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OsEIL1 protects rice growth under NH_4^+ nutrition by regulating OsVTC1-3-dependent N-glycosylation and root NH_4^+ efflux

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Abstract

Rice is known for its superior adaptation to ammonium (NH_4^+) as a nitrogen source. Compared to many other cereals, it displays lower NH_4^+ efflux in roots and higher nitrogen-use efficiency on NH_4^+. A critical role for GDP-mannose pyrophosphorylase (VTC1) in controlling root NH_4^+ fluxes was previously documented in *Arabidopsis*, but the molecular pathways involved in regulating VTC1-dependent NH_4^+ efflux remain unclear. Here, we report that ETHYLENE-INSENSITIVE3-LIKE1 (OsEIL1) acts as a key transcription factor regulating OsVTC1-3-dependent NH_4^+ efflux and protein N-glycosylation in rice grown under NH_4^+ nutrition. We show that *OsEIL1* in rice plays a contrasting role to *Arabidopsis*-homologous ETHYLENE-INSENSITIVE3 (AtEIN3) and maintains rice growth under NH_4^+ by stabilizing protein N-glycosylation and reducing root NH_4^+ efflux. OsEIL1 constrains NH_4^+ efflux by activation of *OsVTC1-3*, but not *OsVTC1-1* or *OsVTC1-8*. OsEIL1 binds directly to the promoter EIN3-binding site (EBS) of OsVTC1-3 in vitro and in vivo and acts to increase the transcription of *OsVTC1-3*. Our work demonstrates an important link between excessive root NH_4^+ efflux and OsVTC1-3-mediated protein N-glycosylation in rice grown under NH_4^+ nutrition and identifies OsEIL1 as a direct genetic regulator of *OsVTC1-3* expression.

KEYWORDS

ammonium, AtEIN3, N-glycosylation, NH_4^+ efflux, OsEIL1, OsVTC1-3, rice

1 | INTRODUCTION

Ammonium (NH_4^+) and nitrate (NO_3^-) are the primary sources of nitrogen (N) for crop plants. Farmers often add N fertilizer in large amounts to satisfy the high N demand of crops. Ammonium-based fertilizers, together with nitrification inhibitors, have been shown to help mitigate the detrimental effects of N leaching and run-off from agricultural fields into natural waters in a variety of circumstances (Coskun et al., 2017; Lu et al., 2019; Z. H. Wang & Li, 2019), and some authors have suggested that NH_4^+ should be employed even more frequently to reduce environmental N pollution (Subbarao & Searchinger, 2021). Such approaches, however, lead to high soil ammonium accumulation in many crop systems (Ferretti et al., 2014; Kronzucker et al., 2003; Maestre et al., 2016) and not infrequently

manifest in toxicity in crops, evident in stunted vegetative growth and decreased yield (Balkos et al., 2010; Jian et al., 2018; G. J. Li et al., 2019; Ma et al., 2016), in addition to other environmental impacts (Y. Li et al., 2016; Min et al., 2021; H. Sun et al., 2020). Indeed, for most plant species, NH_4^+ represents a stressor even at moderate levels of supply, once soil concentrations rise above 0.1–0.5 mmol L^{-1} (Britto & Kronzucker, 2002). Understanding the molecular underpinnings of tolerance to the NH_4^+ source of N is therefore critical. NH_4^+ is the main N source in rice paddy soils, and it is utilized as the major source for N assimilation in rice. Although rice is considered to be a relatively NH_4^+-tolerant species it can also suffer from NH_4^+ toxicity at soil NH_4^+ concentrations that exceed 1 mmol L^{-1} (Balkos et al., 2010; Jia et al., 2020; Jiao et al., 2020; Ma et al., 2016). To obtain maximal grain yields, modern protocols of N fertilizer application have increased the content of NH_4^+ in many paddy soils (S. Yang et al., 2013; Y. Zhang et al., 2017), and it is common for soil-solution concentrations in rice paddies to exceed 1 mmol L^{-1} after fertilization (Liang et al., 2007; Lin et al., 2012), thus exposing rice plants to toxic levels of NH_4^+. N deficiency and NH_4^+ toxicity are widespread in rice production, inhibiting rice growth and decreasing nitrogen use efficiency (NUE) (G. Chen et al., 2013; H. Chen et al., 2021).

Many components have been identified that affect NH_4^+-dependent growth in the model system *Arabidopsis* (Coleto et al., 2021; B. Li et al., 2014; Liu & von Wirén, 2017; Straub et al., 2017; D. D. Sun et al., 2021), but there is still very limited knowledge about the regulatory mechanisms of NH_4^+ tolerance in rice. It is well understood that the capacity for NH_4^+ metabolism via glutamine synthetase in roots is essential to tolerance (Bittsánszky et al., 2015; Britto & Kronzucker, 2002; Givan, 1979; Guan et al., 2016; Ishiyama et al., 2004; Konishi et al., 2017; B. Li et al., 2011). In rice roots, the glutamine synthetase-encoding genes OsGLIN1.1 and OsGLIN1.2 play central roles (Ishiyama et al., 2004). *Indeterminate domain 10* (*OsIDD10*) regulates the induction of several genes involved in N metabolism in rice, including genes encoding glutamine synthetase 2 (Xuan et al., 2013), and *semi dwarf-1* (*OsSD1*) and microRNAs (miR444) link the metabolic response to NH_4^+ in rice to gibberellin and brassinosteroid signaling, respectively (Jiao et al., 2020; B. Wang et al., 2020). Rice responds to ammonium stress by adopting a helical root growth pattern to moderate auxin signaling (Jia et al., 2020). It is also well documented that rice's superior adaptation and tolerance to NH_4^+ compared to other cereals is linked to lower NH_4^+ efflux in roots (Britto & Kronzucker, 2002; Britto et al., 2001; Coskun et al., 2013a; Kronzucker et al., 1998, 2001; Szczerba et al., 2008). It has been suggested that protein N-glycosylation alteration is associated with growth inhibition under NH_4^+ toxicity (Di et al., 2021; Jadid et al., 2011; C. Qin et al., 2008; Tanaka et al., 2015), and GDP-mannose pyrophosphorylase (VTC1) has been reported to be involved in the regulation of *Arabidopsis* growth under NH_4^+-toxicity conditions by regulating protein N-glycosylation. VTC1 mutation results in defective N-glycosylation of proteins, assumed to represent important downstream molecular events leading to growth inhibition under NH_4^+ toxicity (Barth et al., 2010; Kempinski et al., 2011; C. Qin et al., 2008). Mutation of VTC1 has been shown to enhance root NH_4^+ efflux, linking N-glycosylation of proteins in *Arabidopsis* functionally to root NH_4^+ fluxes (Di et al., 2021; Q. Li et al., 2010). However, it remains unclear which molecular pathways are involved in modulating NH_4^+ efflux in rice.

Ethylene production has been shown to increase linearly with tissue NH_4^+ accumulation (Barker, 1999), concurrent with the development of toxicity symptoms (G. J. Li et al., 2013; You & Barker, 2002, 2005). Enhanced ethylene signaling can significantly increase NH_4^+ sensitivity in *Arabidopsis* (H. Chen et al., 2021). Recently, we isolated an *Arabidopsis thaliana* NH_4^+-tolerant mutant, *ammonium tolerance 1 (amot1)*, which exhibits more robust growth under NH_4^+ treatment, and gene cloning revealed *amot1* to be allelic to ethylene-insensitive 3 (AtEIN3) (G. J. Li et al., 2019). Moreover, AtEIN3/AMOT1 could upregulate the expression of genes coding for peroxidases (PODs) (G. J. Li et al., 2019), previously shown to correlate positively with NH_4^+-induced reactive oxygen species (ROS) content changes and cell growth inhibition. The ethylene signal is transmitted via the positive regulator ETHYLENE INSENSITIVE 2 (EIN2) and is further transduced into the nucleus to cause the accumulation of two master transcriptional activators, AtEIN3/EIL1, which initiate transcriptional re-programming in various ethylene responses in *Arabidopsis* (Tao et al., 2015). OsEIN2 is also an essential positive regulator of ethylene responses and a regulator of several agronomic traits in rice (C. Yang, Lu, et al., 2015; Zhao et al., 2021). Rice possesses six EIN3-like homologs, and ETHYLENE INSENSITIVE3-LIKE1 (OsEIL1) and OsEIL2 are suggested to be involved in ethylene signaling (C. Yang, Lu, et al., 2015; C. Yang, Ma, et al., 2015; H. Qin et al., 2017); among these, OsEIL1 shows the highest similarity to AtEIN3 of *Arabidopsis* (C. Yang, Lu, et al., 2015; C. Yang, Ma, et al., 2015; Zhao et al., 2021). These clues prompted us to ask whether rice OsEIL1 may have a function related to regulating NH_4^+ tolerance in rice, similar to *Arabidopsis*-homologous AtEIN3. To study the adaptation of rice to NH_4^+, the role of OsEIL1 in rice was assessed. Surprisingly, we found that loss of function of OsEIL1 enhances the sensitivity of rice to NH_4^+, in fundamental contrast to the roles of AtEIN3 in *Arabidopsis*. Furthermore, this positive regulation by OsEIL1 appears in part to be due to the direct control of GDP-mannose pyrophosphorylase (VTC1) OsVTC1-3-dependent NH_4^+ efflux in roots and to protein N-glycosylation. We show that the loss of OsVTC1-3 function results in increased sensitivity of roots to NH_4^+, in enhanced NH_4^+ efflux, and in reduced N-glycosylation. By contrast, AtEIN3 in *Arabidopsis* does not regulate NH_4^+ fluxes and does not directly regulated *AtVTC1* expression. We demonstrate that, in stark contrast to the negative roles of AtEIN3 in *Arabidopsis*, OsEIL1 participates in the regulation of NH_4^+ efflux and tolerance. OsEIL1 affects NH_4^+ efflux in rice positively by direct regulation of OsVTC1-3 in rice.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

The *Arabidopsis ein3-1*, *vtc1-1* mutants, and *5×EBS:GUS/Col-0* transgenic plants used in this study were as previously reported (Chao

et al., 1997; He et al., 2011; L. Zhang et al., 2021); *Col-0* was the wild type. The overexpressing OsEIL1 (*OsEIL1-OX*) transgenic lines, *oseil1* mutant (*mhz6-2*), *mhz6-3* and *osein2* (*mhz7-2*) of rice (*Oryza sativa* L.) were identified against a Nipponbare (Nip) background (C. Yang, Ma, et al., 2015). The OsVTC1-3 (Os03g0268400) T-DNA knockout mutant *osvtc1-3* (PFG_3C-00707.R) is against a Dongjin (DJ) background and was obtained from: http://signal.salk.edu/cgi-bin/RiceGE. Rice seeds were surface-sterilized with 3% H_2O_2 for 20 min, washed with sterile water, soaked in water for 24 h in the dark, and then transferred to a nylon net floating on 0.5 mM $CaCl_2$ for 3 days. Then, rice seedlings were transferred to 1/2-strength modified Kimura's solution. The solution composition was as follows: 0.5 mM $MgSO_4.7H_2O$, 0.36 mM $CaCl_2{\cdot}2H_2O$, 0.25 mM KCl, 0.2 mM NaH_2PO_4, 9 μM $MnCl_2{\cdot}4H_2O$, 0.5 μM $Na_2MoO_4{\cdot}2H_2O$, 0.3 μM $CuSO_4{\cdot}5H_2O$, 0.7 μM $ZnSO_4{\cdot}7H_2O$, 0.1 mM Fe(III)-EDTA and 50 μM H_3BO_3; 6 mM $NaNO_3$ was added in the nitrate treatment ($+NO_3^-$) and 6 mM NH_4Cl in the ammonium treatment ($+NH_4^+$), according to Ma et al. (2016). The nutrient solution pH was adjusted to 5.5. Plants were grown in a growth chamber, with a 400 μmol m^{-2} s^{-1} light intensity, 65% relative humidity and a 16 h (30°C)/8 h (28°C) day/night cycle. The nutrient solution was exchanged every 2 days. Exogenous 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC) (Sigma) treatment concentrations were according to Mao et al. (2006). The *Arabidopsis* standard growth medium was modified after G. J. Li et al. (2013) and was composed of 0.75 mM KH_2PO_4, 0.75 mM $MgSO_4.7H_2O$, 0.5 mM $CaCl_2{\cdot}2H_2O$, 50 μM Fe(II)-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 0.8% agarose (pH 5.6, adjusted with 1 M NaOH); 2.5 mM $NaNO_3$ was added as the nitrate treatment ($+NO_3^-$) and 2.5 mM NH_4Cl as the ammonium treatment ($+NH_4^+$). The sowing date was set at day 0. *Arabidopsis* was grown, oriented vertically on the surface of the medium in a growth chamber, under a 16 h/8 h day/night cycle, an irradiance of 100 μmol m^{-2} s^{-1}, and a constant temperature of 23 ± 1°C. Five-day-old seedlings were transferred onto $+NO_3^-$ or $+NH_4^+$ medium. After treatments with $+NO_3^-$ or $+NH_4^+$ for 10 days, rice seedlings were harvested and separated into shoots and roots for the determination of biomass (fresh weight). *Arabidopsis* seedlings were transferred onto $+NO_3^-$ or $+NH_4^+$ medium for 6 days, and seedlings were harvested and biomass (fresh weight) was determined using a high-precision balance (0.000001) (XP105; Mettler Toledo).

2.2 | Real-time quantitative polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from *Arabidopsis* shoots or roots and rice root tissues at the indicated time and treatment concentrations. Gene-specific primers for qRT-PCR were designed using Primer-5 software (see Table S1). ACTIN2 was used as the internal reference gene, and relative RNA abundance was normalized to the ACTIN2 internal control [(mRNA)gene/(mRNA) ACTIN2].

2.3 | Measurement of net NH_4^+ fluxes with the NMT system

After treatments with NO_3^- (6 mM) or NH_4^+ (6 mM) for 5 days of rice before measurement, the NMT technique (NMT system BIO-IM; Younger USA, LLC) was used to monitor the net NH_4^+ fluxes at the surface of the root tip elongation zone in both rice and *Arabidopsis*. The NMT system and its use in detecting net NH_4^+ fluxes have been described in detail elsewhere (G. Chen et al., 2013; Q. Li et al., 2010), and the test buffer solution was composed of 0.1 mM $CaCl_2$ and 0.2 mM NH_4Cl (pH 5.5, adjusted with 1 M NaOH) according to previous reports (Bai et al., 2014; Britto & Kronzucker, 2002; G. Chen et al., 2013; Di et al., 2021; Q. Li et al., 2010; Ma et al., 2016; ten Hoopen et al., 2010). The roots of rice and *Arabidopsis* seedlings were equilibrated in the buffer solution for 30 min. All measurements of net NH_4^+ fluxes were carried out at the Xuyue Science and Technology Co., Ltd.

2.4 | NH_4^+ content and N-glycoproteins assays

Roots of each sample were washed in 10 mM $CaSO_4$, frozen in liquid nitrogen, and then extracted with 1 ml of 10 mM formic acid for the NH_4^+ content assay by high-performance liquid chromatography, following derivatization with *o*-phthaldialdehyde (Sigma), as described previously (L. Zhang et al., 2021). The extent of mature N-glycoproteins in $+NO_3^-$ or $+NH_4^+$ treatment for 10 days of rice seedlings was examined using anti-horseradish peroxidase (HRP, 1:200 000; Sigma-Aldrich), and measurements were performed by Jingjie PTMBiolab Co. Ltd.

2.5 | Yeast one-hybrid (Y1H) assay

The Y1H assay was used to examine the binding of OsEIL1 to OsVTC1-1, OsVTC1-3 or OsVTC1-8 using the Matchmaker Gold Yeast One-Hybrid System protocol (Clontech), following the manufacturer's instructions (see Table S2). The full-length CDSs of *OsEIL1* was inserted into the pGADT7 vectors, and the promoter of *OsVTC1-1*, *OsVTC1-3* and *OsVTC1-8* was subcloned into the pAbAi vector. The linearized plasmids of *OsVTC1-1-pro-pAbAi*, *OsVTC1-3-pro-pAbAi* and *OsVTC1-8-pro-pAbAi* were transferred into Y1H yeast, and the positive strain was identified by testing the aureobasidin A (AbA) inhibition. The positive yeast received the transfer of *OsEIL1-pGADT* with an empty vector as the control, and was cultured in SDSD/-uracil/-leucine (SD/-Ura/-Leu)SD/-Ura/-Leu) medium containing different AbA concentration.

2.6 | Histochemical staining and image analysis

Histochemical analysis of β-glucuronidase (GUS) reporter enzyme activity in *Arabidopsis* was performed as described by Weigel and Glazebrook (2002).

2.7 | Electrophoretic mobility shift (EMSA) assays

To produce the OsEIL1 protein, the full-length complementary DNA was inserted into the pET30a expression vector. The resulting vector was expressed in *Escherichia coli* strain BL21. Recombinant protein OsEIL1-N-His was purified using a 6× HIS-Tagged Protein Kit (Kangwei). Oligonucleotide probes of the *OsVTC1-3* promoter were synthesized and labeled with biotin at their 5′ ends with an EMSA probe biotin labeling kit (Beyotime; Table S2). EMSA was performed using the EMSA kit (Pierce). Briefly, biotin-labeled probes were incubated in 1× binding buffer (50 mM KCl, 10 mM EDTA, 2.5% glycerol and 5 mM $MgCl_2$) at 24°C for 25 min with or without proteins. The reactions were added to an unlabeled probe.

2.8 | Transactivation of the target promoter in tobacco leaves

To generate the *proOsVTC1-1:LUC*, *proOsVTC1-3:LUC*, *proOsVTC1-8:LUC* and *proAtVTC1:LUC* reporters, the promoter sequences of *OsVTC1-1*, *OsVTC1-3*, *OsVTC1-8* and *AtVTC1* were inserted into the vector of pGreenII-0800 using a ClonExpress II one-step cloning kit. The *35S:OsEIL1* and *35S:AtEIN3* effectors were created by cloning their coding sequences into the pBI121 vector using a ClonExpress II one-step cloning kit. The empty vector was used a negative control. Transient dual-luciferase assays in 5-week-old *Nicotiana benthamiana* leaves were performed and checked using dual-luciferase assay reagents (Promega). For this analysis, the ratio between LUC and REN activity was measured with three biological replicates from three leaves.

2.9 | Statistical and graphical analyses

The SPSS 13.0 program (SPSS) was used to statistically analyze the data. Details are shown in the figure legends. Graphs were produced using Origin 8.0.

2.10 | Accession numbers

OsEIN2 (Os07g0155600), OsEIL1 (Os03g0324300), OsEIL2 (Os07g0685700), OsEIL3 (Os09g0490200), OsEIL4 (Os08g0508700), OsEIL5 (Os02g0574800), OsEIL6 (Os04g0456900), OsACS2 (Os04g0578000), OsACO5 (Os05g0149400), OsERF002 (Os06g0181700), OsVTC1-1 (Os01g0847200), OsVTC1-3 (Os03g0268400), OsVTC1-8 (Os08g0237200), OsPRX4 (Os01g0270300), OsPRX2 (Os01g0205900), OsPRX16 (Os01g0327400), OsPRX34 (Os03g0121300), OsPRX41 (Os03g0339300), OsPRX59 (Os04g0688100), OsPRX67 (Os05g0134800), OsPRX95 (Os07g0104500), OsPOX3006 (Os07g0677500), OsPRX135 (Os12g0112000), OsBiP1 (Os02g0115900), OsACTIN2 (Os10g0510000), AtEIN3 (At3g20770), AtVTC1 (At2g39770), AtNUDX9 (At3g46200) and AtACTIN2 (At3g18780)

3 | RESULTS

3.1 | Knockout of OsEIL1 results in hypersensitivity to NH_4^+

Our previous results in *Arabidopsis* demonstrated that mutation of AtEIN3 can contribute to NH_4^+ tolerance (G. J. Li et al., 2019). To expand our understanding of the homologs of EIN3 in the response to NH_4^+ in rice, we first created a rooted maximum-likelihood tree with predicted EIN3s from Arabidopsis and rice with available full genome sequences. Phylogenetic analysis indicates that OsEIL1 possesses high similarity to Arabidopsis AtEIN3 (C. Yang, Ma, et al., 2015; Figure S1). We further tested the transcriptional level of six *OsEILs* in response to NH_4^+ of the rice roots using quantitative real-time PCR (qPCR). NH_4^+ treatment significantly induced *OsEIL1* expression, and slightly increased *OsEIL2* expression, but had no effect on *OsEIL3*, *OsEIL4* and *OsEIL6* expression (Figure S2). We further examined the NH_4^+ sensitivity of the *oseil1* mutant (*mhz6-2*) harboring 8-bp insertions at position 418 (C. Yang, Ma, et al., 2015). The *oseil1* was found to exhibit negligible levels of *OsEIL1* transcripts under both $+NO_3^-$ and $+NH_4^+$ conditions (Figure 1d). Under NO_3^- conditions, root growth of *oseil1* plants was superior to that of wild type (Nipponbare, Nip) plants (Figure 1a,c), consistent with the previous finding (H. Qin et al., 2017) that showed *oseil1* possessing a longer root under normal conditions. Following exposure to equal concentrations of NH_4^+, root and shoot growth were inhibited in both genotypes; however, root and shoot biomass were significantly reduced in *oseil1* seedlings following treatment with $+NH_4^+$ compared with Nip seedlings (Figure 1a–c). The relative fresh biomass of roots and shoots was 16% and 15% lower, respectively, in the *oseil1* mutant than in the wild type. We further tested the response of *oseil1* allelic mutant, *mhz6-3* (C. Yang, Ma, et al., 2015), to ammonium, and the *mhz6-3* displayed a similar NH_4^+ sensitive phenotype to *oseil1* (*mhz6-2*) (Figure S3). These observations suggest that the loss of OsEIL1 function renders the mutant more sensitive to NH_4^+ than the wild type, contrasting the role of AtEIN3 in the NH_4^+ response in *Arabidopsis*.

OsEIL1 was strongly upregulated in NH_4^+-treated seedlings (Figure 1d), and a time-course experiment showed *OsEIL1* induction to be sustained in roots in response to NH_4^+ treatment (Figure 1e). Previous reports showed that OsEIL1 can regulate the ethylene response in rice (C. Yang, Lu, et al., 2015; C. Yang, Ma, et al., 2015). Thus, we further tested the expression of ethylene-synthesis- and signaling-pathway-related genes under NH_4^+-treatment in wild-type seedlings (Figure S5a), and showed that *OsACS2*, *OsACO5*, *OsEIN2* and *OsERF002* expression were also significantly induced by NH_4^+. OsEIN2 was identified in previous reports as a central component of ethylene signaling in rice, and, similar to the *oseil1* mutation, the *osein2* mutant also exhibited a more sensitive phenotype to NH_4^+ than the wild type (Figure S5b,c). To determine whether ethylene is required for NH_4^+-induced *OsEIL1* gene expression, we first determined the *OsEIL1* expression level in Nip and the *oseil1* mutant under both NO_3^- and NH_4^+ treatment, and quantified the ethylene

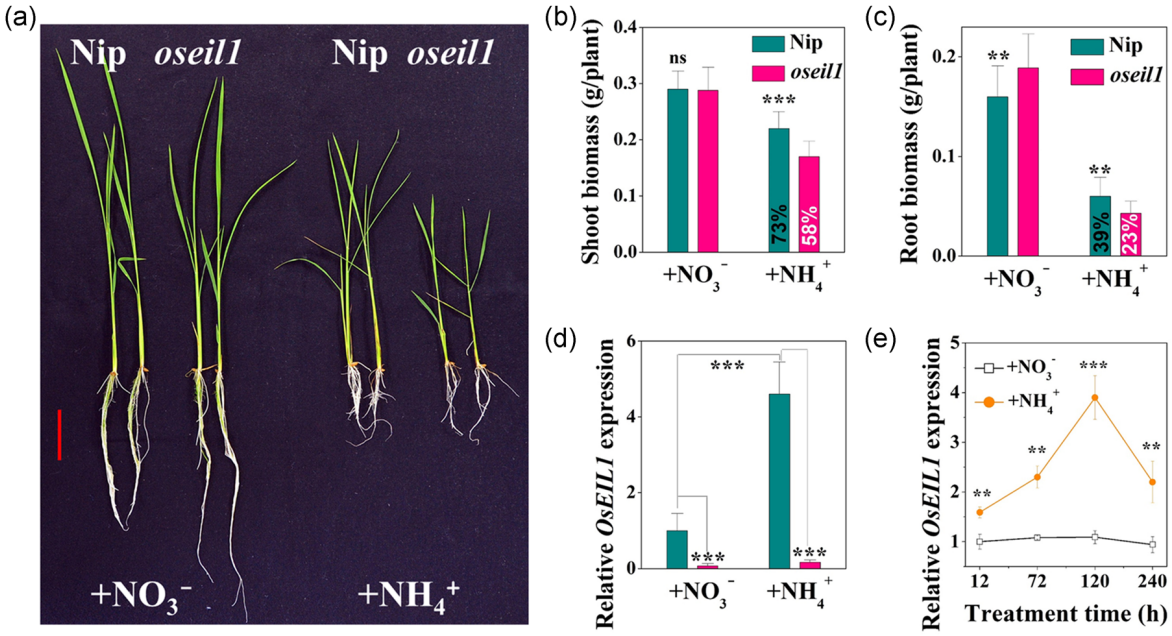

FIGURE 1 The phenotypic response of the rice *oseil1* mutant treated with NH_4^+. (a) Phenotypes of the rice OsEIL1 loss-of-function mutant in response to NH_4^+ treatment. Three-day-old rice seedlings were exposed to 6 mM NH_4Cl ($+NH_4^+$) or 6 mM $NaNO_3$ ($+NO_3^-$) for 10 days, and then pictures were taken. Bar = 5 cm. (b, c) Shoot and root biomass (fresh weight) were determined. Data are the means ± SD ($n \geq 22$ for shoot and root biomass). (d) qRT-PCR analysis of expression of *OsEIL1* in the roots of Nip and the *oseil1* mutant. Data are shown as the means ± SD of at least three biological replicates. (e) Time-dependent expression of OsEIL1 in wild-type rice roots. Rice seedlings were exposed to a $+NO_3^-$ and $+NH_4^+$ solution for different times. Data are shown as the means ± SD of three biological replicates. Significant differences were determined by an independent samples *t* test (**$p < 0.01$, ***$p < 0.001$, and "ns" designates no significance)

biosynthetic precursor 1-aminocyclopropane-1-carboxylic acid (ACC). qPCR analysis showed that ACC treatment had no effect on *OsEIL1* expression (Figure S4a), in agreement with Mao et al. (2006). We also tested *OsEIL1* expression in the *osein2* mutant, and showed that the OsEIN2 mutation had no effect on *OsEIL1* transcription in response to NH_4^+ (Figure S4b).

3.2 | *oseil1* mutation results in elevated NH_4^+ efflux under NH_4^+

AtEIN3 can upregulate the expression of the genes coding for *Arabidopsis* peroxidases (PODs), previously shown to positively correlate with NH_4^+-induced cell growth inhibition (G. J. Li et al., 2019). We first measured the expression of POD-coding genes (Sasaki et al., 2004) in wild type (Nip) and *oseil1* roots. However, expression patterns of POD-coding genes tested under NO_3^- and NH_4^+ conditions were similar, and they were also not significantly affected in the *oseil1* mutant compared with the wild type (Figure S6). The H_2O_2 content was also not significantly affected in the *oseil1* mutant under both NO_3^- and NH_4^+ conditions (Figure S7). NH_4^+ accumulation in roots was significantly higher in *oseil1* seedlings than in wild-type seedlings when cultivated in the presence of NH_4^+ (Figure S8). High unidirectional NH_4^+ fluxes (influx and efflux) in roots have been demonstrated to be strongly and consistently associated with sensitivity to elevated NH_4^+ (Britto et al., 2001; G. Chen et al., 2013; Coskun et al., 2017; Kronzucker et al., 2003). Measurements of net NH_4^+ fluxes at the surface of the elongation zones of wild type and *oseil1* roots, assayed by NMT, revealed NH_4^+ efflux in the roots of both cultivars when supplied with NH_4^+ as the sole N source (Figure 2a,b). NH_4^+ efflux in *oseil1* (*mhz6-2*) and *mhz6-3*, however, was higher than in the wild type (Figures 2c, S3b), pointing to a role of *OsEIL1* in regulating root NH_4^+ effluxes under NH_4^+.

3.3 | OsEIL1 regulates OsVTC1-3 dependent protein N-glycosylation and root NH_4^+ efflux

The results obtained above indicate that OsEIL1 is involved in regulating NH_4^+ efflux in the root under NH_4^+ conditions. OsEIL1 is located in the nucleus (C. Yang, Ma, et al., 2015), and we, therefore, needed to examine how the gene might be engaged in the regulation of root NH_4^+ fluxes. Previous studies have produced evidence indicating a critical role for AtVTC1 in controlling root NH_4^+ fluxes in *Arabidopsis* (Di et al., 2021; Q. Li et al., 2010). There are three VTC1 homologs, designated as OsVTC1-1, OsVTC1-3 and OsVTC1-8, in rice. Previous reports and phylogenetic analyses indicate that OsVTC1-3 has the highest similarity, in terms of functional alignment, to *Arabidopsis* AtVTC1 (H. Qin, Deng, et al., 2016; H. Qin, Wang, et al., 2016; Figure S9). This led us to hypothesize that OsVTC1 might be directly linked to OsEIL1 in the regulatory cascade leading to the control of root NH_4^+ fluxes. To test our hypothesis, we first

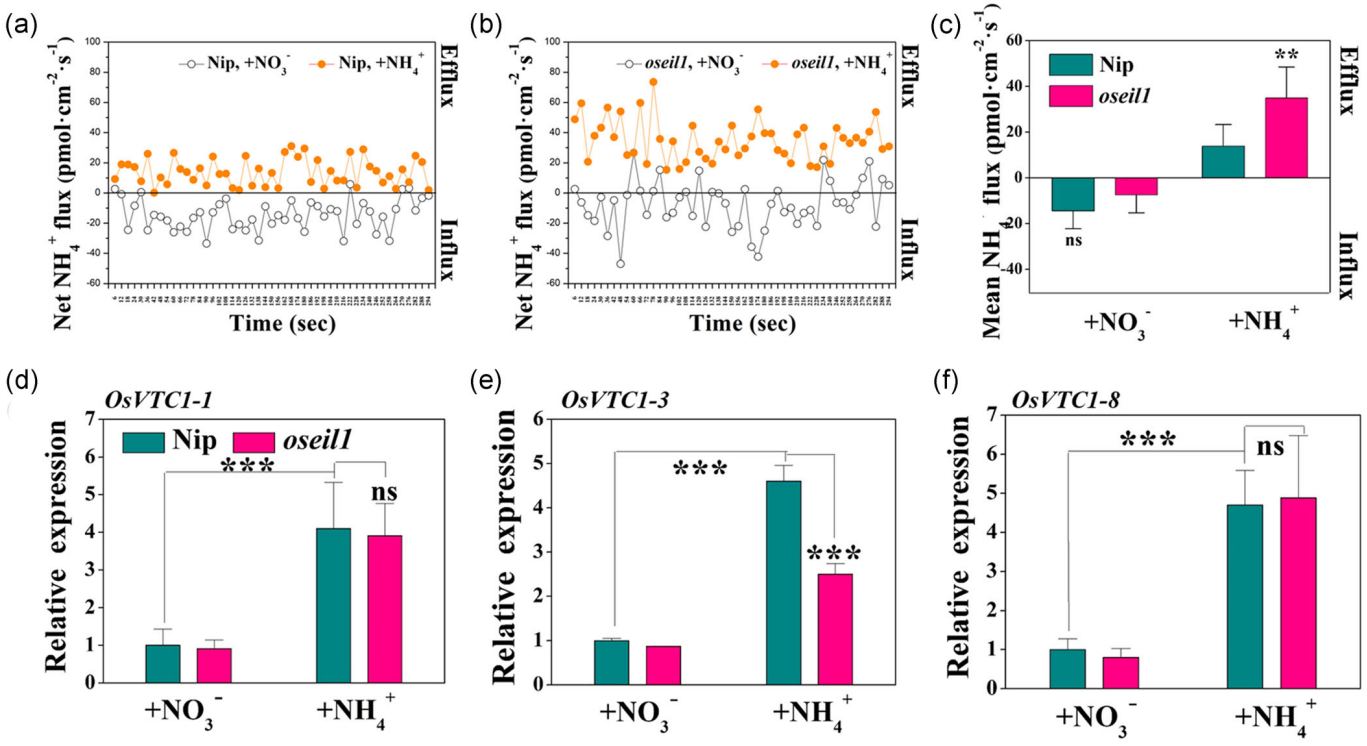

FIGURE 2 The effect of NH_4^+ treatment on net NH_4^+ fluxes and expression of *OsVTC1* sin the rice. (a, b) Net NH_4^+ fluxes of Nip and *oseil1* at the root elongation zone, mean values of fluxes in (a), and (b) are shown in (c). Values are the means ± SD, and more than eight independent seedlings were analyzed in flux experiments. The seedlings were grown on a solution with 6 mM NO_3^- and 6 mM NH_4^+ for 5 d of growth, and then NH_4^+ fluxes were determined. (d) to (f) qRT-PCR analysis of expression of the *OsVTC1-1*, *OsVTC1-3* and *OsVTC1-8* genes in Nip and the *oseil1* mutant root tissues in response to NO_3^- and NH_4^+ for 24 h. Data are shown as the means ± SD of three biological replicates. Significant differences were determined by an independent samples *t* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and "ns" designates no significance) [Color figure can be viewed at wileyonlinelibrary.com]

determined if OsEIL1 regulates the expression levels of OsVTC1 genes. In wild-type seedlings, *OsVTC1-1*, *OsVTC1-3* and *OsVTC1-8* were all induced to a higher level by NH_4^+ treatment, suggesting that OsVTC1s are involved in the response to NH_4^+ (Figure 2d–f). Further experiments showed that the expression of *OsVTC1-3* in roots was much lower in the *oseil1* mutant compared with the wild type in response to NH_4^+ (Figure 2f). However, *OsVTC1-1* and *OsVTC1-8* expression in *oseil1* did not show a significant difference compared with wild type on NH_4^+ (Figure 2d,e). These results indicate that OsEIL1 is involved in NH_4^+-stimulated root expression of *OsVTC1-3*. Thus, OsVTC1-3 appears to be targeting the OsEIL1 gene in the context of regulation of root NH_4^+ fluxes. Several studies have demonstrated that root-medium acidification associated with NH_4^+ uptake, in and of itself, can cause many of the symptoms typically associated with NH_4^+ toxicity (Britto & Kronzucker, 2002; Hachiya et al., 2021; Lager et al., 2010; Meier et al., 2020; Patterson et al., 2010). We, therefore, also examined whether low pH affects the expression of *OsEIL1* and *OsVTC1-3* in Nip and *oseil1* under NO_3^- conditions. However, qPCR analysis showed that low pH treatment had no effect on *OsEIL1* and *OsVTC1-3* expression in both Nip and *oseil1* (Figure S10). Although the transcription of *OsVTC1-3* is much lower in *oseil1* than that of wild type under NH_4^+ (Figure 2f), NH_4^+ nevertheless induced the transcription of *OsVTC1-3*, indicating that other genes are also involved in NH_4^+-dependent *OsVTC1-3* transcription. To further investigate whether ethylene was involved in the regulation of *OsVTC1-3* expression, we examined *OsVTC1-3* transcription in Nip, *oseil1*, and *osein2* with or without ACC. The results show that ACC can induce *OsVTC1-3* expression on NO_3^-, and OsEIN2 and OsEIL1 mutations reduce *OsVTC1-3* expression on NO_3^- plus ACC and NH_4^+ compared with the wild type (Figure S11a).

To identify the precise role of OsVTC1-3 in the regulation of transmembrane NH_4^+ fluxes in response to NH_4^+, we examined seedling growth and root NH_4^+ efflux under NO_3^- and NH_4^+ in the *osvtc1-3* mutant. Consistent with the *oseil1* response, *osvtc1-3* plants exhibited relatively higher inhibition of root and shoot growth than was found in the wild type (Dongjin, DJ) when exposed to NH_4^+ (Figure 3a,b). While on NO_3^-, the average NH_4^+ flux was similar in *osvtc1-3* and wild type (DJ) roots (Figure 3c). However, under NH_4^+ treatment, NH_4^+ efflux in *osvtc1-3* roots was significantly higher than in wild-type roots (Figure 3c). These observations show that the loss of OsVTC1-3 function renders the mutant more sensitive to NH_4^+ than the wild type and that this is linked to elevated NH_4^+ efflux in roots, with similar functions to *AtVTC1* in *Arabidopsis*.

N-glycosylation is one of most common posttranscriptional protein modifications in eukaryotes and affects many processes, from enzyme activities to the folding, stability and intermolecular

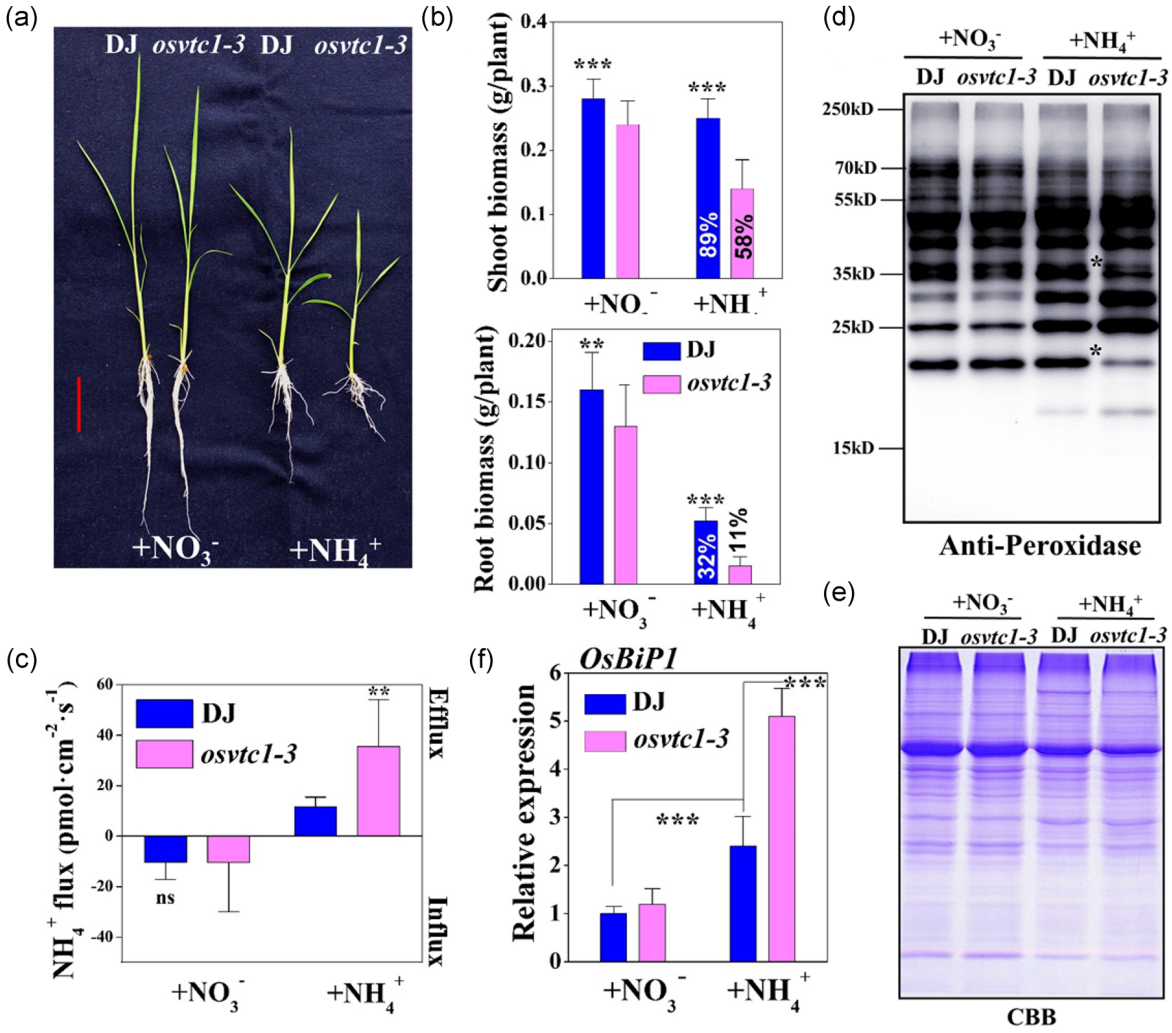

FIGURE 3 Sensitivity of the OsVTC1-3 knockout mutant to NH_4^+ treatment. (a, b) Three-day-old rice seedlings were exposed to 6 mM NH_4Cl ($+NH_4^+$) or 6 mM $NaNO_3$ ($+NO_3^-$) for 10 days, and shoot and root biomass (fresh weight) were determined. Data are the means ± SD ($n \geq 20$ for shoot and root biomass). Bar = 5 cm. (c) Mean values of NH_4^+ fluxes of DJ and *osvtc1-3* at the root elongation zone. Values are the means ± SD, and more than eight independent seedlings were analyzed in flux experiments. The seedlings were grown on a solution with 6 mM NO_3^- and 6 mM NH_4^+ for 5 d, and then NH_4^+ fluxes were determined. (d, e) Protein N-glycosylation levels in DJ and *osvtc1-3* seedlings were evaluated by a ConA-peroxidase reagent, the reaction with which is a characteristic feature of N-glycans. Coomassie Brilliant Blue (CBB) staining of protein gels was used to control for protein loading. The asterisk indicates different specific N-glycoprotein bands. Three-day-old rice seedlings were exposed to 6 mM NH_4Cl ($+NH_4^+$) or 6 mM $NaNO_3$ ($+NO_3^-$) for 10 days, and protein N-glycosylation levels in shoots were determined. (f) qRT-PCR analysis of the expression of *OsBiP1* in DJ and *osvtc1-3* seedlings' root tissues after treatment with 6 mM NO_3^- and 6 mM NH_4^+ for 10 d. Data are shown as the means ± SD of three biological replicates. Significant differences were determined by an independent samples *t* test (**$p < 0.01$, ***$p < 0.001$, and "ns" designates no significance). qRT-PCR, real-time quantitative polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]

interaction of proteins (Zeng et al., 2018). AtVTC1 has been reported to be involved in regulating *Arabidopsis* NH_4^+ efflux by regulating protein N-glycosylation (Di et al., 2021; Q. Li et al., 2010; C. Qin et al., 2008). To identify whether OsVTC1-3 is involved in regulating protein N-glycosylation in rice under NH_4^+, the N-glycosylation levels in seedlings were checked by employing a specific N-glycosylation peroxidase antibody, which directly binds to the oligomannose chains of N-glycoproteins (Di et al., 2021; Strasser et al., 2004). Compared with wild type (DJ), the *osvtc1-3* mutant contains less N-glycoprotein under NH_4^+ treatment, supporting the positive role of OsVTC1-3 in stabilizing protein N-glycosylation in rice, which is similar to the known role of AtVTC1 in *Arabidopsis* (Figure 3d,e). The *oseil1* and *osein2* mutants displayed less *OsVTC1-3* expression and also contained less N-glycoprotein on NH_4^+ than the wild type (Figure S11b). We further tested the expression of immunoglobulin-binding protein (BiP), an isoform of the heat shock protein 70 (Hsp70) chaperone that is strongly induced by N-glycosylation defects in the endoplasmic reticulum (ER) (C. Qin et al., 2008). The rice genome contains at least five OsBiP genes, and OsBiP1 plays the major role (Qian et al., 2021; Wakasa et al., 2011). When exposed to NH_4^+, *OsBiP1* expression was

increased 5.1- and 2.4-fold in *osvtc1-3* and wild type (DJ), compared with their controls, respectively (Figure 3f). Together with the earlier reports of NH_4^+ efflux increases in the *Arabidopsis vtc1-1* mutant (Di et al., 2021; Q. Li et al., 2010), the present data further confirm the positive regulatory role of protein N-glycosylation on NH_4^+ efflux in a major crop.

3.4 | OsVTC1-3 is the direct downstream target of OsEIL1 in the regulation of root NH_4^+ efflux in response to NH_4^+

We then investigated whether OsEIL1 directly regulates the expression of OsVTC1-3. Analysis of the *OsVTC1-3* promoter indicated that it contains two putative EIN3-binding sites (EBSs: ATGTA; P3 and P4; Figure 4a), implying a possible direct interaction between OsEIL1 and OsVTC1-3. The Y1H assay revealed that the EIN3 protein can bind to the full-length promoter and to fragments of OsVTC1-3 (FL, P1, P3 and P4) but not to fragments without or with the mutational binding site (P2, mP3 and mP4; Figure 4b). This was further investigated in-vitro by the EMSA, using the purified N terminus of OsEIL1 (OsEIL1-N). As shown in Figure 4c, two fragments of the P3 and P4 regions of the OsVTC1-3 promoter, but not the mutational EBS motifs (mP3, mP4), were bound by OsEIL1-N. Cold-probe (competitor) competed for OsEIL1-N binding (Figure 4c). These results indicate that OsEIL1 directly binds to the OsVTC1-3 promoter in vitro. The Dual-LUC assays also showed that the OsEIL1 protein firmly binds to the binding sites (P1, P3 and P4) of OsVTC1-3 (Figure 4d). The results show that OsEIL1 activates the expression of *OsVTC1-3*. As shown in Figure 4e, the *OsVTC1-1* and *OsVTC1-8* promoter was not found to be bound by OsEIL1 in the Y1H assay. We also performed dual-luciferase transient transcriptional activity assays in tobacco leaf cells. Expression of OsEIL1 and the REN internal control were driven by the CaMV 35 S promoter, and the OsVTC1-1 and OsVTC1-8 promoter drove that of the LUC reporter gene. The cotransfected effectors did not significantly increase the OsVTC1-1 and OsVTC1-8 promoter-driven LUC activities (Figure 4f). All these findings suggest that OsEIL1 does not directly regulate *OsVTC1-1* and *OsVTC1-8* expression.

To substantiate the premise that root NH_4^+ efflux is modulated via an OsEIL1-OsVTC1 regulatory cascade, we next examined the genetic interaction between OsEIL1 and OsVTC1-3. To determine whether overexpression (OX) of *OsVTC1-3* in the *oseil1* mutant could rescue the NH_4^+ efflux modifications during NH_4^+ treatment, we generated an *OsVTC1-3-OX/oseil1* transgenic line (Figure 5a). Intriguingly, compared with the *oseil1* mutant, *OsVTC1-3* OX in *oseil1* reduced NH_4^+ efflux in the root (Figure 5b), demonstrating that OsVTC1-3 acts genetically downstream of OsEIL1 in the NH_4^+ response. Furthermore, compared with wild type (Nip), the *oseil1* mutant contained less N-glycoprotein under NH_4^+, whereas the *OsVTC1-3-OX/oseil1* transgenic line contained more N-glycoprotein than the *oseil1* mutant (Figure 5c,d). Supportive of this, the *OsVTC1-3-OX/oseil1* transgenic line showed significantly increased growth compared to the *oseil1* mutant under the NH_4^+ condition (Figure 5e,f). The *OsVTC1-3-OX/oseil1* transgenic line also showed a lower *OsBiP1* expression under NH_4^+ than that of *oseil1* mutant (Figure S12). We also measured messenger RNA levels of the OsVTC1-3 gene in the *OsEIL1*-OX constructs in rice (*OsEIL1-OX-(3-5)* and *OsEIL-OX-(4-4)*), with OsEIL1 driven by the cauliflower mosaic virus 35 S promoter, as reported in C. Yang, Ma, et al. (2015), and found that the transcript levels of the OsEIL1 gene were enhanced in the high-expression lines (*OsEIL1-OX-(3-5)*) with NO_3^- treatment (Figure S13a). NH_4^+ treatment-induced OsVTC1-3 expression in the wild type (Nip), while there was no significant difference in expression of *OsVTC1-3* among the wild type, the OsEIL1 low-expression line (*4-4*), and the high-expression line (*3-5*) under the NH_4^+ treatment (Figure S13d). These results suggest that the expression of OsVTC1-3 is responsible for the similarity in the NH_4^+-response phenotype in *OsEIL1-OX* plants and the wild type (Figure S13b,c).

3.5 | AtEIN3 is not involved in regulating NH_4^+ efflux and AtVTC1 expression in *Arabidopsis*

Combining our previous (G. J. Li et al., 2019) and present results suggests that OsEIL1 and AtEIN3 play contrasting roles in the response to NH_4^+ in rice and *Arabidopsis*, in accordance with the differing NH_4^+-toxicity phenotypes (Figure 6a,b). These characteristics prompted us to ask whether *Arabidopsis* AtEIN3 may have a function related to regulating NH_4^+ efflux through direct interaction with AtVTC1. Thus, we first tested whether *AtEIN3* gene expression in *Arabidopsis* was affected by NH_4^+ treatment. However, the expression level of *AtEIN3* was not significantly affected by NH_4^+ (Figure S14a,b). A transgenic reporter line harboring the *GUS* gene driven by five tandem repeats of the AtEIN3-binding site (EBS) followed by a minimal 35S promoter (*5×EBS:GUS/Col-0*) was used to monitor the transcriptional activity of AtEIN3 (He et al., 2011; Stepanova et al., 2007). Interestingly, following NH_4^+ treatment, GUS staining became intensified in seedlings (Figure S14c), indicative of elevated levels of AtEIN3 activity. All these results suggest that the regulation patterns of NH_4^+ on the rice OsEIL1 and *Arabidopsis* EIN3 are different.

The AtEIN3 mutant accumulates less NH_4^+ in tissue under NH_4^+ treatment (Figure S15), consistent with our previous report (G. J. Li et al., 2019). To determine whether AtEIN3 influenced NH_4^+ fluxes in *Arabidopsis* roots, we further monitored the net NH_4^+ flux at the elongation zone as affected by NO_3^- and NH_4^+ in the *Arabidopsis ein3-1* mutant. However, net NH_4^+ effluxes in the root elongation zone did not show significant differences between the wild type (*Col-0*) and *ein3-1* mutant under either NO_3^- or NH_4^+ (Figure 6d). This result indicates that NH_4^+ flux is not affected by AtEIN3. Similar to rice OsVTC1-3, the *Arabidopsis* AtVTC1 mutant also shows higher NH_4^+ efflux (Di et al., 2021; Q. Li et al., 2010; Figure 4d). However, NH_4^+ did not affect *Arabidopsis AtVTC1* expression (Figure 6e), consistent with previous reports (C. Qin et al.,

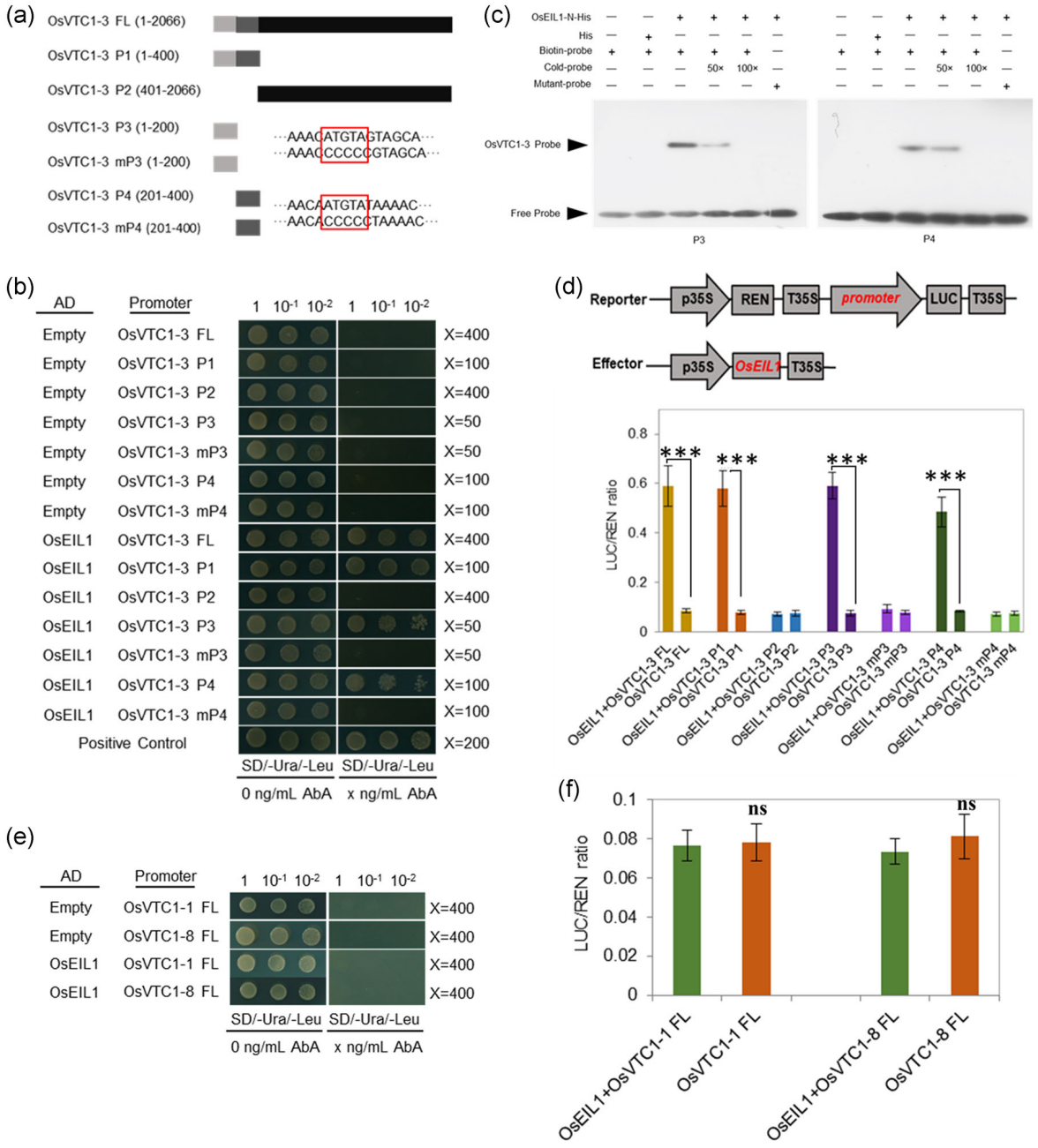

FIGURE 4 *OsVTC1-3* is a direct target gene of OsEIL1. (a) Schematic diagram of the promoter of *OsVTC1-3* and EBSs (ATGTA) fragments (P1, P3 and P4); its mutant versions (mP3, mP4) were used. The sequences containing mutated nucleotides are also shown, and the numbers of brackets indicate the distance away from the start codon. FL, full-length promoter; P1, fragments of EBSs containing P3 and P4; P2, fragments without EBSs; P3, the first EBS binding site; P4, the other EBS binding site. (b) Y1H assay showing OsEIL1 binding to the promoter of the *OsVTC1-3* gene. The yeast expression plasmid pGADT7-OsEIL1 was reintroduced into the yeast strain Y1H Gold carrying the pAbAi-OsVTC1-3 promoter, fragments or their mutant-version vectors. The transformants (with or without dilutions) were screened for growth on yeast synthetic defined medium (SD/-Ura-Leu) in the presence of *X* ng ml^{-1} AbA (antibiotic, the concentration of *X* is indicated on the right) for stringent selection. p53-AbAi/pGAD-p53 was used as a positive control. (c) EMSA showing that OsEIL1 binds to the promoter fragments (P3 and P4) of *OsVTC1-3*. The OsEIL1-N-His recombinant proteins were incubated with biotin-labeled probes in the presence of different amounts of cold DNA competitors. His protein was included as a negative control. (d) Ratios of LUC to REN. Full-length promoter and fragments (P1, P2, P3 and P4) and their mutant versions (mP3 and mP4) were inserted into the reporter vector pGreenII0800-LUC, which carries both LUC and REN ORFs, and OsEIL1 was cloned into the effector vector (pBI121); LUC, Firefly luciferase activity; REN, *Renilla* luciferase activity (control). Data are shown as the means ± SD of five biological replicates. Significant differences were determined by an independent samples *t* test. (e) Y1H assay showing the OsEIL1 does not bind to the promoter of the *OsVTC1-1* and *OsVTC1-8* gene. The yeast expression plasmid pGADT7-OsEIL1 was reintroduced into the yeast strain Y1H Gold carrying the pAbAi-*OsVTC1-1* and *OsVTC1-8* promoter vectors. (f) Ratios of LUC to REN. Full-length promoters of *OsVTC1-1* and *OsVTC1-8* were inserted into the reporter vector pGreenII0800-LUC, which carries both LUC and REN ORFs, and OsEIL1 was cloned into the effector vector (pBI121); LUC, Firefly luciferase activity; REN, *Renilla* luciferase activity (control). Data are shown as the means ± SD of five biological replicates. Significant differences were determined by an independent samples *t* test (***$p < 0.001$, and "ns" designates no significance). EBS, EIN3-binding site; EMSA, electrophoretic mobility shift; Y1H, yeast one-hybrid [Color figure can be viewed at wileyonlinelibrary.com]

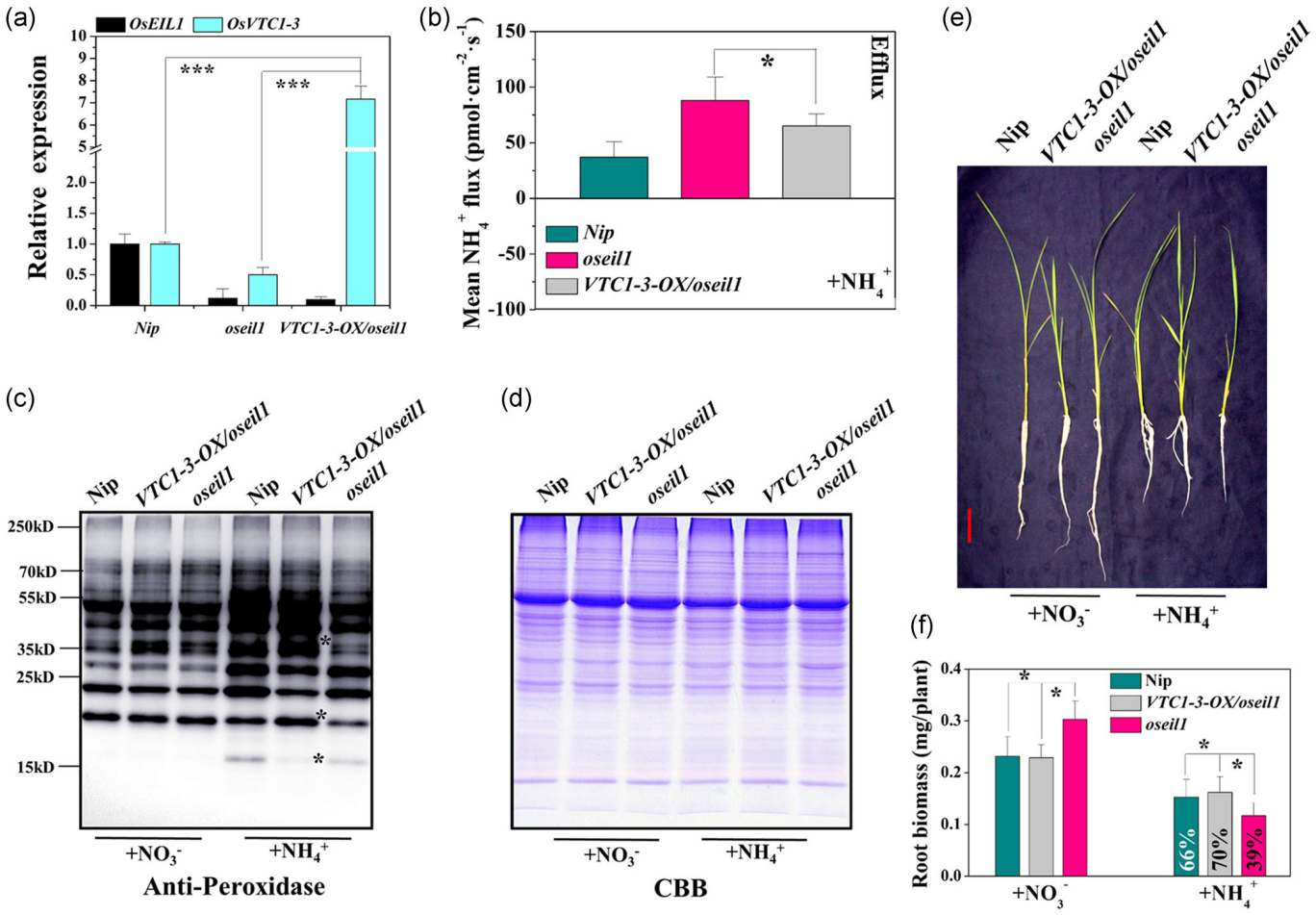

FIGURE 5 *OsVTC1-3* acts downstream of OsEIL1 in the regulation of NH_4^+ efflux in response to NH_4^+. (a) qRT-PCR analysis of OsEIL1 and OsVTC1-3 transcript levels in the indicated genotypes' root tissues. Data are shown as the means ± SD of three biological replicates. (b) Mean values of NH_4^+ fluxes of Nip, *oseil1* and *OsVTC1-3-OX/oseil1* at the root elongation zone are shown. Values are the means ± SD, and more than six independent seedlings were analyzed in flux experiments. The seedlings were grown on a solution with 6 mM NH_4^+ for 5 d, and then NH_4^+ fluxes were measured. Significant differences were determined by an independent samples *t* test (*$p < 0.05$, ***$p < 0.001$). (c,d) The protein N-glycosylation levels in Nip, *oseil1* and *OsVTC1-3-OX/oseil1* seedlings were evaluated by a ConA-peroxidase reagent, the reaction with which is a characteristic feature of N-glycans. Coomassie Brilliant Blue (CBB) staining of protein gels was used to control for protein loading. The asterisk indicates different specific N-glycoprotein bands. Three-day-old rice seedlings were exposed to 6 mM NH_4Cl ($+NH_4^+$) or 6 mM $NaNO_3$ ($+NO_3^-$) for 10 days, and the protein N-glycosylation levels in shoots were determined. (e, f) Three-day-old rice seedlings were exposed to 6 mM NH_4Cl ($+NH_4^+$) or 6 mM $NaNO_3$ ($+NO_3^-$) for 15 days, and root biomass (fresh weight) was determined. Data are the means ± SD ($n = 14$ for root fresh biomass). Bar = 5 cm. qRT-PCR, real-time quantitative polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]

2008; L. Zhang et al., 2021), and there was also no difference between the wild type (*Col-0*) and the *ein3-1* mutant. GDP-D-mannose pyrophosphohydrolase (NUDX9) has been reported to be involved in regulating NH_4^+ efflux in *Arabidopsis* (Di et al., 2021; Tanaka et al., 2015). NH_4^+ treatment certainly induced *NUDX9* expression (Di et al., 2021; Figure S16), but there was also no difference between *Col-0* and the *ein3-1* mutant (Figure S16). To further test whether AtEIN3 directly regulates the expression of *AtVTC1*, the reporter vector pAtVTC1:LUC and the effector vector 35S-AtEIN3 were transiently expressed in tobacco leaves. The cotransfected effectors did not significantly increase the *AtVTC1* promoter-driven LUC activities (Figure 6f). All these findings further demonstrate that AtEIN3 does not directly regulate *AtVTC1* expression.

4 | DISCUSSION

4.1 | The transcription factor OsEIL1 regulates OsVTC1-3-dependent protein N-glycosylation and NH_4^+ efflux in rice

In the high range of NH_4^+ supply (>1 mM), NH_4^+ acquisition is largely mediated by high-capacity, low-affinity transport systems (LATS) (Britto & Kronzucker, 2006), systems that are either specific to NH_4^+, transport NH_3, or are shared with other cations such as K^+ (Glass et al., 2001; Coskun et al., 2013a, 2013b). Low-affinity NH_4^+ influx is accompanied by a significant magnitude of NH_4^+ efflux, often constituting a futile cycle of NH_4^+ ion and NH_3 flux in roots (Britto & Kronzucker, 2002, 2006; Britto et al., 2001; G. Chen et al., 2013;

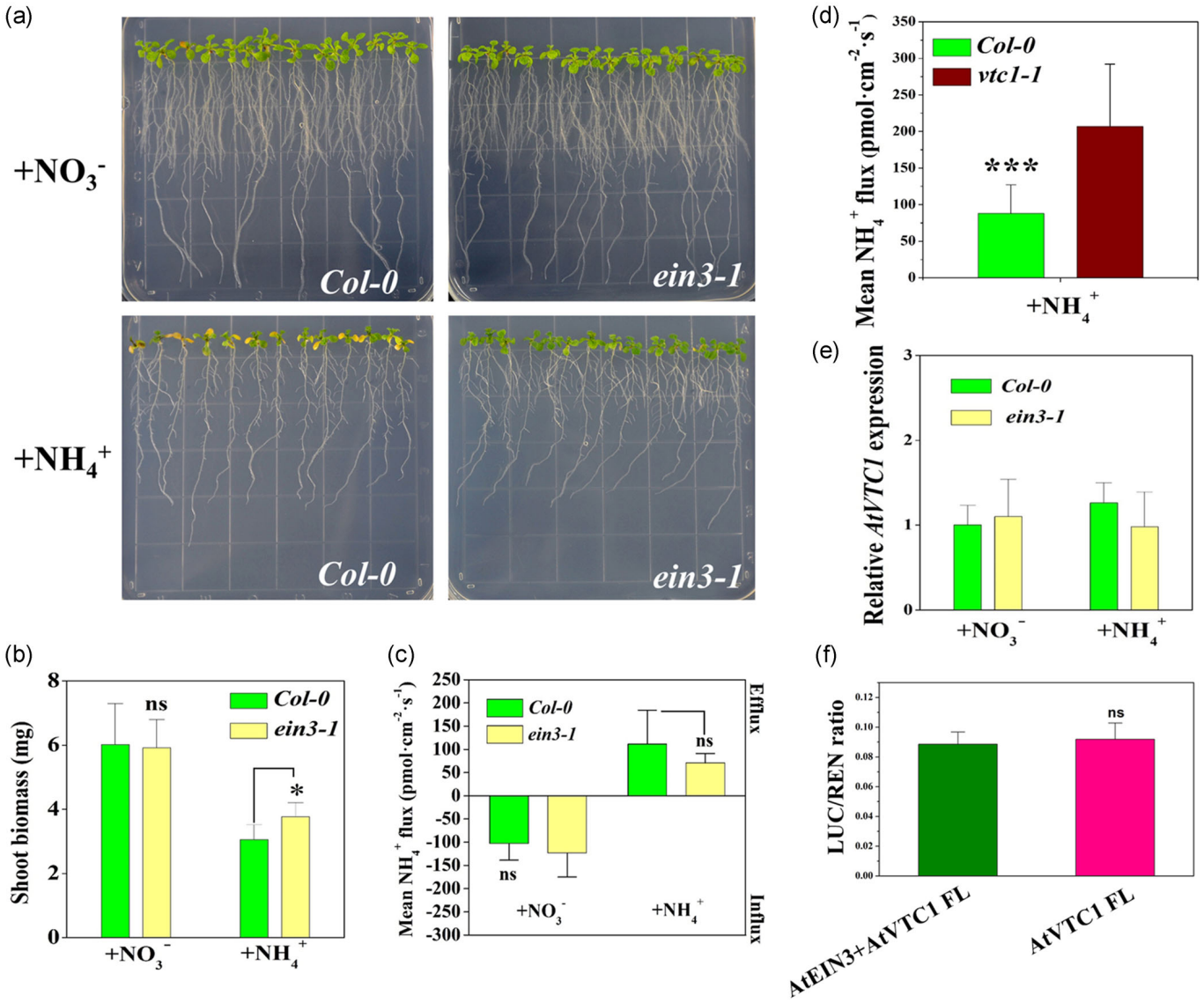

FIGURE 6 AtEIN3 is not involved in regulating NH_4^+ efflux and *AtVTC1* expression in *Arabidopsis*. (a, b) Phenotypes of the *Arabidopsis* AtEIN3 loss-of-function mutant in response to NH_4^+ treatment. Five-day-old plants were transferred to $+NO_3^-$ or $+NH_4^+$ treatment for 6 d, and then pictures were taken (a). Fresh shoot weight (b) of *A. thaliana Col-0* and *ein3-1* mutant plants following treatment $+NO_3^-$ or $+NH_4^+$ for 6 d. Data are the means ± SD of shoot biomass ($n \geq 12$). (c) Net NH_4^+ fluxes of *Col-0* and *ein3-1* at the root elongation zone. Five-day-old plants were transferred to NO_3^- or NH_4^+ treatment for 5 d, and then root NH_4^+ fluxes were determined. Values are the means ± SD, and at least three independent seedlings were analyzed in flux experiments. (d) Mean values of NH_4^+ fluxes of *Col-0* and *vtc1-1* at the root elongation zone. Values are the means ± SD, and eight independent seedlings were analyzed in flux experiments. Five-day-old plants were transferred to NH_4^+ treatment for 5 d, and then root NH_4^+ fluxes were determined. (e) qRT-PCR analysis of expression of the AtVTC1 gene in *Col-0* and the *ein3-1* mutant root tissues in response to NO_3^- and NH_4^+ for 24 h. Data are shown as the means ± SD of three biological replicates. (f) Transient expression assay testing the regulation of AtEIN3 on *AtVTC1* transcription in *Nicotiana benthamiana* leaves. The error bars indicate SD of five independent transient transfections in *N. benthamiana* leaves. FL, full-length promoter. Significant differences were determined by an independent samples *t* test (*$p < 0.05$, ***$p < 0.001$, and "ns" designates no significance). qRT-PCR, real-time quantitative polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]

Coskun et al., 2013a). NH_4^+ efflux from roots results in a reduction of net N uptake, and the process is also commonly associated with elevated respiration rates and therefore has a significant impact on plant performance (Britto et al., 2001; G. Chen et al., 2013; Coskun et al., 2013a; Esteban et al., 2016). Excessive NH_4^+ efflux from roots has been shown to be strongly associated with NH_4^+ toxicity in numerous plants (Britto et al., 2001; B. Li et al., 2014). NUE in rice is also frequently compromised by futile NH_4^+ cycling in roots, and larger NH_4^+ efflux in the elongation zone of the root has been observed in low-NUE cultivars under NH_4^+ treatment (G. Chen et al., 2013; M. Chen et al., 2020). However, rice displays superior adaptation and tolerance to NH_4^+ compared to other cereals because of its lower NH_4^+ efflux (Britto et al., 2001; G. Chen et al., 2013), while it remains unclear which molecular pathways are involved in modulating root

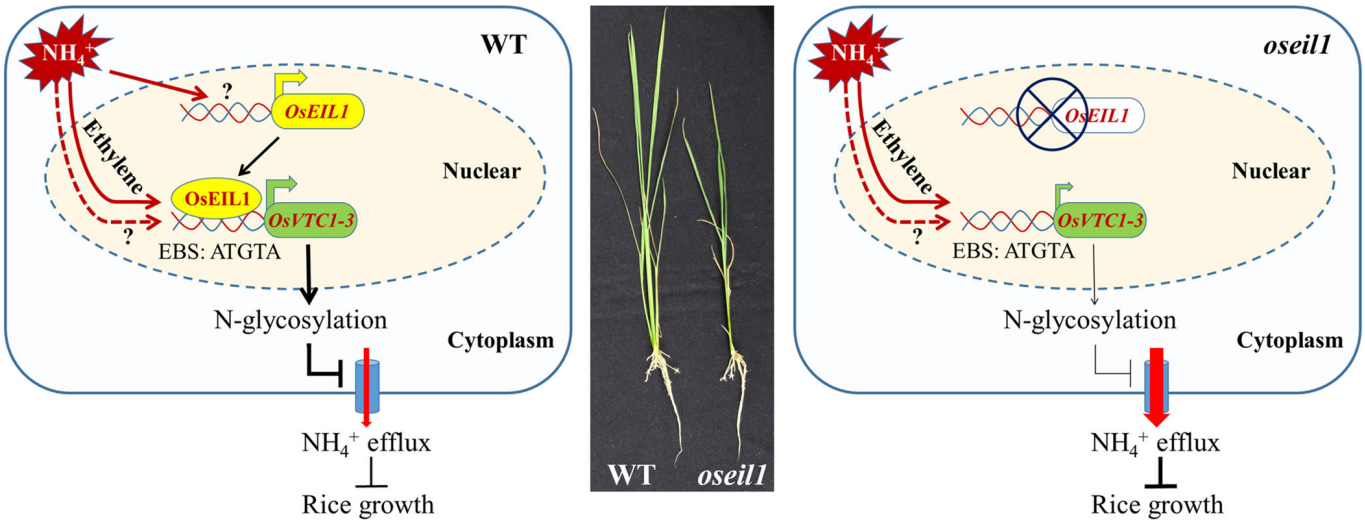

FIGURE 7 A proposed model for the role of OsEIL1 in regulating NH_4^+ efflux in rice under NH_4^+ treatment. Based on our study and previous reports (Britto et al., 2001; Q. Li et al., 2010; H. Qin, Deng, et al., 2016; C. Yang, Ma, et al., 2015), we propose a model to illustrate how OsEIL1 acts in NH_4^+ efflux in rice. In the wild type, the OsEIL1 transcription is induced by NH_4^+, OsEIL1 then binds directly to the promoter EBSs (ATGTA) sites of *OsVTC1-3* and acts to increase the transcription of *OsVTC1-3*. OsVTC1-3 maintains the stability of protein N-glycosylation to constrain NH_4^+ efflux in rice. OsEIL1-mediated regulation of *OsVTC1-3* contributes to the restriction of NH_4^+ efflux in rice seedling roots and to the protection of rice growth under NH_4^+ conditions. However, in the *oseil1* mutant, expression of the OsEIL1-dependent *OsVTC1-3* gene is blocked under the NH_4^+ treatment, although ethylene and/or other signaling pathways continue to stimulate partial *OsVTC1-3* gene expression. Lower *OsVTC1-3* gene expression results in lower N-glycosylation levels and higher NH_4^+ efflux in the *oseil1* mutant [Color figure can be viewed at wileyonlinelibrary.com]

NH_4^+ efflux in rice. In plants, protein N-glycosylation alteration is associated with hypersensitivity to abiotic stresses including NH_4^+ treatment. Recently, two genes, GDP-mannose pyrophosphorylase (VTC1) and GDPD-mannose pyrophosphohydrolase (NUDX9), were linked to the control of excessive NH_4^+ efflux and protein N-glycosylation in *Arabidopsis* under NH_4^+ treatment (Di et al., 2021), and AtVTC1 was shown to play the dominant role (Di et al., 2021). In the *Arabidopsis* NH_4^+-hypersensitive mutant *vtc1*, root NH_4^+ efflux was greatly stimulated, and this was associated with a lower protein N-glycosylation level (Di et al., 2021; Q. Li et al., 2010; C. Qin et al., 2008). The upstream regulator of AtNUDX9 in the response to NH_4^+ has been identified in *Arabidopsis* (Di et al., 2021), but the molecular mechanisms by which plants perceive NH_4^+ stress and transmit stress signals to regulate VTC1-dependent NH_4^+ efflux also remain unclear. Moreover, impaired N-glycosylation results in the activation of the unfolded-protein-response (UPR) signaling pathway, followed by increased protein folding activity and cell death, but the key regulatory elements controlling N-glycosylation under NH_4^+ treatment in rice remain unidentified.

Our study provides evidence that OsVTC1-3 positively regulates protein N-glycosylation to constrain NH_4^+ efflux in rice roots under NH_4^+ treatment (Figure 3), similar to role of AtVTC1 in *Arabidopsis* (Q. Li et al., 2010; C. Qin et al., 2008; Figure 6d). Moreover, the expression of *OsVTC1-3* in response to NH_4^+ requires the cooperation of OsEIL1, and OsEIL1 binding to EBSs (ATGTA) can be shown to be essential for the promoter activity of *OsVTC1-3*. We also demonstrate that OsEIL1 acts as a transcription factor to negatively regulate NH_4^+ efflux in roots of rice, different from the role of AtEIN3 in the NH_4^+ response in *Arabidopsis*. First, the *oseil1* mutant displayed an enhanced NH_4^+-sensitivity phenotype (Figures 1a–c, S3). Second, the *oseil1* mutant possessed higher NH_4^+ efflux (Figure 2b) and lower N-glycosylation levels (Figures 5c,d, S11b). Third, in vivo and in vitro analyses show that OsEIL1 directly activates the expression of *OsVTC1-3* in rice (Figures 2 and 4). Furthermore, genetic analysis demonstrated that OX of *OsVTC1-3* in an *oseil1* background partially rescued the NH_4^+-efflux and N-glycosylation response and NH_4^+-dependent growth in the *oseil1* mutant (Figure 5). These results not only present an important link between excessive NH_4^+ efflux and protein N-glycosylation in rice in the response to NH_4^+ but also identify the critical regulatory element OsEIL1 that controls OsVTC1-3-dependent N-glycosylation under NH_4^+ nutrition in rice. OsEIL1 is one of the key transcriptional regulators of the ethylene signaling pathway in rice. Ethylene (ACC) does not appear to regulate *OsEIL1* expression but led to an induction of *OsVTC1-3* in the absence of NH_4^+ (Figures S4, S11a), while the OsEIN2 mutation also affected OsVTC1-3 expression under both ACC and NH_4^+ (Figure S11a). Consistent with this, the *osein2* mutant contained less N-glycoprotein under NH_4^+ than the wild type (Figure S11b). In *Arabidopsis*, the C-terminal fragment of EIN2 is cleaved in the presence of ethylene and translocated to the nucleus for activation of the EIN3/EIL1 transcriptional cascade (C. Yang, Lu, et al., 2015). Whether and how ethylene also regulates OsEIL1 protein activity to affect *OsVTC1-3* expression under NH_4^+ is worthy of further study. Interestingly, NH_4^+ still induces *OsVTC1-3* expression in the *osein2* mutant (Figure S11b), implying that there are other signaling pathways involved in the regulation of rice *OsVTC1-3* expression under NH_4^+.

Based on our findings, we propose a model to illustrate how OsEIL1 acts in NH_4^+ efflux in rice (Figure 7). OsEIL1 transcription is induced by NH_4^+, OsEIL1 then binds directly to the promoter EBSs (ATGTA) of *OsVTC1-3* and acts to increase the transcription of *OsVTC1-3*. OsVTC1-3 maintains the protein N-glycosylation to constrain NH_4^+ efflux in rice, and OsEIL1-mediated regulation of OsVTC1-3 contributes to a stabilization of protein N-glycosylation and of NH_4^+ efflux in rice seedlings and maintains rice growth under NH_4^+ conditions. H. Qin, Deng, et al. (2016) showed that rice encodes three full-length *Arabidopsis* VTC1 homologs: OsVTC1-1, OsVTC1-3 and OsVTC1-8, but there was no difference in the expression levels of *OsVTC1-1* and *OsVTC1-8* between wild-type and *oesil1* mutant plants (Figure 2), suggesting that *OsVTC1-1* and *OsVTC1-8* might not be involved in the OsEIL1-mediated control of NH_4^+ efflux. We also observed an interesting phenomenon: OX of OsEIL1 did not enhance NH_4^+ tolerance, possibly due to the already high natural abundance of OsEIL1 under NH_4^+ conditions in rice. Alternatively, a limited number of direct downstream targets (i.e., OsVTC1-3; Figure S13d) may restrict the effects of OX of OsEIL1. Moreover, ACC could still induce *OsVTC1-3* expression in the *oseil1* mutant under the NO_3^- condition (Figure S11a), suggesting that ethylene regulates OsVTC1-3 expression via another OsEIL1-independent pathway. C. Yang, Ma, et al. (2015) revealed that OsEIL1 and OsEIL2 coregulated ethylene signaling in rice, and we here show that NH_4^+ also induces *OsEIL2* expression (Figure S2); thus, the role of OsEIL2 in regulating NH_4^+ efflux and NH_4^+ tolerance appears worthy of further investigation.

4.2 | Divergent roles for OsEIL1 and AtEIN3 in the NH_4^+ response of rice and *Arabidopsis*, respectively

In contrast with the observation that OsEIL1 mutation enhances NH_4^+ efflux in rice, the *Arabidopsis atein3-1* mutant showed no difference in NH_4^+ efflux compared with the wild type, *Col-0*. An alternative explanation lies with direct downstream targets. AtVTC1 acts as a key modulator in the regulation of NH_4^+ efflux in *Arabidopsis* (Di et al., 2021; Q. Li et al., 2010; C. Qin et al., 2008), and both NH_4^+ efflux and inhibition of N-glycosylation were more pronounced in the *atvtc1* mutant than in the wild type. In contrast to the observation that OsEIL1 is upregulated by NH_4^+ and binds directly to the promoter EBS sites of OsVTC1-3, AtEIN3 does not directly interact with the AtVTC1 promoter and also has no effect on the AtVTC1 expression (Figure 6e,f). In *Arabidopsis*, in addition to AtVTC1, the AtNUDX9 gene also regulates N-glycosylation, and the *atnudx9* mutant had lower NH_4^+ efflux (Di et al., 2021). AtWRKY46 is a direct negative regulator of AtNUDX9, stabilizing protein N-glycosylation and reducing NH_4^+ efflux (Di et al., 2021). However, AtEIN3 also did not regulate *AtNUDX9* expression under NH_4^+. AtEIN3, can directly bind to promoters of genes coding for PODs to upregulate their expression, which has been shown to correlate positively with NH_4^+-induced ROS accumulation and cell growth inhibition in *Arabidopsis* (G. J. Li et al., 2019; Podgórska et al., 2015). By contrast, in previous work and in our present study, the expression of genes encoding PODs, were not induced by NH_4^+ treatment in either the wild type or the *oseil1* mutant of rice (Hassan et al., 2008; Jalloh et al., 2009; Figure S6). Furthermore, ROS content also did not differ between the wild type and the *oseil1* mutant under the NH_4^+ treatment (Figure S7). In a recent study, dual and opposing roles of AtEIN3 revealed a 'generation conflict' during seed growth in *Arabidopsis*, and AtEIN3 was shown to possess both ethylene-dependent and -independent regulatory functions in seed growth (Heydlauff et al., 2021). The present and previous studies suggest that the functional complexity of the EIN3 gene product and the products of its homologous genes varies with developmental stage and among species. C. Yang, Ma, et al. (2015) also showed that OsEIL1 negatively impacts salt tolerance in rice seedlings, in contrast to the positive impact of AtEIN3 in *Arabidopsis*. Plants may engage a common pathway to produce different phenotypes with the use of various downstream components, and this could occur even within the same species. A good example lies in the fact that different rice varieties respond to flooding distinctly, involving various downstream targets (Groen et al., 2020; Hattori et al., 2009; Xu et al., 2006). The frequency of specific cis motifs, the extent of chromatin accessibility, and the degree of environmental activation are more prevalent in rice (Reynoso et al., 2019). Different natural growing conditions and breeding selection may have led to differences in EIN3 functions. Nitrate is the main N form utilized and assimilated by *Arabidopsis* and by most plants that thrive in well-aerated soils (Britto & Kronzucker, 2002, 2013), and absolute NH_4^+ concentrations in soil solution tend to be low (Liu & von Wirén, 2017; Miller et al., 2007), although they can vary over orders of magnitude (Glass et al., 2001). Thus, NH_4^+ toxicity in such plants is rare under natural circumstances in well-aerated soils, explaining the lack of refined adaptations. It has been suggested that AtEIN3 plays a pivotal role in the regulation of nitrate-use efficiency (De Gernier et al., 2016; J. Wang et al., 2015; Zheng et al., 2013). However, most rice cultivars have traditionally been cultivated under flooded, anaerobic soil conditions (Kirk & Kronzucker, 2000, 2005). Most varieties are well adapted to a semiaquatic habitat (Reynoso et al., 2019), where ammonium is the primary N source and often reaches levels in soil solution that can affect plant growth negatively (see above). Avoidance of excessive NH_4^+ uptake and excessive, energetically burdensome fluxes, is therefore vital to the healthy growth of rice and would present a constant 'selection pressure' in the field. Our results indicate the importance of maintaining regulation via the OsEIL1 gene to confer lower NH_4^+ fluxes and NH_4^+ accumulation in rice.

Taken together, our results show that, in stark contrast to the roles of AtEIN3 in *Arabidopsis*, OsEIL1 restricts NH_4^+ efflux in rice by direct regulation of OsVTC1-3-mediated N-glycosylation. The work identifies the key transcription factor that regulates OsVTC1-3-mediated NH_4^+ efflux and protein N-glycosylation in rice under ammonium conditions. Excessive NH_4^+ efflux from roots has been shown to be strongly associated with lower NUE in rice (G. Chen et al., 2013; M. Chen et al., 2020). Understanding the molecular mechanism of the interactions between the OsEIL1-mediated

pathway and NH_4^+ stress in rice will yield critical insights into the interaction of protein N-glycosylation and the regulation of root ion fluxes and offer a novel clue for improving ammonium utilization efficiency in rice. Furthermore, it will be interesting to analyze the extent to which the differential functional impacts of EIN3 homologs are conserved within various plant lineages in response to ammonium. Functional studies of other monocots and eudicots will help to clarify this issue.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Guangjie Li and Lin Zhang executed the experiments, interpreted data and generated figures. Jinlin Wu and Xiaowei Yue assisted in the experiment. Guangjie Li and Herbert J. Kronzucker were the primary writers of the manuscript. Weiming Shi was involved in the design of experiments, analysis, and interpretation of the data. Herbert J. Kronzucker, Meng Wang, Li Sun and Dongwei Di assisted in the discussion. All authors read and approved of the manuscript.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the article and its supporting information.

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SUPPORTING INFORMATION

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