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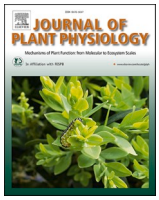

High ammonium inhibits root growth in *Arabidopsis thaliana* by promoting auxin conjugation rather than inhibiting auxin biosynthesis

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ABSTRACT

Ammonium (NH_4^+) inhibits primary root (PR) growth in most plant species when present even at moderate concentrations. Previous studies have shown that transport of indole-3-acetic acid (IAA) is critical to maintaining root elongation under high-NH_4^+ stress. However, the precise regulation of IAA homeostasis under high-NH_4^+ stress (HAS) remains unclear. In this study, qRT-PCR, RNA-seq, free IAA and IAA conjugate and PR elongation measurements were conducted in genetic mutants to investigate the role of IAA biosynthesis and conjugation under HAS. Our data clearly show that HAS decreases free IAA in roots by increasing IAA inactivation but does not decrease IAA biosynthesis, and that the IAA-conjugating genes *GH3.1*, *GH3.2*, *GH3.3*, *GH3.4,* and *GH3.6* function as the key genes in regulating high-NH_4^+ sensitivity in the roots. Furthermore, the analysis of promoter::GUS staining *in situ* and genetic mutants reveals that HAS promotes IAA conjugation in the elongation zone (EZ), which may be responsible for the PR inhibition observed under HAS. This study provides potential new insight into the role of auxin in the improvement of tolerance to NH_4^+.

1. Introduction

Ammonium (NH_4^+) is not only a major nitrogen (N) source but also a critical intermediate in the metabolism of plants (Glass et al., 2002). However, NH_4^+ as a sole N source is frequently harmful to plants when it is provided at moderate to high concentrations (Britto et al., 2001; Britto and Kronzucker, 2002, 2013; Li et al., 2014; Zhang et al., 2021), while species well adapted to the NH_4^+ source are few (Kronzucker et al., 1997). NH_4^+ toxicity is not only observed in terrestrial plants, but is also found in yeast (Hess et al., 2006), cyanobacteria (Drath et al., 2008; Dai et al., 2012), algae (Dai et al., 2012), and animals (Biver et al., 2008). In higher plants, roots are the primary site of contact with NH_4^+, resulting in a severe modification of root architecture, which includes shorter primary roots (Li et al., 2010; Kojima, 2018), abnormal lateral root development (Esteban et al., 2016), and a loss of gravitropism (Zou et al., 2012). Although the effect of NH_4^+ on root development has been previously studied by both genetic or physiological approaches, the precise underlying mechanisms are still unclear.

Auxins are vital to normal plant growth and regulate diverse processes such as root elongation, gravitropic responses, apical dominance, and stresses responses more generally (Di et al., 2016a). The levels and distribution patterns of the active auxin form IAA are tightly controlled by three processes: synthesis, inactivation, and transport (Korasick et al., 2013a,b). Auxin inactivation occurs primarily by conjugating with sugars or amino acids. In *Arabidopsis thaliana* (Arabidopsis), three IAA glucosyltransferases, UGT75D1, UGT84B1, and UGT84B2, catalyse the conjugation of IAA to sugars and further produce either temporary storage forms or irreversibly inactive products (Jackson et al., 2001;

Abbreviations: Arabidopsis, *Arabidopsis thaliana*; NH_4^+, ammonium; HAS, high-NH_4^+ stress; IAA, indole-3-acetic acid; PR, primary root; EZ, elongation zone; MZ, meristem zone; QC, quiescent center.

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Zhang et al., 2016). The production of amide-linked IAA-amino acid conjugates is catalysed by Group II GRETCHEN HAGEN3 (GH3) acyl amido synthetases (GH3.1-GH3.6, GH3.9, and GH3.17) (Staswick et al., 2005). Overexpression or loss-of-function mutations in these IAA-conjugating genes have been shown to alter many IAA-mediated biological processes, such as IAA sensitivity, lateral root development, and stress responses, indicating that IAA conjugation plays a critical role in regulating plant normal growth (Staswick et al., 2005; Zhang et al., 2016; Park et al., 2007).

Understanding of the critical role of IAA in the high-NH_4^+-mediated arrest of root development has greatly improved in recent decades. Studies in the 1990s showed that the Arabidopsis auxin mutants *aux1*, *axr1,* and *axr2* are less sensitive than wild type to the inhibitory effects of NH_4^+ on root growth (Stevens et al., 2004), and that NH_4+, but not NO_3^-, could reduce IAA content in wheat roots (Cao et al., 1993; Kudoyarova et al., 1997). Previous studies have suggested that contact with the root tip (the apical 2 mm) is both necessary and sufficient for primary root (PR) growth inhibition by NH_4^+ stress, and that NH_4^+ targets principally cell elongation (Li et al., 2010; Kempinski et al., 2011). Auxin transport, mediated by AUX1 and PIN2, plays an important role in lateral root development and in root gravitropism (Ke et al., 2015; Li et al., 2011; Zou et al., 2013). Moreover, a recent study showed that a rice cultivar with high endogenous IAA exhibited improved tolerance to high-NH_4^+ stress (HAS) (Di et al., 2018). However, the precise genetic mechanisms and genes involved in this process are not yet clearly known.

The previous studies provide tantalizing hints at the central role of IAA homeostasis under HAS, meriting further investigation. In this study, we address two questions to elucidate the pathways involved in IAA-regulated root growth under high NH_4^+: (i) Which processes contribute to IAA homeostasis under HAS: IAA biosynthesis, IAA conjugation, or both? (ii) Which genes are responsible for the HAS tolerance?

2. Materials and methods

2.1. Plant material and growth conditions

The wild-type Arabidopsis control used in this study was Col-0. Arabidopsis mutants, and accession numbers used in this study are listed in Supplemental Table S2. Seeds of the above strains were cold-treated at 4 °C for 48 h, and then surface-sterilized with 0.1 % $HgCl_2$ before being sown on a normal growth medium, which contained 2 mM KH_2PO_4, 5 mM $NaNO_3$, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM Fe-EDTA, 50 mM H_3BO_3, 12 mM $MnSO_4$, 1 mM $ZnC1_2$, 1 mM $CuSO_4$, 0.2 mM Na_2MoO_4, 1% sucrose and 1% agar (adjusted to pH 5.7 with 0.1 M NaOH) (Li et al., 2010). Germination and plant growth was carried out at 23 °C ± 1 °C with a 16 h light/8 h dark cycle.

2.2. Treatment and measurement of root length

We used two means of measurement to investigate primary root growth inhibition. For whole seedlings supplied with high NH_4^+, surface-sterilized seeds were germinated and grown on media with 15 mM $(NH_4)_2SO_4$. On day 8, primary roots were measured using ImageJ software. For root-supplied NH_4^+, seeds were germinated and grown for 5 days prior to being transferred to the treatment medium. We used segmented agar plates (13 × 13 cm) to ensure that either the shoot or the root was exposed to media containing 15 mM $(NH_4)_2SO_4$, 0.001 μM IAA, and various concentrations (0 μM, 1.5 μM, and 3 μM) of L-Kyn and yucasin (0 μM, 5 μM, and 10 μM). We employed ImageJ to measure the primary root lengths after treatment for three days.

2.3. Histochemical GUS staining

For *p*DR5::GUS staining, 5-day-old seedlings containing a *GUS* marker were transferred to fresh agar plates, grown for 3 days and then incubated in 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) and 50 mM potassium phosphate buffer, pH 7.5, with 0.1 % v/v Triton X-100 for GUS staining at 37 ℃ for 4 h before observation.

For promoter::GUS staining, 5-day-old transgenic seedlings were treated with 15 mM $(NH_4)_2SO_4$ for 3 d prior to GUS staining (6 h).

2.4. Microscopic analysis of root elongation

To measure the length of the elongation zone, meristem zone, and root cap, images were obtained using an Olympus BX51 microscope equipped with differential interference contrast (DIC) optics and an Olympus DP71 camera. The length from the first elongated cell to the first root hair was defined as EZ, and the length from the QC to the first elongated cell was defined as MZ. Measurements were taken at least twice.

2.5. RNA isolation and qRT-PCR

RNA was extracted using the FastPure Plant Total RNA Isolation Kit (Polysaccharides& Polyphenolics –rich) (RC401, Vazyme Biotech Co., Ltd). 1 μg of total RNA was used to synthesize the first-strand cDNA by using a HiScript 1st-Strand cDNA Synthesis Kit (R111-01; Vazyme Biotech Co., Ltd), and then the diluted cDNA (20 times) was used for real-time PCR. The qRT-PCR was performed as previously described (Di et al., 2018; Sun et al., 2020a).The primers are listed in Table S3.

For analysing NH_4^+-induced gene transcription, 8-day-old seedlings transferred to liquid media were treated with 30 mM NH_4^+ for different lengths of time (15 min, 30 min, 60 min, 120 min, 240 min, and 360 min), and then roots were used for RNA extraction.

2.6. RNA-sequencing analysis

5-day-old seedlings were transferred to new media with or without 30 mM NH_4^+ for another 5 days, then the roots of 10-day-old seedlings were collected for RNA extraction using the TRIzol reagent (Invitogen, USA). The methods for RNA-seq were described in the previous study in detail (Sun et al., 2017). DEGs (different expressed genes) and GO (Gene ontology) were performed as in a previous study (Sun et al., 2020b).

2.7. Determination of IAA concentration

200 mg of Arabidopsis roots were ground to powder in liquid nitrogen and then extracted with 80 % MeOH containing internal standards ($^2H_2$-IAA) at -20 °C for 16 h. After centrifugation at 15,000 × *g* for 15 min, the supernatant was collected and dried by evaporation. The residue was reconstituted using 5 % NH_4OH and loaded onto an Oasis MAX (Waters) cartridge. The cartridge was sequentially washed with 5 % NH_4OH, water and MeOH, and then eluted with 5 % FA in MeOH. The eluent was evaporated and re-dissolved in 80 % MeOH for LC–MS/MS analysis.

LC–MS/MS analysis was performed using a UPLC system (Waters) coupled to a 6500 QTrap system (AB SCIEX). LC separation used a BEH C18 column (1.7 mm, 2.1 × 100 mm; Waters) with mobile phase 0.1 % formic acid (A), and ACN (B). The gradient was set to an initial 2 % B and increased to 50 % B within 6 min. IAA, IAA-Asp, IAA-Leu/Ile, and IAA-Glu were detected in MRM mode with transitions 176/130, 291/130, 289/130, and 305/130, respectively.

2.8. Statistical analysis

Data sets were analysed using Prism 6 software (GraphPad Software). Comparisons between pairs of groups were made using a Student's *t*-test. All values were presented as means ± SD. Comparisons between multiple groups were conducted using two-way ANOVA tests.

3. Results

3.1. Disrupting IAA homeostasis in roots but not in shoots is responsible for NH_4^+-inhibited PR growth

We previously demonstrated that the root tip is both necessary and sufficient for plants to respond to high NH_4^+, and that high NH_4^+ decreases the auxin-response reporter *p*DR5::GUS in roots (Li et al., 2010). In this study, we first investigated in detail where and how IAA homeostasis is disrupted under NH_4^+ stress. A segmented agar plate system that permitted the supply of NH_4^+ to shoots and/or roots in isolation was used (Fig. 1A). To determine the distribution of IAA, we monitored both *p*DR5::GUS and *p*DR5::GFP under varying NH_4^+-supply modes. 15 mM $(NH_4)_2SO_4$ supplied to whole seedlings decreased the intensity of GUS and GFP in both roots and shoots, while root-supplied or shoot-supplied NH_4^+ only reduced GUS staining or GFP fluorescence within the directly NH_4^+-supplied organs (Figs. 1B and S1). In addition, root-supplied and whole-seedling-supplied seedlings exhibited similar primary root inhibition (35 % *vs* 44 %), but shoot-supplied seedlings showed weaker inhibition of PR growth (~15 %; Fig. 1C). However, when we applied low concentrations of exogenous IAA to roots, root-supplied IAA could partially rescue the NH_4^+-mediated root inhibition (Fig. 1B and C). These results show that high NH_4^+ mainly decreases the free IAA content in tissues directly in contact with NH_4^+, and that the changes of free IAA content in roots are critical to the PR growth inhibition induced by high NH_4^+.

3.2. IAA biosynthesis plays a positive role in root growth under HAS

To explore which processes, IAA biosynthesis, conjugation, or both, are involved in the inhibition of PR growth by high NH_4^+, we first performed qRT-PCR to analyze the biosynthetic genes of IAA and Trp, a precursor of IAA in plants, in the roots with or without high NH_4^+. Our results show that most of the IAA and Trp biosynthetic genes were upregulated 1.27~2.76-fold under HAS (Fig. 2A–C), except for *INS*, *TAR2*, *YUC7,* and *YUC8/CKRC2*, suggesting that IAA biosynthesis may be involved in regulating IAA accumulation under HAS.

To further investigate the role of IAA biosynthesis under HAS, we then examined root growth in mutants disrupted in IAA biosynthesis under HAS (Di et al., 2016a) (Fig. 2A). These mutants showed varied tolerance to high NH_4^+ (Fig. 3). Some of the mutants exhibited increased sensitivity to high NH_4^+, *e.g. trp2*, *trp3*, *trp4/wei7*, *trp5/wei2*, *taa1/ckrc1*, *yuc3,* and *yuc5*, while others showed similar, or slight increased, sensitivity to HAS compared with their wild-type controls (Figs. 3A,B and S2).

Gene redundancy may result in a weak or inconspicuous mutative phenotype. To further clarify the role of IAA biosynthesis in maintaining PR growth during HAS, we further examined two chemical inhibitors, L-kynurenine (Kyn) and 5-(4-chlorophenyl)-4H-1,2,4-triazole-3-thiol (yucasin), which act as inhibitors of TAA1/TARs and YUCs activity, respectively (He et al., 2011; Nishimura et al., 2014) (Fig. 3C). The relative root growth of Col grown on high-NH_4^+ media with 1.5 μM Kyn, 3 μM Kyn, 5 μM yucasin, and 10 μM yucasin, was 44.5 %, 33.2 %, 55.2 %, and 40.3 %, respectively, revealing higher sensitivity to HAS compared with Col without any inhibitors (69.2 %) (Figs. 3B, E and S3, S4). In addition, compared with Col and the single mutants *cyp79b2* (68.0 %) and *cyp79b3* (67.8 %), the relative root growth of the double mutant *cyp79b2cyp79b3* was 58.1 %, also exhibiting elevated sensitivity to HAS (Fig. 3D). We conclude that IAA biosynthesis exerts a positive impact on primary root growth under HAS conditions.

3.3. IAA conjugation regulated by IAA-conjugating proteins contributes to PR sensitivity under HAS

RNA-seq data has shown that high NH_4^+ can upregulate most genes encoding IAA-conjugation proteins (Table S1 and Fig. S5). To further clarify the role of IAA conjugation under HAS, we then performed qRT-PCR to investigate the induction of IAA-conjugating genes after different treatment time under HAS. Our data show that, after high-NH_4^+ treatment for 15 min, transcription of *GH3.2* and *GH3.17* was upregulated 2-fold compared with untreated plants, but other IAA-conjugating genes were also induced by high-NH_4^+ treatment for 30 min (Fig. 4). Moreover, most of the IAA-conjugating genes were strongly induced when treatment time was extended to 2 h (Fig. 4). In addition, except for *GH3.5* and *GH3.17*, the transcription levels of most IAA-conjugating genes were still elevated more than 2-fold compared to untreated plants after 6 h of treatment (Fig. 4). Furthermore, *p*DR5::GUS transcription levels exhibited a significant decrease after 1-h high-NH_4^+ treatment (Fig. 4). These data suggest that high NH_4^+ induces the transcription of IAA-conjugating genes, and that these IAA-conjugating genes may function differently dependent on treatment time.

To further examine the roles of IAA-conjugating genes, we then measured the PR length of IAA-conjugating mutants under both control

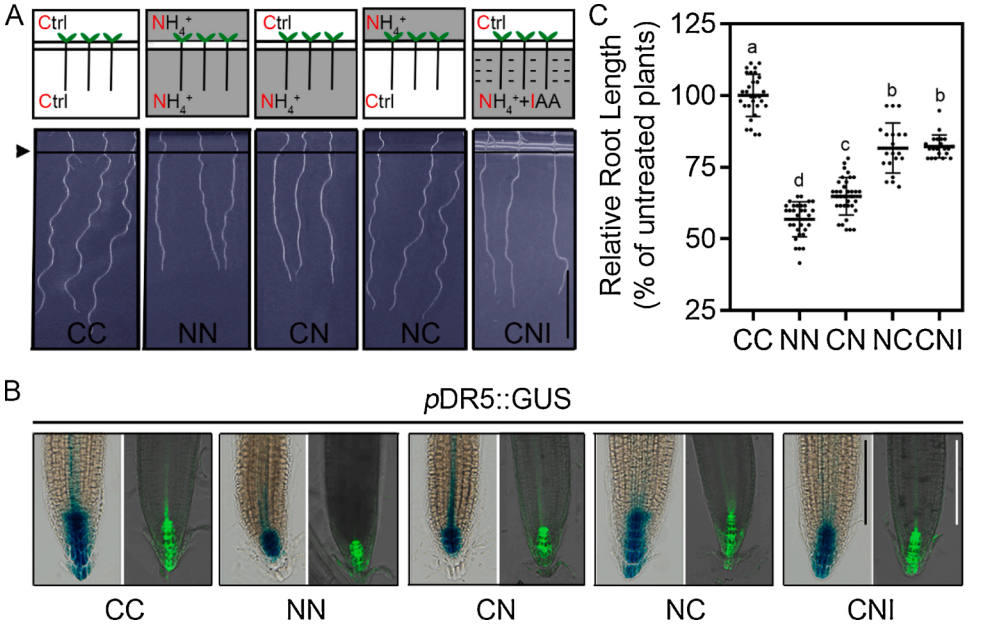

Fig. 1. High NH_4^+ inhibits Arabidopsis PR growth by decreasing free IAA in roots. **(A)** Phenotype of roots after 3-d growth on segmented agar plates with roots and/or shoots supplied with 15 mM $(NH_4)_2SO_4$ (+/-) 0.001 μM IAA. Ctrl indicates control, NH_4^+ indicates Ctrl medium with 15 mM $(NH_4)_2SO_4$ (Bar = 1 cm). Black triangle indicates start point after transfer; **(B)** GUS staining and GFP fluorescence in roots with root/shoot-supplied NH_4^+ and/or IAA, after staining for 1 h of the PR tip or shoot of Arabidopsis (for GUS staining). Bar = 1 mm. One representative sample is shown for each set of parameters. **(C)** Relative PR length of 5-day-old untreated Col (wild-type) Arabidopsis seedlings grown on segmented agar plates with NH_4^+ and/or IAA for another 3 d. Error bars indicate ± SD. Lowercase letters indicate significant differences at $P < 0.05$, according to ANOVA, followed by Tukey's multiple comparison test (n = 15). In (C) 100 % corresponds to 30.1 ± 2.22 cm for Col-0.

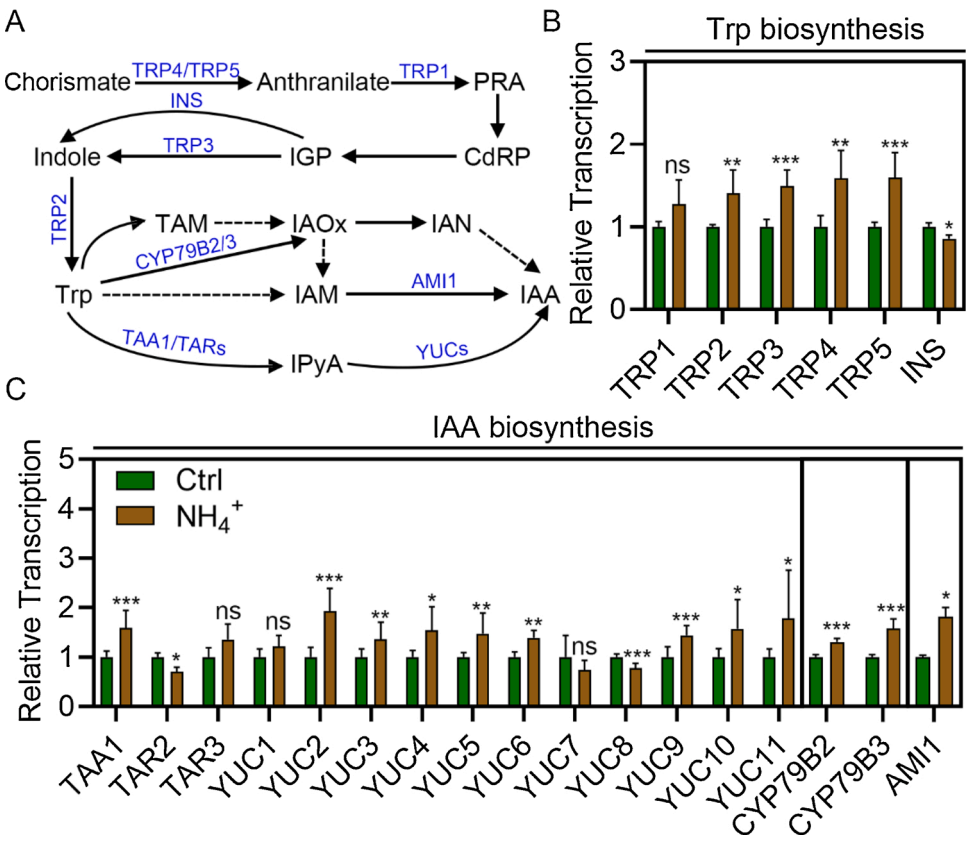

Fig. 2. Transcription analysis of genes involved in IAA biosynthesis and IAA conjugation. **(A)** The genes involved in IAA biosynthesis. Blue letter: genes involved in Trp or IAA biosynthesis; black letter: metabolites involved in Trp or IAA biosynthesis; solid line: identified pathway; dashed line: speculative pathway; **(B** and **C)** Relative transcription levels of Trp biosynthetic genes **(B)** and IAA biosynthetic genes **(C)** in roots after 15 mM $(NH_4)_2SO_4$ treatment for 6 h. Data are means of three biological replicates. Error bars indicate ± SD. $0.05 > {}^{*}P > 0.01$; $^{***}P < 0.001$ (*t*-test). ns indicates not significant.

and high-NH_4^+ conditions. The relative root length of *gh3.1* (74.6 %), *gh3.2* (73.2 %), *gh3.3* (73.5 %), *gh3.4* (75.3 %), and *gh3.6* (71.3 %) was greater, and that of *gh3.5* (69.8 %), *gh3.9* (70.4 %), *gh3.17* (70.2 %), *ugt84b1* (70.4 %), and *ugt84b2* (70.6 %) slightly greater, than that of Col (68.0 %), while the relative root length of UGT75D1*ox* (*UGT75D1* overexpression line) was reduced by 47.3 % compared with Col (Fig. 5A-B and Fig. S6). Together, these data suggest that IAA-conjugating genes play a negative role in response to HAS.

We further directly determined the contents of free IAA and of several IAA-conjugates (IAA-Asp, IAA-Glu, and IAA-Leu/Ile) in the roots under both control and HAS. Our data show that free IAA was 27.66 pg mg^{-1} under control conditions, but decreased to 19.66 pg mg^{-1} upon exposure to high NH_4^+ (Fig. 6A). The contents of IAA-Asp (1.12 pg mg^{-1}), IAA-Glu (3.66 pg mg^{-1}) and IAA-Leu/Ile (0.4 pg mg^{-1}) under control increased to 2.64 pg mg^{-1}, 7.10 pg mg^{-1}, and 1.07 pg mg^{-1}, respectively, after high-NH_4^+ treatment (Fig. 6B–D). Overall, it is clearly demonstrated that HAS decreases free IAA content by promoting IAA conjugation in the roots.

3.4. *High-NH_4^+-induced IAA conjugation in the elongation zone might be responsible for PR inhibition*

Our previous study had shown that primary root growth inhibition under high NH_4^+ was mostly due to inhibition in the elongation zone (EZ) (Li et al., 2010), but the precise pathway remains unknown. We further measured the length of the meristem zone (MZ) and EZ of some auxin-related mutants, with altered NH_4^+ sensitivity, namely *trp4/wei7*, *trp5/wei2*, *taa1/ckrc1*, *gh3.3*, *gh3.6*, and UGT75D1*ox* under both control and high-NH_4^+ conditions (Figs. 3A anf B, 5 B). MZ and EZ length of *trp4/wei7*, *trp5/wei2*, and *taa1/ckrc1* were shorter than Col, whilst it was slightly longer and shorter in the *gh3.3* and *gh3.6* lines under control, respectively (Fig. 7A and B). When exposed to high NH_4^+, the relative MZ length (high-NH_4^+/Ctrl) in *trp4/wei7*, *trp5/wei2*, and *taa1/ckrc1* was 65.5 %, 64.6 %, and 63.3 %, *i.e.* displaying slightly higher sensitivity than Col (72.2 %), while the relative EZ length in *trp4/wei7*, *trp5/wei2*, *taa1/ckrc1,* and Col was 37.1 %, 46.7 %, 40.5 %, and 57.9 %, respectively, supporting the positive role of IAA biosynthesis in regulating MZ and EZ growth under HAS (Fig. 7A). Furthermore, the relative MZ length of *gh3.3* (73.8 %) and *gh3.6* (73.9 %) was slightly higher than that of Col (72.2 %), while the relative EZ length of the two genotypes (70.6 % in *gh3.3* and 66.5 % in *gh3.6*, respectively) was higher than in Col (Fig. 7B). Consistent with this, the *UGT75D1* overexpression line also exhibited increased sensitivity to high-NH_4^+ in both the MZ and EZ (Fig. 7A and B).

To further test this conclusion, we then examined promoter::GUS staining for these selected genes involved in Trp biosynthesis (*TRP4/WEI7*, *TRP5/WEI2*), IAA biosynthesis (*TAA1/CKRC1*), and IAA conjugation (*GH3.3*, *GH3.6*, *UGT75D1*). We found that the Trp biosynthetic genes *TRP4/WEI7* and *TRP5/WEI2* were mainly expressed in the quiescent center (QC) and were upregulated by high NH_4^+ (Fig. 7C). However, the IAA-biosynthetic gene *TAA1/CKRC1* was mainly expressed in the EZ under control conditions and was induced at greater intensity following high-NH_4^+ treatment (Fig. 7C). In addition, the expression of TAA1/CKRC1 was also observed in the QC after high-NH_4^+ treatment (Fig. 7C). Moreover, the IAA-conjugating genes *GH3.3* and *GH3.6* were mainly expressed in the EZ and QC, and GUS staining revealed induction in the EZ but not in the QC, suggesting a special role for *GH3.3* and *GH3.6* in the EZ under HAS (Fig. 7C). Furthermore, we found that *p*DR5::GUS staining in the QC was similar between the control and high-NH_4^+ conditions; however, GUS staining in the EZ was obviously decreased under HAS, indicating the important role of free IAA in regulating EZ growth under such conditions (Fig. 7C). In addition, we also found that *p*UGT75D1::GUS was mainly expressed in the QC and MZ under control conditions, exhibiting strong induction in the QC and MZ, with further amplification in the EZ under HAS (Fig. 7C), indicating UGT75D1-mediated IAA conjugation mainly acts in the MZ and EZ, and especially the MZ, under high-NH_4^+ stress. Overall, these

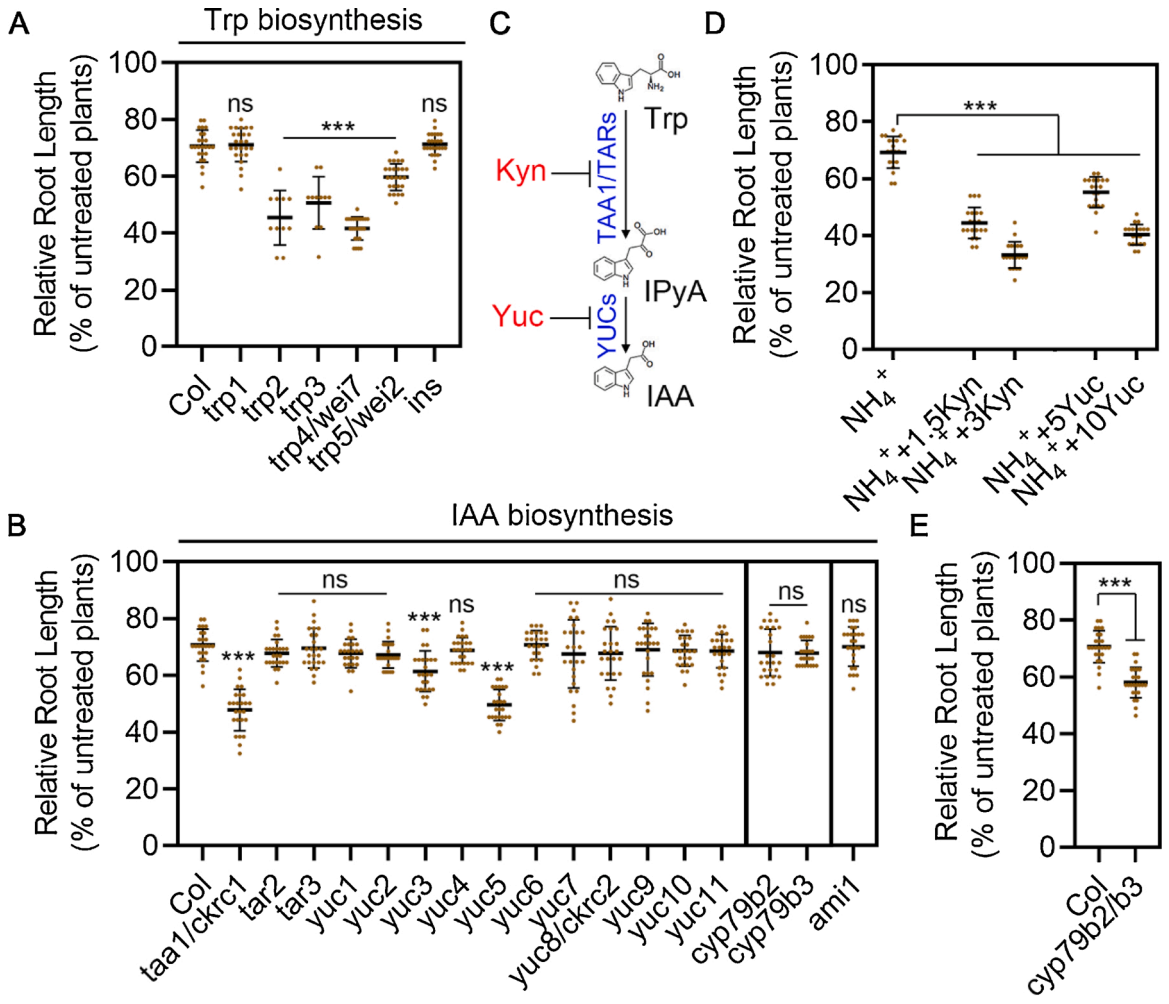

Fig. 3. IAA biosynthesis is involved in the regulation of IAA homeostasis under NH_4^+ stress.
(A and **B)** Relative PR length of Trp-biosynthesis mutant seedlings **(A)** and IAA-biosynthesis mutant seedlings **(B)** grown on medium with or without 15 mM $(NH_4)_2SO_4$. **(C)** The functional location of IAA biosynthesis inhibitor L-Kyn and Yucasin; (D) Effect of adding the TAA1/CKRC1 inhibitor L-Kyn and YUC inhibitor yucasin on the inhibition of root growth under high NH_4^+. Shown are mean values ± SD with n = 25; Asterisks indicate significant differences at ***, $P < 0.01$ (*t*-test). ns indicates not significant.

data show that high NH_4^+ upregulates the transcription of IAA-conjugating genes and further promotes the IAA conjugation in the EZ of roots, which may be responsible for PR inhibition under high NH_4^+.

4. Discussion

4.1. Elevated IAA conjugation rather than decreased IAA biosynthesis is responsible for the reduction in free IAA in roots under HAS

Active auxin IAA levels are controlled by the transport, biosynthesis, and conversion (Korasick et al., 2013a,b). It is well known that NH_4^+ stress can decrease free IAA content in roots (Li et al., 2010; Liu et al., 2013; Konishi et al., 2017; Di et al., 2018). However, the precise process involved in the pathway has remain obscured. In this study, we show that high NH_4^+ can reduce the availability of free IAA in Arabidopsis roots (Figs. 1B and 6 A), and further that this effect mainly occurs in the tissues directly exposed to NH_4^+. The decrease in free IAA content in roots is critically responsible for PR inhibition under HAS (Figs. 1 and S1).

Our previous study had suggested that auxin transport is regulated by AUX1 and PIN2, engaged in auxin influx and efflux, and is not involved in PR growth inhibition (Li et al., 2010). In this study, we analysed the transcription levels of genes involved in IAA biosynthesis and conjugation and found that most of the IAA-conjugating genes were induced by high NH_4^+. Interestingly, the IAA-biosynthetic genes (including Trp-biosynthetic genes) (*e.g.* upregulated *TRP1*, *2*, *3*, *4*, *5*, *TAA1*, *YUC2*, *3*, *4*, *5*, *6*, *9*, *10*, *11 vs.* downregulated *TAR2*, *YUC8* and *INS*) exhibited different responses to high NH_4^+ (Fig. 2C). However, the inhibitors L-Kyn and yucasin could aggravate the sensitivity to high NH_4^+, supporting the positive role of IAA biosynthesis in NH_4^+ tolerance (Fig. 3D). The increased transcriptional response to HAS may result from feedback regulation of YUC locally (Suzuki et al., 2015).

In addition, we measured free IAA and IAA conjugates in 8-day-old Arabidopsis roots (Fig. 6). Consistent with reduced *p*DR5::GUS or *p*DR5::GFP expression in the root-tip region (Figs. 1C and 7 C), free IAA content decreased under HAS (Fig. 6A). Furthermore, concentrations of several IAA conjugates were also strongly increased by high NH_4^+ (Fig. 6B–D). We conclude that root-supplied NH_4^+ disrupts IAA homeostasis in roots by promoting IAA conjugation rather than by inhibiting IAA biosynthesis.

Most of the IAA in plants is stored in conjugated forms, which participate in IAA homeostasis *via* the storage and release of free IAA. Besides IAA biosynthesis, the conversion of IAA conjugates to free IAA is also an important mechanism for maintaining active IAA levels and plays an important roles in the regulation of root development and responses to abiotic stress (*e.g.* temperature, drought, and salt stress) (Ding

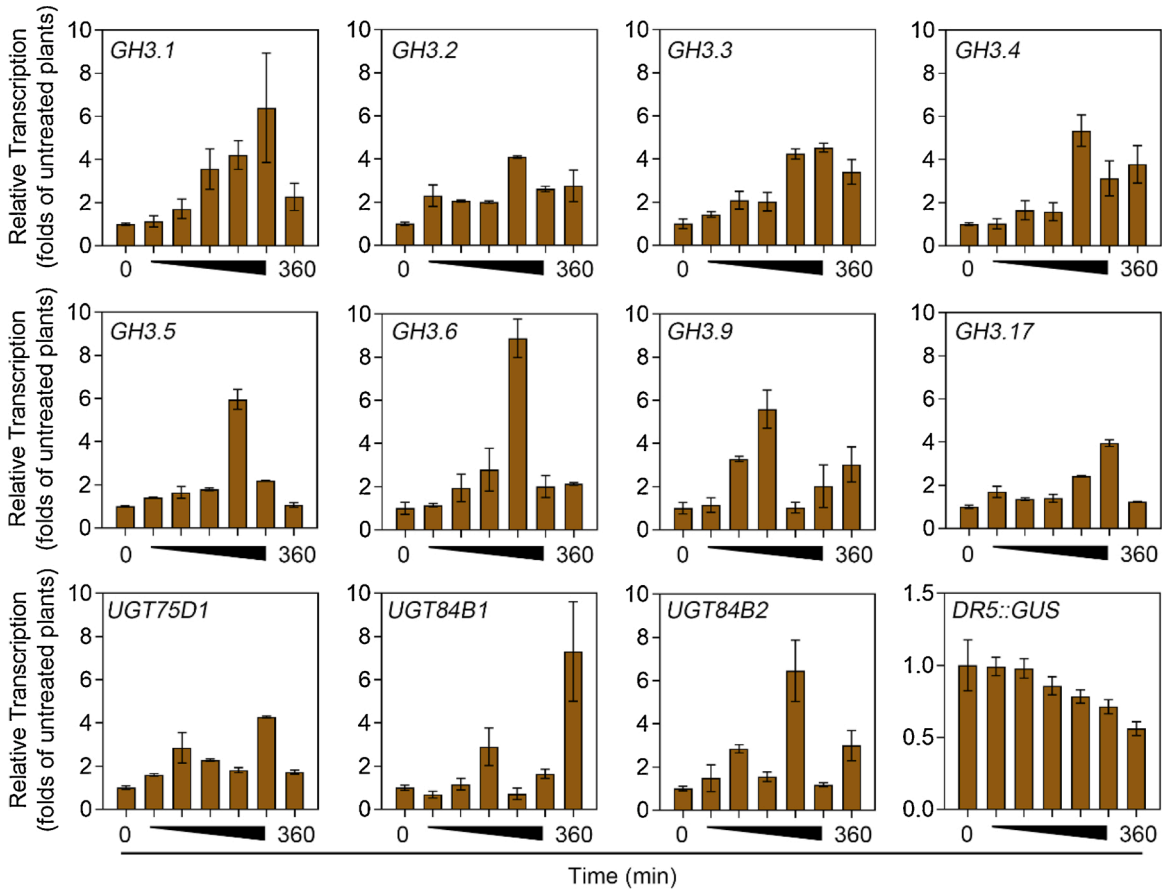

Fig. 4. Relative transcription of IAA-conjugating genes after various periods of time of high-NH_4^+ stress treatment.
8-day-old seedlings were treated with 15 mM $(NH_4)_2SO_4$ for various periods of time (0, 15, 30, 60, 120, 240, 360 min) before roots were collected for RNA extraction. Data are the means of three replicates. Error bars indicate ± SD.

et al., 2015; Khan and Stone, 2007; Zhang et al., 2007; Branka Salopek-Sondi et al., 2015). The present study clearly shows that NH_4^+ stress induces the transcription of genes coding for IAA-conjugation proteins, leading to enhanced IAA conjugation, decreased free IAA content, and decreased PR growth.

4.2. *GH3.1, GH 3.2, GH3.3, GH3.4, and GH3.6 play an important role in regulating the high NH_4^+ sensitivity of roots*

In Arabidopsis, three IAA glucosyltransferases (UGT75D1, UGT84B1, and UGT84B2) and eight GH3 amido synthetases (GH3.1-GH3.6, GH3.9, and GH3.17) have been reported to catalyse the conjugation of IAA to sugars or amino acids (Jackson et al., 2001; Zhang et al., 2016; Stawick et al., 2005). We analysed the transcription of these genes under control and HAS conditions, and the data show that these genes are all induced by high NH_4^+, revealing their participation in the plant response to high NH_4^+ (Fig. 4). Moreover, the single mutations in *GH3.1*, *GH3.2*, *GH3.3*, *GH3.4,* and *GH3.6* resulted in increased high-NH_4^+ tolerance in the roots, supporting their important function in the NH_4^+ response (Fig. 5). In addition, our data also show that these IAA-conjugating genes exhibit differential transcription abundance in roots, suggesting that the localisation of expression in roots, in particular in the EZ, of these genes is important in staging a response under HAS (Fig. S7). However, it is not as yet known why plants need to induce IAA-conjugating genes under HAS. We propose the following two reasons: 1) The upregulation of the IAA-conjugating genes may be a reflection of plant injury incurred by exposure to excessive ammonium, rather than representing an active induction for the purpose of acclimation. The induction of IAA-conjugating genes by HAS could then result in less free IAA accumulation in roots and inhibit the root growth under HAS; 2) The induction of these genes functions as a feedback signal to activate the upstream signaling components of the NH_4^+ response, so as to improve plant tolerance to ammonium stress.

Considering the positive role of IAA in response to high NH_4^+, we aimed at examining the impact of increases in endogenous IAA contents on NH_4^+ tolerance, and, to this end, examined both mutation and inhibition of IAA-conjugating genes, rationalizing as follows: 1) Mutation of IAA biosynthetic genes leads to higher sensitivity to HAS (Fig. 3); 2) Overexpression of IAA-biosynthetic genes results in developmental defects (Di et al., 2016b; Zhao et al., 2001); 3) Mutation or inhibition of some IAA-conjugating genes promotes PR growth under control conditions and increases NH_4^+ tolerance (Figs. 4 and 7A and B).

4.3. *IAA conjugation in the EZ may be responsible for PR inhibition under HAS*

PR growth is determined by two processes: cell division in the MZ and cell elongation in the EZ (Scheres et al., 2002). Our previous results have shown that PR inhibition under high NH_4^+ is mostly due to the repression of EZ growth (Li et al., 2010). The critical role of IAA in regulating EZ elongation has been widely investigated, and there are several studies that have linked exposure to high NH_4^+ to decreased IAA content in root tips (Di et al., 2018; Li et al., 2011; Liu et al., 2013). In our study, HAS decreased *p*DR5::GUS staining in the root, including in the MZ and EZ, and particularly in the EZ, indicating a role for IAA in regulating EZ elongation under HAS (Fig. 7C). To identify whether IAA biosynthesis or conjugation is involved in this regulation, we then analysed the EZ and MZ length of both IAA biosynthesis- and

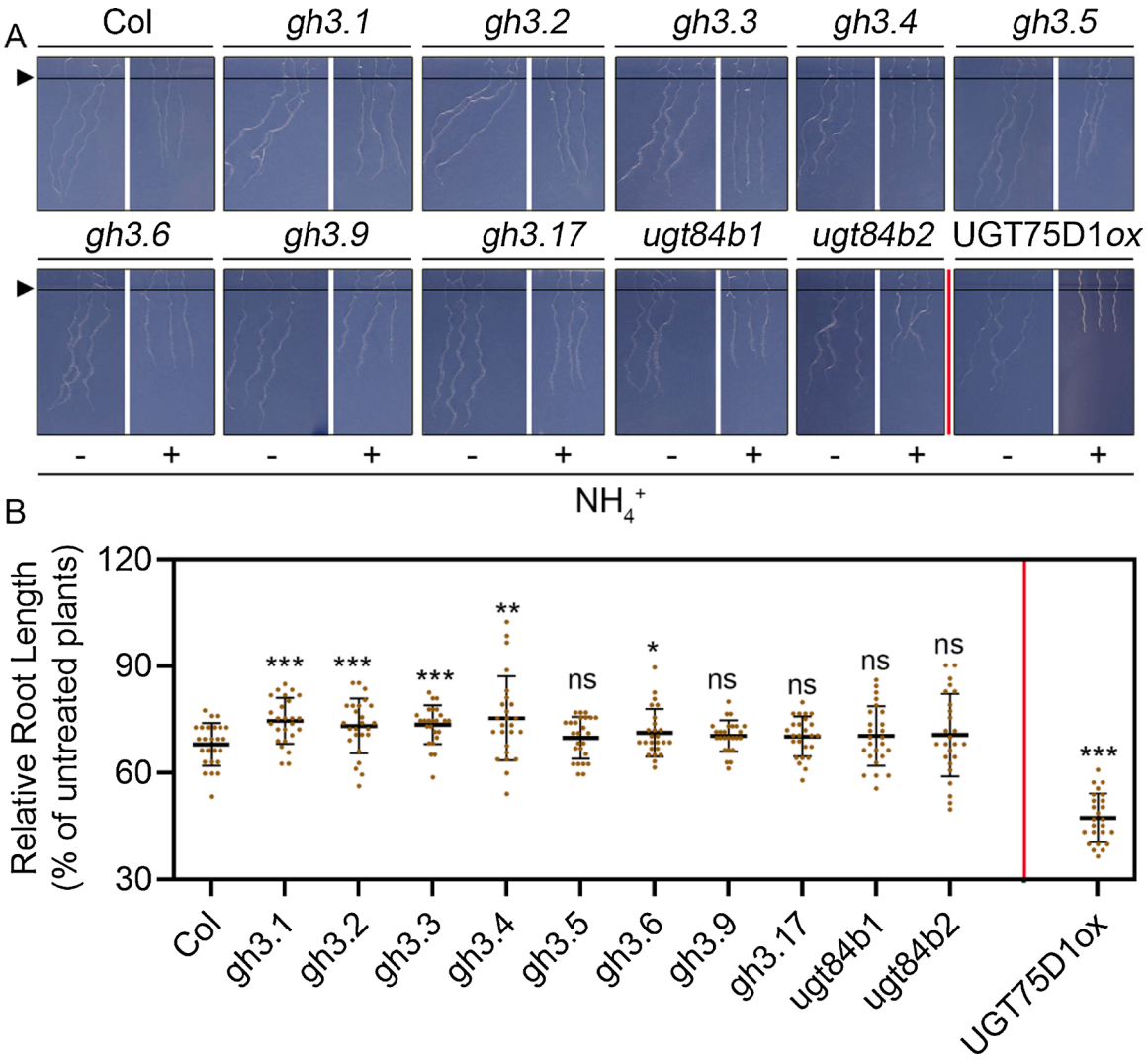

Fig. 5. Mutation of IAA-conjugating genes resulted in higher tolerance to high-NH_4^+ stress.
(A) Phenotype of IAA-conjugating mutants grown on media with or without 15 mM $(NH_4)_2SO_4$; **(B)** Root length of IAA-conjugating mutants grown on media with or without 15 mM $(NH_4)_2SO_4$. 5-day-old untreated seedlings were transferred to a medium with or without 15 mM $(NH_4)_2SO_4$, then grown for 3 days prior to measurement. Values shown are the means ± SD with n = 25. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*t*-test). ns indicates not significant. Black triangle indicates start point after transferring.

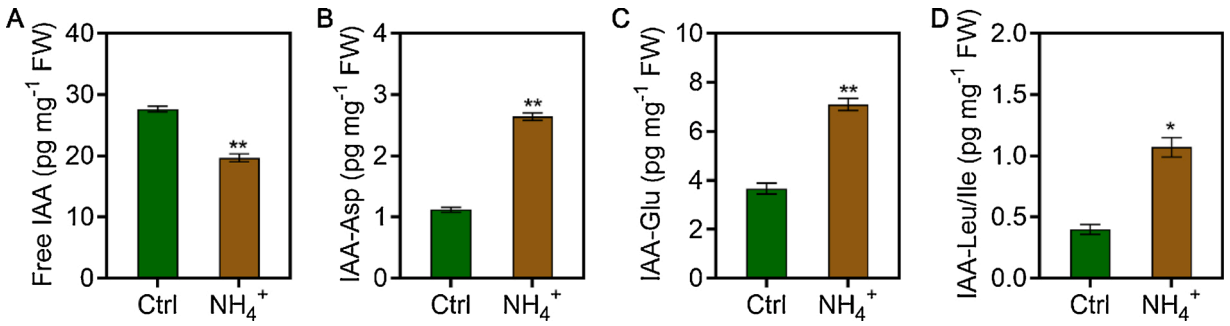

Fig. 6. High-NH_4^+ stress decreases free IAA and increases IAA conjugates in Col roots.
(A) Free IAA content, IAA-Asp content **(B)**, IAA-Glu content **(C),** and IAA-Leu/Ile content **(D)** in Col roots grown on media with or without high NH_4^+. Root samples were prepared from 5-day-old seedlings with or without 15 mM $(NH_4)_2SO_4$ treatment for 3 days. Values shown are the means ± SD with n = 3. *, $P < 0.05$; **, $P < 0.01$ (*t*-test). ns indicates not significant.

IAA-conjugating mutants, which exhibit altered NH_4^+ sensitivity. Our data suggest that mutation of these genes only slightly altered NH_4^+ sensitivity in the MZ. However, NH_4^+ sensitivity of the EZ was increased in IAA biosynthesis mutants (*trp4/wei7*, *trp5/wei2,* and *taa1/ckrc1*) but decreased in IAA-conjugating mutants (*gh3.3* and *gh3.6*), underscoring the positive role of IAA in EZ elongation under HAS (Fig. 7A and B).

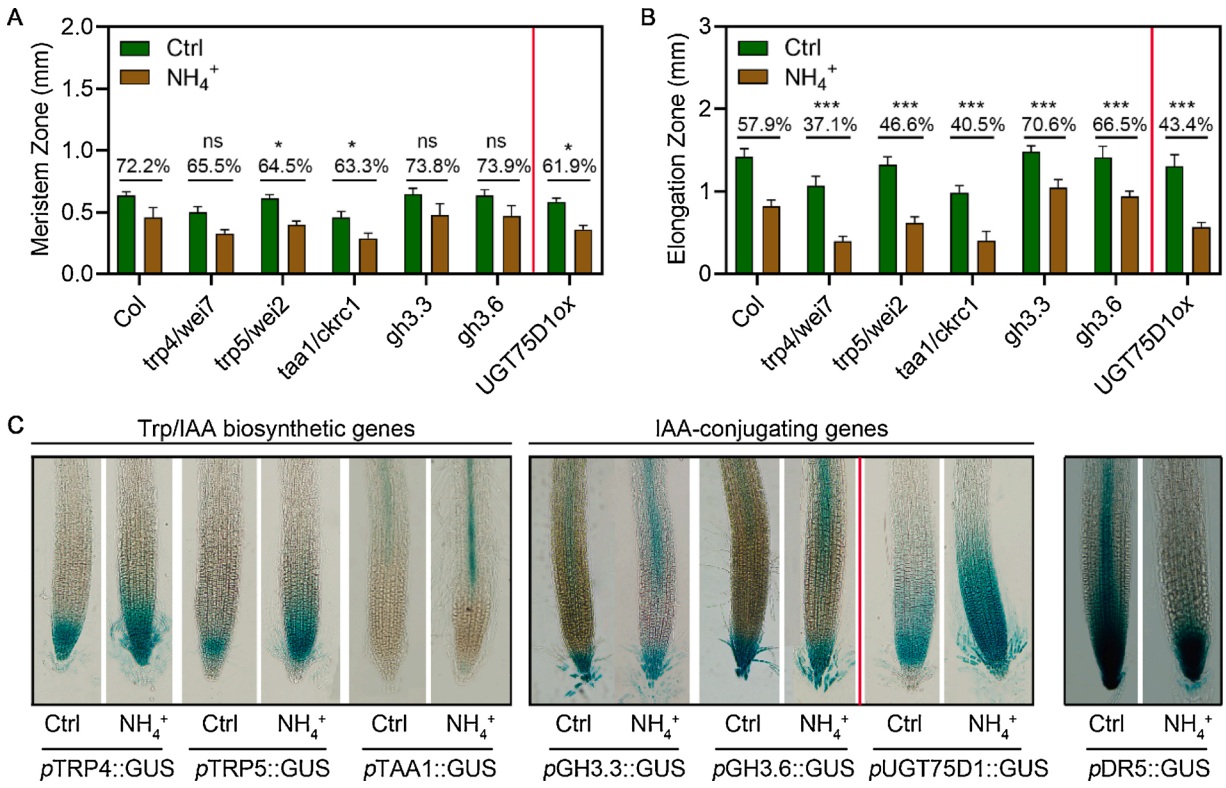

Fig. 7. High NH_4^+ induces IAA-conjugating genes in the elongation zone.
(A and **B)** The length of MZ **(A)** and EZ **(B)** of *trp4/wei7*, *trp5/wei2*, *taa1/ckrc1*, *gh3.3*, *gh3.6,* and UGT75D1*ox* grown on media with or without 15 mM $(NH_4)_2SO_4$. 5-day-old untreated seedlings were transferred to a medium with or without 15 mM $(NH_4)_2SO_4$, then grown for 3 days prior to measurement. Values shown are the means ± SD with n = 25. *, $P < 0.05$; ***, $P < 0.001$ (*t*-test). ns indicates not significant. **(C)** GUS staining of *p*romoter::GUS of genes involved in IAA homeostasis. 5-day-old seedlings with promoter::GUS were transferred to fresh media with or without 15 mM $(NH_4)_2SO_4$ for 3 days prior to staining (bar = 1 mm).

Furthermore, we found that the IAA-conjugating genes *GH3.3* and *GH3.6* were mainly expressed in the EZ and QC under control conditions, but were induced in the EZ under HAS. *UGT75D1* was mainly expressed in the MZ and QC under control, and showed high intensity in the MZ, EZ, and QC, revealing the role of IAA conjugation in regulating EZ elongation under high NH_4^+ (Fig. 7C). By contrast, the Trp-biosynthetic genes *TRP4/WEI7* and *TRP5/WEI2* were mainly expressed in the QC and were upregulated under HAS, while the IAA-biosynthetic genes *TAA1/CKRC1* were expressed in the EZ and QC and were upregulated by high NH_4^+ (Zhou et al., 2011) (Fig. 7C). HAS mainly depressed *p*DR5::GUS staining in the EZ, but not in the MZ and QC (Fig. 7C), and we, therefore, suggest that IAA conjugation is associated with NH_4^+-induced EZ inhibition and that IAA biosynthesis plays a positive role in maintaining EZ elongation under HAS. It was previously shown that elevated NH_4^+ efflux in relation to the function of VTC1, a gene encoding GDP-mannose pyrophosphorylase (GMPase), in the EZ was also involved in regulating NH_4^+ sensitivity in Arabidopsis (Qin et al., 2008; Barth et al., 2010; Li et al., 2010). The *vtc1-1* mutant also exhibited a lower free IAA contents in roots under HAS, pointing at a possible relationship between NH_4^+ efflux and IAA in the EZ (Barth et al., 2010). In addition, many studies have shown that IAA stimulates the activity of H^+-ATPases *via* phosphorylation modification (Shen et al., 2006; Takahashi et al., 2012; Wang et al., 2016; Meier et al., 2020), and the H^+-ATPase inhibitor vanadate indeed influences NH_4^+ fluxes in roots (Luo et al., 2013). Together, these data imply a potential role for IAA in VTC1-dependent NH_4^+ fluxes, which, given their known centrality to NH_4^+ toxicity more generally (Britto et al., 2001; Kronzucker et al. 2003; Balkos et al., 2010; Li et al., 2010; Coskun et al., 2013), should be determined directly in future studies.

Clarification of the mechanism underpinning PR growth inhibition under HAS is obviously critical to understanding NH_4^+ tolerance in plants. Here, we identify that high NH_4^+ leads to the inhibition of IAA conversion in the roots to suppress PR elongation, which provides a potential new target for plant improvement and a new option for screening for NH_4^+-tolerant genotypes.

5. Conclusion

In the present study, we performed qRT-PCR, RNA-seq, and measurements of free IAA and IAA conjugates and PR elongation in genetic mutants to clarify the role of IAA biosynthesis and conjugation under HAS. Our results clearly show that HAS decreases free IAA in roots by increasing IAA inactivation but not by decreasing IAA biosynthesis. In addition, the phenotypic analysis of IAA-conjugating mutants suggests that the IAA-conjugating genes *GH3.1*, *GH3.2*, *GH3.3*, *GH3.4,* and *GH3.6* function as the key genes in regulating NH_4^+ sensitivity in roots. Furthermore, promoter::GUS staining *in situ* and MZ/EZ length measurement in genetic mutants indicates that HAS mainly promotes IAA conjugation in the EZ, which may be responsible for the PR inhibition under high NH_4^+.

CRediT authorship contribution statement

Dong-Wei Di: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Visualization. **Guangjie Li:** Methodology, Writing - review & editing. **Li Sun:** Methodology, Writing - original draft. **Jingjing Wu:** Methodology, Investigation. **Meng Wang:** Writing - original draft. **Herbert J. Kronzucker:** Writing - review & editing. **Shuang Fang:** Investigation. **Jinfang Chu:** Investigation. **Weiming Shi:** Conceptualization, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2021.153415.

References

Balkos, K.D., et al., 2010. Optimization of ammonium acquisition and metabolism by potassium in rice (*Oryza sativa* L. Cv. IR-72). Plant Cell Environ. 33 (1), 23–34. https://doi.org/10.1111/j.1365-3040.2009.02046.x.

Barth, C., et al., 2010. A mutation in GDP-mannose pyrophosphorylase causes conditional hypersensitivity to ammonium, resulting in Arabidopsis root growth inhibition, altered ammonium metabolism, and hormone homeostasis. J. Exp. Bot. 61 (2), 379–394. https://doi.org/10.1093/jxb/erp310.

Biver, S., et al., 2008. A role for Rhesus factor Rhcg in renal ammonium excretion and male fertility. Nature 456 (7220), 339–343. https://doi.org/10.1038/nature07518.

Branka Salopek-Sondi, S.P., et al., 2015. Improvement of root architecture under abiotic stress through control of auxin homeostasis in Arabidopsis and Brassica crops. Endocyt Cell Res. 26, 100–111.

Britto, D.T., Kronzucker, H.J., 2002. NH_4^+ toxicity in higher plants: a critical review. J. Plant Physiol. 159 (6), 567–584. https://doi.org/10.1078/0176-1617-0774.

Britto, D.T., et al., 2001. Futile transmembraneNH_4^+ cycling: a cellular hypothesis to explain ammonium toxicity in plants. Proc. Natl. Acad. Sci. U.S.A. 98, 4255–4258. https://doi.org/10.1073/pnas.061034698.

Britto, D.T., et al., 2013. Ecological significance and complexity of N-source preference in plants. Ann. Bot. 112 (6), 957–963. https://doi.org/10.1093/aob/mct157.

Cao, Y., et al., 1993. Ammonium inhibition of Arabidopsis root growth can be reversed by potassium and by auxin resistance mutations *aux1*, *axr1*, and *axr2*. Plant Physiol. 102 (3), 983–989. https://doi.org/10.1104/pp.102.3.983.

Coskun, D., et al., 2013. Rapid ammonia gas transport accounts for futile transmembrane cycling under NH_3/NH_4^+ toxicity in plant roots. Plant Physiol. 163 (4), 1859–1867. https://doi.org/10.1104/pp.113.225961.

Dai, G.Z., et al., 2012. Ammonia may play an important role in the succession of cyanobacterial blooms and the distribution of common algal species in shallow freshwater lakes. Glob. Change Biol. Bioenergy 18 (5), 1571–1581. https://doi.org/10.1111/j.1365-2486.2012.02638.x.

Di, D.W., et al., 2016a. The biosynthesis of auxin: how many paths truly lead to IAA? Plant Growth Regul. 78, 275–285. https://doi.org/10.1007/s10725-015-0103-5.

Di, D.W., et al., 2016b. Functional roles of Arabidopsis *CKRC2/YUCCA8* gene and the involvement of PIF4 in the regulation of auxin biosynthesis by cytokinin. Sci. Rep. 6, 36866. https://doi.org/10.1038/srep36866.

Di, D.W., et al., 2018. Involvement of auxin in the regulation of ammonium tolerance in rice (*Oryza sativa* L.). Plant Soil 432 (1-2), 373–387. https://doi.org/10.1007/s11104-018-3813-4.

Ding, Z.J., et al., 2015. Transcription factor WRKY46 modulates the development of Arabidopsis lateral roots in osmotic/salt stress conditions via regulation of ABA signaling and auxin homeostasis. Plant J. 84 (1), 56–69. https://doi.org/10.1111/tpj.12958.

Drath, M., et al., 2008. Ammonia triggers photodamage of photosystem II in the *cyanobacterium Synechocystis* sp strain PCC 6803. Plant Physiol. 147 (1), 206–215. https://doi.org/10.1104/pp.108.117218.

Esteban, R., et al., 2016. Review: mechanisms of ammonium toxicity and the quest for tolerance. Plant Sci. 248, 92–101. https://doi.org/10.1016/j.plantsci.2016.04.008.

Glass, A.D.M., et al., 2002. The regulation of nitrate and ammonium transport systems in plants. J. Exp. Bot. 53, 855–864. https://doi.org/10.1093/jexbot/53.370.855.

He, W., et al., 2011. A small-molecule screen identifies L-kynurenine as a competitive inhibitor of TAA1/TAR activity in ethylene-directed auxin biosynthesis and root growth in Arabidopsis. Plant Cell 23 (11), 3944–3960. https://doi.org/10.1105/tpc.111.089029.

Hess, D.C., et al., 2006. Ammonium toxicity and potassium limitation in yeast. PLoS Biol. 4 (11), e351. https://doi.org/10.1371/journal.pbio.0040351.

Jackson, R.G., et al., 2001. Identification and biochemical characterization of an Arabidopsis indole-3-acetic acid glucosyltransferase. J. Biol. Chem. 276 (6), 4350–4356. https://doi.org/10.1074/jbc.M006185200.

Ke, L., et al., 2015. Differential roles of PIN1 and PIN2 in root meristem maintenance under low-B conditions in *Arabidopsis thaliana*. Plant Cell Physiol. 56 (6), 1205–1214. https://doi.org/10.1093/pcp/pcv047.

Kempinski, C.F., et al., 2011. Toward the mechanism of NH_4^+ sensitivity mediated by Arabidopsis GDP-mannose pyrophosphorylase. Plant Cell Environ. 34 (5), 847–858. https://doi.org/10.1111/j.1365-3040.2011.02290.x.

Khan, S., Stone, J.M., 2007. *Arabidopsis thaliana* GH3.9 influences primary root growth. Planta 226 (1), 21–34. https://doi.org/10.1007/s00425-006-0462-2.

Kojima, S., 2018. The possible interaction of ammonium and auxin polar transport on root system architectures in the two ecotypes of *Arabidopsis thaliana*. Soil Sci. Plant Nutr. 64, 616–622. https://doi.org/10.1080/00380768.2018.1507603.

Konishi, N., et al., 2017. Contribution of two glutamine synthetase isozymes to ammonium assimilation in Arabidopsis roots. J. Exp. Bot. 68, 613–625. https://doi.org/10.1093/jxb/erw454.

Korasick, D.A., et al., 2013a. Auxin biosynthesis and storage forms. J. Exp. Bot. 64 (9), 2541–2555. https://doi.org/10.1093/jxb/ert080.

Korasick, D.A., et al., 2013b. Auxin biosynthesis and storage forms. J. Exp. Bot. 64 (9), 2541–2555. https://doi.org/10.1093/jxb/ert080.

Kronzucker, H.J., et al., 1997. Conifer root discrimination against soil nitrate and the ecology of forest succession. Nature 385, 59–61. https://doi.org/10.1038/385059a0.

Kudoyarova, G.R., et al., 1997. Comparison of the effects of nitrate and ammonium forms of nitrogen on auxin content in roots and the growth of plants under different temperature conditions. Plant Growth Regul. 23 (3), 207–208. https://doi.org/10.1023/A:1005990725068.

Li, Q., et al., 2010. Root growth inhibition by NH_4^+ in Arabidopsis is mediated by the root tip and is linked to NH_4^+ efflux and GMPase activity. Plant Cell Environ. 33 (9), 1529–1542. https://doi.org/10.1111/j.1365-3040.2010.02162.x.

Li, B.H., et al., 2011. Shoot-supplied ammonium targets the root auxin influx carrier AUX1 and inhibits lateral root emergence in Arabidopsis. Plant Cell Environ. 34 (6), 933–946. https://doi.org/10.1111/j.1365-3040.2011.02295.x.

Li, B.H., et al., 2014. Ammonium stress in Arabidopsis: signaling, genetic loci, and physiological targets. Trends Plant Sci. 19, 107–114. https://doi.org/10.1016/j.tplants.2013.09.004.

Liu, Y., et al., 2013. Ammonium inhibits primary root growth by reducing the length of meristem and elongation zone and decreasing elemental expansion rate in the root apex in *Arabidopsis thaliana*. PLoS One 8 (4), e60131. https://doi.org/10.1371/journal.pone.0061031.

Luo, J., et al., 2013. Net fluxes of ammonium and nitrate in association with H^+ fluxes in fine roots of *Populus popularis*. Planta 237 (4), 919–931. https://doi.org/10.2037/42568887.

Meier, M., et al., 2020. Auxin-mediated root branching is determined by the form of available nitrogen. Nat. Plants 6, 1136–1145. https://doi.org/10.1038/s41477-020-00756-2.

Nishimura, T., et al., 2014. Yucasin is a potent inhibitor of YUCCA, a key enzyme in auxin biosynthesis. Plant J. 77 (3), 352–366. https://doi.org/10.1111/tpj.12399.

Park, J.E., et al., 2007. An Arabidopsis GH3 gene, encoding an auxin-conjugating enzyme, mediates phytochrome B-regulated light signals in hypocotyl growth. Plant Cell Physiol. 48 (10) https://doi.org/10.1093/pcp/pcm086, 1514-1514.

Qin, C., et al., 2008. GDP-mannose pyrophosphorylase is a genetic determinant of ammonium sensitivity in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. U.S.A. 105 (47), 18308–18313. https://doi.org/10.1073/pnas.0806168105.

Scheres, B., Benfey, P., Dolan, L., 2002. Root development. In: Somerville, C.R., Meyerowitz, E.M. (Eds.), The Arabidopsis Book. American society of Plant Biologists, Rockville, MD, USA.

Shen, H., et al., 2006. Root plasma membrane H^+-ATPase is involved in the adaptation of soybean to phosphorus starvation. J. Exp. Bot. 57 (6), 1353–1362. https://doi.org/10.1093/jxb/erj111.

Staswick, P.E., et al., 2005. Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. Plant Cell 17 (2), 616–627. https://doi.org/10.1105/tpc.104.026690.

Stevens, C.J., et al., 2004. Impact of nitrogen deposition on the species richness of grasslands. Science 303 (5665), 1876–1879. https://doi.org/10.1126/science.1094678.

Sun, L., et al., 2017. Spatio-temporal dynamics in global rice gene expression (*Oryza sativa* L.) in response to high ammonium stress. J. Plant Physiol. 212, 94–104. https://doi.org/10.1016/j.jplph.2017.02.006.

Sun, L., et al., 2020a. Endogenous ABA alleviates rice ammonium toxicity by reducing ROS and free ammonium via regulation of the SAPK9-bZIP20 pathway. J. Exp. Bot. 71 (15), 4562–4577. https://doi.org/10.1093/jxb/eraa076/5736877.

Sun, L., et al., 2020b. Transcriptome analysis of rice (*Oryza sativa* L.) in response to ammonium resupply reveals the involvement of phytohormone signaling and the transcription factor OsJAZ9 in reprogramming of nitrogen uptake and metabolism. J. Plant Physiol. 246–247. https://doi.org/10.1016/j.jplph.2020.153137, 153137.

Suzuki, M., et al., 2015. Transcriptional feedback regulation of YUCCA genes in response to auxin levels in Arabidopsis. Plant Cell Rep. 34 (8), 1343–1352. https://doi.org/10.1007/s00299-015-1791-z.

Takahashi, K., et al., 2012. Auxin activates the plasma membrane H^+-ATPase by phosphorylation during hypocotyl elongation in Arabidopsis. Plant Physiol. 159 (2), 632–641. https://doi.org/10.2307/41549885.

Wang, P., et al., 2016. Auxin enhances aluminium-induced citrate exudation through upregulation of GmMATE and activation of the plasma membrane H^+-ATPase in soybean roots. Ann. Bot. 118 (5), 933–940. https://doi.org/10.1093/aob/mcw133.

Zhang, Z., et al., 2007. Dual regulation role of GH3.5 in salicylic acid and auxin signaling during Arabidopsis-*Pseudomonas syringae* interaction. Plant Physiol. 145 (2), 450–464. https://doi.org/10.1104/pp.107.106021.

Zhang, G.Z., et al., 2016. Ectopic expression of UGT75D1, a glycosyltransferase preferring indole-3-butyric acid, modulates cotyledon development and stress tolerance in seed germination of *Arabidopsis thaliana*. Plant Mol. Biol. 90 (1-2), 77–93. https://doi.org/10.1007/s11103-015-0395-x.

Zhang, L., et al., 2021. Induction of S-nitrosoglutathione reductase protects root growth from ammonium toxicity by regulating potassium homeostasis in Arabidopsis and rice. J. Exp. Bot. erab140. https://doi.org/10.1093/jxb/erab140.

Zhao, Y., et al., 2001. A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science 291, 306–309. https://doi.org/10.1126/science.291.5502.306.

Zhou, Z., et al., 2011. Functional characterization of the *CKRC1/TAA1* gene and dissection of hormonal actions in the Arabidopsis root. Plant J. 66 (3), 516–527. https://doi.org/10.1111/j.1365-313X.2011.04509.x.

Zou, N., et al., 2012. Ammonium-induced loss of root gravitropism is related to auxin distribution and TRH1 function, and is uncoupled from the inhibition of root elongation in Arabidopsis. J. Exp. Bot. 63 (10), 3777–3788. https://doi.org/10.1093/jxb/ers068.

Zou, N., et al., 2013. GSA-1/ARG1 protects root gravitropism in Arabidopsis under ammonium stress. New Phytol. 200 (1), 97–111. https://doi.org/10.1111/nph.12365.