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TITLE:
OsNAR2.1 interaction with OsNIT1 and OsNIT2 functions in root-growth responses to nitrate and ammonium

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One-sentence summary: A nitrate transport accessory protein interacts with two nitrilase proteins to regulate root-growth responses to nitrate and ammonium supply.

AUTHOR CONTRIBUTIONS
G.X. and X.F. conceived the research; M.S. performed the experiments; J.C., H.Q., and L.L. provided technical assistance to the experiments; M.S and G.X. analyzed the data and wrote the article.

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ABSTRACT

The nitrate transport accessory protein OsNAR2 plays a critical role in root-growth responses to nitrate and nitrate acquisition in rice (*Oryza sativa*). In this study, a pull-down assay combined with yeast two-hybrid and co-immunoprecipitation analyses revealed that OsNAR2.1 interacts with OsNIT1 and OsNIT2. Moreover, an in vitro nitrilase activity assay indicated that indole-3-acetonitrile (IAN) is hydrolyzed to indole-3-acetic acid (IAA) by OsNIT1, the activity of which was enhanced 3–4-fold by OsNIT2 and in excess of 5–8-fold by OsNAR2.1. Knockout (KO) of OsNAR2.1 was accompanied by repressed expression of both OsNIT1 and OsNIT2, whereas KO of OsNIT1 and OsNIT2 in the osnit1 and osnit2 mutant lines did not affect expression of OsNAR2.1 or the root nitrate acquisition rate. osnit1 and osnit2 displayed decreased primary root length and lateral root density. Double KO of OsNAR2.1 and OsNIT2 caused further decreases in lateral root density under nitrate supply. Ammonium supply repressed OsNAR2.1 expression whereas it upregulated OsNIT1 and OsNIT2 expression. Both osnit1 and osnit2 showed root growth hypersensitivity to external ammonium; however, less root growth sensitivity to external IAN, higher expression of three IAA-amido synthetase (*GH3*) genes, and a lower rate of $^3$H-IAA movement towards the roots were observed. Taken together, we conclude that the interaction of OsNIT1 and OsNIT2 activated by OsNAR2.1 and nitrogen supply is essential for maintaining root growth possibly via altering the IAA ratio of free to conjugate forms and facilitating its transportation.

Keywords: ammonium; auxin; indole-3-acetonitrile; nitrate; nitrilase; *Oryza sativa*; roots.
INTRODUCTION

Plants display high root growth plasticity in primary root (PR) elongation, particularly lateral root (LR) initiation and elongation, to adapt to variation in nutrient and water supply (Drew et al., 1975; Gojon et al., 2011; Gruber et al., 2013). Two major forms of nitrogen (N) available for plant root uptake are nitrate (NO$_3^-$) and ammonium (NH$_4^+$). Nitrate is the major form of N available for dry land crop species and is also an important source of N for paddy rice (Kirk and Kronzucker, 2005; Li et al., 2008; Xu et al., 2012). Moreover, nitrate serves as a signaling molecule in various plant developmental processes including the determination of root architecture (Perilli et al., 2012; Wierzba and Tax, 2013; Vidal et al., 2013; O’Brien et al., 2016). In Arabidopsis, several key molecules have been identified that are involved in the regulation of LR initiation and development by localized nitrate, such as the transcription factor ANR1 (Zhang et al., 1998), the nitrate transceptor NRT1.1 (Krouk et al., 2010), miR393, the auxin receptor AFB3, and their downstream target NAC4 (Vidal et al., 2010; 2013). The \textit{nac4} mutation results in altered LR growth but not PR growth in response to nitrate (Vidal et al., 2010). Low N in roots represses the accumulation of auxin which controls LR formation (Vidal et al., 2010; Wang et al., 2019). A gradual reduction in auxin levels is closely related to enhanced differentiation of distal stem cells in root tips in response to low nitrate (Wang et al., 2019). Moreover, NRT1.1 protein behaves like a root auxin transporter under low nitrate supply (Krouk et al., 2010).

Unlike nitrate, ammonium commonly mediates inhibition of both PR and LR elongation in most plant species (Li et al., 2010; Rogato et al., 2010; Li et al., 2014). The presence of ammonium systematically alters root development, affecting processes including elongation, gravitropism, and LR branching (Lima et al., 2010; Li et al., 2014). The root lengths of the auxin-resistant mutants \textit{aux1}, \textit{axr1}, and \textit{axr2} are less affected by high levels of ammonium compared with wild type (WT), indicating that auxin may be involved in ammonium-induced root length reduction (Cao et al., 1993). However, several lines of evidence have suggested that the root growth reduction induced by ammonium supply is associated with ammonium efflux in the
root elongation zone, independent of the auxin pathways (Li et al., 2010; Liu et al., 2013). The combinatorial effect of nitrate on ammonium-regulated root system architecture suggests that ammonium regulates PR elongation and LR branching by distinct pathways (Liu and von Wiren, 2017).

The main form of plant auxin is indole-3-acetic acid (IAA), for which several pathways for tryptophan-dependent IAA biosynthesis have been proposed in plants (Woodward et al., 2005; Sugawara et al., 2009; Korasick et al., 2013; Abu-Zaitoon, 2014). Indole-3-acetonitrile (IAN) has been identified as one of two key intermediates in indole-3-acetaldoxime (IAOx)-mediated IAA biosynthesis (Sugawara et al., 2009). IAN conversion during basal IAA production requires nitrilases for the hydrolysis of nitriles (Müller et al., 1998; Park et al., 2003; Lehmann et al., 2017). Nitrilase isogenes have been identified in almost all the major plant families (Piotrowski, 2008). For example, the relatively small nitrilase family in Arabidopsis, comprising four members (NIT1–4), has gained diverse biological functions in nitrile metabolism (Piotrowski, 2008). Two groups of nitrilases that catalyze the conversion of IAN to IAA, namely NIT1 in Arabidopsis and ZmNIT2 in maize (Zea mays) (NIT4 orthologs of Arabidopsis), have been characterized thus far. Mutation of NIT1 was shown to decrease both plant sensitivity to IAN treatment (Normanly et al., 1997) and total IAA concentration without affecting the concentration of free IAA (Lehmann et al., 2017). Over-expression of NIT1 resulted in drastic changes of both free IAA and IAN levels, resulting in a phenotype characterized by shorter PR and increased LR number (Lehmann et al., 2017). Maize kernels contain an endogenous nitrilase with activity towards the substrate IAN (Park et al., 2003). ZmNIT2 can hydrolyze IAN to IAA with 7–20-fold higher activity than AtNIT1/2/3 (Park et al., 2003).

As opposed to IAA production, the proposed major role of the nitrilase 4 (NIT4) sub-family of angiosperms is catalyzing the conversion of β-cyanoalanine to aspartic acid and asparagine for detoxification of hydrogen cyanide (Piotrowski et al., 2001; Jenrich et al., 2007; Piotrowski, 2008). Poaceae possesses two different NIT4 orthologs (NIT4A and NIT4B). The rice (Oryza sativa) genome contains two NIT4 orthologs, namely OsNIT4A and OsNIT4B that were renamed as OsNIT1 and OsNIT2
by Ding et al (2008), which are investigated in this study. Changes in OsNIT1 and OsNIT2 expression are similar to the changes in total IAA after bacterial infection (Ding et al., 2008), indirectly suggesting that OsNIT1 and OsNIT2 affect IAA accumulation. However, Sugawara et al. (2009) detected the presence of IAN in Arabidopsis, but not in the seedlings of maize, rice, or tobacco (Nicotiana tabacum). These data suggest a species- or organ-specific contribution of nitrilase to IAA production in plants.

Nitrate Assimilation Related family (NAR2) proteins have been considered as important components of nitrate transporters (NRT) in plants (Tong et al., 2005). In Arabidopsis, six of the seven AtNRT2 members require AtNAR2.1 as a component of high-affinity nitrate transporters (Kotur et al., 2012). Disruption of AtNAR2.1 causes an almost complete loss of inducible high-affinity nitrate influx (Orsel et al., 2006). In rice, OsNAR2.1 interacts with OsNRT2.1/2.2/2.3a and plays a broad role in root acquisition of nitrate in response to both low- and high-nitrate supply (Feng et al., 2011; Yan et al., 2011; Liu et al., 2014). Knockout (KO) of OsNAR2.1 causes reduced LR density and PR growth in nitrate medium (Huang et al., 2015). Notably, osnar2.1 mutants display decreased auxin distribution in the roots and the accompanying root phenotype can be largely complemented by external NAA (Huang et al., 2015), suggesting that OsNAR2.1 functions in both nitrate uptake and auxin-mediated nitrate signaling. Therefore, the function of the OsNAR2.1 regulatory pathway in plant auxin signaling is an intriguing question.

In this study, through a pull-down assay combined with yeast-two-hybrid and co-immunoprecipitation analyses, we show that OsNAR2.1 directly interacts with the two nitrilase proteins OsNIT1 and OsNIT2 in rice. In addition to demonstrating that OsNIT1 and OsNIT2 potentially encode IAN hydrolysis enzymes, we provide a number of physiological, biochemical, and genetic lines of evidence indicating that OsNAR2.1 contributes to auxin-responsive, nitrate-regulated root growth via activation of OsNIT1 and OsNIT2.
RESULTS

OsNAR2.1 interacts with OsNIT1 and OsNIT2 in roots under nitrate supply

We first conducted a preliminary pull-down assay of nitrate-treated seedlings of rice WT (cv. Nipponbare) using an OsNAR2.1-GST tag fusion protein (Supplemental Figure S1A). The extracted proteins revealed several known functional proteins that potentially interact with OsNAR2.1 (Supplemental Table S1). Considering that OsNAR2.1 is involved in root responses to nitrate and auxin (Huang et al., 2015), we focused on two candidate nitrilases (EC 3.5.5.1), as this is the enzyme involved in IAA synthesis and detoxification in Arabidopsis and maize (Piotrowski et al., 2008).

The predicted genes encoding nitrilase in the rice genome are OsNIT1 (OsNIT4A, LOC_Os02g42350) and OsNIT2 (OsNIT4B, LOC_Os02g42330), which are orthologs of Arabidopsis AtNITs and maize ZmNITs (Jenrich et al., 2007; Piotrowski, 2008).

We subsequently tested for interaction between OsNAR2.1 and OsNIT1/2 through yeast two-hybrid (Y2H) assays (Figure 1A) as well as co-immunoprecipitation (Co-IP) assays (Figure 1B) and mass spectrometry analysis (Supplemental Figure S1B, S1C). The results of these analyses confirmed that OsNAR2.1 interacts with OsNIT1 and OsNIT2 at the protein level.

To further confirm the interaction of these proteins in rice, we detected their tissue localization under the same experimental conditions. In-situ hybridization analysis showed that OsNIT1, OsNIT2, and OsNAR2.1 were abundantly expressed in root tips and LRs (Figure 1C). We have previously shown that OsNAR2.1 is expressed mainly in the roots, especially in root tips and LRs under nitrate supply (Feng et al., 2011). In this study, we found that OsNIT1, OsNIT2, and OsNAR2.1 were each transcriptionally upregulated in the roots by nitrate supply, and that gene expression induction was further enhanced by increasing nitrate supply (Figure 1D). In addition, sub-cellular localization analysis showed that OsNIT1 and OsNIT2 had a wide cellular distribution (Supplemental Figure S2), which is similar to the localization of OsNAR2.1 (Liu et al., 2014).

Knockout of OsNIT1 or OsNIT2 results in the same root phenotype as the
To examine the roles of OsNIT1 and OsNIT2 in root-growth responses to nitrate, we examined the root phenotypes of osnit1 and osnit2 mutants generated by CRISPR-Cas9 editing and T-DNA insertional mutagenesis. Three independent mutant lines for each gene were selected for detailed analysis (Supplemental Figure S3). It has been shown that osnar2.1 exhibits a phenotype of short PRs and low root density under low nitrate supply (Huang et al., 2015). In this study, we observed that the root

![Image](https://example.com/image.png)

**Figure 1. OsNAR2.1 interacted with OsNIT1 and OsNIT2 at nitrate supplied roots.**

*osnar2.1 mutant under nitrate supply*

To examine the roles of OsNIT1 and OsNIT2 in root-growth responses to nitrate, we examined the root phenotypes of osnit1 and osnit2 mutants generated by CRISPR-Cas9 editing and T-DNA insertional mutagenesis. Three independent mutant lines for each gene were selected for detailed analysis (Supplemental Figure S3). It has been shown that osnar2.1 exhibits a phenotype of short PRs and low root density under low nitrate supply (Huang et al., 2015). In this study, we observed that the root
phenotypes of osnit1, osnit2, and osnar2.1 mutants in the Nipponbare background were similar under 0.25 mM nitrate supply. Under nitrate supply, inactivation of either OsNAR2.1, OsNIT1, or OsNIT2 decreased PR growth by 15–25% and LR density by 16–26% (Figure 2A, 2C, 2D). OsNIT1 KO in the Hwayoung background (osnit1-1) also resulted in significantly reduced PR length and LR density. Moreover, the

Figure 2. Knockout of OsNIT1 or OsNIT2 resulted in the same root phenotype with osnar2.1 mutant at nitrate supply but did not alter nitrate uptake rate of unit weight root.
expression levels of OsNIT1 and OsNIT2 in the roots of the osnar2.1 mutant line were largely repressed under nitrate supply (Figure 2B) relative to their expression levels in WT, indicating that OsNAR2.1 is necessary for nitrate-regulated expression of OsNIT1 and OsNIT2 in rice roots.

To further confirm that OsNIT1 and OsNIT2 share the same function as OsNAR2.1 in root responses to nitrate, we crossed osnit2-2 and osnar2.1-1 and obtained a homozygous hybrid mutant line. Interestingly, under nitrate supply, double KO of OsNIT2 and OsNAR2.1 resulted in similar PR length inhibition as seen in osnar2.1, whereas considerably reduced LR density was observed compared to osnar2.1 or osnit2 (Figure 2E and 2F). Due to unknown reasons, we could not isolate a double mutant line of osnit1 and osnar2.1 in this study. The additive effect of OsNIT2 on OsNAR2.1 function in nitrate regulation of root growth supports an interaction between OsNAR2.1 and OsNIT2, and possibly OsNIT1 (Figure 1).

Knockout of OsNIT1 and OsNIT2 does not alter nitrate uptake rate per root unit weight

We previously reported that inactivation of OsNAR2.1 limits root nitrate acquisition under a broad range of nitrate supply, which is the result of OsNAR2.1 being an essential partner protein for the function of several OsNRT2 nitrate transporters (Yan et al., 2011). Therefore, we also investigated the roles of OsNIT1 and OsNIT2 in OsNAR2.1 expression and root nitrate uptake. In contrast to the inactivation of OsNAR2.1, which led to the repression of OsNIT1 and OsNIT2 expression (Figure 2B), no significant changes in expression levels of OsNAR2.1, OsNRT2.1, or OsNRT2.3 were observed in either osnit1 or osnit2 mutants (Figure 2G). Moreover, KO of OsNIT1 did not affect the expression of OsNIT2 and vice versa (Supplemental Figure S4), indicating that both OsNIT1 and OsNIT2 are downstream components of the OsNAR2.1 regulatory pathway. We further determined that the root unit weight $^{15}$NO$_3^-$ influx rate of osnit1 and osnit2 lines were comparable with WT in a short-term (5 min) assay (Figure 2H), even though the total amount of $^{15}$NO$_3^-$ per plant in osnit1 and osnit2 was decreased by approximately 20% due to the smaller root size of the
mutants (Figure 2H). These results indicate that *OsNAR2.1* is upstream of *OsNIT1* and
*OsNIT2* in a nitrate signaling pathway, and that *OsNIT1* and *OsNIT2* interaction with
*OsNAR2.1* does not directly contribute to root nitrate uptake.

**Knockout of *OsNIT1* or *OsNIT2* decreases root growth sensitivity to IAN, but not
to NAA**

Since the presence and contribution of IAN to IAA synthesis in rice is questionable
(Sugawara et al., 2009), we analyzed the effect IAN and NAA (naphthylacetic acid, IAA analogue) on rice root growth. In WT plants, PR length was slightly stimulated by low (0.01 μM) IAN treatment but inhibited by high (10 μM) IAN and 0.1 μM NAA treatment, whereas LR density and the expression levels of *OsNIT1* and *OsNIT2* did not display distinct sensitivity to low external IAN and NAA treatment (Supplemental Figures S5A, S5B, S5C).

The KO mutants of *OsNIT1* showed less sensitivity to external IAN in both the Nipponbare and Hwayoung backgrounds, and exhibited phenotypes of longer PRs and reduced LR density (Figure 3A, 3B, 3C). The PR length and LR density in *osnit1* was approximately 140% longer and 40% less, respectively, than its Nipponbare WT (Figure 3B, 3C). The *osnit2* mutants showed a similar phenotype regarding changes in PR length as *osnit1*, but the effect was less substantial. KO of *OsNIT1* or *OsNIT2* did not affect root responses to external NAA (Figure 3B, 3C). These data indicate that IAN, which can be hydrolyzed by NIT proteins, may play a regulatory role in rice root growth.

**Knockout of *OsNIT1* or *OsNIT2* decreases acropetal IAA transport, but does not change root total IAA concentration**

In view of the substantial responses of rice root growth to external IAN and that both OsNIT1 and OsNIT2 were shown to be involved in these responses, we attempted to analyze the effect of *OsNIT1* or *OsNIT2* KO on IAN and IAA concentration in rice. We did not detect IAN in WT Nipponbare seedlings (Supplemental Figure S6A), indicating that IAN was either not present, present at levels below our detection limits,
or that IAN is rapidly hydrolyzed in rice. In addition, the total concentration of IAA in the roots of osnit1 and osnit2 mutants and WT was similar (Figure 4A), indicating that OsNIT1 and OsNIT2 are not major influential factors for total IAA synthesis or accumulation in rice seedlings.

We also compared [3H]-IAA transport in the roots of WT and mutant plants under nitrate supply. Notably, as shown by the amounts of [3H]-IAA in 0–3 cm root-tip sections, KO of OsNAR2.1 significantly restricted both root-ward and shoot-ward movement of [3H]-IAA (Figure 4B,C), whereas KO of OsNIT1 or OsNIT2 caused inhibition of [3H]-IAA root-ward movement alone (Figure 4B,C). Since PR length in each of the three mutant lines was shorter than that in the WT (Figure 2), the [3H]-IAA transport distance from the site of application at the root-shoot junction to the root tip was in effect shorter in the mutants, and therefore,
the effect of OsNIT1 or OsNIT2 disruption on acropetal transport of [3H]-IAA in the roots is theoretically larger than that implied by the data shown in Figure 4.

To confirm that the loss of OsNIT1 or OsNIT2 function can alter auxin distribution or auxin forms, we analyzed the expression of five auxin efflux transporter-encoding genes and three IAA-amido synthetase-encoding genes, which may prevent free IAA accumulation in rice (Xu et al., 2005; Ding et al., 2008; Wang et al., 2009; Zhang et al., 2009; Du et al., 2012; Wang et al., 2017). In comparison to WT, both osnit1 and osnit2 lines exhibited repressed expression of OsPIN1c and
OsPIN1d an up-regulated expression of OsPIN2, OsGH3-2, OsGH3-8, and OsGH3-13 in their roots (Figure 4D). The expression of OsPIN1a and OsPIN1b was not significantly affected in the mutants (Supplemental Figure S6B). These data support the hypothesis that OsNIT1 or OsNIT2 regulate root growth via alteration of auxin root-ward transport and local auxin distribution.

OsNIT1 displays IAN hydrolysis activity, which is significantly improved by co-expression of OsNIT2 and OsNAR2.1

It has been shown that some NIT enzymes like sorghum SbNIT4A and SbNIT4B2 may form heteromeric complexes to achieve high catalytic activity (Jenrich et al., 2007). Therefore, we tested whether the interaction between OsNIT1, OsNIT2, and OsNAR2.1 caused enhanced IAN hydrolysis activity in vitro.

First, we observed that OsNIT1 and OsNIT2 interacted with each other in a Y2H assay (Figure 5A), similar to the previously reported interaction between SbNIT4A and SbNIT4B2 (Jenrich et al., 2007). A short-term in vitro assay of IAN conversion to IAA quantified by liquid chromatography indicated that OsNIT1, but not OsNIT2 or OsNAR2.1, performed IAN hydrolysis (Figure 5B). Remarkably, co-expression of OsNIT1 and OsNIT2 enhanced OsNIT1 IAN hydrolysis activity by 3.2-fold, which was further increased by 5.3-fold following the co-expression of OsNIT1 and OsNIT2 together with OsNAR2.1 (Figure 5B, Supplemental Figure S7). To confirm OsNIT1 function in hydrolyzing IAN to IAA and the activation of OsNIT1 enzymatic activity by OsNIT2 and OsNAR2.1, we performed the conversion rate assay using four different concentrations of IAN under the same experiment conditions except for an extension of reaction time from 1 h to 2 h. The results showed that the Michaelis-Menten equation could be used to predict the kinetics of OsNIT1 hydrolysis activity in the conversion of IAN to IAA (Supplemental Figure S8). The additional presence of OsNIT2 or both OsNIT2 and OsNAR2.1 increased OsNIT1 enzyme affinity for IAN, resulting in a 3.8–4.7-fold or 5.6–8.2-fold increase in IAA production rate, respectively (Supplemental Table S2). These results indicate the biological significance of the interaction between OsNAR2.1 and the two NIT
proteins in rice nitrilase activity.

Unlike OsNAR2.1, OsNIT1 and OsNIT2 are upregulated by ammonium and function in ammonium root-growth responses

We previously reported that expression of OsNAR2.1 is activated by nitrate whereas it is repressed by ammonium (Feng et al., 2011). Moreover, there are no significant root phenotypic differences between OsNAR2.1-mutant and WT plants under ammonium supply (Yan et al., 2011; Liu et al., 2014; Huang et al., 2015). However, we noticed that the expression levels of both OsNIT1 and OsNIT2 were upregulated in the roots by ammonium (Figure 6A). To further confirm these expression dynamics, we detected OsNIT1 and OsNIT2 tissue localization in rice under the same experimental conditions. In-situ hybridization analyses showed that OsNIT1 and OsNIT2 were abundantly expressed in root tips and LRs under ammonium supply, whereas OsNAR2.1 expression was very faint in comparison (Figure 6B).

We subsequently supplied different genotypes with 0.25 mM NH$_4^+$ and observed that the osnit1 and osnit2 mutants in the Nipponbare background consistently displayed a phenotype of shorter PRs and lower LR root density, both of which were approximately 35–45% less than in WT (Figure 6C). By contrast, KO of OsNAR2.1 in
both the Hwayoung and Nipponbare backgrounds did not alter the expression of OsNIT1 and OsNIT2 (Figure 6D) or root growth under ammonium supply (Figure 6E, 6F). Moreover, double mutation of OsNAR2.1 and OsNIT2 resulted in a similar root phenotype as osnit2 (Figure 6G, 6H), confirming that OsNAR2.1 is not involved in the role that NIT proteins play in root responses to ammonium supply.

Figure 6. Knockout of OsNIT1 and OsNIT2 decreased both primary root and lateral root growth at ammonium supply.
To further verify that OsNIT1 and OsNIT2 function in regulating root responses to ammonium, we investigated acropetal transport of $[^3\text{H}^+]-\text{IAA}$ in the corresponding mutant lines. In agreement with the repression of OsNAR2.1 expression by ammonium supply, there was no significant difference in either acropetal or basipetal transport of IAA in osnar2.1 compared to WT (Figure 6I, 6J). Moreover, inactivation of OsNIT1 or OsNIT2 did not significantly affect IAA basipetal (shoot-ward) transport in roots under ammonium supply (Figure 6J), comparable to that seen in roots under nitrate supply (Figure 4C). Remarkably, in osnit1 and osnit2, the amount of $[^3\text{H}^+]-\text{IAA}$ transported from its site of application at the root-shoot junction to the 0–3mm root tip section was less than that in WT (Figure 6I). Since the ammonium-supplied mutant lines showed much shorter PR length than WT (Figure 6C, 6E), these data confirm that OsNIT1 and OsNIT2 play regulatory roles in root responses to both ammonium and nitrate.
DISCUSSION

For efficient acquisition of N with varied forms and concentrations, plants develop sophisticated regulatory pathways in altering root morphology, architecture, N transport and assimilation. We have previously reported that OsNAR2.1 in rice is activated by nitrate and inhibited by ammonium (Feng et al., 2011). As a component of nitrate transporters, OsNAR2.1 contributes to both root nitrate acquisition and nitrate regulated root growth (Yan et al., 2011; Liu et al., 2014; Huang et al., 2015). In this study, we identified that OsNIT1 and OsNIT2 are two new interacting proteins of OsNAR2.1 and found that they are the down-stream components of OsNAR2.1 regulation pathway in the root response to nitrate supply. Moreover, ammonium supply could activate the interaction of OsNIT1 and OsNIT2 for maintaining root growth in rice.

The role of OsNIT1 and OsNIT2 as the components of nitrilase enzyme in catalyzing IAN conversion and IAA distribution

Previously, two groups of nitrilases that can catalyze the conversion of IAN to IAA, NIT1 orthologs in Brassicaceae and NIT4 orthologs in maize (Zea mays) have been characterized (Osswald et al., 2002; Park et al., 2003; Ishikawa et al., 2007; Kriechbaumer et al., 2007). In this study, we detected that OsNIT1 alone belonging to NIT4 sub-family could also have the activity to hydrolyze IAN (Figure 5B; Supplemental Figure S8; Supplemental Table S2), whereas the velocity was slow and similar to that catalyzed previously by NIT1 and NIT4 in Arabidopsis, maize, Brassica rapa, and Sorghum (Osswald et al., 2002; Park et al., 2003; Ishikawa et al., 2007; Jenrich et al., 2007; Kriechbaumer et al., 2007). In addition, the Km of SbNIT4A/B2 with IAN was 0.16 mM (Jenrich et al., 2007), which is comparable to Km (0.65 mM) of OsNIT1/2/OsNAR2.1 (Supplemental Figure S8), indicating the similar NIT enzyme activity in rice and Sorghum bicolor. Moreover, as showed in Arabidopsis, nit1 mutants were resistant to external IAN, indicating that the slow IAN hydrolysis activity of NIT1 was sufficient to produce an auxin-overproduction phenotype (Normanly et al., 1997). Similarly, we observed that osnit2, in particular...
osnit1, displayed a stimulated PR growth and a reduced LR growth as compared to WT in the presence of exogenous IAN (Figure 3A, 3B), indicating that inactivation of OsNIT1 or OsNIT2 could prevent auxin-overproduction in rice (Supplemental Figure S5B). Notably, IAN was not detectable in the seedlings of rice (cv. Nipponbare) in this study (Supplemental Figure S6A), the same as reported by Sugawara et al (2009). In addition, KO of OsNIT1 or OsNIT2 did not significantly affect total IAA content in the roots of seedlings (Figure 4A). These results indicated that nitrilases may not be the key enzymes in IAA biosynthesis as proposed by Piotrowski et al (2008) but affect auxin-related regulatory pathways in plants.

Sorghum bicolor contains three NIT4 isoforms SbNIT4A, SbNIT4B1, and SbNIT4B2 (Jenrich et al., 2007). Interestingly, each isoform of SbNIT4 did not possess the enzymatic activity in hydrolyzing β-cyanoalanine whereas the heteromeric complexes of SbNIT4A/B1 and SbNIT4A/B2 showed high activity in catalyzing the hydrolysis of β-cyanoalanine (Jenrich et al., 2007). The SbNIT4A/B2 complex could also catalyze the conversion of IAN to IAA (Jenrich et al., 2007). The assay of site-specific mutagenesis of the active cysteine residue demonstrates that hydrolysis of β-cyanoalanine is catalyzed by the SbNIT4A isoform in both complexes whereas hydrolysis of IAN occurs at the SbNIT4B2 isoform (Jenrich et al., 2007). In maize, ZmNIT2 is expressed in auxin synthesizing tissues and shows efficient activity in hydrolyzing IAN to IAA (Kriechbaumer et al., 2007). Notable, ZmNIT2 could have an additional enzymatic function in turnover of β-cyanoalanine when it forms heteromers with the orthologs ZmNIT1 (Kriechbaumer et al., 2007). Interestingly, the IAN hydrolysis activity of OsNIT1 was also strongly enhanced by presence of OsNIT2 (Figure 5A, 5B). Taken together, we predict that the basic function of Poaceae nitrilase in catalyzing either IAN to IAA or hydrolyzing β-cyanoalanine is determined by one NIT component and enhanced by the other.

It has been shown that inactivation of NITI resulted in decrease of total IAA, but not free IAA in the seedlings of Arabidopsis (Lehmann et al., 2017). In this study, we found that KO of OsNITI or OsNIT2 did not change the concentration of total IAA (Figure 4A), but confined the acropetal transport of ³H-IAA in the roots (Figure 4B).
In comparison to WT, *osnit1* and *osnit2* mutants showed upregulated expression of three IAA-amido synthetase (GH3) genes (Figure 4D) and down-regulation of two putative IAA efflux transporter genes *OsPIN1c* and *OsPIN1d* expression in the nitrate supplied roots (Figure 4D). Notably, *OsPIN1c* and *ld* are orthologs genes of *AtPIN1* expressed in Arabidopsis vascular tissue (Gälweiler et al., 1998). Double mutation of *OsPIN1c* and *OsPIN1d* resulted in a short PR phenotype (Li et al., 2019). Therefore, we hypothesize that *OsPIN1c* and *OsPIN1d* are also involved in rice root IAA distribution. It can be deduced that the activation of OsNIT1 and OsNIT2 mediates root growth partially via altering the IAA ratio of free to conjugate forms and transportation.

*Interaction between OsNIT1 and OsNIT2 and activation by OsNAR2.1 contribute to nitrate regulated root growth in rice*

Root architecture is shaped through N interactions with PR and LRs. It is known that interactions with auxin signaling are important to N regulation of root branching (Lavenus et al., 2013; Forde, 2014). In this study, we found that KO of either *OsNIT1* or *OsNIT2* resulted in shorter length of PR and lower LR density at nitrate supply condition (Figure 2). In comparison to their WT, the mutants showed less sensitivity of the root growth to external IAN (Figure 3A, 3B) and lower rate of root-ward movement of $^3$H-IAA (Figure 4B). However, the contribution of OsNIT1 and OsNIT2 to maintain root growth seems different to their putative orthologs in Arabidopsis. KO of *AtNIT1* and *AtNIT2* did not affect the length of PR whereas over-expression of *AtNIT1* resulted in shorter PR and higher LR density (Lehmann et al., 2017).

Notably, the impaired root growth of both *osnit1* and *osnit2* mutants was similar to that of *osnar2.1* mutant (Figure 2A, 2C and 2D). Previously, we have shown that knockdown of *OsNAR2.1* inhibited LR formation under nitrate supply (Huang et al., 2015). The *osnar2.1* mutant showed significant less LRs than WT co-occurring with decreased auxin transport from shoots to roots even at similar N concentrations in their roots (Huang et al., 2015), suggesting that OsNAR2.1 probably functions in nitrate-signaling in addition to nitrate uptake (Yan et al., 2011; Liu et al., 2014).
this study, we found that OsNAR2.1, OsNIT1 and OsNIT2 are co-localized in the root tissues (Figure 1C) and all of them showed nitrate-enhanced expression in the roots (Figure 1D). We provide robust evidence that the three proteins can interact with each other. The presence of OsNAR2.1 and OsNIT2 enhanced the enzyme activity of OsNIT1 (Figure 5B). The preliminary pull-down assay (Supplemental Figure S1) together with yeast-two-hybrid assay (Figure 1A), co-immunoprecipitation assay (Figure 1B) and its Mass Spectrometry analysis (Supplemental Figure S1B, S1C) all support this notion.

We also found that OsNAR2.1 is at nitrate regulatory upstream of OsNIT1 and OsNIT2. KO of OsNAR2.1 repressed the expression of OsNIT1 and OsNIT2 (Figure 2B), whereas inactivation of OsNIT1 or OsNIT2 did not affect the expression of OsNAR2.1 (Figure 2G). OsNAR2.1 enhanced the enzyme activity of OsNIT1 and OsNIT2 (Figure 5B) and functions in root nitrate acquisition at wide range (Yan et al., 2011; Liu et al., 2014), whereas OsNIT1 and OsNIT2 mutations did not affect root nitrate uptake rate (Figure 2H). Nevertheless, the double mutation of OsNAR2.1 and OsNIT2 (osnit2-2×osnar2.1-1) showed a smaller LR density than the two parental mutants under nitrate conditions (Figure 2F). Since OsNAR2.1 is essential to activate the nitrate uptake functions of OsNRT2.1, OsNRT2.2 and OsNRT2.3a in rice (Yan et al., 2011; Liu et al., 2014), it is not so surprised that the regulatory role of OsNAR2.1 in occurrence of LRs is broader than that of OsNIT2, and probably OsNIT1 as well. Therefore, our results demonstrated that OsNAR2.1 could regulate root formation not only by interacting with OsNIT1 and OsNIT2 but also through the nitrate uptake (Figure 7).

OsNIT1 and OsNIT2 also play a regulatory role on root growth that is partially independent from OsNAR2.1. In osnar2.1 mutant, both the activities of NIT1 and the NIT1+NIT2 complex might be impaired but remain significant (Figure 2B). Since OsNIT1 alone showed an IAN to IAA conversion activity and OsNIT2 could enhance the OsNIT1 activity in hydrolyzing IAN (Figure 5A), it is not surprising that osnar2.1 or osnit2 single mutant has a weaker phenotype than their double osnit2×osnar2.1
Remarkably, the role of OsNAR2.1 in nitrate uptake was independent from OsNIT1 and OsNIT2 (Figure 2G and 2H), whereas the role of OsNIT1 and OsNIT2 in the regulation of root growth can be enhanced by OsNAR2.1 (Figure 5B, Supplemental Figure S7 and Supplemental Table S2). We found that OsNAR2.1 may have other interacting proteins besides nitrilase and nitrate transporters as shown in pull down and Co-IP assay (Supplemental Figure S1 and Supplemental Table S1). Therefore, the nitrate signaling role of OsNAR2.1 not only relies on its interaction with the OsNITs and nitrate transporters, but also other unknown regulators.

Independent role of OsNIT1 and OsNIT2 in maintaining ammonium-supplied root growth

It has been shown that ammonium triggered LR branching and inhibition of PR and LR elongation (Liu et al., 2013; Zou et al., 2013), which was the same as that we observed in this study (Figure 6). The repression of PIN2 altered auxin distribution in the root apices exposed to ammonium suggested the auxin involvement in the ammonium repression of root growth (Liu et al., 2013; Zou et al., 2013). Since OsNAR2.1 was repressed when ammonium was provided as only N source (Feng et
al., 2011; Figure 6A), it was not surprising that KO of OsNAR2.1 did not significantly affect the root phenotypes at ammonium supply condition (Figure 6C-H). However, ammonium supply enhanced expression of both OsNIT1 and OsNIT2, and their inactivation repressed acropetal transport of \(^{3}\)H-IAA and limited both PR length and LR densities (Figure 6). The data clearly demonstrated that OsNIT1 and OsNIT2 have independent roles via activation of nitrilase in maintaining root growth which is basically not regulated by OsNAR2.1. There might be other ammonium-induced proteins which activate the enzyme activity of OsNIT1 and/or OsNIT2 for altering the form and transportation of IAA. This speculation is worth to be further characterized in future.

MATERIALS AND METHODS

Plant Materials and Growth Conditions. Rice (Oryza sativa L. ssp. japonica) of the Nipponbare background was used for physiological experiments and rice transformation (mutant constructed by Crispr Cas9 system). The osnit1-1 T-DNA insertion mutant (Line ID PFG_1C-01739.R) with a genetic background of japonica cv. Hwayoung was obtained from PFG. For hydroponic experiments of nearly 1-month duration, which formed the basis of most of the experiments in this paper (except for the 10-day hydroponic experiment described below), rice seeds were surface sterilized in a 30% (v/v) NaClO solution for 30 min, washed, and germinated on 1/2 MS medium for 3 days at 25°C in darkness and for another 5 days in growth-chamber conditions as follows: 14-h light/10-h dark photo cycle, day/night temperatures of 30°C/24°C, and relative humidity of approximately 60%. The air in the growth room was refreshed every 6 h. Hydroponic experiments were performed using the rice normal nutrient solution from IRRI. Nitrate treatment used Ca(NO\(_3\))\(_2\) instead of NH\(_4\)NO\(_3\), whereas ammonium treatment used (NH\(_4\))\(_2\)SO\(_4\) instead of NH\(_4\)NO\(_3\). Twenty seedlings were grown in each culture vessel containing 7.5 L nutrient solution, and the solution was changed every two days. Following germination for 8 days, rice seedlings were initially treated with 1/2 strength nutrient solution for 6 days, then transferred to full-strength culture solution containing either
1.25 mM NH₄NO₃, 1.25 mM Ca(NO₃)₂, or 1.25 mM (NH₄)₂SO₄ for another 7 days before treatments or sampling. For 10-day hydroponic experiments, rice seeds were surface sterilized in a 30% (v/v) NaClO solution for 30 min, washed, and germinated in deionized water for 3 days at 25°C in darkness. Seeds displaying a comparable extent of germination (just had a white tip) were transferred to hydroponic media containing either 0.125 mM Ca(NO₃)₂, 0.125 mM (NH₄)₂SO₄, or other experimental treatments as described in the figure legends for another 7 days, and maintained in the growth-chamber conditions described above. Thirty seedlings were grown in each culture vessel containing nearly 3 L solution, and the solution was changed every two days.

**Pull Down assay.** Details of OsNAR2.1-GST tag fusion protein generation are provided below in the ‘Preparation of recombinant proteins’ section. Target proteins were purified using ProteinIso™ GST Resin (TRANSGEN BIOTECH, DP201, Beijing). Total protein of rice (cv. Nipponbare) roots which were treated with nitrate was extracted with Plant Protein Extraction Kit (CWBIO, CW0885S, Beijing). Total protein was incubated with the OsNAR2.1-GST fusion protein for 4 h at 4°C. Bound proteins were collected and subjected to two-dimensional electrophoresis (2-DE). The gel was recovered and analyzed for each gel point protein.

**Yeast two-hybrid assay.** The interactions between OsNAR2.1 and either OsNIT1 or OsNIT2 were tested using the DUAL membrane pairwise interaction kit (Yan et al., 2011). HIS3 and ADE2 were used as reporter genes in the yeast strain NMY51, with each strain carrying a pair of bait and prey plasmids (pBT3-C and pPR3-N are the control vectors with no cloned cDNA).

Full-length cDNA of *OsNAR2.1* was cloned into pBT3-C (LEU2, KanR) (Liu et al., 2014) and *OsNIT1* and *OsNIT2* cDNAs were cloned into pPR3-N (TRP1, AmpR) (primers detailed in Supplemental Table S3), and expression vectors were co-transformed into yeast strain NMY51 (MATα his3 trp1 leu2 ade2 LYS2::HIS3 ura3::lacZ ade2::ADE2 GAL4) using the DS Yeast transformation kit (Dualsystems
Biotech, Shanghai). Transformed colonies were selected in SD-LW medium and incubated for growth of positive transformants. For growth assays, several independent positive transformants were selected and grown in SD-LW liquid medium at 30°C overnight. Culture concentrations were adjusted to OD546 = 0.8 and diluted 10, 100, and 1000 times. Five microliters of each dilution was spotted on to SD-LW and SD-AHLW solid media, respectively, and incubated at 30 °C for 2.5 days.

**Co-IP assay.** Root protein was extracted from rice (cv. Nipponbare) OsNAR2.1+His and His over-expression lines using a Plant Protein Extraction Kit (CWBIO, CW0885S, Beijing). Total root protein was passed through ProteinIso Ni-NTA Resin (TRANSGEN, DP101, Beijing) to obtain binding protein (Elution Buffer: 300 mM NaCl, 50 mM NaH$_2$PO$_4$, 400 mM imidazole, 10 mM Tris base, pH 8.0). Anti-His analysis for testing OsNAR2.1 interaction with other proteins after IP) was performed by Native Page and all other analyses were SDS-PAGE. Anti-NIT2 was purified from a rabbit injected with a specific peptide chain of OsNIT2 (amino acid sequence EKNSAAKSDGISRT). A portion of the sample was subjected to immunoblot analysis using indicated antibodies, and the remaining sample was incubated with 10 mM DTT, 55 mM ammonium iodoacetate, and 1 µg trypsin for enzymatic hydrolysis overnight. Afterwards, the polypeptide was desalted by a C18 column, then drained and dissolved with 15 ul of Loading Buffer (0.1%(v/v) formic acid, 3%(v/v) acetonitrile). The peptide was analyzed by LC-MS/MS (ekspert™ nanoLC; AB Sciex Triple TOF 5600-plus).

**RNA In Situ Hybridization.** Longitudinal sections of root tips and mature roots of WT (cv. Nipponbare) seedlings with a length of about 10 mm were fixed in FAA solution (1.85% (v/v) formaldehyde, 5% (v/v) acetic acid, and 63% (v/v) ethanol), dehydrated with a mixture of ethanol and 1-butanol, and then embedded in paraffin. The embedded sections were sliced (10-µm thickness) using a microtome (LEICA RM2235). The full-length cDNA sequences of OsNAR2.1, OsNIT1, and OsNIT2 were cloned into pENTR-D-TOPO (primers detailed in Supplemental Table S3). Digoxin
(DIG)-labeled RNA probes in antisense orientation were synthesized using T7 RNA polymerase, with each linearized plasmid DNA used as a template, using the DIG RNA labeling kit as described previously (Ishiyama et al., 2004). RNA in situ hybridization with DIG-labeled RNA probes was performed as previously described (Ishiyama et al., 2004).

RNA extraction, cDNA synthesis, and RT-qPCR. Total RNA was extracted from plant samples using Trizol reagent (Invitrogen, Shanghai) according to the manufacturer’s instructions. First-strand cDNA was synthesized from total RNA using the HIScript II Reverse Transcriptase with gDNA wiper (Vazyme, R223-01, Nanjing). Reverse transcription quantitative PCR (RT-qPCR) was performed using the AceQ qPCR SYBR Green Master Mix (Vazyme, Q111-01, Nanjing) on the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Shanghai) according to the manufacturer’s instructions. Relative expression level of each sample was determined by normalizing it to the amount of OsActin1 (LOC_Os03g50885) detected in the same sample and presented as $2^{-\Delta \Delta CT}$. All primers used for RT-qPCR are detailed in Supplemental Table S3.

Determination of $^{15}$N-NO$_3^-$ uptake rate. Rice seedlings were first planted in IRRI solution containing 1.25 mM NH$_4$NO$_3$, then they were deprived of N for 3 days. Next, plants were first transferred into 0.1 mM CaSO$_4$ for 1 min, then to a complete nutrient solution containing 0.25 mM $^{15}$NO$_3^-$ (Ca$^{(15}$NO$_3^-)_2$) for 5 min, and finally to 0.1 mM CaSO$_4$ for 1 min. Then, we used paper to blot excess water from the plants. The shoots and roots were separated. Root samples were placed in an oven at 105°C for 30 min to inactivate the enzymes, and further dried to a constant weight at 70°C. After recording their dry weights, the samples were ground into powder using a ball mill. The Isotope Ratio Mass Spectrometer system (Flash 2000 HT, Thermo Fisher Scientific, Germany) was used to determine the $^{15}$N content of the samples.

Determination of total IAA and IAN. Samples were ground to a powder with liquid...
nitrogen and freeze-dried. IAA measurement was performed as described below. The above dry powder was added with 1 ng of $[^{13}\text{C}_6]\text{-IAA}$ and extracted three times with 80% (v/v) methanol. After the extraction was concentrated, ethyl acetate containing 5% (v/v) acetic acid was added, and the extraction was collected again. Extractions were freeze-dried once more, following which 10 μl of pyridine and 40 μl of BSTFA were added and reacted at 80°C for 30 min. Finally, 50 μl of n-hexane was added for mass spectrometry using the method of Hor-A-MRM.M. The instrument used was GC-QqQ MS (7890a-5975b, Agilent) and the column was DB-5ms (30 m × 0.25 mm × 0.10 μm, Agilent) (Novák et al., 2012). IAN measurement: The above dry powder was extracted three times with methanol, and the extraction was concentrated, and then subjected to liquid phase analysis by the method of GB/T 16631-2008 high performance liquid chromatography. The instrument used was the Agilent 1200 high performance liquid chromatograph (Sugawara et al., 2009).

$^3\text{H}$-IAA transport assay. Acropetal and basipetal auxin transport was assayed in excised seminal roots as described by Lewis and Munday (2009) with minor modifications. For acropetal auxin transport, agar mixtures containing 0.7% (w/v) agar, 0.04% (v/v) $[^3\text{H}]$-IAA (26.0 Ci/mmol), 10 μM cold IAA, 2% DMSO, and 25 mM MES (pH 5.5) were prepared in a scintillation vial. Following shoot excision 1 cm above the root-shoot junction, 20-μl agar droplets of the $[^3\text{H}]$-IAA solution was applied to the cut surface. After a 6-h incubation in 60–70% humidity at 25°C in darkness, root segments were excised at distances of 0–1, 1–2, and 2–3 cm from the root apex and weighed. Then the root segments were immediately placed in scintillation solution (3 ml) for 12 h. For basipetal auxin transport, 20-μl agar droplets (agar mixtures as described above) of the $[^3\text{H}]$-IAA solution was applied to root segments excised at distances of 0–3, 3–6, 6–9, 9–12, and 12–15 mm distance from the root apex. The root segments were digested with perchloric acid and then immediately placed in scintillation solution (3 ml) for 12 h. The amount of radioactivity of $[^3\text{H}]$ in each sample was determined using a Beckman LS6500.
Preparation of recombinant proteins. To obtain recombinant proteins, the plasmids MBP-OsNIT1, OsNIT2-6×His, and OsNAR2.1-6×His (primers detailed in Supplemental Table S3) were transformed into *E. coli* strain Transetta (DE3) (TRANSGEN BIOTECH, CD801-01, Beijing). The bacterial cells were cultivated at 37°C shaking at 150–200 rpm. At an A$_{600}$ of 0.6–0.8, the bacterial cells were induced by 0.5 mM IPTG at 16°C for 16 h. Cells were collected and disrupted using ultrasonic cell breakers (Fisher Scientific, Model 120, Shanghai) on ice. The recombinant proteins were affinity-purified with amylose resin high flow (NEB, E8022, Beijing) or ProteinIso™ Ni-NTA Resin (TRANSGEN, DP101, Beijing) according to manufacturer’s instructions.

Nitrilase activity assays. Assays were carried out in a volume of 50 μl solution containing 50 mM KPi (pH 8.0), 1 mM DTT, 3 mM ATP (important), 3 mM substrate IAN, and 2 μg for each purified enzyme except for OsNIT1, for which 6 μg was used since the western blot result showed many bands. IAN conversion rate (consumed IAN content / total IAN content × 100%) was used as a measure of enzymatic activity as analyzed by HPLC. Reaction time was 1–2 h at 28°C, with 200 μl methanol added to stop reactions. An aliquot (15 μl) of the diluted sample was injected into the HPLC system (Agilent 1200LC) equipped with a ZORBAX C18 SB-Aq column. The flow rate was 0.8 ml/min, and the sample was eluted with 0.1% (v/v) H$_3$PO$_4$ (5 min), followed by a linear methanol gradient to 40% (v/v) methanol in 7 min and holding at this composition for an additional 18 min. The column effluent was monitored at 280 nm. Under these conditions, the retention times of IAA and IAN were 23.161 and 28.684 min, respectively.

Subcellular Localization. For subcellular localization constructs, the full-length open reading frames (ORFs) of *OsNIT1* and *OsNIT2* were amplified and subcloned into the intermediate vectors pSAT6A-EGFP-N1 and pSAT6-EGFP-C1 to generate OsNIT1-GFP, GFP-OsNIT1, OsNIT2-GFP, and GFP-OsNIT2 vectors. All vectors were introduced into the final expression vector pRCS2-ocs-nptII with the rare cutter.
PI-PspI. The constructs were transformed into rice protoplasts by the polyethylene glycol-mediated method. The isolation and transformation of rice protoplast was performed as described previously (Jia et al., 2011). In brief, 10 μg plasmid DNA of each construct was transformed into 0.2-ml protoplast suspension. HDEL fusion mCherry served as an endoplasmic reticulum marker. After incubation at 28°C in darkness for 12–15 h, fluorescence signals in rice protoplasts were detected. Confocal microscopy images were taken using a TCS SP8 X confocal laser scanning microscope (Leica). Excitation/emission wavelengths were 488 nm/495–556 nm for GFP and 587 nm/600–650 nm for mCherry.

Accession Numbers
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: LOC_Os02g42350 (OsNIT1), LOC_Os02g42330 (OsNIT2), LOC_Os02g38230 (OsNAR2.1), LOC_Os02g02170 (OsNRT2.1), LOC_Os01g50820 (OsNRT2.3), LOC_Os06g12610 (OsPIN1a), LOC_Os02g50960 (OsPIN1b), LOC_Os11g04190 (OsPIN1c), LOC_Os12g04000 (OsPIN1d), LOC_Os06g44970 (OsPIN2), LOC_Os01g55940 (OsGH3-2), LOC_Os07g40290 (OsGH3-8), LOC_Os11g32520 (OsGH3-13).

SUPPLEMENTAL DATA
Supplemental Figure S1. OsNAR2.1 interacts with OsNIT1 and OsNIT2.
Supplemental Figure S2. Subcellular Localization Analysis of OsNIT1 and OsNIT2.
Supplemental Figure S3. Characterization of different genotypes used in this paper.
Supplemental Figure S4. Gene expression pattern of OsNIT1 in osnit2 and OsNIT2 in osnit1.
Supplemental Figure S5. Selection of appropriate IAN treatment concentration.
Supplemental Figure S6. Extracted IAN was not detectable in the seedlings of Nipponbare wild-type and expression levels of OsPIN1a and OsPIN1b were unchanged in osnit1 or osnit2.
Supplemental Figure S7. The enzyme activity of OsNIT1 and OsNIT2 for IAN hydrolysis.

Supplemental Figure S8. The kinetics of IAN conversion rate to IAA by NIT enzyme.

Supplemental Table S1. List of proteins identified by pull-down assay.

Supplemental Table S2. The enzyme activity for conversion of IAN to IAA at four substrate concentration ranges after a 2-h reaction time.

Supplemental Table S3. The primers used in this paper.

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FIGURE LEGENDS

Figure 1. OsNAR2.1 interacts with OsNIT1 and OsNIT2 following nitrate supply to roots.

A, Interaction test of OsNAR2.1 with OsNIT1 and OsNIT2 using the DUAL membrane pairwise interaction kit. B, Interaction test of OsNAR2.1 with OsNIT1 and OsNIT2 using Co-IP. OE11 and OE16, two independent lines over-expressing OsNAR2.1 with 6×His tag; Negative, control line segregated from OsNAR2.1 with 6×His tag over-expression lines; pUbi::His, control line over-expressing 6×His tag only. Immunoblots were developed with anti-His antibody to detect OsNAR2.1 expression and with anti-NIT2 to detect OsNIT2 expression. Anti-HSP used as positive control. C and D, Analysis of OsNAR2.1, OsNIT1, and OsNIT2 expression patterns in the roots of rice seedings (cv. Nipponbare) supplied with different concentration of Ca(NO₃)₂.
OsNIT2 revealed by RNA in situ hybridization in root tip and mature root area supplied with 2.5 mM nitrate. All detection probes were based on the whole anti-sense strand. Negative control materials were incubated with water. Scale bar in mature is 500 μm; scale bar in root tip is 200 μm. D, Relative expression levels of OsNAR2.1, OsNIT1, and OsNIT2 in the roots treated with different nitrate concentrations as quantified by RT-qPCR. OsActin (LOC_Os03g50885) was used as an internal control. The values in D represent means ± SE of three biological replicates.

**Figure 2. Knockout of OsNIT1 or OsNIT2 results in the same root phenotype as the osnar2.1 mutant following nitrate supply but does not alter root nitrate uptake rate.**

A, B, C, and D, Seeds were first germinated in deionized water for 3 days, then were transferred to hydroponic media containing 0.125 mM Ca(NO₃)₂ for a further 7 days. HY, Hwayoung; Nip, Nipponbare; WT, wild type; nit1-1, the knockout (KO) mutant of OsNIT1 in the Hwayoung genetic background; nit1-2, nit1-3, nit2-1, nit2-2, nit2-3, nar2.1-1, nar2.1-2, and nar2.1-3, individual KO mutant lines of OsNIT1, OsNIT2, and OsNAR2.1 genes in the Nipponbare genetic background. A, Root phenotype of different lines. Scale bar, 1 cm. B, Effect of OsNAR2.1 KO on expression levels of OsNIT1 and OsNIT2 in roots analyzed by RT-qPCR. OsActin was used as an internal control. C and D, PR, primary root; LR, lateral root. Others are same to A. The values in B, C, and D represent means ± SE of three (B) and six (C and D) biological replicates, respectively (***P≤0.01). E and F, Seeds were germinated and grown under the same conditions as in A. nit2×nar2.1, homozygous hybrid of nit2-2 (abbreviated as nit2 in E and F) and nar2.1-1 (abbreviated as nar2.1 in E and F). Others are the same to C and D. Scale bar in E is 1 cm. The values in F represent means ± SE of seven biological replicates (*P≤0.05, ** P≤0.01). G, Relative expression levels of OsNAR2.1, OsNRT2.1, and OsNRT2.3 in the roots of Nipponbare wild-type (WT) and the OsNIT1 and OsNIT2 KO mutants supplied with 1.25 mM Ca(NO₃)₂ for one week. H, The ¹⁵N absorption rate per root unit weight and total ¹⁵N content of WT, osnit1, and osnit2 mutants supplied with 0.125 mM Ca(¹⁵NO₃)₂ for 5 minutes. The values
represent means ± SE of three biological replicates (*$P \leq 0.05$); ns: not significant.

**Figure 3.** Knockout of *OsNIT1* or *OsNIT2* decreases root growth sensitivity to indole-3-acetonitrile, but not to naphthylacetic acid.

Seeds were first germinated in deionized water for three days, then were transferred to the hydroponic media containing 0.125 mM Ca(NO$_3$)$_2$ with 10 μM IAN or 0.1 μM NAA for another 7 days before being photographed and sampled for the measurement of root growth and LR density. The genotypes are the same as those used and described in Figure 2A. IAN: indole-3-acetonitrile; NAA: naphthylacetic acid. Scale bar, 1 cm. Others are same to Figure 2. The data in B and C represent means ± SE of six biological replicates (*$P \leq 0.05$, **$P \leq 0.01$); ns: not significant.

**Figure 4.** Knockout of *OsNIT1* or *OsNIT2* alters expression of auxin efflux transporter *OsPIN* genes and IAA amido synthetase *OsGH3* genes, and decreases acropetal IAA transport, but does not affect root total IAA concentration.

A and D, Wild type (WT; cv. Nipponbare), *osnit1*, and *osnit2* plants were grown in IRRI solution containing 1.25 mM Ca(NO$_3$)$_2$ for one week before being sampled for gene expression and IAA concentration analyses. A, Total IAA concentration in the roots. The roots of five seedlings were taken and mixed as one sample. B and C, WT, *osnit1*, *osnit2*, and *osnar2.1* plants were grown in IRRI solution containing 1.25 mM Ca(NO$_3$)$_2$ for 10 days before being sampled for [3H]IAA transport analysis. B, Acropetal transport; C, Basipetal transport. D, Relative expression levels of auxin efflux transporter *OsPIN* genes and IAA amido synthetase *OsGH3* genes quantified by RT-qPCR. *OsActin* was used as an internal control. Data represent means ± SE of three biological replicates (* and lowercase letters represent statistically significant difference at $P \leq 0.05$ level); ns, not significant.

**Figure 5.** Co-presence of OsNAR2.1, OsNIT1m, and OsNIT2 improves OsNIT1-mediated IAN hydrolysis.

A, Interaction test between OsNIT1 and OsNIT2 using the DUAL membrane pairwise
interaction kit. B, OsNIT1 and OsNIT2 enzyme activity. IAN conversion rate
(consumed IAN content / total IAN content × 100%) was used as a measure of
enzymatic activity by HPLC. Reaction time was 1 h at 28°C. Both the 6×His and
MBP tag proteins were used as negative controls. The data represent means ± SE of
four biological replicates (different lowercase letters represents statistically significant
difference at $P \leq 0.05$ level).

Figure 6. Knockout of OsNIT1 and OsNIT2 decreases both primary root and
lateral root growth following supply of ammonium.

A and B, Expression levels and patterns of OsNAR2.1, OsNIT1, and OsNIT2 in the
roots of rice seedlings (cv. Nipponbare) in response to growth in IRRI solution
containing 1.25 mM NH$_4$NO$_3$ (AN) or 1.25 mM (NH$_4$)$_2$SO$_4$ (A). A, Relative gene
expression levels under the above experimental conditions quantified by RT-qPCR.
OsActin was used as an internal control. The values in A represent means ± SE of
three biological replicates. B, Expression patterns of OsNAR2.1, OsNIT1, and OsNIT2
determined by RNA in situ hybridization in root tips and mature root area supplied
with 2.5 mM ammonium. All detection probes were based on the whole anti-sense
strand. Scale bar in mature is 500 μm; scale bar in root tip is 200 μm. C, D, E, and F,
Seeds were first germinated in deionized water for three days, then transferred to
hydroponic media containing 0.125 mM (NH$_4$)$_2$SO$_4$ for another 7 days. Genotypes are
the same as those used and described in Figure 2A. C, Scale bar, 1 cm. D, Effect of
OsNAR2.1 knockout (KO) on expression levels of OsNIT1 and OsNIT2 in roots
determined by RT-qPCR. OsActin was used as an internal control. C and D, PR,
primary root; LR, lateral root. Others are the same to Figure 2A. The values in D, E,
and F represent means ± SE of three (D) and six (E and F) biological replicates
(***$P \leq 0.01$). G and H, Seeds were germinated and grown under the same conditions as
described for C. Genotypes are the same as those used and described in Figure 2E.
Others are same to Figure 2C and 2D. Scale bar in G is 1 cm. The values in H
represent means ± SE of seven biological replicates (***$P \leq 0.01$; ns: not significant. I
and J, Wild type (WT; cv. Nipponbare) and KO mutant plants were grown in IRRI
solution containing 1.25 mM (NH₄)₂SO₄ for 10 days before being sampled for [³H]IAA transport analysis. I, Acropetal transport; J, Basipetal transport. Data represent means ± SE of three biological replicates (P<0.05); ns: not significant.

Figure 7. Model for the role of OsNAR2.1, OsNIT1, and OsNIT2 in mediating root growth in rice.

Interaction of OsNAR2.1 with OsNRT2.1, OsNRT2.2, and OsNRT2.3a and their effect on root nitrate uptake were previously reported (Feng et al., 2011; Yan et al., 2011; Liu et al., 2014). All other noted functions are demonstrated in this study.
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