Nitric Oxide Affects Rice Root Growth by Regulating Auxin Transport Under Nitrate Supply

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Nitrogen (N) is a major essential nutrient for plant growth, and rice is an important food crop globally. Although ammonium (NH$_4^+$) is the main N source for rice, nitrate (NO$_3^-$) is also absorbed and utilized. Rice responds to NO$_3^-$ supply by changing root morphology. However, the mechanisms of rice root growth and formation under NO$_3^-$ supply are unclear. Nitric oxide (NO) and auxin are important regulators of root growth and development under NO$_3^-$ supply. How the interactions between NO and auxin in regulating root growth in response to NO$_3^-$ are unknown. In this study, the levels of indole-3-acetic acid (IAA) and NO in roots, and the responses of lateral roots (LRs) and seminal roots (SRs) to NH$_4^+$ and NO$_3^-$, were investigated using wild-type (WT) rice, as well as osnia2 and ospin1b mutants. NO$_3^-$ supply promoted LR formation and SR elongation. The effects of NO donor and NO inhibitor/scavenger supply on NO levels and the root morphology of WT and nia2 mutants under NH$_4^+$ or NO$_3^-$ supply suggest that NO$_3^-$-induced NO is generated by the nitrate reductase (NR) pathway rather than the NO synthase (NOS)-like pathway. IAA levels, [$^3$H] IAA transport, and PIN gene expression in roots were enhanced under NO$_3^-$ relative to NH$_4^+$ supply. These results suggest that NO$_3^-$ regulates auxin transport in roots. Application of SNP under NH$_4^+$ supply, or of cPTIO under NO$_3^-$ supply, resulted in auxin levels in roots similar to those under NO$_3^-$ and NH$_4^+$ supply, respectively. Compared to WT, the roots of the ospin1b mutant had lower auxin levels, fewer LRs, and shorter SRs. Thus, NO affects root growth by regulating auxin transport in response to NO$_3^-$ supply. Overall, our findings suggest that NO$_3^-$ influences LR formation and SR elongation by regulating auxin transport via a mechanism involving NO.

Keywords: auxin, nitrate (NO$_3^-$), nitric oxide (NO), rice, root

INTRODUCTION

Nitrogen (N) is a major essential nutrient for plant growth (Stitt, 1999). Ammonium (NH$_4^+$) and nitrate (NO$_3^-$) are the major sources of N for plants (Kronzucker et al., 2000). Rice (Oryza sativa L.) is a major staple food globally. NH$_4^+$ is the main form of N in paddy soil (Wang et al., 1993). Rice roots are exposed to both NH$_4^+$ and NO$_3^-$, and are efficient at acquiring NO$_3^-$ by nitrification in the rhizosphere (Kirk and Kronzucker, 2005; Duan et al., 2007). It has been predicted that 40% of the total N acquired by rice roots grown under flooded conditions is NO$_3^-$ (Kronzucker et al., 2000; Kirk and Kronzucker, 2005). However, most previous studies on root
growth and formation in response to NO$_3^-$ have focused on upland plants such as *Arabidopsis* and maize, and so further work is needed.

Plants have various mechanisms to adapt to NO$_3^-$ supply, such as plasticity of root growth (Patterson et al., 2016; Shahzad and Amtmann, 2017; Sun C. et al., 2017). Localized NO$_3^-$ supply stimulates the initiation and/or elongation of lateral roots (LRs) (Drew and Saker, 1975; Zhang and Forde, 1998; Friml et al., 2003). In *Arabidopsis*, the local stimulation of LR growth is caused by NO$_3^-$ functioning as a signal rather than as a nutrient (Zhang and Forde, 1998). Nitrate transporters, transcription factors, and micro-RNAs regulate root growth and formation in response to NO$_3^-$ (Remans et al., 2006; Vidal et al., 2010; Trevisan et al., 2011, 2012; Zhao et al., 2012, 2013; Alvarez et al., 2014; Yan et al., 2014; Huang et al., 2015). For example, NRT1.1, which encodes an NO$_3^-$ transporter, reportedly regulates the stimulatory effects of NO$_3^-$ on LR growth and development (Zhang and Forde, 1998; Zhang et al., 1999; Remans et al., 2006). AINRT2.1 is involved in the response of roots to low NO$_3^-$ supply, mainly in LR formation (Little et al., 2005; Remans et al., 2006). Knockdown of OsNAR2.1, a partner protein of the high-affinity nitrate transporter, inhibits LR formation in response to nitrate (Huang et al., 2015). NO$_3^-$ regulates root growth by posttranscriptional regulation of the NRT1.1/NPF6.3 (Bouguyon et al., 2016). NPF7.3/NRT1.5, a nitrate transporter, is involved in LR formation in *Arabidopsis* (Zheng et al., 2016). miR444a plays key roles in nitrate-dependent LR elongation and nitrate accumulation by downregulating the expression of ANR1-like genes in the NO$_3^-$ signalling pathway in rice (Yan et al., 2014). miR393/AFB3, an NO$_3^-$-responsive module, regulates LR density in response to external and internal N concentrations in *Arabidopsis* (Vidal et al., 2010; Vidal et al., 2013). The transcript levels of four ANR1-like genes, OsMADS25, OsMADS27, OsMADS57 and OsMADS61, as well as TGA1/TGA4 and CPC, are influenced by NO$_3^-$ supply and regulate root growth and formation (Yu et al., 2014; Canales et al., 2017; Sun et al., 2018). However, how plants sense external nitrate and the signal transduction system that influences root system development are remain unclear.

In addition to environmental conditions, the root growth of plant is regulated by plant hormones, such as auxin. Most auxin is synthesized in aboveground tissues by *YUCCA* family genes (Stepanova et al., 2011; Zhao, 2012) and is transported by auxin carriers, such as AUX1/LAX family (auxin-influx carriers), and ABCB/PGP and PIN family (auxin-efflux carriers) (Friml, 2003; Friml et al., 2003; Blakseele et al., 2005; Zazimalova et al., 2010; Peret et al., 2012; Bhosale et al., 2018; Giri et al., 2018). Auxin plays a key role in root growth in response to NO$_3^-$ (Zhang et al., 1999; Zhang and Mi, 2005; Krouk et al., 2010). Localized NO$_3^-$ supply does not stimulate LR elongation in axr4, an auxin-insensitive mutant, which suggests that NO$_3^-$ regulates LR growth via auxin signaling pathways (Zhang et al., 1999). The NO$_3^-$ and auxin signaling pathways are linked by their effect on auxin transport through AtNRT1.1 (Krouk et al., 2010). Liu et al. (2010) suggested that in LRs, NO$_3^-$-fed compartments have lower auxin levels than NO$_3^-$-free compartments, and localized NO$_3^-$ supply inhibits auxin transport from shoot to root in maize. Knockdown of OsNAR2.1 decreases LR formation by inhibiting auxin transport from shoots to roots (Huang et al., 2015). However, the roles of auxin transport in regulating LR growth under NO$_3^-$ supply are more complex.

Nitric oxide (NO), as a signaling molecule, is involved in the growth and formation of the root system under NO$_3^-$ supply (Manoli et al., 2014; Trevisan et al., 2014; Sun et al., 2015; Kan et al., 2016). NO synthase-like (NOS-like) and nitrate reductase (NR) are the two key NO production pathways in plants. The NOS of plant has not been identified (Crawford, 2006; Moreau et al., 2008, 2010; Gas et al., 2009; Gupta et al., 2011), although studies that have used inhibitors of the animal NOS enzyme have demonstrated the involvement of the L-arginine pathway in the production of NO (Zhao et al., 2007). Moreau et al. (2008) suggested that *Arabidopsis* AtNOS1 does not possess NOS activity, as it is a GTPase, and renamed it NO-associated enzyme (AtNOA1). Despite the lack of clarity on the role of AtNOS, the roots of noa1 mutants (formerly Atnos1) have lower NO levels than WT (Guo and Crawford, 2005; Schlicht et al., 2013). In plants, the NR pathway mediates NO generation, and the nitrate concentration in roots influences the production of NO by regulating NR activity (Yamasaki et al., 1999; Meyer et al., 2005; Yamasaki, 2005). The levels of nitrate and nitrite are important determinants of NR-induced NO generation (Vanin et al., 2004). NO is a nitrate-related signal generated by the NR pathway that regulates root growth and formation (Zhao et al., 2007; Manoli et al., 2014; Trevisan et al., 2014; Sun et al., 2015). However, the mechanism by which NO regulates the root system architecture requires further investigation.

The interactions between NO and auxin in regulating root growth are closely linked (Correa-Aragunde et al., 2004; Fernández-Marcos et al., 2011; Jin et al., 2011; Chen and Kao, 2012; Sun H. et al., 2017). Application of SNP (a NO donor) and IAA/IBA (exogenous auxin) increased the lateral root (LR) formation. This effect of SNP and IBA were significantly inhibited by cPTIO (a NO scavenger) (Jin et al., 2011; Chen and Kao, 2012; Sun H. et al., 2017), suggesting that NO may act downstream of auxin in regulation of LR development. However, the interaction between NO and auxin in regulating root elongation is different from affecting LR formation. NO inhibited the elongation of roots by decreasing acropetal auxin transport in *Arabidopsis* and rice (Fernández-Marcos et al., 2011; Sun H. et al., 2017), suggesting that the interactions between auxin and NO in regulating root growth are complex and unclear.

Rice, an important food crop globally, is an ideal model for studying plant root growth because of its small genome size and availability of its complete genome sequence and well-characterized related mutants (Feng et al., 2002; Sasaki et al., 2002). In this study, we evaluated LR formation and the length of seminal roots (SRs) of rice and measured auxin concentrations, *DR5::GUS* activity, [H] indole-3-acetic acid (IAA) transport, and NO levels under NH$_4^+$ and NO$_3^-$ supply. The results suggest that NO influences rice root growth by regulating auxin transport in response to NO$_3^-$. 

MATERIALS AND METHODS

Plant Materials

The Nipponbare and Dongjin (DJ) ecotype of rice were used in this study. osnia2-1 and osnia2-2 mutant lines (Sun et al., 2016) and ospin1b-1 and ospin1b-2 mutant lines (Sun H. et al., 2017) with the japonica cv. Dongjin ecotype were also used.

Plant Growth

Rice seedlings were grown at day/night temperatures of 30°C/18°C under natural light in a greenhouse. Seven-days-old seedlings of uniform size and vigor were transplanted into holes in a lid placed over the top of pots (four holes per lid and three seedlings per hole). Nutrient solutions ranging from one fourth (2 days), one third (2 days), and a half (2 days) to full strength (1 day) were applied for 1 week, followed by full-strength nutrient solution for 1 week. The chemical composition of International Rice Research Institute (IRRI) nutrient solution was (mM): 2.5 (NH$_4^+$), 0.3 KH$_2$PO$_4$, 0.35 K$_2$SO$_4$, 1.0 CaCl$_2$, 1.0 MgSO$_4$-7H$_2$O, 0.5 Na$_2$SiO$_3$; and (µM) 9.0 MnCl$_2$, 0.39 (NH$_4$)$_2$Mo$_7$O$_{24}$, 20.0 H$_3$BO$_3$, 0.77 ZnSO$_4$, and 0.32 CuSO$_4$ (pH 5.5).

The treatments applied were as follows: 100 nM indole-3-acetic acid (IAA), auxin transport inhibitor 300 nM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), and 100 µM L-NAME (NG-nitro-arginine methyl ester) (Sun H. et al., 2017).

Root System Architecture

The previous experiments (Sun et al., 2014) and the preliminary experiments suggested that the elongation of root (seminal root and adventitious root) and the lateral root (LR) number of seminal root/adventitious root was increased under NO$_3^-$ and NO$_2^-$ supply. The seminal root here is the first and longest root from embryo and functions mainly during the early stages of rice. Therefore, SRs and the numbers of LRs on SRs were used to evaluate the effects of NH$_4^+$ and NO$_3^-$ on the root system. The length of SR was measured with a ruler. LRs were enumerated visually. To visualize the formation of LR primordia, pDR5::GUS, a specific reporter that contains seven repeats of a synthetic auxin response element and reflects in vivo auxin levels (Ulimasov et al., 1997), were transformed into rice plants. After staining roots in β-glucuronidase (GUS) buffer for 2 h, LR primordia were enumerated using a stereomicroscope (Olympus SZX16) according to Sun H. et al. (2017). All experiments included eight replicates.

Determination of Total N Concentration

The shoots and roots were separated from rice plants, and heated at 105°C for 30 min to kill the enzyme activities, followed by desiccation at 70°C for 48 h to a constant weight. The desiccated samples were ground into powder, and about 0.05 g of the powder was digested using 5 mL of 98% H$_2$SO$_4$ and about 1 mL of 30% H$_2$O$_2$ at 270°C for 30 min. The digested liquid was diluted to 100 mL with distilled water after cooling. The total N concentration of rice plants was analyzed using the Kjeldahl method. A 5 mL aliquot from the 100 mL digested liquid was determined by a colorimetric continuous flow analysis (Autoanalyzer 3; Bran+Luebbe, Germany) (Li et al., 2008). All experiments included eight replicates.

Determination of IAA Levels

Indole-3-acetic acid levels of roots were determined as described previously (Lu et al., 2009). Fresh samples (0.5 g) were frozen in liquid N$_2$. IAA levels were analyzed by high-performance liquid chromatography (HPLC).

To assess auxin distribution, rice plants were transformed with the pDR5::GUS constructs using Agrobacterium tumefaciens (strain EHA105). The roots were subjected to GUS staining. Stained plant tissues were photographed using a stereomicroscope (Olympus SZX16) equipped with a color CCD camera. All experiments included eight replicates.

[^H]IAA-Transport

Shoot-to-root auxin transport in rice plants was assayed according to Song et al. (2013).[^H]IAA polar transport was assayed in root samples under NH$_4^+$ and NO$_3^-$ supply. The[^H]IAA solution contained 0.5 µM[^H]IAA (20 Ci mmol$^{-1}$) in 2% dimethyl sulfoxide (DMSO), 25 mM MES (pH 5.2), and 0.25% agar.

Shoot to root auxin transport in intact plants was monitored as follows.[^H]IAA solution (20 µL) was applied to the cut surface after rice shoots were removed at 2 cm above the junction of shoot and root. After an 18 h (overnight) incubation in darkness, two root segments, namely all the lateral root (LR) region and the root tip (RT), were weighed and incubated in 4 mL of scintillation solution.[^H]IAA radioactivity was detected using a multipurpose scintillation counter (LS6500; Beckman-Coulter, Fullerton, CA, United States).

The assay for acropetal (3–6 cm from the root tip) and basipetal (0–3 cm from the root tip) auxin transport was performed.[^H]IAA solution (3 µL) was applied to the root tip placed horizontally on a plastic film. After incubation in a humid, dark environment for 18 h (overnight), root segments were cut into two parts: (1) the distal 1 cm from the root tip and (2) the remaining 2 cm.[^H]IAA radioactivity was measured in the 2 cm long segments. All experiments included five replicates.

Cortical Cell Length Analysis

Cortical cell length was analyzed as described by Jia et al. (2008). Cortical cells were visualized under a microscope (Olympus SZX16) equipped with a color CCD camera. The average cortical cell length of the maturation zone of SRs was determined using a mixture of 40–60 cortical cells at about 6 cortical cell layers (on per longitudinal section) with eight replicates in the maturation zone.

pCYCB1;1::GUS Construct

The pCYCB1;1::GUS fusion construct was generated as described by Colón-Carmona et al. (1999), and transformed into rice plants.
Plants were stained for GUS activity in the root tips (RTs) for 2 h at 37°C. The RTs were subjected to histochemical GUS staining and photographed using a microscope (Olympus SZX16) equipped with a color CCD camera. All experiments included eight replicates.

**Measurement of NO Levels in Roots**
Nitric oxide was imaged by staining with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) under an epifluorescence microscope. The roots were soaked with 10 µM DAF-FM DA in 20 mM HEPES-NaOH buffer (pH 7.5) for 30 min in the dark. The roots were washed three times in fresh buffer and immediately visualized with a stereomicroscope (Olympus SZX16; excitation 488 nm, emission 495–575 nm) equipped with a color CCD camera. Green fluorescence intensity was quantified as described by Guo and Crawford (2005) using Photoshop software (Adobe Systems, San Jose, CA, United States). All experiments included eight replicates.

**Measurement of Nitrate Reductase (NR) Activity in Roots**
Nitrate reductase activity in rice roots was analyzed by Ogawa et al. (1999). The assay mixture contained 25 mM K3PO4 buffer (pH 7.5), 10 mM KNO3, 0.2 mM NADH, 5 mM NaHCO3, and 5 µL extract in a final volume of 0.5 mL. The assays were conducted at 30°C for 15 min. The reaction was terminated by adding 50 µL of 0.5 M Zn(CH3COO)2, and excess NADH was oxidized by adding 50 µL of 0.15 mM phenazine methosulphate. The mixture was centrifuged at 10,000 × g for 5 min. The NO2− level was quantified by combining 500 µL supernatant with 250 µL of 1% sulfanilamide prepared in 1.5 N HCl and 250 µL of 0.02% N-(1-naphthyl)ethylene-diamine dihydrochloride, and the absorbance at 540 nm was read using a spectrophotometer. All experiments included five replicates.

**Quantitative Reverse Transcription-Polymerase Chain Reaction**
Total RNA was isolated from the roots of rice plants under NH4+ or NO3− supply for 14 days. The RNA extraction, reverse transcription, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) methods were as described by Jia et al. (2011). All experiments with three replicates. The primer sets for PINs, YUCCAs, NOA, NIA1, NIA2, and CYCB1;1 are listed in Supplementary Tables 1–3.

**Data Analysis**
Data were pooled to calculate means and standard errors (SEs) and subjected to one-way analysis of variance (ANOVA), followed by a Ryan–Eynot–Gabriel–Welch F-test at P < 0.05 to determine the statistical significance of differences between treatments. All statistical evaluations were conducted using SPSS (version 11.0) statistical software (SPSS Inc., Chicago, IL, United States). All experiments included three independent biological replicates.

**RESULTS**
**NO3− Regulates LR Formation and SR Elongation**
Compared to under NH4+ supply, the number of LRs and SR length were increased by 28 and 20%, respectively, under NO3−.

**FIGURE 1** The lateral root (LR) number and seminal root (SR) length in wild-type (Nipponbare) rice seedlings. Seedlings were grown in hydroponic media containing NH4+ and NO3− for 14 days. (A) The morphology of the rice plants; (B) LR number; (C) SR length. Data are means ± SE and bars with different letters indicate significant difference at P < 0.05 tested with ANOVA.
supply (Figure 1). However, the total N concentration in shoots and roots were decreased by about 20% under NO$_3^-$ relative to under NH$_4^+$ supply. These results suggest that the root growth and total N concentration of rice plants are regulated by NO$_3^-$ (Supplementary Figure 1).

**NO Is Generated by the NR Pathway and Is Involved in LR Formation and SR Elongation Under NO$_3^-$ Supply**

To determine whether NO regulates LR formation and SR elongation under NO$_3^-$ supply, we analyzed NO-associated green fluorescence in SRs (LR region and RT) (Figures 2A,B). Compared to NH$_4^+$, NO-associated green fluorescence signals in RTs and the LR regions were stronger under NO$_3^-$ supply, which suggests that production of NO in roots is induced by NO$_3^-$.

We examined the functions of an NO donor (SNP) and NO scavenger (cPTIO) in root elongation and LR formation under NO$_3^-$ supply. Application of SNP under NH$_4^+$ supply significantly increased the NO-associated green fluorescence signal in SRs, the number of LRs, and the SR length to levels similar to those under NO$_3^-$ supply (Figure 3). However, the number of LRs, and the SR length did not respond to SNP under NO$_3^-$ supply (Supplementary Figure 2). Treatment with cPTIO under NO$_3^-$ supply markedly decreased the NO-associated green fluorescence signal, the number of LRs, and the SR length (Figure 3). Thus, NO production in rice roots is enhanced by NO$_3^-$ and is involved in LR formation and SR elongation.

Nitrate reductase activity in rice roots was assessed under NH$_4^+$ and NO$_3^-$ supply. NR activity increased by 119% in roots under NO$_3^-$ supply relative to NH$_4^+$ supply (Figure 2C). The expression of NIA2 was significantly higher under NO$_3^-$ supply than under NH$_4^+$ supply. However, compared with NIA2, the expression of NIA1 had less differences between NH$_4^+$ and NO$_3^-$ . The transcript level of NO-associated (NOA) (a homolog of NOA1 in Arabidopsis) in roots was similar under NH$_4^+$ supply and NO$_3^-$ supply (Figure 2D). These results suggest that NO generation is enhanced by NR rather than the NOS-like pathway under NO$_3^-$ supply.

Application of the NR inhibitor Tu (25 µM) decreased the NO-associated green fluorescence signal, the number of LRs, and the SR length under NO$_3^-$ . However, treatment of rice plants with the NOS inhibitor L-NAME (100 µM) under NO$_3^-$ supply did not influence any of the parameters (Figure 3). These results confirm that NO is generated by NR rather than NOS-like under NO$_3^-$ supply.

### Figures

**FIGURE 2** | Accumulation of nitric oxide (NO), relative nitrate reductase (NR) activity, and qRT-PCR analysis of NO-associated (NOA), NIA1, NIA2 genes in wild-type (Nipponbare) rice seedlings. Seedlings were grown in hydroponic medium containing NH$_4^+$ and NO$_3^-$ for 14 days. **(A)** Photographs of NO production shown as green fluorescence in the lateral root (LR) and root tip (RT); **(B)** NO production expressed as fluorescence intensity relative to the roots; **(C)** Relative nitrate reductase (NR) activity in the roots; **(D)** Relative expression of NOA, NIA1 and NIA2 in roots. Bar = 1 mm. Data are means ± SE and bars with different letters indicate significant difference at $P < 0.05$ tested with ANOVA.
FIGURE 3 | Accumulation of nitric oxide (NO) and root morphology in wild-type (Nipponbare). Seedlings were grown in hydroponic medium containing NH$_4^+$ and NO$_3^-$ in addition to SNP (10 µM), cPTIO (100 µM), Tu (25 µM) and L-NAME (100 µM) for 14 days. (A–C), Photographs of NO production shown as green fluorescence in the lateral root (LR) and root tip (RT) (A), and NO production expressed as fluorescence intensity relative to lateral root (B) and root tip (C); (D), Lateral root (LR) number; (E), Seminal root (SR) length. Bar = 1 mm. Data are means ± SE and bars with different letters indicate significant difference at $P < 0.05$ tested with ANOVA.

The *osnia2-1* and *osnia2-2* mutant lines have reduced NR activity (Sun et al., 2016). All parameters of both nia2 mutant lines were similar to those of WT plants under NH$_4^+$ supply, but significantly lower under NO$_3^-$ supply (*Figure 4*). Application of SNP to nia2 mutants under NO$_3^-$ supply increased the number of LRs and the SR length to levels similar to those in the WT (Supplementary Figure 3). Moreover, treatment of WT with Tu decreased the number of LRs and SR length to levels similar to those in the nia2 mutants (Supplementary Figure 3), confirming that NO is produced via the NIA2-dependent NR pathway under NO$_3^-$ supply.

**Auxin Levels in Roots Are Regulated by NO$_3^-$**

We measured endogenous IAA concentrations in the LR region and RT. The endogenous IAA concentrations were 75 and 91% higher in the LR region and RT, respectively, under NO$_3^-$ relative to NH$_4^+$ (Figure 5A). We investigated the effects of NH$_4^+$ and NO$_3^-$ on auxin status in rice with transgenic plants transformed with the *pDR5::GUS* constructs. *DR5::GUS* activity was more widely distributed in the LR region and RT under NO$_3^-$ relative to NH$_4^+$ supply (Figure 5C). This was consistent with the IAA concentration results. [H]$^3$ IAA transport from shoots to
roots was significantly higher in roots under NO$_3^-$ relative to NH$_4^+$ supply. Basipetal transport and acropetal transport of [$^3$H]IAA were higher under NO$_3^-$ relative to NH$_4^+$ supply (Figures 5B,D). Therefore, polar auxin transport was increased under NO$_3^-$ supply.

**Auxin Is Involved in SR Elongation and LR Formation**  
We examined the number of LRs and the SR length after application of IAA and NPA (Figure 6). Application of IAA (100 nM) under NH$_4^+$ supply increased DR5::GUS expression in roots, the number of LRs, and the SR length to levels similar to those under NO$_3^-$ supply. The effects of application of IAA (100 nM) on DR5::GUS expression in roots and root morphology was of lesser magnitude under NO$_3^-$ supply (Figure 6). These results suggest that SR elongation and LR formation are regulated by auxin transport under NO$_3^-$ supply.

**Expression of OsPIN Family Genes and Root Morphology of Spin1b Mutants**  
We analyzed the expression of the PIN1-10 auxin transport genes in roots (Figure 7). Compared to under NH$_4^+$ supply, the expression levels of PIN genes in roots were upregulated under NO$_3^-$ supply (Figure 7). The expression level of OsPIN1b is the highest of the nine OsPIN genes in rice root (Wang et al., 2009; Sun H. et al., 2017). Therefore, OsPIN1b was used as a target gene in subsequent analyses.

The spin1b-1 and spin1b-2 mutant lines have reduced auxin levels in LRs and the RT (Sun H. et al., 2017). The IAA concentration in roots of the spin1b-1 mutant did not differ

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**FIGURE 4** Root morphology and accumulation of nitric oxide (NO) in nia2 mutants and wild-type (DJ) the rice seedlings. Seedlings were grown in hydroponic medium containing NH$_4^+$ and NO$_3^-$ for 14 days. **(A)**, The morphology of the rice plants; **(B)**, LR number; **(C)**, SR length; **(D)**, Photographs of NO production shown as green fluorescence in the lateral root (LR) and root tip (RT); **(E-F)**, NO production expressed as fluorescence intensity relative to lateral root **(E)** and root tip **(F)**. Bar = 1 mm. Data are means ± SE and bars with different letters indicate significant difference at $P < 0.05$ tested with ANOVA.
NO Regulates Auxin Transport Under NO$_3^-$ Supply

Both NO and auxin are involved in regulation of root growth in response to NO$_3^-$ supply, so we investigated the effects of their interaction. Application of SNP under NH$_4^+$ supply increased DR5::GUS activity and $[^3]$H IAA activity in roots to levels similar to those under NO$_3^-$ supply. Moreover, treatment with cPTIO under NO$_3^-$ supply decreased DR5::GUS expression and $[^3]$H IAA activity in roots to levels similar to those under NH$_4^+$ supply (Figures 9A,B). However, application of IAA to roots did not affect the levels of NO in LR and RT under NH$_4^+$ condition (Supplementary Figure 6). These results suggest that NO regulates auxin transport under NO$_3^-$ supply. The expression of YUCCA1-8 in the first leaf had no differences under NH$_4^+$ with or without SNP (Supplementary Figure 4A). However, compared with NH$_4^+$, application of SNP up-regulated the levels of OsPIN1b and OsPIN1d gene expression (Supplementary Figure 4B).

To determine the effects of duration of NO exposure for auxin buildup and root architecture change. The levels of DR5::GUS, LR number and SR length were examined over 16 days under NH$_4^+$ with or without SNP supply (Supplementary Figure 5). The results showed that the levels of DR5::GUS in LR region and RT were increased from 2 and 1 days, respectively, under SNP supply relative to application of NH$_4^+$ alone (Supplementary Figures 5A,B). Compared with sole NH$_4^+$ supply, the LR number and SR length were increased from 10 days under SNP treatment (Supplementary Figures 5C,D).

LR Primordia Formation and Root Meristem Activity Under NO$_3^-$ Supply

To determine the mechanism by which NO$_3^-$ regulates LR formation and SR elongation, we enumerated LR primordia, determined the lengths of epidermal cells in the maturity zone, and assayed CYCB1;1::GUS activity in the RT (Figure 10).
DR5::GUS activity and root morphology in wild-type (DJ). Seedlings were grown in hydroponic medium containing NH$_4^+$ and NO$_3^-$ in addition to IAA (100 nM) and NPA (300 nM) for 14 days. (A), DR5::GUS activity in the lateral root zone (LR) and root tip (RT); (B), Lateral root (LR) number; (C), Seminal root (SR) length. Bar = 1 mm. Data are means ± SE and bars with different letters indicate significant difference at $P < 0.05$ tested with ANOVA.

The number of LR primordia increased by 61% under NO$_3^-$ relative to NH$_4^+$ supply, which suggests that LR formation is dependent on LR primordia (Figures 10A–D,K). The lengths of epidermal cells did not differ between NH$_4^+$ and NO$_3^-$ supply (Figures 10E–H,M), which suggests that the promotion of root elongation by NO$_3^-$ was not due to changes in cell elongation. We used transgenic plants expressing the pCYCB1;1::GUS construct to assess the cyclic activity of cells in the root meristem. CYCB1;1::GUS activity and CYCB1;1 expression in the root meristem were increased under NO$_3^-$ relative to NH$_4^+$ supply (Figures 10I,J,L). Therefore, NO$_3^-$ affected LR formation by increasing LR primordia formation and promoted root elongation mainly by increasing root meristem activity rather than the elongation of epidermal cells in the maturity zone.

**DISCUSSION**

The regulation of root elongation and formation in response to NO$_3^-$ supply is important for the growth of plants. Two examples of the plasticity of root growth and development are promotion of root elongation and LR growth under NO$_3^-$ supply. In upland species such as maize and Arabidopsis, the root length is increased under NO$_3^-$ supply (Liu et al., 2013; Manoli et al., 2014) and localized NO$_3^-$ supply stimulates LR elongation (Zhang and Forde, 1998; Friml et al., 2003). In rice, localized NO$_3^-$ supply may stimulate LR elongation relative to no NO$_3^-$ supply (Wang et al., 2002). In this study, compared to NH$_4^+$, NO$_3^-$ supply increased the SR length and stimulated the formation of LRs (Figure 1), which suggests that the functions of NO$_3^-$ in regulating rice root growth and development are similar in maize and Arabidopsis.

Several lines of studies suggested that NO had two strategies in plants response to NO$_3^-$ supply. Firstly, NO as a signaling molecule functions in the regulation of root growth and formation in plants under NO$_3^-$ condition (Manoli et al., 2014; Sun et al., 2015). Manoli et al. (2014) suggested that the NO$_3^-$-induced increase in root length is dependent on the NO signaling pathway. Sun et al. (2015) showed that NO is induced by partial nitrate nutrition (PNN) and is involved in LR formation in rice. Secondly, NO enhanced N uptake by increasing the expression of N transport genes under PNN (Sun et al., 2015). In this study, application of SNP increased the SR length and LR number under NH$_4^+$. Treatment with cPTIO...
under NO$_3^-$ supply decreased the SR length and the number of LR (Figure 3). These results confirm that NO is involved in LR formation and SR elongation in the presence of NO$_3^-$.

The concentrations of total N in rice plants were decreased under NO$_3^-$ relative to NH$_4^+$ (Supplementary Figure 1), suggesting NH$_4^+$ is the main N source for rice. NO was induced by PNN condition and NO could enhance the N uptake in rice (Sun et al., 2015). In this study, NO production was induced by NO$_3^-$ maybe a strategy for rice plants to obtain more N.
NOS-like and NR pathways participated in NO production in plants (Wilson et al., 2008). In Arabidopsis, the gene of AtNOSI did not regulate NOS activity, therefore, it was renamed NO-associated enzyme (NOA1) (Moreau et al., 2008). The NO levels were significantly decrease in the root of noa1 mutant (formerly NOS1) relative to WT plants (Guo and Crawford, 2005). Besides NOA1-dependent pathway, NIA1 was involved in NR-regulated NO production in plants (Bright et al., 2006; Zhao et al., 2009). NIA2 expression is higher than that of NIA1 (Fan et al., 2007; Sun et al., 2015). Sun et al. (2015) reported that the NO generated by
NIA2-dependent NR increases LR formation in rice. In this study, NR activity and NIA2 expression were significantly higher under NO$_3^-$ supply relative to NH$_4^+$ supply. Moreover, the regulation of SR elongation and LR formation by NO$_3^-$ was inhibited by Tu (NR inhibitor) but not by L-NAME (NOS inhibitor) (Figures 2D, 3), which suggests that NO regulated root growth and formation under NO$_3^-$ supply main via the NR pathway. The changes in the root morphology and NO-associated green fluorescence signal of nia2 mutants were little affected by NO$_3^-$ (Figure 4). This suggests that NO is produced by the NR pathway rather than the NOS-like pathway and is involved in regulation of root growth under NO$_3^-$ supply.

Auxin distribution in the LR region is regulated by auxin transport, and auxin controls LR initiation and elongation in response to NO$_3^-$ supply (Grieneisen et al., 2007; Vanneste and Friml, 2009; Krouk et al., 2010; Song et al., 2013). Application of a low concentration of NO$_3^-$ affects LR growth by regulating auxin transport (Krouk et al., 2010). Liu et al. (2010) reported that local application of NO$_3^-$ reduces acropetal and basipetal transport compared to N-free treatment, and decreases auxin distribution in the LR region to a level more suitable for LR elongation in maize. Song et al. (2011, 2013) found that auxin synthesis and auxin transport from shoot to root are higher under (PNN treatment relative to application of NH$_4^+$ alone in a high-NO$_3^-$-response rice cultivar. The polarity of auxin transport is determined by the asymmetric localisation of the AUX1 and PIN auxin influx and efflux facilitators (Kramer, 2004). PIN proteins are the main auxin efflux carriers in plants (Friml et al., 2003; Wisniewska et al., 2006). Song et al. (2013) reported that PIN5b expression is upregulated under PNN relative to NH$_4^+$ supply. In Arabidopsis, PIN2 expression is upregulated in roots under NO$_3^-$ supply compared to NH$_4^+$ (Liu et al., 2013). In this study, the auxin levels in LR and RT were higher under NO$_3^-$ relative to NO$_3^-$ supply (Figures 5B,D), which suggests that the auxin distribution in roots is regulated by NO$_3^-$ supply. [H] IAA transport and PIN family gene expression were increased under NO$_3^-$ relative to NH$_4^+$ supply (Figures 5B,D, 7), which suggests that PIN genes are involved in auxin transport under NO$_3^-$ supply.

NO and auxin help regulate root growth and formation (Jin et al., 2011; Chen and Kao, 2012). NO acts downstream of auxin in regulating lateral root formation (Chen et al., 2010; Jin et al., 2011; Cao et al., 2017) and affects root elongation by regulating polar auxin transport (Fernández-Marcos et al., 2011). In rice, NO functions downstream of auxin in regulating LR formation but inhibits elongation of root by decreasing auxin levels in root tips under Fe deficiency (Sun H. et al., 2017). However, Manoli et al. (2016) found that the NO-mediated root apex responses to NO$_3^-$ are regulated by auxin in maize. These results suggest that the interactions between auxin and NO in regulating root growth are complex. In this study, application of SNP under NH$_4^+$ supply increased the auxin levels in roots, and treatment with cPTIO under NO$_3^-$ supply decreased the auxin levels in the roots (Figure 9). Thus, NO is involved in NO$_3^-$-regulated auxin transport in roots. However, treatment with IAA did not affect the level of NO in roots under NH$_4^+$ supply, consistent with the previous report by Sun H. et al. (2017). These results suggested that NO maybe act upstream of auxin in regulating root growth and formation. The expression of PIN1b and PIN1d in roots were up-regulated under SNP supply relative to application of NH$_4^+$ alone. However, the expression of YUCAs in the first leaf had no changes between NH$_4^+$ and NH$_4^+$ in addition to SNP (Supplementary Figure 4), suggesting that NO increased auxin levels in root mainly by regulating auxin transport but not auxin synthesis. Compared to WT, roots of the pin1b mutant had lower auxin levels, fewer LRs, and shorter SRs (Figure 8). Moreover, the root morphology of the pin1b mutant had less changes between NH$_4^+$ and NO$_3^-$ (Figures 8D,E). Therefore, NO$_3^-$ affects root growth by regulating root auxin transport via a mechanism involving NO. And these results suggest that the interactions between auxin and NO in regulating root growth in response to NO$_3^-$ supply are different from Fe deficiency.

Lateral root formation is dependent on LR primordia initiation under NO$_3^-$ supply (Song et al., 2013; Sun et al., 2015). In this study, the number of LR primordia was higher under NO$_3^-$ supply compared to NH$_4^+$ supply. Root length depends on two basal formation processes: cell division in the RT meristem and the length of root cells in the maturity zone (Scheres et al., 2002). The activity of meristematic cells in the root meristem affects root elongation (Bilou et al., 2005). NO$_3^-$ supply increases root meristem activity by regulating the expression of CYCB1;1 in Arabidopsis (Liu et al., 2013). In this study, NO$_3^-$ supply increased pCYCB1;1::GUS construct and CYCB1;1 expression levels in the RT but did not affect the length of mature cells (Figure 10). These findings suggest that SR elongation is regulated by increasing cell division in the root meristem zone under NO$_3^-$ relative to NH$_4^+$ supply.

CONCLUSION

In conclusion, NO is generated mainly by the NR pathway and induces LR formation and SR elongation by regulating auxin transport in the presence of NO$_3^-$ . NO$_3^-$ influences LR formation by increasing the number of LR primordia, and root elongation by increasing root meristem activity.

AUTHOR CONTRIBUTIONS

HS and FF performed the experiments and wrote the paper, JL analyzed the data, QZ designed the experiment.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00659/full#supplementary-material
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