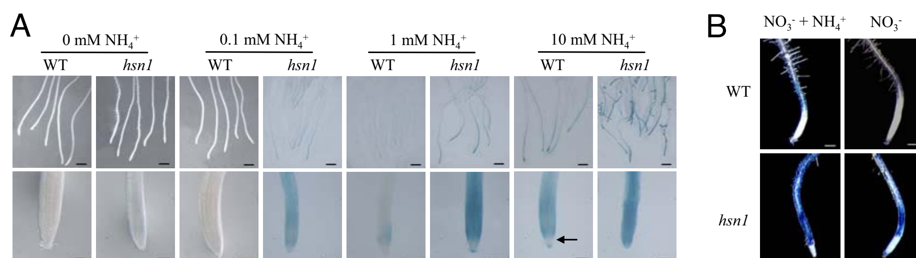

Fig. 4. Comparative analyses of UPR and cell death in the roots of WT *Arabidopsis* and *hsn1* seedlings (10 DAG). (*A*) UPR in the roots (*Upper*) and root tips (*Lower*) was revealed by staining of the biomarker BiP–GUS that is frequently used in monitoring UPR in eukaryotic cells (21, 22). BiP–GUS was transferred into WT and *hsn*1 backgrounds through genetic crossing. The marker-tagged lines were cultured on modified MS media with a constant level of NO_3^- (20 mM) but rising concentrations of NH_4^+. The arrow indicates the root apical meristematic region. (Bars: *Upper*, 1 mm; *Lower*, 0.1 mm.) (*B*) WT *Arabidopsis* and *hsn1* were grown on NO_3^- + NH_4^+ or NO_3^- medium. Cell death in the roots was revealed by the blue precipitates produced by histochemical staining with Evans blue. (Bars: 0.2 mm.)

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role in the inhibition of GMPase activity by NH_4^+. However, further studies are needed to verify this hypothesis. Of the 2 consequences of decreased GMPase activity, defective protein glycosylation in the roots rather than lowered AsA content is responsible for the growth inhibition of WT, *hsn1*, and *vtc1* seedlings by NH_4^+. This finding is compatible with the recent demonstration that oxidative stress does not contribute significantly to plant sensitivity to NH_4^+ (23).

In eukaryotic organisms, the accumulation of unfolded or misfolded proteins in the ER activates UPR signal-transduction pathways, which leads to increased protein folding capacity and cell death (to minimize the damage by the cells overaccumulating unfolded proteins; ref. 24). In agreement with previous reports (19, 20), this work revealed that defective N-glycosylation of proteins, observed in WT *Arabidopsis* and *hsn1* roots grown in the presence of NH_4^+, rendered UPR and cell death. Importantly, UPR and cell death were more severe in *hsn1* roots than in those of WT control in the presence of NH_4^+, which correlates tightly with the much larger decrease of GMPase activity in *hsn1* roots and the enhanced inhibition of *hsn1* root growth by NH_4^+. Thus, UPR and cell death are intensified in the *Arabidopsis* mutants with debilitated GMPase activity levels on the medium containing NH_4^+. In addition to this work, the association among disturbed protein N-glycosylation, augment of UPR and occurrence of cell death in the roots has been found in the *Arabidopsis* mutant with a defect in the *STT3a* gene encoding an essential subunit of the oligosaccharyltransferase complex (25). Interestingly, the *stt3a-1* mutant displays increased sensitivity to salt/osmotic stress (25). Thus, it seems likely that the same paradigm (i.e., alteration of protein N-glycosylation, initiation of UPR, and occurrence of cell death) may be used to regulate the responses of *Arabidopsis* to different environmental stimuli (e.g., salt/osmotic stress, NH_4^+ toxicity).

Our data also provide useful clues for explaining several interesting observations in this work. First, the root growth of WT *Arabidopsis* seedlings was inhibited on NO_3^- + NH_4^+ medium relative to that attained on NO_3^- medium. This inhibition correlates with a significant reduction of GMPase activity and the alteration of N-glycosylation of proteins in the WT roots cultured on NO_3^- + NH_4^+ medium, indicating a decrease of GMPase activity, and disturbance of protein N-glycosylation may at least be partly involved in the inhibition of WT *Arabidopsis* root growth by NH_4^+. Second, the roots of *hsn1* and *vtc1* seedlings were shorter than those of WT controls on NO_3^- medium (Fig. 2*C*). This phenomenon is accompanied by the substantially lower GMPase activity levels in the roots of the 2 mutants compared with that in the roots of WT control on NO_3^- medium (Fig. 2*D Right*). Moreover, on this medium, the roots of *vtc1* seedlings were significantly shorter than those of *hsn1* (Fig. 2*C*), which correlates with an even lower GMPase activity level in *vtc1* roots (Fig. 2*D Right*). Together, these data indicate that the shorter root phenotype of *hsn1* and *vtc1* seedlings on NO_3^- medium is the result of constitutively decreased GMPase activity. Third, in either WT *Arabidopsis* or GMPase mutants, the roots were more pronouncedly inhibited by NH_4^+ than the shoots. Interestingly, on NO_3^- + NH_4^+ medium, the level of GMPase activity in the root tissues was generally lower than that in the aerial tissues (Fig. 2*D*). This result may be partly responsible for the enhanced susceptibility of root growth to NH_4^+. Because retarded root growth is a common and severe symptom of NH_4^+ sensitivity in higher plants (26, 27), it will be important to examine whether the inhibition of root growth by NH_4^+ in other plant species also involves a relatively lower level of GMPase activity in the root cells in future studies.

A further interesting observation in this work is that *hsn1* and WT control did not differ significantly in the NH_4^+ content in either the shoot or root tissues on NO_3^- + NH_4^+ medium (Fig. 1*E*), indicating that NH_4^+ content does not play a major part in the hypersensitivity of *hsn1* to NH_4^+ and in the enhanced inhibition of *Arabidopsis* roots by NH_4^+. In line with this observation, a previous study has shown that the transport process of NH_4^+ across the plasma membrane is an important step that distinguishes NH_4^+-sensitive plant species from those with NH_4^+ tolerance (2). Nevertheless, the NH_4^+ contents in the root and shoot tissues of the seedlings cultured on NO_3^- + NH_4^+ medium both are significantly higher than those of the seedlings cultured on NO_3^- medium. Whether these differences would contribute to the retarded *Arabidopsis* growth in the presence of NH_4^+ is a matter for future investigations.

In summary, the molecular genetic and biochemical investigations in this work have revealed that the NH_4^+ sensitivities of WT GMPase and its mutants result in profound changes in cellular activities and cell survival in *Arabidopsis* roots. Decreased GMPase activity and its consequences (defective protein N-glycosylation, activation of UPR, and cell death in the roots) are important molecular events in the NH_4^+ sensitivity of *Arabidopsis*. Although the responses of higher plants to NH_4^+ are complex and multiple mechanisms may be involved (1), the insights gained in this work may offer clues for future attempts to understand more completely the molecular genetic basis underlying the variations of NH_4^+ sensitivity among different plant families and species.

Materials and Methods

Plant Materials and Growth Conditions. The plant materials used in this work included WT *A. thaliana* (Col-0 and L*er* ecotypes) and genetic mutants derived from Col-0 ecotype. Seed germination and seedling growth were accomplished by using modified MS media, with the 2 most frequently used ones being NO_3^- + NH_4^+ medium (with 10 mM NH_4^+ and 20 mM NO_3^-, provided by KNO_3 and NH_4NO_3, respectively, as nitrogen sources) and NO_3^- medium (with 20 mM NO_3^-, provided by KNO_3, as sole nitrogen source). The above media all were supplemented with 0.5% (wt/vol) sucrose and 0.5 g/L Mes, adjusted to pH 5.7, and solidified with 1% agar-agar (Fisons). *Arabidopsis* growth took place in a growth cabinet (Percival Scientific), preset with a 16-h light/8-h dark photoperiod, 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, and a constant temperature of 20 °C.

Chemical Reagents and Molecular Methods. Unless otherwise stated, the chemicals used in this work were purchased from Sigma. The standard molecular and biochemical methods for nucleic acid manipulations, PCR amplifications, and protein investigations with SDS/PAGE and immunoblotting followed those described (28). The cloning experiments all were verified by DNA sequencing to exclude inadvertent introductions of sequence errors.

Ammonium Assay. NH_4^+ content was determined colorimetrically at 410 nm after reaction with Nessler's reagent (29).

Map-Based Cloning. A F_2 population derived from a cross between *hsn1* mutant and L*er* ecotype was used for map-based cloning. Markers used in the initial mapping analysis were selected from The *Arabidopsis* Information Resource web site (www.*Arabidopsis*.org). Several new simple sequence length polymorphism (SSLP) markers were developed at the bottom of chromosome 2 based on differences between the Col-0 and L*er* (available at www.*Arabidopsis*.org/Cereon/index.jsp) genomic sequences. The candidate gene was mapped between the 2 new SSLP markers H13 and H16. The primers for amplifying H13 were 5′-CCACCTCATTCCCGTAGTG-3′ and 5′-CCTCGGTA GATTCATGGTCAACTG-3′, and those for H16 were 5′-TGAACCGACAGTGTGACCTTT-3′ and 5′-AGTAAGAATCACGTTAATGAATGG-3′.

Complementation Assay. A 4.2-kb genomic DNA fragment containing WT GMPase coding region and 2.7 kb of 5′ flanking sequence (upstream of the ATG start codon) was amplified by using the primers 5′-TGGTGGCGTGTATTTGTCAAAA-3′ and 5′-CAGCGTTTTGATTGATGCTTATTC-3′. The resulted fragment was blunt-ended and cloned into SmaI-digested pCAMBIA1300, creating the transformation construct for the complementation assay. Nine independent homozygous and genetically stable transgenic lines were developed based on hygromycine resistance. The 9 lines all complemented *hsn1* phenotype although only data from 2 representatives (*WTGMP/hsn1–3*, *WTGMP/hsn1–8*) are presented.

GMPase Activity Assay. GMPase activity in the supernatant was measured by monitoring the production of GDP-D-mannose from D-mannose-1-P and GTP. The extraction of total proteins and determination of protein concentrations were accomplished as detailed (30). The assay mixture contained 10 μg of total protein, 50 mM Tris·HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM GTP, and 1 mM D-mannose-1-P in a total volume of 50 μL. Incubations were done at 37 °C for 1 h. The reaction was stopped by boiling for 2 min followed by centrifugation to remove the precipitated proteins. Reaction products were analyzed by HPLC by using a C18 Bondapack column (Waters; 3.9 × 300 mm, 10-μm particle size) as described (31).

The above assay conditions were also used in the experiments comparing the activities of recombinant WT GMPase and hsn1 enzymes at 3 pH regimes (pH 6.5, 7.5, and 8.5). The cDNA coding sequences of WT GMPase and hsn1 were amplified by RT-PCR using the primers 5′-CTCATATGATGAAGGCACTCATTC-3′ and 5′-CTAAGCTTCATCACTATCTCTGG-3′ (the underlined nucleotides form the restriction sites for NdeI and HindIII, respectively), cleaved with NdeI and HindIII, and cloned into the bacterial expression vector pET30a. The resulted constructs were expressed in the bacterial cells, with the overexpressed proteins purified according to a published protocol (30). The purified recombinant protein (WT GMPase or hsn1 mutant, all in 100 ng) was used to initiate the enzyme assay. The different pH regimes in the enzyme reactions were created by using the Tris·HCl solutions with pH values adjusted to 6.5, 7.5, and 8.5.

N-Glycoprotein Analysis. Peroxidase-conjugated Con A was purchased from Sigma. A mouse polyclonal antiserum against *Arabidopsis* PDI was developed as follows. The PDI cDNA was amplified by PCR using the primers 5′-CTCATATGACCGATACCATCAACAAACACG-3′ and 5′-CACTCGAGGTGGTTGTTTGGATCTTTGTC-3′ (the underlined nucleotides form the restriction sites for NdeI and XhoI, respectively). The bacterial expression and purification of *Arabidopsis* PDI were then performed as described above. The purified protein was used as an antigen to develop PDI-specific polyclonal antibodies in mouse (30).

Total protein samples were prepared as described above. Affinodetection of N-glycoproteins was carried out by using the Con A-peroxidase method (32). The PDI-specific antiserum described above was used to reveal the N-glycosylation status of PDI following a published procedure (33).

Detection of UPR in *Arabidopsis* Roots. UPR was monitored by histochemical staining of the biomarker BiP–GUS that was rapidly activated by ER stress (21). The transgenic *Arabidopsis* line harboring BiP-GUS (GUS expression cassette directed by *BiP2* gene promoter) was derived from a previous publication (21). By genetic crossing, the BiP–GUS marker was transferred into *hsn1* background. Histochemical GUS staining was performed following a published protocol (34).

Cell Death Assay. Cell death was examined by using Evans blue, a compound that selectively enters the dead cells as a result of their freely permeable plasma membranes (35). The root tips of 10 DAG seedlings were submerged in 20 mL of 1% (wt/vol) Evans blue water solution for 10 min, washed with distilled water for 2 h, and then photographed as above.

For additional methods see *SI Text*.

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