OsEIL1 is involved in the response to heterogeneous high ammonium in rice: A split-root analysis

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ABSTRACT

Ammonium (NH_4^+) concentrations in rice fields show heterogeneous spatial distribution under the combined influences of nitrogen fertilizer application and modern agronomic practices. However, the characteristics and mechanisms of rice roots in response to heterogeneous NH_4^+ supply are not well understood. Here, we found a systemic response of rice roots to heterogeneous and high (10 mM) NH_4^+ supply using a split-root experiment, and show root growth on the NH_4^+-free (NO_3^-) side was also inhibited by localized high-NH_4^+ supply. Moreover, *OsEIL1* (encoding a core transcription factor in the ethylene signaling pathway) was found to be involved in the response of rice roots to heterogeneous NH_4^+. *OsEIL1* mutation significantly increased the inhibitory effect of localized high-NH_4^+ on root growth of the NO_3^- side, as well as significantly increased NH_4^+ efflux there. Furthermore, our results indicate that the mitigating effect of OsEIL1 on NH_4^+ efflux is related to the regulated expression of *OsVTC1-3* (encoding a GDP-mannose pyrophosphorylase). These findings provide insight into the mechanisms by which *OsEIL1* responds to heterogeneous high NH_4^+ and contribute to our understanding of rice adaptation to heterogeneous NH_4^+ supply.

1. Introduction

Ammonium (NH_4^+) and nitrate (NO_3^-) are the primary inorganic nitrogen (N) sources for crops. Ammonium-based fertilizers are routinely applied to soils, and in some circumstances, recommended over NO_3^- fertilizers to mitigate leaching and soil run-off and induce biological nitrification inhibition via root exudates (Coskun et al., 2017a; Li et al., 2022; Min et al., 2021b; Zhang et al., 2021). These agronomic measures and strategies, however, can result in the accumulation of high levels of NH_4^+ in various crop systems, leading to plant toxicity (Britto and Kronzucker, 2002; Ferretti et al., 2014; Kronzucker et al., 2003; Maestre et al., 2016; Min et al., 2021a; Sun et al., 2020). They can also overlook important innate differences in N-source preference across species (e.g. rice is well documented to have adapted to NH_4^+ as a primary N source, although, like many plants, can also effectively utilize NO_3^- and benefits from a mixed NH_4^+-NO_3^- supply; Kronzucker et al., 1999, 2000; Britto and Kronzucker, 2002, 2013; Kirk and Kronzucker, 2005). Nevertheless, to obtain maximal grain yields, modern protocols of N fertilizer application have increased the content of NH_4^+ in many paddy soils, and rice is indeed commonly exposed to potentially toxic NH_4^+ concentrations (Britto and Kronzucker, 2002, 2013; Balkos et al., 2010; Jia et al., 2020; Li et al., 2022; Ma et al., 2016; Zhang et al., 2021).

Spatial distribution of N in soils is heterogeneous due to a combination of fertilizer practices and natural processes, including microbial N transformations (e.g. nitrification and denitrification; Li et al., 2016; Coskun et al., 2017b; Lu et al., 2020). In a typical paddy field, $[NH_4^+]$ decreases with increased soil depth (Zheng et al., 2020; Zhu et al., 2019). For instance, after urea application to the topsoil, the maximum $[NH_4^+]$ ($\sim$2.3–13 mM) occurred at the soil surface and declined with soil depth (Liu et al., 2016). Over time, soil $[NH_4^+]$ can also decline rapidly due to ammonia volatilization or nitrification (Liu et al., 2016; Song et al., 2022). But modern agronomic practices could bring about more significant or various heterogeneous distribution of nutrients in the field. For example, the application of nitrification inhibitors can keep soil NH_4^+ concentrations higher over time (Lu et al., 2019). Controlled-release urea application, on the other hand, reduces soil $[NH_4^+]$ and NH_3 volatilization from the surface layer of paddy fields and maintains a higher $[NH_4^+]$ in the deeper layers of the soil (Zheng et al., 2020). In one study on "one-time root-zone" fertilizer application (fertilizer is applied once into 10 cm-deep holes positioned 5 cm from the rice seedling's roots), NH_4^+ continuously rose as the soil depth increased and the maximum value was observed at the fertilization zone, and the maximum average $[NH_4^+]$ for the "one-time root-zone" fertilizer application after 30, 60, and 90 days was 60, 26, and 3.5 mM, respectively (Liu et al., 2016). Indeed, compared to manual topsoil dressing, "side-deep" fertilization (when transplanting rice seedlings, fertilizer can be incorporated side-deep, at 5–6 cm of soil depth near rice roots) of urea and controlled-release urea maintained a higher NH_4^+ concentration ($\sim$4.3–6.2 mM; Min et al., 2021b; Zhu et al., 2019). Thus, heterogeneous high NH_4^+ is widely present in paddies, but the biological mechanisms underpinning the rice root response to such heterogeneous supply remain largely unknown.

Recent studies investigating the tolerance to NH_4^+ in plants have revealed the crucial role of root NH_4^+ efflux (Britto and Kronzucker, 2002; Britto et al., 2001; Li et al., 2022). In Arabidopsis, mutations in *VTC1* (encoding a GDP-mannose pyrophosphorylase) have been found to result in defective N-glycosylation of proteins, which is presumed to be a pivotal downstream molecular role that leads to growth inhibition under NH_4^+ toxicity (Barth et al., 2010; Di et al., 2021; Li et al., 2010; Qin et al., 2008; Xiao et al., 2023). In rice, the *osvtc1-3* mutation has been shown to enhance root NH_4^+ efflux. Furthermore, OsEIL1, a core transcription factor in the ethylene signaling pathway in rice, was shown to directly regulate the expression of *OsVTC1-3* (Li et al., 2019, 2022). OsEIL1 constrains NH_4^+ efflux by activating transcription of *OsVTC1-3* via direct binding to its promoter (Li et al., 2022). However, whether the above mechanisms are involved in response to heterogeneous high NH_4^+ remains unclear. To address this, we conducted a split-root experiment (with one half exposed to high NH_4^+, the other to NO_3^-) and observed that *OsEIL1* and *OsVTC1-3* were also induced in roots of the NO_3^- half. Moreover, *OsEIL1* plays a mitigating role against NH_4^+ toxicity in NO_3^--supplied roots via the regulation of *OsVTC1-3* and downstream NH_4^+ efflux. These findings provide insight into the specific mechanisms by which *OsEIL1* and *OsVTC1-3* respond to heterogeneous high NH_4^+.

2. Materials and method

2.1. Plant materials and growth conditions

Rice (*Oryza sativa* L.) mutants *oseil1-1* (*mhz6-3*) and *oseil1-2* (*mhz6-2*) were produced from a Nipponbare wild-type (WT) background (Yang et al., 2015). The mutant *osvtc1-3* is against a Dongjin wild-type background and was obtained from: http://signal.salk.edu/cgi-bin/RiceGE (Li et al., 2022).The seedlings were supplied with a half-strength modified Kimura's solution (0.5 mM $MgSO_4 \cdot 7H_2O$, 0.36 mM $CaCl_2 \cdot 2H_2O$, 0.25 mM KCl, 0.2 mM NaH_2PO_4, 0.1 mM Fe(II)-EDTA, 50 μM H_3BO_3, 9 μM $MnCl_2 \cdot 4H_2O$, 0.7 μM $ZnSO_4 \cdot 7H_2O$, 0.5 μM $Na_2MoO_4 \cdot 2H_2O$, and 0.3 μM $CuSO_4 \cdot 5H_2O$). 10 mM $NaNO_3$ was added in the nitrate treatment (NT) and 10 mM NH_4Cl in the ammonium

treatment (AM), according to Wang et al. (2020). The nutrient solution pH was adjusted to 5.5. Plants were grown in growth chambers supplied with 16/8 h (28/25 °C) day/night cycles and a light intensity of 400 μmol m^{-2} s^{-1}.

2.2. Split-root experimental setup

Rice seeds were surface sterilized with 10% H_2O_2 for 30 min, then washed thoroughly with distilled water and germinated in distilled water at 28 °C for 48 h in darkness. Seedlings were then transferred to the growth chamber and placed on nylon nets floating on 0.5 mM $CaCl_2$ as the day 0 and kept in darkness for 1 day followed by normal light. On the third day, the primary root was trimmed with sterile scissors leaving only 3 cm. On the sixth day, crown roots were split in half and supplied with nutrient solutions with varying N treatments. A plastic pot with separate compartments was used to culture rice seedlings for split-root experiments. The nutrient solution was exchanged every 2 days. Root length and NH_4^+ efflux was measured after rice seedlings were incubated in the treated nutrient solution for 10 days. Roots length was measured with a ruler after the roots were straightened on each side. Representative rice seedlings from the measurement population were selected for photographs (CANON EOS X7i). Root samples for qRT-PCR, on the other hand, were obtained at 36, 72, and 120 h of nutrient solution treatment and stored at −80 °C.

2.3. RNA isolation and qRT-PCR

Rice tissues were ground using a plant tissue ball grinder (Wonbio-800, Shanghai Onebio Biotech Co., Ltd) at cryogenic temperatures in liquid nitrogen. Total RNA was extracted using the FastPure Plant Total RNA Isolation Kit (RC411, Vazyme Biotech Co., Ltd). The HiScript 1 st-Strand cDNA Synthesis Kit (R111-01, Vazyme Biotech Co., Ltd) was used for cDNA synthesis. Synthesized cDNAs and ChamQ Universal SYBR qPCR Master Mix Kit (Q711-02, Vazyme Biotech Co., Ltd) were used in qRT-PCR to characterize the transcript levels of relevant genes. Light-Cycler 480 Real-Time PCR system (Roche), referring to the manufacturer's instructions, was used in qRT-PCR. The primers are listed in Table S1.

2.4. Transactivation of the target promoter in tobacco leaves

Transient expression was analyzed according to the method of Yang et al. (2000). A four-week-old *Nicotiana benthamiana* plant was used for infiltration. The *Promoter-OsEIL1-GUS* construct was transformed individually into *Agrobaterium tumefaciens* strain EHA105. *A. tumefaciens* cells were infiltrated into the abaxial surface of *N. benthamiana* leaves using a 2-mL syringe. *N. benthamiana* plants were grown under dark conditions in the greenhouse for 48 h following infiltration. Histochemical staining analysis of β-glucuronidase (GUS) reporter was performed as described by Weigel and Glazebrook (2002). The quantitative analysis of GUS activity in *N. benthamiana* leaves were determined according to the method of Jefferson et al. (1987).

2.5. Measurement of net NH_4^+ flux with the non-invasive micro-test technology (NMT) system

NMT (NMT system BIO-IM; Younger USA, LLC) was used to measure net NH_4^+ fluxes at the surface of the root tip elongation zone as previously described (Chen et al., 2013; Li et al., 2010, 2022). In brief, the roots of rice seedlings were incubated in the treated nutrient solution for 10 days, and then equilibrated in the buffer solution for 30 min, which was composed of 0.1 mM $CaCl_2$ and 0.2 mM NH_4Cl (pH 5.5, adjusted with 1 M NaOH). All measurements of net NH_4^+ fluxes were carried out at the Bapu Science and Technology Co., Ltd (Shanghai, China).

2.6. Statistical analysis

The IBM SPSS Statistics 26 (SPSS) and Microsoft EXCEL programs were used to statistically analyze the data. Details are shown in the figure legends. Graphs were produced using GraphPad Prism 8.

3. Results

3.1. Localized high NH_4^+ results in a systemic inhibition of rice root growth

We observed a notable negative impact of high-NH_4^+ supply on root length in the WT, whereas the same concentration of NO_3^- did not significantly affect root growth (Fig. S1). We further conducted a split-root experiment to investigate the response of rice roots to localized high-NH_4^+ supply. When NH_4^+ was supplied on both sides of the split root

(AM-AM), root length decreased by approximately 50% relative to homogeneous NO_3^- controls (NT-NT). Interestingly, root length decreased by 22% on the NO_3^- side (NT-AM, NT) of a NT-AM split, and by 44% on the NH_4^+ side (NT-AM, AM), compared to the NT-NT control (Fig. 1A–C). These results suggest a systemic inhibitory response of root growth to localized high-NH_4^+ supply.

3.2. The oseil1 mutant is more sensitive to localized high NH_4^+

Recent studies have shown that the *oseil1* mutant exhibits significantly higher biomass than WT when supplied with NO_3^-, and the opposite response when supplied with NH_4^+ (Li et al., 2022). This heightened sensitivity to NH_4^+ in *oseil1* prompted our investigation into the involvement of *OsEIL1* in the response to spatially heterogeneous high-NH_4^+ supply. We first used two *oseil1* mutant lines with significantly
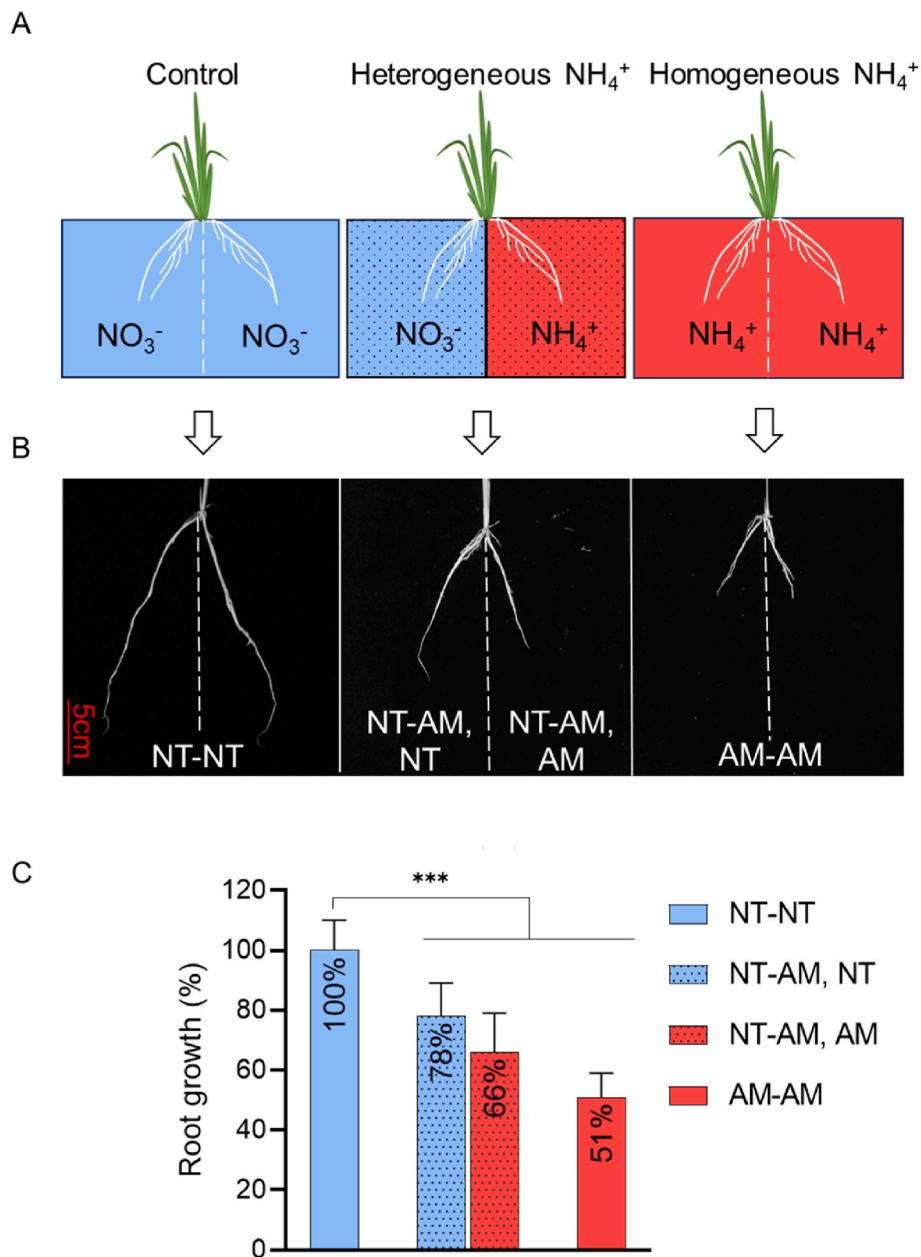

Fig. 1. Root phenotypes of the rice Nipponbare WT under split-root N conditions. (A, B) Root phenotypes in response to NO_3^--NO_3^- (NT-NT; Control), NO_3^--NH_4^+ (NT-AM), and NH_4^+-NH_4^+ (AM-AM). For the mixed-N condition, NT-AM, NT denotes the NO_3^- half and NT-AM, AM denotes the NH_4^+ half. Scale bar = 5 cm. (C) Root growth was determined in (A). Data are the means ± SD (n ≥ 11). Root growth (%) is presented relative to Control (NT-NT; 16.3 ± 1.55 cm). Significant differences were determined by an independent sample *t-test* (***$P < 0.001$).

lower *OsEIL1* expression than WT for a split-root experiment (Fig. 2). We observed greater root growth inhibition in the *oseil1* mutants to high NH_4^+ compared to WT. Under homogeneous NH_4^+ supply (AM-AM), both *oseil1-1* and *oseil1-2* mutants exhibited a root length decrease of approximately 70%, whereas the WT only decreased by c. 55%, relative to NT-NT controls (Fig. 2B and C). For localized NH_4^+ supply (NT-AM, AM), the *oseil1* mutants displayed a c. 60% decrease in root growth, whereas WT showed a c. 40% decrease, relative to NT-NT controls. On the NO_3^- side (NT-AM, NT), both *oseil1* mutants displayed a c. 40% decrease, compared to c. 20% in WT (Fig. 2B and C). Similarly, localized NH_4^+ supply (NT-AM, AM) decreased the root biomass of the NO_3^- side (NT-AM, NT) in both WT and *oseil1-1* mutant, with a greater reduction in the latter (Fig. S2). These findings highlight the crucial role of *OsEIL1* in response to localized high NH_4^+ and the systemic stunting of root growth.

3.3. Localized high NH_4^+ induces a systemic up-regulation of OsEIL1 expression

In AM-AM roots of the WT, there was a significant increase in *OsEIL1* expression compared to NT-NT controls, consistent with previous reports (Li et al., 2022). Additionally, the expression of *OsEIL1* gradually increased over time, with approximately 6-fold up-regulation at 36 and 72 h, and nearly 20-fold up-regulation at 120 h (Fig. S3A). Similar results were also found in tobacco leaf experiments. Heterologous expression of *ProOsEIL1-GUS* in tobacco leaf and the GUSase activity were enhanced by NH_4^+ induction (Fig. S3B). In NT-AM roots, a significant upregulation of *OsEIL1* was observed on the NH_4^+ side (NT-AM, AM). On the NO_3^- side (NT-AM, NT), initial upregulation of *OsEIL1* was not significant by 36 h, but was at 72 and 120 h (Fig. 3). These findings demonstrate a systemic response of *OsEIL1* expression to local high NH_4^+.

Furthermore, we examined the expressions of *OsACS2*, *OsACO5*, and *OsEIN2*, which are upstream genes involved in the ethylene signaling pathway. The expression patterns of all three genes were very similar to those of *OsEIL1*. Between 36 and 120 h, the expression of *OsACS2*, *OsACO5*, and *OsEIN2* was significantly induced in both AM-AM and NT-AM, AM roots. In NT-AM, NT roots, their expression was not significantly different at 36 h but gradually increased at 72 and 120 h. These findings suggest that *OsACS2*, *OsACO5*, and *OsEIN2*, as upstream players in the ethylene signaling pathway, are also involved in the response of
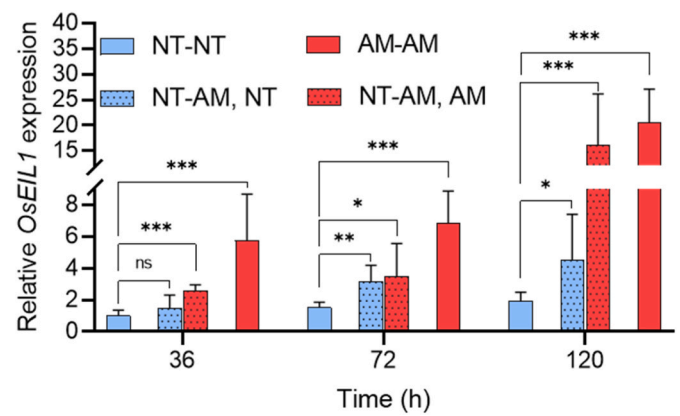

Fig. 3. *OsEIL1* expression in response to split-root N treatments. qRT-PCR analysis of time-dependent expression of *OsEIL1* under different N treatments (see Fig. 1 for an explanation of labels). Data are the means ± SD (n ≥ 4). Significant differences were determined by an independent samples *t*-test (*$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.001; and "ns", no significance).

rice to localized NH_4^+ supply (Fig. S4).

3.4. OsEIL1 plays a role in regulating NH_4^+ efflux in response to localized high NH_4^+

Previous studies have identified root NH_4^+ efflux as a critical trait for high-NH_4^+ tolerance, and it has been shown that *OsEIL1* regulates NH_4^+ efflux in rice (Britto and Kronzucker, 2002; Britto et al., 2001; Di et al., 2021; Kronzucker et al., 2003; Li et al., 2010, 2022). To further investigate the involvement of *OsEIL1* in regulating NH_4^+ efflux in response to localized high NH_4^+, NH_4^+ efflux was measured in the *oseil1-1* mutant and WT by split-root experiments using the NMT assay. In AM-AM roots, NH_4^+ efflux was higher in *oseil1* compared to WT, consistent with the findings of Li et al. (2022). However, *oseil1* exhibited nearly double the NH_4^+ efflux on the localized NH_4^+ side (NT-AM, AM) compared to WT. This pattern was also observed on the NT-AM, NT side, where *oseil1* displayed approximately double the NH_4^+ efflux compared to WT (Fig. 4). These results provide evidence for the involvement of *OsEIL1* in
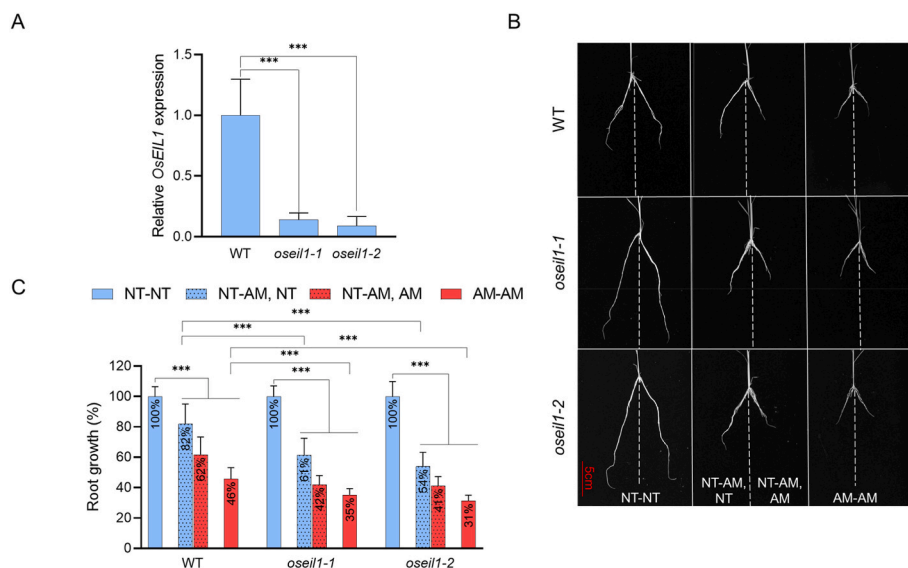

Fig. 2. Root growth responses of the *oseil1* mutant to split-root N conditions. (A) qRT-PCR analysis of expression of *OsEIL1* in the roots of Nipponbare WT and the *oseil1* loss-of-function mutants. Data are shown as the means ± SD of at least six biological replicates. (B) Root phenotypes of WT and *oseil1* mutants in response to NT-NT, NT-AM, and AM-AM treatments (see Fig. 1 for an explanation of labels). Scale bar = 5 cm. (C) Root growth of WT and *oseil1* mutants. Data are the means ± SD (n ≥ 11). Root growth (%) is presented relative to the Control (NT-NT) condition for each genotype (16.7 ± 1.08 cm for WT, 26.7 ± 1.77 cm for *oseil1-1*, and 26.3 ± 2.59 cm for *oseil1-2*). Significant differences were determined by an independent sample *t*-test (***$P <$ 0.001).
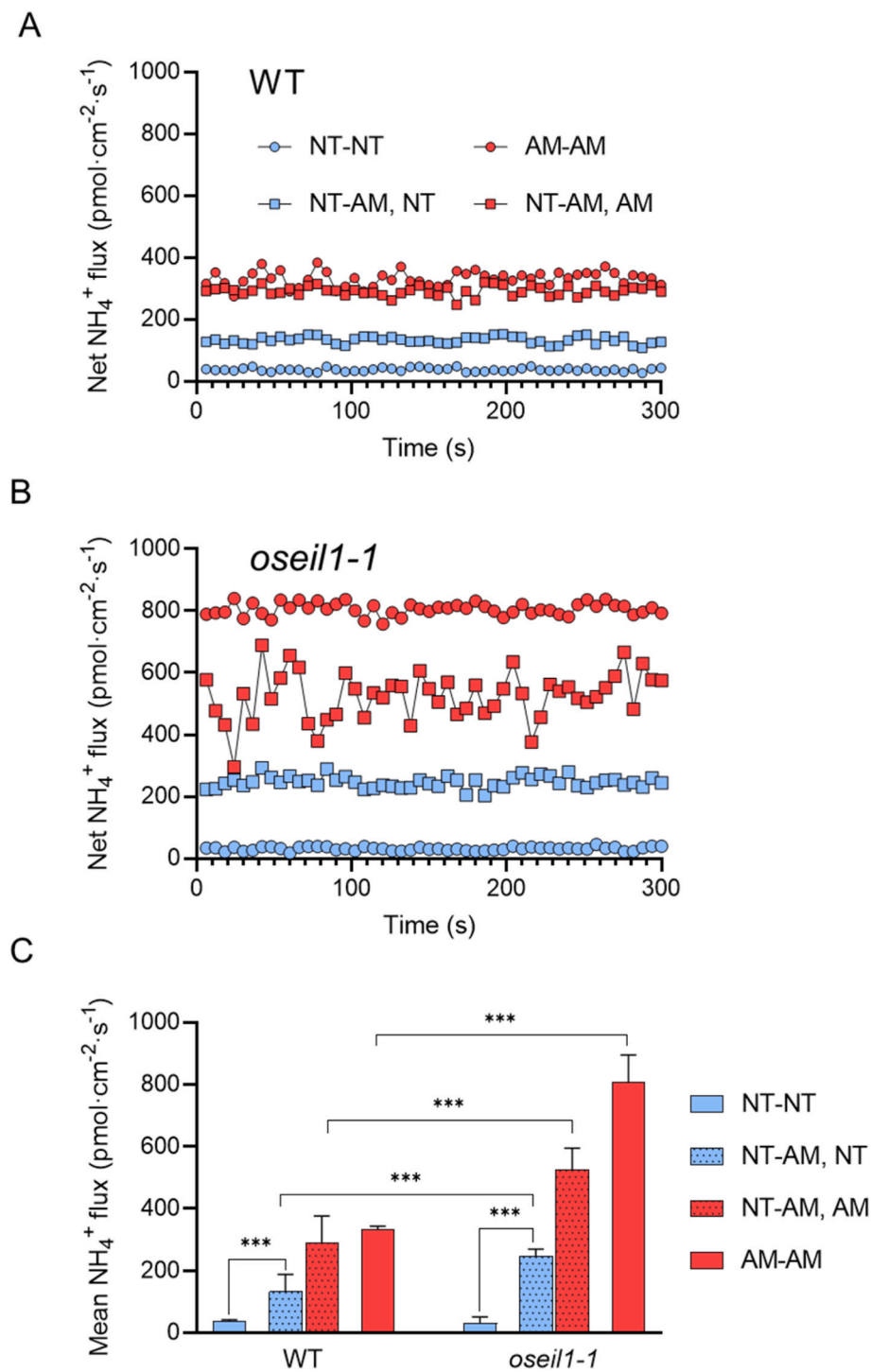
Fig. 4. Net NH_4^+ root fluxes in response to split-root N treatments. (A, B) Net NH_4^+ fluxes in WT and *oseil1-1* at the root elongation zone. Mean flux values $\pm$ SD (n $\geq$ 8) in (A) and (B) are shown in (C). See Fig. 1 for an explanation of labels. Significant differences were determined by an independent sample *t-test* (***$P < 0.001$; and "ns", significance).

the response of rice to localized high NH_4^+ supply through the regulation of NH_4^+ efflux.

3.5. OsVTC1-3 expression is regulated by OsEIL1 in response to localized high NH_4^+

The results from our previous report (Li et al., 2022) show that *OsVTC1-3* is directly regulated by OsEIL1, which affects NH_4^+ efflux from rice roots. To further clarify the role of OsEIL1 in regulating NH_4^+ efflux

in response to localized high NH_4^+, we also examined the expression of *OsVTC1-3* in the split-root assay.

Compared to the NT-NT condition, and like *OsEIL1*, *OsVTC1-3* expression was significantly upregulated in AM-AM and NT-AM, AM roots in the WT (Fig. 5). Furthermore, the expression levels progressively increased from 36 to 120 h (Fig. 5A). In NT-AM, NT roots, *OsVTC1-3* was not yet significantly induced at 36 h, but showed approximately 2-fold upregulation at 72 and 120 h, relative to NT-NT (Fig. 5A).
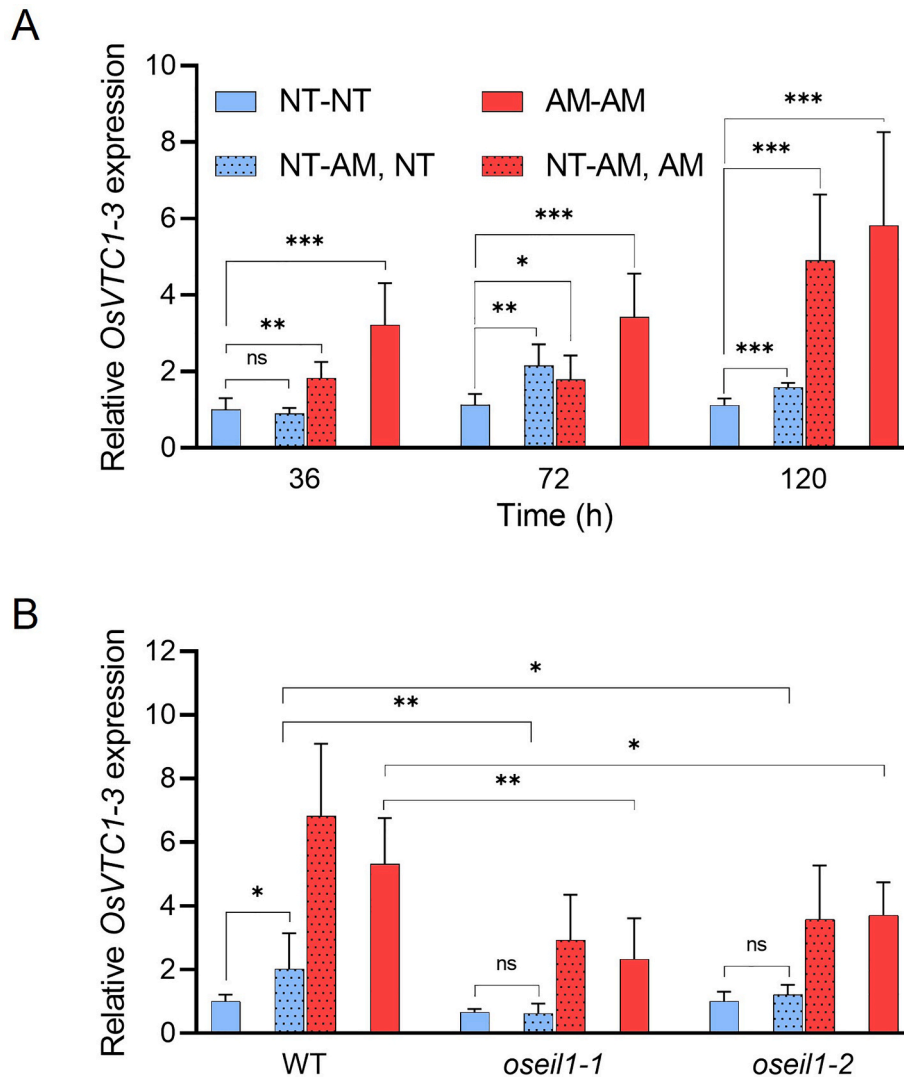
Fig. 5. *OsVTC1-3* expression in response to split-root N treatments. (A) qRT-PCR analysis of *OsVTC1-3* expression in WT roots in response to split-root N treatments over time (see Fig. 1 for an explanation of labels). (B) qRT-PCR analysis of *OsVTC1-3* expression in WT and *oseil1* mutants in response to split-root N treatments (t = 120 h). Data are shown as the means ± SD (n ≥ 4). Significant differences were determined by an independent sample *t-test* (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and "ns", no significance).

We also examined the expression of *OsVTC1-3* in the *oseil1* mutant under split-root conditions. Here, *OsVTC1-3* expression was induced ~2- to 4-fold in AM-AM and NT-AM, AM roots, relative to NT-NT (Fig. 5B). By contrast, in WT roots, *OsVTC1-3* expression was induced ~6-fold in both cases. Moreover, while the expression of *OsVTC1-3* in the WT was elevated on the NT-AM, NT side, the *oseil1* mutant did not show a significant difference in *OsVTC1-3* expression compared to NT-NT. We further confirmed the role of *OsVTC1-3* in regulating root growth under split-root conditions using the *osvtc1-3* mutant, and observed greater reductions in root biomass for *osvtc1-3* relative to WT in the NT-AM, NT side (Fig. S5).

4. Discussion

The input of large amounts of nitrogen fertilizers in paddies and the promotion of new agronomic measures such as "one-time root zone" fertilization, "side-deep" fertilization, and nitrification inhibitor application, have made the spatial and temporal heterogeneous distribution of high NH_4^+ more pronounced (Li et al., 2016; Liu et al., 2016; Song et al., 2022; Zhu et al., 2019). High concentrations of NH_4^+ expose plants to NH_4^+ toxicity and cause significant inhibitions to root growth.

However, it has not been clearly investigated whether the exposure of rice to heterogeneous high NH_4^+ causes only localized root growth inhibition or systemic inhibition, nor has the underlying molecular mechanisms been explored.

In our study, we demonstrated that, in rice with heterogeneous high-NH_4^+ supply (NT-AM), in addition to roots on the NH_4^+ side (NT-AM, AM) showing growth suppression, the other, NO_3^- side (NT-AM, NT), also showed significant root-length reductions (Fig. 1C). In other words, rice seedlings responded to localized high NH_4^+ by bringing about systemic root-growth inhibition. Unlike rice, other plants can display only local growth suppression in response to heterogeneous NH_4^+ supply. For example, in Arabidopsis, root growth inhibition was only observed on the high-NH_4^+ side (Li et al., 2011). Similarly, in *Lotus japonicus*, primary root growth was only inhibited on the localized high-NH_4^+ side (Rogato et al., 2010), and root biomass was inhibited only on the localized high-NH_4^+ side in tobacco (Walch-Liu et al., 2001). In tomato, root growth decreased significantly on the NT-AM, AM side, whereas root growth in fact significantly increased on the NT-AM, NT side (Dong et al., 2012). The response of different species to heterogeneous high NH_4^+ may be influenced by factors such as differing nutrient utilization efficiencies, nutrient mobility, and the adaptive evolution to plant

habitats more generally (e.g. drylands vs. paddy fields). Additionally, the complexity of the ecological environments in which these plants have naturally evolved may contribute to their development of complex systemic and localized responses systems to adapt to their surroundings.

Split-root experiments have been instrumental in revealing differing N-source specialization of species, as well as mixed N-source synergisms. In a maize split-root experiment, Schortemeyer and Feil (1996) observed superior growth in NT roots and AM-induced growth suppression whether applied homogeneously (AM-AM) or not (NT-AM). Interestingly, NO_3^--NH_4^+ synergism on growth was only observed if supplied evenly across the roots, as opposed to being supplied to spatially separated root halves (Schortemeyer et al., 1993; Schortemeyer and Feil, 1996). Similarly, in soybean, when NH_4^+ and NO_3^- were supplied in combination to the whole-root system, the cumulative NH_4^+ to NO_3^- uptake ratio was higher compared to that of the split-root system (Chaillou et al., 1994). In wheat, a 1:1 NO_3^-:NH_4^+ exposure to both sides of a split-root setup produced higher shoot dry weight, shoot N content, and NH_4^+:NO_3^- uptake ratio compared to spatially separated N sources (Feil, 1994). Split-root experiments have also revealed interesting stress physiology responses vis-à-vis N-source preference. In rice under osmotic (PEG) stress, higher water uptake in NT-AM, AM roots was associated with more lateral roots and lower cell death, aerenchyma formation, and lignification compared to NT-AM, NT roots (Gao et al., 2020).

Previous studies have found that *OsEIL1*, a core transcription factor of the ethylene signaling pathway, is involved in the ethylene signaling process in response to high NH_4^+ in rice. The *oseil1* mutant was found to be more sensitive to high NH_4^+ stress compared to WT (Li et al., 2022). Furthermore, in our study, split-root conditions also demonstrated that the *oseil1* mutant was not only more sensitive than WT to AM-AM and NT-AM, AM, but also root growth was more sensitive to systemic high NH_4^+ stress, i.e. NT-AM, NT (Fig. 2B and C). It suggests that *OsEIL1* is involved in the signaling process of the systemic response to heterogeneous high-NH_4^+ stress in rice. The results of qRT-PCR also indicate that *OsEIL1* is induced by high NH_4^+ and systematically up-regulated by heterogeneous high-NH_4^+ stress on the NT-AM, NT side (Fig. 3). Upstream of *OsEIL1*, *OsACS2*, *OsACO5*, and *OsEIN2* also shared a similar expression pattern with *OsEIL1* (Fig. S4), suggesting that the systemic response of rice to high NH_4^+ can be regulated by upstream ethylene signaling to regulate *OsEIL1* and, thus, downstream processes. The adaptation and tolerance of rice to ammonium is also correlated with its capacity for NH_4^+ efflux (Britto et al., 2001). It was also demonstrated that *OsEIL1* responds to ammonium stress by regulating NH_4^+ efflux (Li et al., 2022). Consistently, the *oseil1* mutants have an intensification of NH_4^+ efflux, during high-NH_4^+ stress, compared to WT in this study (Fig. 4), and this resulted in *oseil1* mutants being more sensitive to high-NH_4^+ stress (Fig. 2). However, it remains unknown whether the systemic sensitivity caused by localized high NH_4^+ in terms of root division also leads to changes in systemic NH_4^+ efflux. In our experimental results, it was confirmed that, even without direct exposure to high NH_4^+, NH_4^+ efflux was significantly increased compared with control. And further, the *oseil1* mutant also showed more NH_4^+ efflux on the NO_3^- side in the mixed-N-provision treatment than occurred in WT under the same conditions (Fig. 4), suggesting that systemic sensitization to high NH_4^+ also plays out at the level of NH_4^+ efflux. Thus, the presence of *OsEIL1* facilitates the maintenance of NH_4^+ efflux in rice roots subjected to high NH_4^+ and mitigates the intensity of NH_4^+ efflux and toxic phenotypes.

In Arabidopsis, GDP-mannose pyrophosphorylase (AtVTC1) has been shown to regulate NH_4^+ efflux during high-NH_4^+ stress. Due to less N-glycosylation of proteins, the *atvtc1* mutant is hypersensitive to NH_4^+, and NH_4^+ efflux from the roots is greatly stimulated (Di et al., 2021; Li et al., 2010, 2022; Qin et al., 2008). Similarly, rice OsVTC1-3 also affects NH_4^+ efflux by influencing the level of protein N-glycosylation, and, consequently, NH_4^+ efflux. It was demonstrated that OsEIL1 in rice acts as a transcription factor, capable of directly regulating downstream *OsVTC1-3* expression. Our experiments here demonstrate that *OsVTC1-3*

is also up-regulated on the NO_3^- side in the mixed-N-provision treatment as rice systemically responds to high NH_4^+, with indeed significantly greater expression on that side than in the control (Fig. 5A). By contrast, under split-root conditions, the *oseil1* mutant had no induction of *OsVTC1-3* while the elevation of *OsVTC1-3* on the NO_3^- side in the mixed-N-provision treatment was induced in WT (Fig. 5B). Consistent with the *oseil1* mutant, the root biomass on the NT-AM, NT side of *osvtc1-3* was reduced to a greater extent compared to WT (Fig. S5). Thus, the ability of OsEIL1 to participate in the systemic response of rice to high NH_4^+ by regulating *OsVTC1-3*, as well as the synergistic tendency of the expression patterns of *OsEIL1* and *OsVTC1-3* with NH_4^+ efflux, all support the conclusion that the systemic response of rice to heterogeneously provided high NH_4^+ can be regulated by the *OsEIL1-OsVTC1-3*-mediated NH_4^+-efflux pathway.

5. Conclusion

Our study provides valuable insights into the complex interplay of genes and signaling pathways involved in the response of rice to heterogeneously provided high NH_4^+. The findings highlight the role of OsEIL1 as a key regulator, modulating NH_4^+ efflux and the expression of *OsVTC1-3* in response to localized high NH_4^+. Additionally, the involvement of upstream ethylene signaling genes, such as *OsACS2*, *OsACO5*, and *OsEIN2*, suggests their possible regulatory roles in the *OsEIL1-OsVTC1-3* pathway during high-NH_4^+ treatment. Further exploration of this intricate network of genes and their interactions can contribute to a better understanding of plant responses to heterogeneous nutrient stresses, ultimately aiding in the development of strategies for optimizing nutrient uptake and utilization in crops.

CRediT authorship contribution statement

Jinlin Wu: Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Devrim Coskun:** Writing – review & editing. **Guangjie Li:** Writing – review & editing, Methodology, Conceptualization. **Zhaoyue Wang:** Investigation, Data curation. **Herbert J. Kronzucker:** Writing – review & editing. **Weiming Shi:** Writing – review & editing, Project administration, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jplph.2024.154205.

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