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RESEARCH ARTICLE

PIN5 is involved in regulating NH_4^+ efflux and primary root growth under high-ammonium stress via mediating intracellular auxin transport

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Abstract

Background and Aims Ammonium (NH_4^+) is an important nitrogen (N) source in many ecosystems and agricultural systems but excessive NH_4^+ is toxic to root growth and development, especially when NH_4^+ is the sole N source. Previous studies have shown that polar auxin (indole-3-acetic acid, IAA) transport mediated by PIN2 and AUX1 is critical for maintaining lateral root development under high-NH_4^+ stress. However, the regulation of subcellular IAA homeostasis under high-NH_4^+ stress has remained unclear.

Methods Knockout mutants for the intracellular IAA transporter PIN5 and the plasma-membrane H^+-ATPases AHA1 and AHA2 were used, and primary root length, transmembrane H^+ fluxes, and NH_4^+ fluxes in the roots were determined.

Results We show that high NH_4^+ disturbs the subcellular IAA homeostasis by upregulating the expression of PIN5. Knockout of *PIN5* resulted in elevated cytoplastic IAA accumulation and reduced NH_4^+ efflux under high-NH_4^+ stress. Furthermore, we show that NH_4^+ treatment promotes H^+ efflux at the root elongation zone, in turn promoting NH_4^+ efflux, with the involvement of PIN5-mediated intracellular IAA transport. Moreover, stabilization of rhizosphere pH reduced NH_4^+ efflux and promoted primary root growth in the *pin5* mutant under high NH_4^+.

Conclusion Our findings provide a mechanistic explanation for the role of subcellular IAA homeostasis in response to high-NH_4^+ stress through the coordinated regulation of NH_4^+ efflux and H^+ efflux.

Keywords PIN5 · Ammonium · Intracellular auxin homeostasis · H^+ fluxes · NH_4^+ fluxes

Abbreviations

ER	Endoplasmic Reticulum
EZ	Elongation Zone
H^+	Proton
IAA	Indole-3-Acetic Acid

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IAA_{cyt}	Cytosolic IAA
Kyn	L-kynurenine
MES	2-(N-morpholino) Ethanesulfonic Acid
MZ	Meristem Zone
NH_4^+	Ammonium
NO_3^-	Nitrate
PM	Plasma Membrane
PR	Primary Root
VA	Vanadate

Introduction

In soil, ammonium (NH_4^+) and nitrate (NO_3^-) are the dominant forms of inorganic and active N (Bai et al. 2014; Britto and Kronzucker 2013; Kronzucker et al. 1997, 2003). NH_4^+ is a preferred N source for plants when present in soil solution at low to intermediate concentrations (typically below several millimolar; (Britto and Kronzucker 2013; Kronzucker et al. 2003), but higher concentrations of NH_4^+ are toxic to most terrestrial plants (Britto and Kronzucker 2002; Coskun et al. 2013; Szczerba et al. 2008). NH_4^+ toxicity is not only observed in terrestrial plants (Di et al. 2018; Liu et al. 2022a), but also in cyanobacteria (Dai et al. 2012; Drath et al. 2008), algae (Dai et al. 2012), yeast (Hess et al. 2006), and animals (Biver et al. 2008). In terrestrial plants, the root system acts as the initial NH_4^+ sensor, and the signals initiating NH_4^+ toxicity appear at the root level, usually coupled to a profound modification of root architecture, such as a shorter primary root (PR) (Di et al. 2021a, 2018; Dominguez-Valdivia et al. 2008; Li et al. 2010; Liu et al. 2022a, 2022b; Sun et al. 2020), abnormal lateral root development (Esteban et al. 2016; Li et al. 2011), and a loss of gravitropism (Zou et al. 2013). Although the toxicity of NH_4^+ on root development has been previously studied by both genetic or physiological approaches, the underlying mechanisms remain unclear (Li et al. 2014).

Current knowledge of how NH_4^+ toxicity affects plant PR growth includes rhizosphere acidification, disruptions in the balance of essential nutrient ions ($Fe^{2+/3+}$, K^+, PO_4^{3-} etc.), hormone (auxin, ethylene, abscisic acid etc.) disturbance, futile NH_4^+ cycling, and impaired protein glycosylation (Britto and Kronzucker 2002; Di et al. 2021b; Li et al. 2010; Liu et al. 2022a, 2022b). Of these, elevated NH_4^+ fluxes have been linked to alterations in auxin and ethylene signaling and to protein glycosylation (Di et al. 2021b; Li et al. 2022, 2010). Our previous studies showed that high NH_4^+ principally inhibits elongation zone (EZ) length of PR and that this inhibition is associated with elevated root NH_4^+ fluxes, and a mutation of *VTC1*, a gene encoding GDP-mannose pyrophosphorylase, was shown to enhance NH_4^+ efflux at the EZ (Li et al. 2010). Subsequently, we identified WRKY46 as the first transcription factor involved in the regulation of NH_4^+ efflux. WRKY46 could inhibit NH_4^+ efflux by stabilizing protein glycosylation through direct binding and inhibition of the transcription of *NUDX9*, a gene encoding GDP-D-mannose pyrophosphohydrolase involved in protein glycosylation (Di et al. 2021b). Our group also identified another transcription factor, OsEIL1, which activates *OsVTC1.3* (a gene homologous to *AtVTC1*) and negatively regulates NH_4^+ efflux in rice (Li et al. 2022). In addition, we found that WRKY46 could inhibit NH_4^+ efflux by directly binding to IAA-conjugating genes and inhibiting their transcription, thereby increasing the level of free IAA under high-NH_4^+ stress (Di et al. 2021b). Interestingly, VTC1 and NUDX9 were also involved in regulating the level of free IAA under high-NH_4^+ stress, and the reduction of free IAA levels was also responsible for the regulation of NH_4^+ efflux (Barth et al. 2010; Di et al. 2021b). As is well known, many IAA-conjugating enzymes are located in the endoplasmic reticulum (ER), but it remains unknown whether the auxin transporters that mediate IAA transport between the ER and the cytosol are directly implicated in the plant response to high-NH_4^+ stress (Mravec et al. 2009; Simon et al. 2016).

In *Arabidopsis*, there are eight members of the PIN family and these are divided into two groups, including plasma-membrane (PM)-localized PIN1, PIN4, and PIN7, and ER-localized PIN5, PIN6 (also function on PM), and PIN8 (Dal Bosco et al. 2012; Ding et al. 2012; Mravec et al. 2009; Simon et al. 2016). PIN5 controls subcellular auxin homeostasis by mediating auxin transport from the cytosol to the ER lumen, and knockout of *PIN5* results in more free IAA accumulation in rosette leaves and roots (Mravec et al. 2009). Therefore, we wished to investigate whether PIN5-mediated intracellular IAA transport is essentially implicated in the response to high-NH_4^+ stress.

Many studies have suggested that cytosolic IAA (IAA_{cyt}) enhances the phosphorylation of PM H^+-ATPases, which then promotes cell elongation by acid growth (Li et al. 2005, 2021; Lin et al. 2021; Wang et al. 2016). Moreover, acidification of the rhizosphere through activation of the PM H^+-ATPases is an important component of the toxicity effect of high NH_4^+ (von Wiren et al. 2000). Recent studies have shown that NH_4^+ uptake leading to H^+ efflux and rhizosphere acidification mobilizes rhizosphere Fe^{2+}, resulting in accumulation of Fe^{2+} in the phloem, which, in turn, induces ROS and inhibits PR growth (Liu et al. 2022a, 2022b). At the same time, NH_4^+-induced ROS can activate the expression of the *PDX1.1* gene in roots and provide non-phosphorylated vitamin B6 to the root system to alleviate NH_4^+-induced oxidative stress and maintain PR growth (Liu et al. 2022b). In addition, it has been reported that the NO_3^- efflux channel protein SLAH3 and the NO_3^- transporter NRT1.1 are involved in nitrate-dependent detoxification of NH_4^+ in plants through inhibition of rhizosphere acidification via promoting H^+ influx (Xiao et al. 2022; Ye et al. 2021), confirming many older studies demonstrating the alleviatory effect of NO_3^- in the context of NH_4^+ toxicity (Britto and Kronzucker 2002; 2005; Kronzucker et al. 1999). Moreover, NO_3^- can also increase tolerance to high NH_4^+ by preventing NH_4^+ efflux (Babourina et al. 2007). Hence, it was interesting to explore how IAA_{cyt} regulates H^+ flux and NH_4^+ efflux under high-NH_4^+ stress, and whether this occurs in a coordinated manner.

Here, we present data that show that high NH_4^+ up-regulates the transcription and expression level of PIN5 and decreases IAA_{cyt} accumulation. Knockout of *PIN5* resulted in more IAA_{cyt} accumulation and less NH_4^+ efflux under high-NH_4^+ stress. In addition, high-NH_4^+ treatment stimulated H^+ efflux into the rhizosphere, which in turn promoted NH_4^+ efflux, involving PIN5-mediated intracellular IAA transport. Moreover, inhibition of H^+ efflux decreased NH_4^+ efflux and promoted PR growth in the *pin5* mutant under high NH_4^+. Together, the results provide a mechanistic explanation for the role of intracellular IAA transport in response to high-NH_4^+ stress through the coordinated regulation of NH_4^+ efflux and H^+ fluxes in roots.

Materials and methods

Plant material and growth conditions

The wild type used was Col-0. The materials *pin5*, PIN5*ox*, and PIN5::PIN5::GFP were obtained from Prof. Xiaofeng Li (Lanzhou University). *pin5*/*p*DR5::GUS and PIN5*ox*/*p*DR5::GUS were generated by crossing *p*DR5::GUS with *pin5* and PIN5ox, respectively. *aha1-7* (SALK_065288) and *aha2-5* (SALK_022010) in the Col background were obtained from AraShare (a nonprofit Arabidopsis share center, http://www.arashare.cn). Seeds were cold-treated at 4 °C for 48 h, and then surface-sterilized with 0.1% $HgCl_2$ prior to being sown onto normal growth medium (Di et al. 2016). The medium and growth conditions are as described in our previous study (Di et al. 2021a): 2 mM KH_2PO_4, 5 mM $NaNO_3$, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM Fe-EDTA, 50 µM H_3BO_3, 12 µM $MnSO_4$, 1 µM $ZnCl_2$, 1 µM $CuSO_4$, 0.2 µM Na_2MoO_4, 1% sucrose, and 1% agar (adjusted to pH 5.7 with 1 M NaOH). Germination and plant growth was carried out at 23 °C ± 1 °C with a 16 h light/8 h dark cycle.

Treatment and root length analysis

To investigate the root growth inhibition, 5-day-old seedlings were transferred to new medium with different ions or molecules (15 mM NH_4Cl; 30 mM NH_4Cl; 60 mM NH_4Cl; 5 µM / 10 µM / 50 µM VA; 30 mM NH_4Cl + 5 µM / 10 µM / 50 µM VA). After growth for another three days, PR length was determined by ImageJ software.

RNA Isolation, RT-PCR and qRT-PCR

RNA was extracted from roots (~50 mg) using the reagent TRIzol (Sangon Biotech Co. Ltd, Shanghai, China). Reverse transcription was performed using a HiScript 1st Strand cDNA Synthesis Kit (R111-01; Vazyme Biotech Co., Ltd). 1 µg of total RNA was used to synthesize the first-strand cDNA and was diluted 20 times for retro-translation PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR) (Di et al. 2021a).

For qRT-PCR, the reagent ChamQ SYBR qPCR Master Mix (Q311-02, Vazyme Biotech Co., Ltd) was used to amplify in a LightCycler® 480II (Roche). The results were normalized relative to *ACTIN 8* (*AT1G49240*). The

method has been detailed in our previous study (Di et al. 2021a). The primers are as follows: qRT-ACT8-F: TGT GACAATGGTACTGGAATGG; qRT-ACT8-R: TTG GATTGTGCTTCATCACC; qRT-ACT8-F: AAATAT AGCAACAAAGGAAGC; qRT-ACT8-R: TCTAAA CTCTAAGACAAACAA.

Western blotting

For concentration experiments, 8-day-old PIN5::PIN5::GFP seedlings were treated with NH_4Cl at varying concentrations (0 mM, 15 mM, 30 mM, and 60 mM) for four hours prior to total protein extraction. For time-course experiment, 8-day-old PIN5::PIN5::GFP seedlings were treated with 30 mM NH_4 Cl for different times (0.5 h, 1 h, 2 h, 4 h, and 72 h) prior to total protein extraction. Proteins were detected by Western blotting using a mouse anti-GFP primary antibody (1: 1000; Abcam) and subsequently with a Sheep Anti-Rabbit IgG H&L (HRP) conjugated antibody (1: 5000; Abcam). Anti-Rubisco was used as the control.

Histochemical GUS staining

For *p*DR5::GUS staining, 5-day-old Col/*p*DR5::GUS, *pin5*/*p*DR5::GUS, and PIN5*ox*/*p*DR5::GUS seedings were transferred to fresh medium with or without 30 mM NH_4Cl. After growth for three days, the seedlings were used for GUS staining at 30 ℃ for 3 h prior to observation, as described in our previous study (Di et al. 2021b).

Determination of free IAA concentration and IAA conjugates

For determining free IAA and IAA conjugates (IAA-Glu and IAA-Asp), whole roots were collected (200 mg freeze-dried roots per sample, three biological repeats). Details of sample pretreatment and IAA determination were reported in previous studies (Di et al. 2021a, 2021b). IAA and IAA conjugates contents were determined by Nanjing WeBiolotech Testing Technology Co., Ltd.

Measurement of net NH_4^+ flux and H^+ flux using the NMT system

Net fluxes of NH_4^+ and H^+ were measured by Non-invasive Micro-test Technology (NMT, NMT100/200 Series; YoungerUSA, LLC, Amherst, MA01002, USA; Xuyue Company, Beijing, China). The principle of this instrument is as detailed in our previous study (Di et al. 2021b).

Net flux of H^+ was measured in basal media at the EZ and MZ (0.1 mM KCl, 0.1 mM $CaCl_2$, 2.5 mM $Ca(NO_3)_2$, pH 5.7) over 3 min to ensure steady values, and then 600 mM NH_4Cl was introduced to reach a final NH_4^+ concentration of 30 mM, and measured for another 10 min (600 mM NH_4Cl, 0.1 mM KCl, 0.1 mM $CaCl_2$, 2.5 mM $Ca(NO_3)_2$, pH 5.7).

For NH_4^+ flux measurements, the method was as detailed in our previous study (Di et al. 2021b). 7-day-old seedlings were transferred to fresh media with or without 30 mM NH_4Cl, 30 mM NH_4Cl+5 nM IAA, 30 mM NH_4Cl+1.5 μM Kyn, 5 μM VA, 30 mM NH_4Cl+5 μM VA and then grown for 24 h before measurement. Net NH_4^+ flux was measured in basal media at the EZ and/or MZ (0.2 mM NH_4Cl, 0.1 mM $CaCl_2$, pH 5.7) for 5 min. All measurements were carried out at Xuyue Company. (Beijing, China).

Statistical analysis

Datasets were analyzed using Prism 6 software (GraphPad Software). Comparisons between two groups were made using Student's t- test. Values of P denote differences significant at $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$, respectively. Comparisons among multiple groups were made by two-way ANOVA tests, and $P<0.05$ were considered significant. All values are presented as means±SD.

Accession numbers

PIN5 (*AT5G16530*), *AHA1* (AT2G18960), *AHA2* (AT4G30190), and *ACTIN8* (*AT1G49240*).

Results

The *pin5* mutant exhibits elevated tolerance to high-NH_4^+ stress

To investigate the function of intracellular auxin homeostasis in response to high NH_4^+, we observed and analyzed the phenotypes of the knockout mutant *pin5* and the overexpression line PIN5*ox* grown on medium with or without NH_4^+. When grown on NH_4^+-free medium, PR length in both *pin5* and PIN5*ox* were slightly shorter than their wild type, Col (Fig. 1a). When

introducing high NH_4^+ into the medium, relative PR in *pin5* was 71.9% and 19.8% in PIN5*ox*, more tolerant and sensitive compared with Col (60.2%), respectively, indicating that PIN5 is involved in response to high NH_4^+ (Fig. 1b). Furthermore, we also analyzed gene transcription and protein expression levels of PIN5 under high NH_4^+. Our results show high NH_4^+ can upregulate the transcription and expression of *PIN5*, and this is increasingly more pronounced after increasing the NH_4^+ concentrations or the treatment time (Fig. 1c-f). Thus, we conclude that PIN5 is directly implicated in high-NH_4^+ sensitivity by up-regulating its gene transcription and protein expression. The data suggest that PIN5 plays a negative-regulatory role in maintaining PR elongation under high-NH_4^+ stress.

PIN5 is involved in the regulation of free IAA and IAA conjugate content under high NH_4^+

To determine whether PIN5 is involved in the regulation of free IAA contents in roots under high-NH_4^+ stress, we first observed GUS staining in Col/*p*DR5::GUS, *pin5*/*p*DR5::GUS, and PIN5*ox*/*p*DR5::GUS grown on high-NH_4^+ medium. The results show that *pin5*/*p*DR5::GUS and PIN5*ox*/*p*DR5::GUS exhibit less and more GUS staining density reduction compared with Col/*p*DR5::GUS (Fig. 2a), respectively, indicating that high NH_4^+ decreases free IAA contents, partially dependent on PIN5. Furthermore, we then directly measured free-IAA and IAA-conjugate (IAA-Glu and IAA-Asp) contents in Col, *pin5,* and PIN5*ox* roots. Under high-NH_4^+ stress, the free IAA decrease in *pin5*

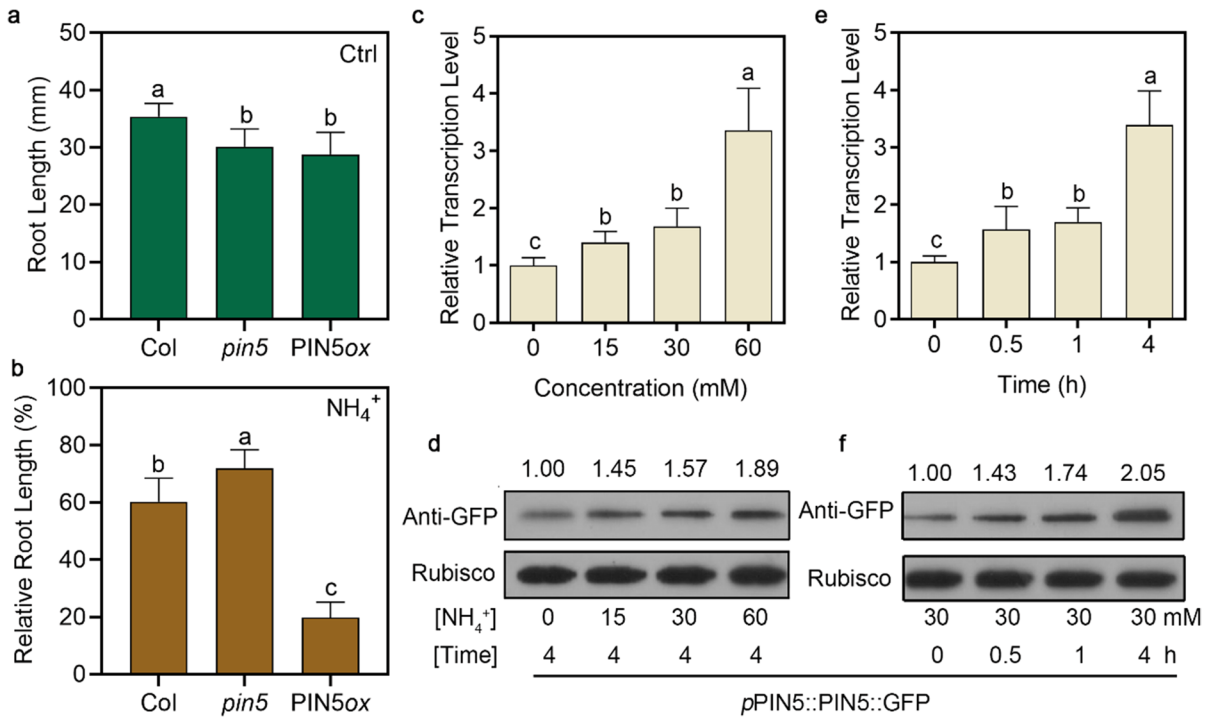

Fig. 1 PIN5 is responsible for high-NH_4^+ tolerance. **a** Quantification of PR length of Col, *pin5,* and PIN5*ox* seedlings grown on control medium; **b** Relative PR length of Col, *pin5,* and PIN5*ox* seedlings grown on medium with 30 mM NH_4Cl compared with untreated seedlings. Five-day-old seedlings were transferred to fresh medium with or without 30 mM NH_4Cl and grown for another three days before phenotypic observation and PR-length measurement. Mean ± SD ($n \geq 25$); **c-d** Relative transcription levels (**c**) and expression levels (**d**) of *PIN5* in seedlings after NH_4Cl treatment at varying concentrations for four hours. Data are the means of three biological replicates. Error bars indicate ± SD; 8-day-old seedlings were treated with various concentrations (0 mM, 15 mM, 30 mM, and 60 mM) of NH_4^+ for four hours, and then seedlings were collected for RNA isolation and total protein extraction; **e–f** different time treatment with high NH_4^+ increased PIN5 transcription levels (**e**) and protein content (**f**). 8-day-old seedlings were treated with high NH_4^+ for different times (0 h, 0.5 h, 1 h, and/or 4 h), and then seedlings were collected for RNA isolation and total protein extraction. PIN5::PIN5::GFP protein accumulation was analysed by Western blotting using anti-GFP antibodies. Error bars with different letters represent statistically significant differences ($P < 0.05$, Duncan's test)

(8.2%) was lower, while that in PIN5*ox* (37.6%) was higher than in Col (20.5%) (Fig. 2b). The increase in IAA-Asp and IAA-Glu contents in Col was 69.6% and 40.1%, while they decreased to 43.6% and 38.3% in *pin5* but increased to 87.1% and 48.6% in PIN5*ox*, respectively (Fig. 2c-d), showing that the transport of free IAA from cytoplasm to ER mediated by PIN5 is essential for promotion of IAA conjugation induced by high-NH_4^+ stress.

PIN5 positively regulates root NH_4^+ efflux under high-NH_4^+ stress

Our previous studies have shown that IAA negatively regulates NH_4^+ efflux in the roots (Di et al. 2021b). As PIN5 is involved in regulating free IAA levels under high-NH_4^+ stress, we asked whether PIN5 inhibition of PR growth under high-NH_4^+ stress is related to NH_4^+ efflux (Figs. 1b and 2b). We directly measured NH_4^+ fluxes in the meristem zone (MZ) and in the EZ of Col and *pin5* roots. Under high-NH_4^+ conditions, NH_4^+ effluxes increased in these two genotypes (Fig. 3). At MZ, the increase in NH_4^+ efflux in *pin5* (81.36 pmol cm^{-2} s^{-1}) was slightly lower compared to Col (106.39 pmol cm^{-2} s^{-1}) after high-NH_4^+ treatment. At EZ, the NH_4^+ efflux was 345.22 pmol cm^{-2} s^{-1} in Col, while inhibited NH_4^+ efflux was observed under high-NH_4^+ stress in *pin5* (207.09 pmol cm^{-2} s^{-1}), suggesting a positive role for PIN5 in regulating NH_4^+ efflux under high$^-$$NH_4^+$ stress (Fig. 3).

Mutation of PIN5 promotes H^+ efflux at the EZ of roots under high-NH_4^+ stress

Previous studies have shown that IAA stimulates PM H^+-ATPase activity and that NH_4^+ uptake is coupled

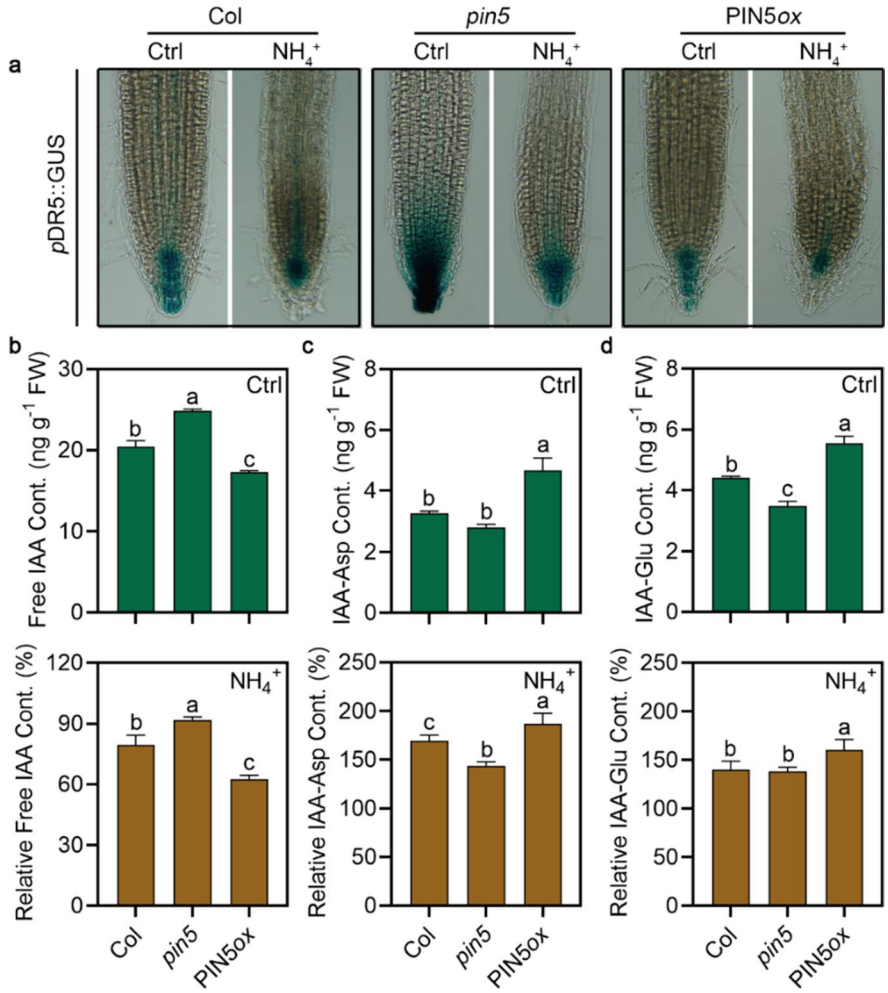

Fig. 2 Free-IAA and IAA-conjugate content determination in Col, *pin5,* and PIN5*ox* roots. (**a**) DR5::GUS staining in Col, *pin5,* and PIN5*ox*; (**b**) free IAA contents; (**c**) IAA-Asp contents and (**d**) IAA-Glu contents grown on control and 30 mM NH_4Cl medium (% of control). 5-day-old seedlings were transferred to fresh media with or without 30 mM NH_4Cl stress and then grown for another three days. Roots were collected for GUS staining and determination of free IAA and IAA conjugates. Data are the means of three biological replicates. Error bars indicate ± SD

to H^+ efflux (Li et al. 2021; von Wiren et al. 2000). To investigate the influence of IAA_{cyt} accumulation due to the *PIN5* mutation on H^+ efflux under high-NH_4^+ stress, we measured H^+ efflux at the MZ and EZ of Col and *pin5* roots. Under control conditions, the MZ of Col and *pin5* exhibited H^+ influx, while the EZ exhibited slight H^+ efflux (Fig. 4 a-d). After introducing high NH_4^+, both MZ and EZ of Col and *pin5* exhibited H^+ efflux. At the MZ, the enhanced H^+ effluxes were similar in Col (44.62 pmol cm^{-2} s^{-1}) and *pin5* (37.01 pmol cm^{-2} s^{-1}), while EZ of *pin5* (156.28 pmol cm^{-2} s^{-1}) showed significantly enhanced H^+ efflux compared with Col (71.83 pmol cm^{-2} s^{-1}) (Fig. 4 a-d), indicating that PIN5-mediated IAA transport from cytoplasm to ER plays a key role in regulating H^+ efflux at EZ induced by high-NH_4^+ stress.

To further examine the role of free IAA in regulating NH_4^+-induced H^+ efflux, we then added a low dose of exogenous IAA and the IAA biosynthesis inhibitor L-Kyn to the medium and measured the H^+ fluxes at the EZ following high-NH_4^+ treatment. Under control conditions, Col seedlings with L-Kyn treatment (Col/ + Kyn) exhibited small H^+ influx, while Col without or with IAA (Col/ + IAA) treatment exhibited H^+ efflux. However, when high NH_4^+ was introduced, H^+ efflux in Col/ + Kyn (53.44 pmol cm^{-2} s^{-1}) was weakened while, in Col/ + IAA (91.55 pmol cm^{-2} s^{-1}), it was enhanced compared to Col (71.83 pmol cm^{-2} s^{-1}), revealing a positive role for IAA in regulating H^+ efflux under high-NH_4^+ stress (Fig. 4f-g). Taken together, the data suggest that high NH_4^+ induces H^+ efflux in both the MZ and EZ and that PIN5-mediated intracellular IAA transport functions in regulating H^+ efflux at the EZ of roots.

Increased rhizosphere H^+ concentration promotes NH_4^+ efflux at the EZ under high-NH_4^+ stress

Given the complex relationship between NH_4^+-IAA and NH_4^+ and H^+ fluxes, it was interesting to know whether there is a correlation between rhizosphere H^+ concentration and NH_4^+ efflux. We therefore selected the PM H^+-ATPase knockout mutants *aha1-7* and *aha2-5* to test their NH_4^+ efflux at the EZ. Under control

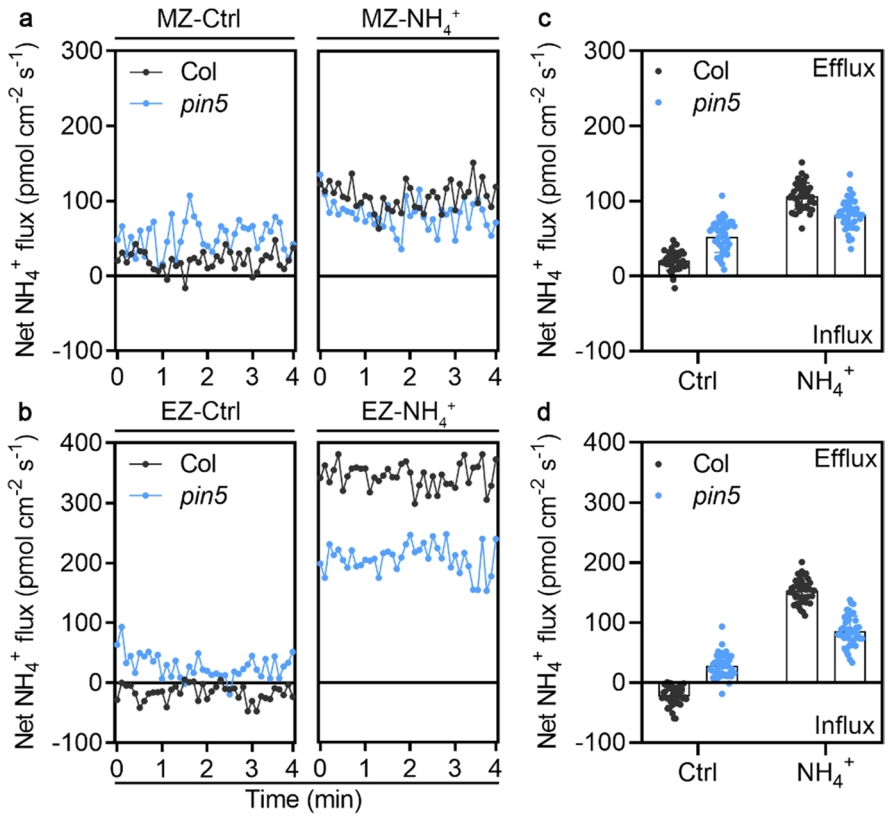

Fig. 3 PIN5 positively regulates NH_4^+ fluxes in the roots, especially at the EZ. NH_4^+ fluxes in Col and *pin5* in the MZ (**a**) and EZ (**b**). (**c**-**d**) Mean NH_4^+ fluxes in (**a**) and (**b**), respectively. 7-day-old seedlings were transferred to fresh medium with or without 30 mM NH_4Cl and grown another 12 h before NH_4^+ fluxes measurement ($n \geq 6$)

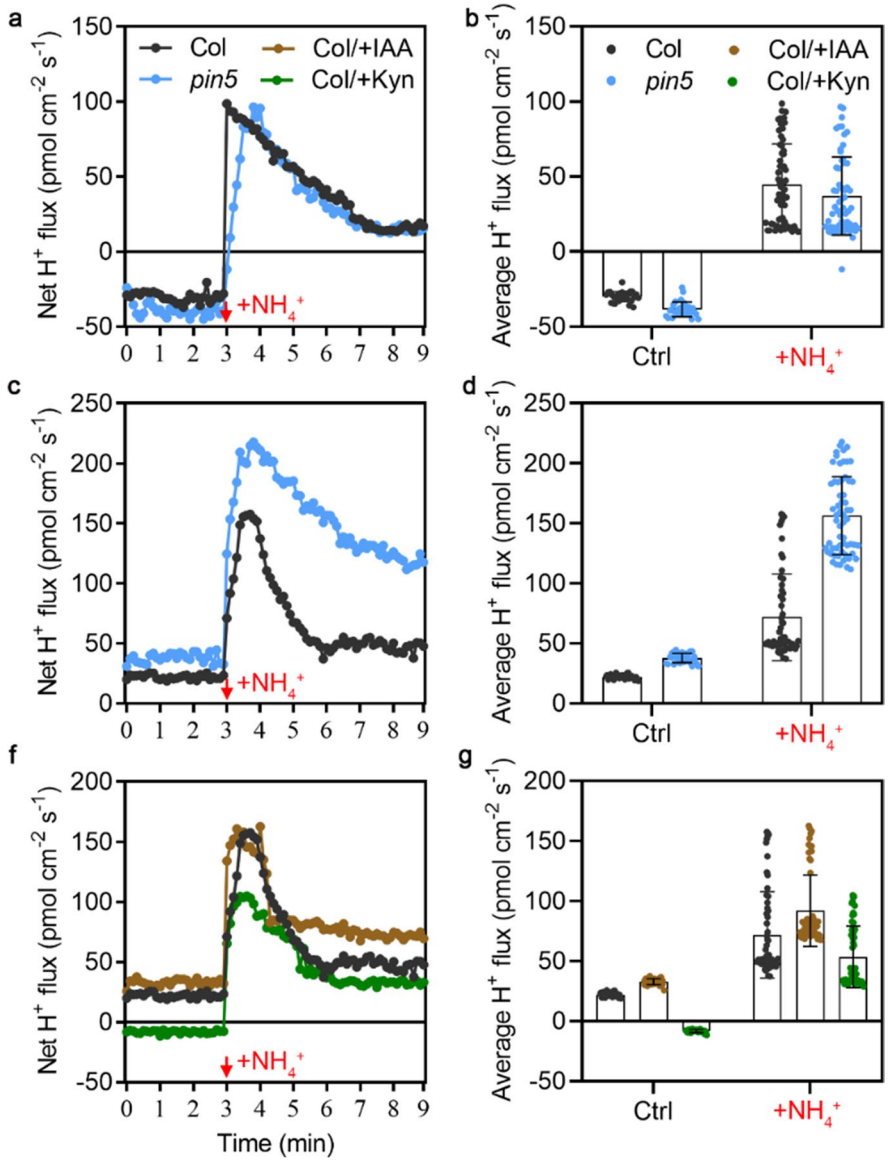

Fig. 4 PIN5 is involved in regulating H^+ efflux at the EZ of roots under high-NH_4^+ stress. (**a**-**c**) High-NH_4^+ treatment (indicated by the red arrow) promoted H^+ efflux at the MZ (**a**) and EZ (**c**) of both Col and the *pin5* mutant; (**b**) mean H^+ flux values in (**a**); (**d**) mean H^+ flux value in (**b**); (**e**) high NH_4^+ treatment (indicated by the red arrow) promoted H^+ efflux at the EZ of Col, Col supplied with IAA or Kyn; (**f**) mean H^+ flux value in (**e**). Values shown are the means ± SD ($n \geq 6$)

conditions, *aha1-7* (-62.16 pmol cm^{-2} s^{-1}) and *aha2-5* (-66.70 pmol cm^{-2} s^{-1}) exhibited higher net NH_4^+ influx compared with Col (-41.92 pmol cm^{-2} s^{-1}) (Fig. 6a-b). Under high-NH_4^+ conditions, the NH_4^+ effluxes were 190.98 pmol cm^{-2} s^{-1} and 173.53 pmol cm^{-2} s^{-1} in *aha1-7* and *aha2-5*, respectively, and 409.56 pmol cm^{-2} s^{-1} in Col, suggesting that increased rhizosphere H^+ concentrations mediated by AHA1 and AHA2 under high-NH_4^+ conditions promote NH_4^+ efflux at the EZ of roots. Furthermore, we also analyzed root growth of *aha1-7* and *aha2-5* under high-NH_4^+ stress. Compared with the 45.66% PR growth inhibition ratio in Col at 30 mM NH_4Cl, PR length of the mutants *aha1-7* and *aha2-5* was only inhibited 38.56% and 34.35%, respectively, indicating a negative role for AHA1 and AHA2 in protecting PR growth under high-NH_4^+ stress (Fig. 6c).

Inhibition of H^+ efflux in *pin5* decreases NH_4^+ efflux and maintains PR growth under high-NH_4^+ stress

To further investigate how PIN5 regulates H^+ efflux and NH_4^+ efflux, we added the PM H^+-ATPase

inhibitor vanadate (VA) to the medium and measured NH_4^+ efflux at EZ of Col and *pin5*. Under control conditions, Col exhibited small NH_4^+ influx (-41.92 pmol cm^{-2} s^{-1}), while in *pin5* NH_4^+ efflux was 50.56 pmol cm^{-2} s^{-1}, indicating that excess endogenous IAA_{cyt} promotes NH_4^+ efflux (Figs. 3b, 6a-b). When VA was introduced to the medium, *pin5* exhibited NH_4^+ influx, similar to Col grown on medium with vanadate (-63.21 pmol cm^{-2} s^{-1} vs -37.60 pmol cm^{-2} s^{-1}), revealing that increased NH_4^+ efflux in *pin5* results from enhanced H^+ efflux at EZ of roots under control conditions (Fig. 6a-b). Furthermore, we also tested the NH_4^+ fluxes of *pin5* and Col grown on high-NH_4^+ medium with or without VA. NH_4^+ effluxes in Col and *pin5* grown on high-NH_4^+ medium with VA were 313.99 pmol cm^{-2} s^{-1} and 116.17 pmol cm^{-2} s^{-1}, 76.67% and 44.03% of those seen when growth occurred on high-NH_4^+ medium, respectively, implying excessive H^+ efflux induced by the *PIN5* mutation is an important factor limiting its ability to inhibit NH_4^+ efflux (Fig. 6a-b). In addition, VA also decreased PR-growth inhibition at 30 mM NH_4^+ in *pin5* (30.96%) compared with Col (52.95%) (Fig. 6c-d). Similar results were also found with exogenous IAA supplementation under high NH_4^+ (Fig. S1). Taken together, our data suggest that PIN5 is involved as a key factor in the response to high-NH_4^+ stress by decreasing IAA_{cyt} accumulation and then NH_4^+ efflux.

Discussion

Futile NH_4^+ fluxes in roots induced by high-NH_4^+ stress are an important diagnostic of NH_4^+ toxicity (Britto et al. 2001; Chen et al. 2013; Coskun et al. 2013; Szczerba et al. 2008). Recently, several genes involved in regulating NH_4^+ efflux in Arabidopsis and rice have been reported, namely *VTC1*, *NUDX9*, *GH3.6*, *UGT75D1*, *WRKY46,* and *EIL1*, and mutations of these genes do not affect NH_4^+ accumulation, implying that high-NH_4^+-induced futile NH_4^+ cycling is independent of NH_4^+ metabolism (Di et al. 2021b; Li et al. 2022, 2010). Our previous studies have shown that high NH_4^+ induces NH_4^+ efflux via promoting IAA conjugation and that the transcription factor WRKY46 is involved through directly inhibiting the transcription of genes coding for the synthesis of IAA-conjugation products (*GH3.1*, *GH3.3*, *UGT75D1* and *UGT84B2*) (Di et al. 2021b, 2018). In addition, VTC1 and NUDX9 regulation of NH_4^+ efflux partially depends on free IAA content under high NH_4^+, revealing the important role of free IAA content in regulating NH_4^+ efflux under high-NH_4^+ stress (Di et al. 2021b; Li et al. 2010). In *Arabidopsis*, *PIN5* encodes an atypical ER-localized PIN protein and regulates intracellular IAA homeostasis and metabolism (Mravec et al. 2009). The important role of the ER in regulating IAA homeostasis has been highlighted due to the presence of auxin-metabolism enzymes (Bartel 1997; Mravec et al. 2009). However, whether PIN5 is also involved in the response to high-NH_4^+ stress is still unclear. Here, we report that PIN5 is responsible for regulating H^+ efflux and NH_4^+ efflux via inhibiting IAA_{cyt} accumulation under high-NH_4^+ stress.

PIN5 participates in regulating NH_4^+ efflux by promoting IAA transport from cytoplasm to ER under high-NH_4^+ conditions

Intracellular auxin transport plays a crucial role during plant growth and development (Ding et al. 2012; Mravec et al. 2009; Simon et al. 2016). Previous studies have reported that PIN5 mediates IAA transport from cytoplasm to ER, where IAA-metabolism enzymes are compartmentalized, and that the mutation of *PIN5* leads to increased free IAA_{cyt} and stunted PR growth under normal conditions (Mravec et al. 2009; Woodward and Bartel 2005). Our own recent studies also suggest that high NH_4^+ decreases free IAA in roots by promoting IAA metabolism, followed by PR growth inhibition (Di et al. 2021a, 2021b, 2018; Liu et al. 2013). To investigate the role of PIN5 during the root response to high NH_4^+, we measured the PR length of *pin5* and PIN5*ox* grown on control and high-NH_4^+ conditions (Fig. 1a-b). Compared with Col, the knockout mutant *pin5* and the overexpression line PIN5*ox* exhibited reduced and elevated sensitivity to high-NH_4^+ stress, respectively, revealing the negative role of PIN5 in response to high-NH_4^+ stress (Fig. 1a -b). As PIN5, located at the ER membrane, mediates IAA transport from cytoplasm to ER and regulates intracellular IAA metabolism (Mravec et al. 2009), we then directly measured the content of free IAA and of IAA conjugates (IAA-Glu and IAA-Asp) in the roots of Col, *pin5,* and PIN5*ox*. Compared with Col, *pin5* accumulated more

free IAA and fewer IAA conjugates under high-NH_4^+ stress and, by contrast, PIN5*ox* accumulated less free IAA and more IAA conjugates (Fig. 2b-d). In addition, the gene transcription and protein expression of PIN5 was also upregulated by high-NH_4^+ stress (Fig. 1c-f), suggesting that the promotion of IAA conjugation by high NH_4^+ is partially dependent on upregulation of *PIN5* in roots.

Our previous study showed that free IAA inhibits NH_4^+ efflux in roots, especially at the EZ (Di et al. 2021b). To identify whether the increased NH_4^+ tolerance in the knockout mutant *pin5* is associated with decreased NH_4^+ fluxes in roots, we directly measured NH_4^+ fluxes in *pin5* and Col under control and high-NH_4^+ conditions. The data show that the induction of high NH_4^+ efflux by high NH_4^+ is significantly weakened in *pin5* compared with Col, especially at the EZ, revealing that PIN5-mediated intracellular IAA transport is also involved in regulating NH_4^+ efflux under high-NH_4^+ stress (Fig. 3). We speculate that high NH_4^+ reduces the IAA content in both the MZ and root cap, which, in turn, leads to a decrease in acropetal IAA transport to the EZ. In addition, our previous studies also showed that high NH_4^+ promotes the transcription of IAA-conjugating genes while reducing *p*DR5::GUS staining in roots, especially in the EZ (Di et al. 2021a; b). Consequently, lower IAA accelerates the efflux of NH_4^+ and H^+ (Di et al. 2021b).

PIN5 is also involved in regulating H^+ efflux under high-NH_4^+ stress

The recent study suggests that cell-surface-localized TMK can directly interact with PM H^+-ATPases when IAA is present, activating their phosphorylation, and increase H^+ efflux from the cytoplasm to the external medium and promote cell-wall acidification, illustrating a positive-regulatory role for IAA in the promotion of H^+ efflux from cells to apoplast (Lin et al. 2021). Furthermore, due to an increase in IAA_{cyt}, the promotion of H^+ efflux at high NH_4^+ is strengthened in *pin5* compared with Col (Fig. 4a-d), implying that the PIN5-mediated reduction in free IAA_{cyt} induced under high NH_4^+ may function as a "brake" of H^+ efflux resulting from NH_4^+ absorption (Fig. 4). In line with this assumption, we also found that exogenous application of VA or MES could more efficiently rescue PR growth inhibition induced under high NH_4^+ in *pin5* compared with that in Col (Figs. 6d and S1). Interestingly, our results also show that 5 μM VA could slightly alleviate NH_4^+ toxicity, while 10 μM VA further enhanced PR growth inhibition under high NH_4^+, indicating that rhizospheric H^+ concentration is critical to PR growth under control and high-NH_4^+ conditions (Fig. 6c-d). Collectively, rapid and flexible intracellular IAA transport between cytoplasm and ER, mediated by PIN5, may protect the PR growth in high-NH_4^+ environments via coordinated regulation of H^+ fluxes and NH_4^+ fluxes.

Taken together, results from previous studies and those in our current findings suggest that high NH_4^+ can regulate H^+ efflux in two seemingly antagonistic ways, and two distinct mechanisms may exist for the regulation of H^+ efflux under high NH_4^+: i) during NH_4^+ uptake by roots, high NH_4^+ stimulates H^+-pump activity from the cytoplasm, favouring the electrochemical gradient for NH_4^+ transport through the PM; ii) once NH_4^+ accumulates in the roots, excess NH_4^+ reduces free IAA_{cyt} content by increasing IAA transport from cytoplasm to ER and prompting IAA conjugation, and then decreases phosphorylation of PM H^+-ATPases, inhibiting H^+ efflux (Figs. 4b-c; 6a-b) (Di et al. 2021a, 2021b). A question that needs continued research is whether free IAA_{cyt} content affects the NH_4^+ activation of the PM H^+-ATPase system to promote H^+ efflux.

The inhibition of PR growth due to NH_4^+-induced rhizospheric acidification is linked to increased NH_4^+ efflux

As NH_4^+ absorption is accompanied by enhanced AMT-coupled PM H^+-ATPase activity, leading to increased H^+ efflux from cells to the apoplast (Hachiya and Sakakibara 2017; Kempinski et al. 2011; Pearson and Stewart 1993; Yuan et al. 2007a, 2007b), many studies have repeatedly verified that medium acidification and/or intracellular pH disturbance is a key cause of toxic symptoms induced by high NH_4^+ (Barker et al. 1966; Bligny et al. 1997; Britto and Kronzucker 2002; 2005; Britto et al. 2001; Gerendas et al. 1997). In addition, RNA-seq analysis has suggested that 20%-41% of NH_4^+-responsive genes are upregulated by low pH and that seedlings grown on acidic pH medium can mimic high-NH_4^+ toxicity, indicating that at least a part of the NH_4^+-triggered physiological response results from rhizosphere pH decreases (Sun et al. 2017; Zheng et al.

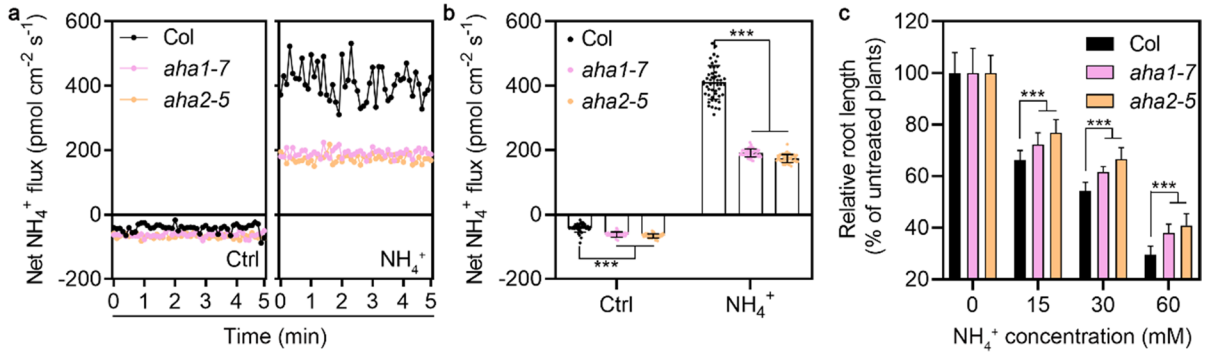

Fig. 5 Decreased rhizosphere H^+ concentration mediated by mutation of the PM H^+-ATPase genes *AHA1* and *AHA2* is responsible for high-NH_4^+-induced NH_4^+ efflux and PR growth inhibition. (**a**) NH_4^+ fluxes of Col, *aha1-7,* and *aha2-5* in the EZ. (**b**) Mean NH_4^+ fluxes in (**a**). 7-day-old seedlings were transferred to fresh medium with or without 30 mM NH_4Cl and grown another 12 h before NH_4^+ flux measurement ($n \geq 6$). (**c**) PR length of Col, *aha1-7,* and *aha2-5* grown on medium with different concentrations of NH_4^+ (0, 15, 30, and 60 mM). Five-day-old seedlings were transferred to fresh medium with different concentrations of NH_4Cl (0, 15, 30 and 60 mM) and grown for another three days before phenotypic observation and PR-length measurement. Mean ± SD ($n \geq 25$). $^{***}P < 0.001$ (t-test)

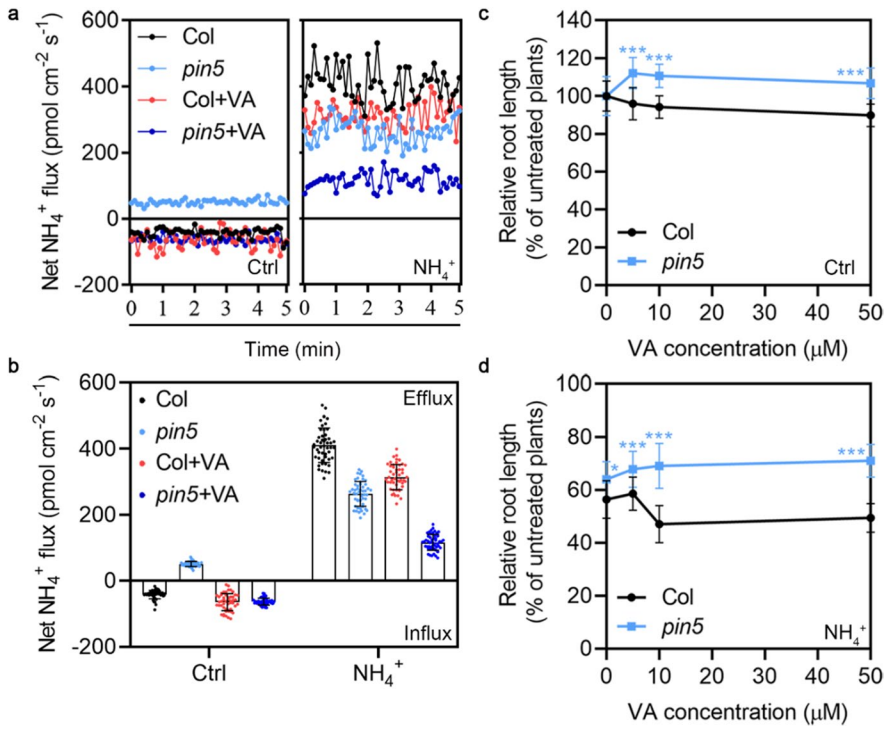

Fig. 6 The inhibitory effect of PIN5 under high-NH_4^+ stress is partially dependent on the PM H^+-ATPase. (**a**) NH_4^+ fluxes of Col and *pin5* in the EZ grown on control, NH_4^+, VA, and NH_4^+ + VA medium. (**b**) Mean NH_4^+ fluxes in (**a**). 7-day-old seedlings were transferred to different fresh media (control, 30 mM NH_4Cl, 5 μM VA, and NH_4Cl + 5 μM VA) and grown another 12 h before NH_4^+ fluxes measurement ($n \geq 6$). (**c**-**d**) PR length of Col and *pin5* grown on control and VA (**c**); and NH_4^+ and NH_4^+ + VA medium (**d**). Five-day-old seedlings were transferred to fresh media (control, 30 mM NH_4Cl, 5 μM/10 μM/50 μM VA and NH_4Cl + 5 μM/10 μM/50 μM VA) and grown for another three days before phenotypic observation and PR-length measurement. Mean ± SD ($n \geq 25$). $^{*}P < 0.05$ and. $^{***}P < 0.001$ (t-test)

2015; Zhu et al. 2021), while it is, however, also clear that rhizosphere or cytosolic pH disturbance alone is not sufficient to explain NH_4^+ toxicity (Britto and Kronzucker 2002). In addition, we show that mutations in the genes *AHA1* and *AHA2*, which encode PM H^+-ATPases in *Arabidopsis*, have increased tolerance to high-NH_4^+ stress (Fig. 5c). Similar results were also observed when the growth medium was supplemented with VA or MES (Figs. 6d and S1) (Jia et al. 2020; Tian et al. 2021), suggesting that, compared with cytosolic acidification, rhizosphere acidification is more critical to the development of NH_4^+ toxicity (Kong et al. 2022). However, the mechanism by which enhanced rhizosphere acidification inhibits PR growth is still elusive.

Previous studies proposed that alkalinization of the medium by NO_3^- absorption and assimilation may play a critical role during detoxification of high NH_4^+ (Hachiya et al. 2012), as the alleviatory role of NO_3^- in NH_4^+ toxicity is well established (Kronzucker et al. 1999). Recently, several studies have demonstrated that NRT1.1 interacts with SLAH3 to form a functional unit to regulate NO_3^- transport (influx/efflux) and H^+ influx and to alleviate NH_4^+ toxicity in roots (Du et al. 2021; Sun et al. 2021; Xiao et al. 2022; Zheng et al. 2015). In addition, NO_3^- could also reduce futile NH_4^+ fluxes and thereby increase NH_4^+ tolerance (Babourina et al. 2007). Interestingly, NRT1.1 functions as a dual carrier, which mediates the uptake of both NO_3^- and IAA (Krouk et al. 2010). Moreover, NO_3^- inhibits NRT1.1-dependent IAA transport, while IAA positively regulates NRT1.1 transcription (Krouk et al. 2010). Considering that IAA transport from cytoplasm to ER is mediated by PIN5 under high-NH_4^+ stress, IAA_{cyt} may function as a "mediator" in the cross-talk among NO_3^-/NH_4^+/H^+ fluxes at the PM under high-NH_4^+ stress. However, how rhizosphere acidification affects NH_4^+ flux is still only partially understood. Here, we show that the NH_4^+ efflux in *aha1-7* and *aha2-5* are both decreased compared with Col (Fig. 5a-b), suggesting that rhizosphere H^+ inhibits excess NH_4^+ efflux. In addition, we also show that additions of a low dose of the PM H^+-ATPase inhibitor VA can inhibit the NH_4^+ efflux and promote PR growth in both Col and *pin5*, especially in *pin5* grown under high-NH_4^+ conditions (Fig. 6a-b), underscoring that high-NH_4^+-stress-induced PR growth inhibition is partially due to enhanced rhizosphere acidification, and that the increased NH_4^+ efflux may be an important reason for the inhibition of PR growth by rhizosphere acidification.

Previous work has shown that mutation of *VTC1* can confer hypersensitivity to high NH_4^+, especially in terms of PR growth (Qin et al. 2008). A subsequent study then identified that the increased PR growth inhibition in *vtc1-1* is linked to elevated NH_4^+ efflux at the EZ (Li et al. 2010). Further studies also showed that mutation of *VTC1* promotes NH_4^+efflux, in partial dependence on a decrease in free IAA content (Barth et al. 2010; Di et al. 2021b), while it has been reported that an increase in the medium pH (pH 7.0) can partially rescue the PR growth of *vtc1-1* under high-NH_4^+ stress (Kempinski et al. 2011). Hence, it is interesting, in future studies, to investigate whether the mutation of *VTC1* influences rhizosphere acidification via glycosylation of H^+-secretion-related proteins and induces NH_4^+ efflux.

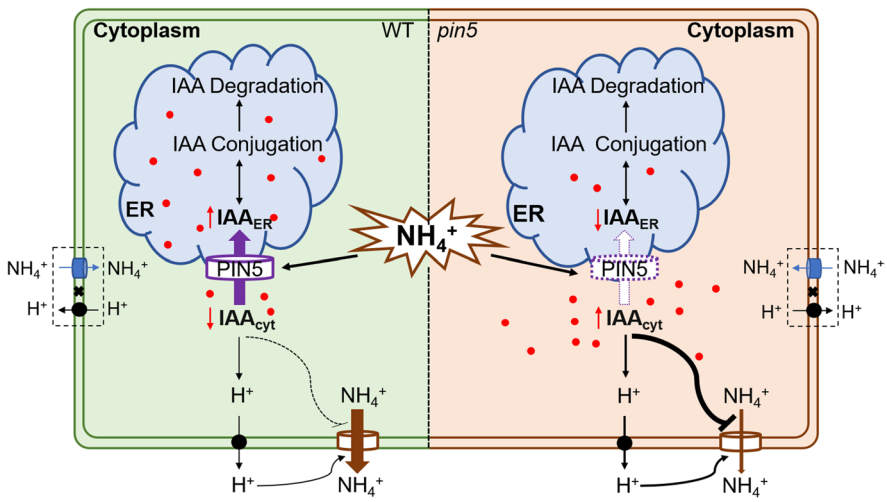

Fig. 7 Proposed working model of PIN5 in response to high-NH_4^+ stress

In summary, increased endogenous IAA acts positively on PR growth under high-NH_4^+ stress by reducing NH_4^+efflux, and this effect can be strengthened when pH is buffered. Clarification of the correlations between IAA; H^+ flux, and NH_4^+ flux in response to high-NH_4^+ stress will be able to offer a new theoretical foundation for breeders to screen for new NH_4^+-tolerant crop cultivars. Our investigation also suggests that additions of IAA-related compounds and buffer agents to NH_4^+ fertilizer formulations may present viable routes for improving crop NH_4^+ tolerance in the future (Fig. 7).

Under high-NH_4^+ stress, NH_4^+ upregulates the transcription and expression of PIN5, increasing IAA transport from cytoplasm to ER, and decreases free IAA accumulation in cytoplasm (IAA_{cyt}). Reduced IAA_{cyt} leads to a weaker inhibition of NH_4^+ efflux and a weaker facilitation of H^+ efflux, effectuating an increase in NH_4^+ efflux. However, when PIN5 is mutated, high-NH_4^+-induced intracellular IAA transport is partially disrupted, resulting in increased IAA_{cyt} accumulation, which leads to an inhibition of NH_4^+ efflux and intensified H^+ efflux. Concurrently, enhanced H^+ efflux may activate NH_4^+ efflux through an as yet unidentified mechanism. Taken together, NH_4^+ efflux is significantly inhibited in *pin5* compared to the wild type (WT). The blue cylinder represents NH_4^+ influx transporters, the black circle represents PM H^+-ATPases, and the cross indicates the interaction between NH_4^+ absorption and H^+ efflux. The brown cylinder indicates the NH_4^+ efflux system. The red dots represent free IAA.

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Author's Contributions W. S. and D-W. D. designed the research; D-W. D., J. W. and M. M. performed the research and analyzed the data; D-W. D., W. S., G. L., M.W. and H. J. K. wrote the paper.

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Data Availability The data that support the findings of this study are available in ScienceDB at https://doi.org/10.57760/sciencedb.07082.

Declarations

Competing interests 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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