Nitrate Modulates Lateral Root Formation by Regulating the Auxin Response and Transport in Rice

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Abstract: Nitrate (NO$_3^-$) plays a pivotal role in stimulating lateral root (LR) formation and growth in plants. However, the role of NO$_3^-$ in modulating rice LR formation and the signalling pathways involved in this process remain unclear. Phenotypic and genetic analyses of rice were used to explore the role of strigolactones (SLs) and auxin in NO$_3^-$-modulated LR formation in rice. Compared with ammonium (NH$_4^+$), NO$_3^-$ stimulated LR initiation due to higher short-term root IAA levels. However, this stimulation vanished after 7 d, and the LR density was reduced, in parallel with the auxin levels. Application of the exogenous auxin α-naphthylacetic acid to NH$_4^+$-treated rice plants promoted LR initiation to levels similar to those under NO$_3^-$ at 7 d; conversely, the application of the SL analogue GR24 to NH$_4^+$-treated rice inhibited LR initiation to levels similar to those under NO$_3^-$ supply by reducing the root auxin levels at 10 d. D10 and D14 mutations caused loss of sensitivity of the LR formation response to NO$_3^-$, which is regulated by nitrate reductase double mutant suggested that the local stimulation of LR elongation is a consequence of the NO$_3^-$ ion acting as a signal rather than a nutrient. Nitrate transporters, transcription factors, and micro-RNAs are involved in NO$_3^-$-modulated LR growth and development [14–19]. LR growth is regulated by both environmental conditions and intrinsic developmental regulators, such as plant hormones [20]. Auxin plays a dominant role in the specification of founder cells that give rise to LR initiation and the later stages of LR development and is involved in NO$_3^-$-modulated LR growth [10,13,14,20,21].

Keywords: ammonium; auxin; lateral root (LR); nitrate; rice; strigolactones (SLs)

1. Introduction

Plants have various mechanisms to adapt to nutrient supply conditions, especially plastic root development [1–7]. Lateral roots (LRs) are generally more sensitive to nutrient conditions than that are primary/adventitious roots in plants [8,9]. The LRs develop from founder cells in the pericycle, the outermost layer of the vascular cylinder (stele) of the roots [10].

Nitrogen (N) is an essential macronutrient for plant growth and crop productivity. Changes in N supplied in the nutrient medium induce plasticity in LR initiation and elongation [5,11–14]. A striking example of plasticity in LR development is seen in the response of Arabidopsis to localised NO$_3^-$ treatment via the stimulation of LR elongation. Studies of an Arabidopsis nitrate reductase double mutant suggested that the local stimulation of LR elongation is a consequence of the NO$_3^-$ ion acting as a signal rather than a nutrient. Nitrate transporters, transcription factors, and micro-RNAs are involved in NO$_3^-$-modulated LR growth and development [14–19]. LR growth is regulated by both environmental conditions and intrinsic developmental regulators, such as plant hormones [20]. Auxin plays a dominant role in the specification of founder cells that give rise to LR initiation and the later stages of LR development and is involved in NO$_3^-$-modulated LR growth [10,13,14,20,21].
Localised NO\textsuperscript{−3} supply does not stimulate LR elongation in axr4, an auxin-insensitive mutant, which suggests that NO\textsuperscript{−3} regulates LR growth via auxin signalling pathways [21]. NRT1.1 is a key component of NO\textsuperscript{−3}-sensing system that enables the plant to detect and exploit NO\textsuperscript{−3} [22]. The NO\textsuperscript{−3} and auxin signalling pathways are also linked by their effect on auxin transport via AtNRT1.1 (CHL1/NPF6.3) [23]. Local high levels of NO\textsuperscript{−3} promoted Arabidopsis LR development as a result of auxin accumulation in the LR primordia and tip [24]. However, inconsistent results have been reported in maize [25], although localised NO\textsuperscript{−3}-induced LR elongation has been observed, NO\textsuperscript{−3}-fed compartments have lower auxin levels compared with NO\textsuperscript{−3}-free compartments, and localised NO\textsuperscript{−3} supply inhibits auxin transport from shoot to root. A positive effect of low NO\textsuperscript{−3} on Arabidopsis LR formation required more auxin accumulation in LR primordia [20], consistent with the result in maize [26]; however, LR formation in rice was inhibited by low NO\textsuperscript{−3}, which was closely linked to lower auxin contents. The role of auxin in NO\textsuperscript{−3}-regulated LR growth remains unclear.

Strigolactones (SLs) are phytohormones involved in the growth and formation of LR in several plant species [27–30]. Compared with the wild-type (WT), Arabidopsis with mutations associated with SL synthesis and signalling had higher LR densities [27]. However, there was no difference in LR density between WT and d mutants in rice [30]. Application of GR24 decreased the LR density in both Arabidopsis and rice [27,30]. SLs are also involved in NO\textsuperscript{−3}-regulated root elongation by modulating PIN1b gene expression [7]. Therefore, the mechanisms by which SLs regulate LR growth in response to NO\textsuperscript{−3} supply are more complex and require further investigation.

Studies of LR growth in response to NO\textsuperscript{−3} have focused on the upland model plant Arabidopsis, and research in other plants, especially crop plants, is needed. Rice (Oryza sativa L.) is a major staple food globally, and NH\textsubscript{4}\textsuperscript{+} provides the main source of N for rice in paddy soil [31]. Interestingly, it has been predicted that 40% of the N acquired by rice roots is NO\textsuperscript{−3} due to nitrification occurring at the root surface, even in flooded conditions [32,33]. Increasing numbers of Chinese farmers are practicing intermittent flooding during rice cultivation, which increases NO\textsuperscript{−3} within the soil horizon. Although NO\textsuperscript{−3} plays a pivotal role in regulating root architecture by stimulating the initiation and elongation of LRs, the role of NO\textsuperscript{−3} in modulating LR growth in rice and the signalling pathways involved in this process remain unclear. Therefore, to evaluate the mechanisms of NO\textsuperscript{−3}-modulated LR formation in rice, we compared the time course of LR formation, auxin content, and DR5::GUS activity of rice in response to NO\textsuperscript{−3} and NH\textsubscript{4}\textsuperscript{+}.

2. Results

2.1. Nitrate Regulated LR Formation in Rice

Compared with NH\textsubscript{4}\textsuperscript{+} conditions, the number of LRs in the seminal root increased under NO\textsuperscript{−3} treatment within 7 d (Figure 1A,B). However, the LR number was lower under NO\textsuperscript{−3} than NH\textsubscript{4}\textsuperscript{+} treatment after 10 d (Figure 1B). There was no difference in LR density between NH\textsubscript{4}\textsuperscript{+} and NO\textsuperscript{−3} conditions before 7 d. Surprisingly, LR density was lower under NO\textsuperscript{−3} than NH\textsubscript{4}\textsuperscript{+} treatment after 8 d (Figure 1C,D). These results suggest that NO\textsuperscript{−3} supply stimulates LR formation for a short period (within 7 d), but this stimulatory effect disappears after 7 d.
2.2. Auxin Is Involved in NO\textsubscript{3}\textsuperscript{-}-Modulated LR Formation

Abundant evidence suggests that auxin has a close relationship with LR development [3,10,13,21]. To understand temporal changes in auxin-responsive genes to NO\textsubscript{3}\textsuperscript{-}, DR5::GUS and the expression of AUXIN RESPONSE FACTOR 1 (ARF1) in roots under NH\textsubscript{4}\textsuperscript{+}, NO\textsubscript{3}\textsuperscript{-}, and NH\textsubscript{4}\textsuperscript{+} plus α-naphthylacetic acid (NAA) treatments were analysed from 0 to 12 h (Figure 2). The expression of DR5::GUS in roots was induced by NO\textsubscript{3}\textsuperscript{-} and NH\textsubscript{4}\textsuperscript{+} plus NAA over the entire experiment compared with NH\textsubscript{4}\textsuperscript{+} nutrition (Figure 2A). Compared with NH\textsubscript{4}\textsuperscript{+}, the expression of OsARF1 was upregulated by both NO\textsubscript{3}\textsuperscript{-} and NH\textsubscript{4}\textsuperscript{+} plus NAA (Figure 2B).

To assess the roles of auxin in NO\textsubscript{3}\textsuperscript{-}-induced LR formation in rice, we examined the LR number in response to exogenous application of NAA under NH\textsubscript{4}\textsuperscript{+} at 7 d. Application of NH\textsubscript{4}\textsuperscript{+} plus NAA significantly increased the LR number at 7 d, to the same level as that under NO\textsubscript{3}\textsuperscript{-} conditions (Figure 2C).

To determine whether ARF1 is involved in NO\textsubscript{3}\textsuperscript{-}-promoted rice LR formation in the short term, we used arf1 mutant. The T-DNA insertion mutant of arf1 is shown in Supplementary Figure S1. Compared with WT plants (DJ), LR number and density were
decreased in the arf1 mutant under both NH$_4^+$ and NO$_3^-$ conditions (Figure 3), indicating that ARF1 is involved in NO$_3^-$-induced LR formation in rice.

**Figure 2.** Histochemical localization of DR5::GUS and qRT-PCR analysis of ARF1 gene, lateral root (LR) number in rice plants. Rice seedlings were grown in hydroponic media containing NH$_4^+$, NO$_3^-$, and NH$_4^+$+NAA treatments. Bar = 1 mm. (A) DR5::GUS in LR region; (B) Relative expression of ARF1 over time; (C) LR number. Data are means ± SE, and bars with different letters indicate significant difference between treatments at $p < 0.05$. h = hours.
Figure 3. The lateral root (LR) number and LR density in arf1 mutant plants. Seedlings were grown in a hydroponic media containing NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{-} conditions for 7 d. (A) The lateral root morphology in seminal root of the rice plants. (B) LR number; (C) LR density. Data are means ± SE, and bars with different letters indicate significant difference between treatments at \(p < 0.05\). d = days.

2.3. SLs Are Also Involved in NO\textsubscript{3}\textsuperscript{-}-Modulated LR Formation

Compared with WT plants, the root morphology of d10 (SL biosynthesis mutant) and d14 (SL-responsive mutant) plants, including LR density, was less responsive to NO\textsubscript{3}\textsuperscript{-} (Figure 4A,B). For example, the LR density under the two treatments was similar between \(d\) mutants and NH\textsubscript{4}\textsuperscript{+}-treated WT plants at 10 d. Interestingly, the LR density of \(d\) mutants was less responsive to NO\textsubscript{3}\textsuperscript{-} supply, ultimately resulting in a greater LR density, compared with WT plants regardless of the treatment at 10 d (Figure 4A). These results suggest that SLs are involved in NO\textsubscript{3}\textsuperscript{-}-modulated LR formation in rice.
Based on the LR density of $d$ mutants in response to NO$_3^-$, we speculated that the IAA content is higher in the roots of $d$ mutants at 10 d (Figure 4B). Compared with NH$_4^+$, NO$_3^-$ treatment reduced the IAA levels in the roots of WT plants. IAA levels were similar between WT and $d$ mutants under NH$_4^+$ conditions, but were higher in $d$ mutants than WT plants under NO$_3^-$ conditions (Figure 4B). We examined whether exogenous application of the SL analogue GR24 affects the IAA levels and LR formation (Figure 5). The application of GR24 in NH$_4^+$-treated rice reduced $DR5::GUS$ expression and IAA levels in roots (Figure 5A,B), and inhibited LR formation to levels similar to those under NO$_3^-$ at 10 d (Figure 5C). Conversely, treatment with NAA plus NO$_3^-$ significantly increased the LR density to the same level as that under NH$_4^+$ at 10 d (Figure 5D). These results indicate that NO$_3^-$ inhibited LR formation, probably by decreasing auxin levels in roots in the long term, and SLs may be involved in this process.

Figure 5. Histochemical localization of $DR5::GUS$, IAA content, and LR density in rice plants. Rice seedlings were grown in hydroponic media containing NH$_4^+$, NO$_3^-$, and NH$_4^+$+GR24 for 10 d. (A) $DR5::GUS$ in LR region; (B) IAA content in LR region; (C,D) LR density. (A) Bar = 1 mm. Data are means ± SE, and bars with different letters indicate significant difference between treatments at $p < 0.05$. d = days.
2.4. OsPIN2 Is Involved in NO\textsubscript{3}\textsuperscript{−}-Modulated Auxin Levels and LR Formation in Rice

A previous study showed that SLs regulate LR formation by inhibiting auxin transport, with involvement of PIN proteins [30]. In this study, compared with NH\textsubscript{4}\textsuperscript{+}, NO\textsubscript{3}\textsuperscript{−} treatment downregulated the expression of PIN2 and proPIN2::GUS at 10 d (Figure 6A,C). PIN2 expression in roots was significantly higher in the \textit{d14} mutant than WT plants under both treatments (Figure 6B). PIN2 expression was downregulated by NH\textsubscript{4}\textsuperscript{+} plus GR24 compared with NH\textsubscript{4}\textsuperscript{+} treatment at 10 d (Figure 6C). These results suggest that SLs participate in the NO\textsubscript{3}\textsuperscript{−}-induced inhibition of PIN2 transcription gene in rice.

Figure 6. Histochemical localization of proPIN2::GUS and OsPIN2 expression in rice plants. Rice seedlings were grown in hydroponic media containing NH\textsubscript{4}\textsuperscript{+}, NO\textsubscript{3}\textsuperscript{−}, and NH\textsubscript{4}\textsuperscript{+} + GR24 for 10 d. (A) Localization of proPIN2::GUS; Bar = 0.5 mm. (B) OsPIN2 expression in WT and \textit{d14} mutant under NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{−}; (C) OsPIN2 expression in WT under NH\textsubscript{4}\textsuperscript{+}, NO\textsubscript{3}\textsuperscript{−}, and NH\textsubscript{4}\textsuperscript{+} + GR24 conditions. Data are means ± SE, and bars with different letters indicate significant difference between treatments at \(p < 0.05\).

To determine the functions of PIN2 in NO\textsubscript{3}\textsuperscript{−}-modulated LR formation, we assessed the LR number and density in \textit{pin2} mutants in response to NO\textsubscript{3}\textsuperscript{−} at 10 d (Supplementary Figure S2; Figure 7). Compared with WT plants, the two \textit{pin2} mutant lines exhibited less responsiveness of the LR number and density to NO\textsubscript{3}\textsuperscript{−} and fewer and less dense LRs under the two treatments at 10 d (Figure 7B,C). This implies that OsPIN2 is also involved in rice LR formation modulated by NO\textsubscript{3}\textsuperscript{−} application long-term.
Figure 7. The lateral root (LR) number and LR density in pin2 mutant plants. Seedlings were grown in a hydroponic media containing NH$_4^+$ and NO$_3^-$ for 10 d. (A) The lateral root morphology in seminal root of the rice plants. (B) LR number; (C) LR density. Data are means ± SE, and bars with different letters indicate significant difference between treatments at $p < 0.05$. d = days.

3. Discussion

Nitrogen is a major plant nutrient, and crops strongly depend on fertilization programs, affecting environmental quality. The identification of crop cultivars with more efficient nutrient acquisition continues to be a priority for plant scientists [34,35]. LRs are crucial for the detection and uptake of N in plants [24,36]. Nitrate triggers several molecular and physiological events, including LR growth, leading to the overall response of the plant to its availability [11,12,37]. Several molecular components of NO$_3^-$-regulated LR growth have been identified, mostly in the model plant Arabidopsis thaliana. However, the role of NO$_3^-$ in modulating LR formation and growth in rice and the signalling pathways involved in this process remain unclear. In this study, we found that NO$_3^-$-induced LR growth depends on the auxin response and transport in roots, with the involvement of SLs.

Studies of an Arabidopsis nitrate reductase double mutant suggested that the local stimulation of LR elongation was a consequence of the NO$_3^-$ ion acting as a signal, rather than a nutrient. The AtANR1 and AtNRT1.1 genes, which encode a transcription factor and a dual NO$_3^-$ transporter, respectively, were proposed to consecutively regulate the stimulatory effect of NO$_3^-$ on LR elongation [11,21,23]. In rice, LR formation was less sensitive to localised NO$_3^-$ supply in osnar2.1 mutants than WT plants, suggesting that OsNAR2.1 is involved in a NO$_3^-$ signalling pathway that modulates LR formation [15]. Here, we also found that NO$_3^-$ induced LR formation, probably via its signalling pathway. As shown in Figure 1, NO$_3^-$ may induce LR formation by triggering systemic signals that influence LR growth compared with NH$_4^+$.

Localised NO$_3^-$ supply did not stimulate LR elongation in the auxin-insensitive mutant, which suggests that NO$_3^-$ regulates LR growth via auxin signalling pathways [21]. To illustrate the mechanism of the nitrate-specific effects on rice LR formation within 7 d, the expression of ARF1 (auxin-responsive gene) and DR5::GUS was evaluated in response to two N forms (Figure 2). After 3 h of treatment, ARF1 expression in rice roots was higher under NO$_3^-$ than under NH$_4^+$, which coincided with the higher DR5::GUS expression in the
LR zone under NO$_3^-$ treatment. NO$_3^-$ enhanced ARF1 expression within hours, suggesting that auxin triggers a systemic signal to participate in NO$_3^-$-induced LR formation in rice. Exogenous application of NAA under NH$_4^+$ supply restored the LR number to a level similar to that under NO$_3^-$ supply within 7 d (Figure 2), and the LR number and density were lower in the arf1 mutant relative to WT plants at 7 d (Figure 3B,C), which further demonstrates that auxin participates in specific NO$_3^-$-induced LR formation.

SLs have been suggested to modulate auxin transport in the regulation of root growth [27,30]. In Arabidopsis, SLs modulated local auxin levels, and the net result of the SL action depended on the auxin status of the plant [27]. In rice, GR24 application markedly reduced auxin transport to levels equivalent to those under N-deficient conditions, which in turn reduced the LR density [30]. A previous study showed that NO$_3^-$ application enhanced SL signalling from 7 d in rice [7]. The SL levels in root exudates are regulated by N stress [30]. In this study (Figure 5), application of GR24 to rice plants under NH$_4^+$ treatment inhibited LR initiation to the same levels as those under NO$_3^-$ treatment by reducing IAA levels in roots at 10 d. Conversely, compared with NO$_3^-$ conditions, NAA treatment of NO$_3^-$-treated rice prevented the downtrend in LR initiation to the same levels as those in NH$_4^+$-treated rice plants at 10 d (Figure 5). This indicated that the NO$_3^-$ supply increased SL production after 7 d and inhibited LR formation by decreasing auxin levels in the LR region, consistent with the previous report [7,30]. This suggests that SLs are involved in NO$_3^-$-inhibited LR formation by reducing auxin transport in roots in the long term.

The influence of SLs on auxin transport is mediated by PIN expression [3,7,27,30]. For example, SLs increased the rate of PIN1 removal from the plasma membrane and altered the polarization of PIN2 in the plasma membrane in Arabidopsis [38]. Similarly, relative PIN expression in rice roots was significantly decreased under LN conditions, after GR24 application [30]. SLs participated in NO$_3^-$-induced rice root elongation by modulating PIN1b transcription [7]. In this study, PIN2 expression was inhibited by NO$_3^-$ treatment long-term (Figure 6A,C), suggesting that PIN2 is involved in NO$_3^-$-modulated auxin polar transport to play an important role in LR development. Compared with NH$_4^+$ treatment, PIN2 expression was downregulated under NO$_3^-$ or NH$_4^+$ plus GR24 treatment at 10 d (Figure 6). Furthermore, the expression of PIN2 was significantly upregulated in the d14 mutant compared with the WT (Figure 6B). These results suggest that NO$_3^-$ inhibits auxin transport by regulating PIN2 expression in roots, with the involvement of SLs. Compared with WT plants, mutations in D genes that eliminate the inhibition of SLs on auxin transport led to higher auxin levels in the LR region and no response of LR formation to NO$_3^-$ relative to NH$_4^+$ (Figure 4). The lower LR number and density were recorded in the pin2 mutants relative to WT plants under both NH$_4^+$ and NO$_3^-$ supplies (Figure 7). These results further demonstrate that the effect of LR formation regulated by NO$_3^-$ depends on auxin response and transport in roots.

4. Materials and Methods

4.1. Plant Materials

The d10 (SL biosynthesis mutant) and d14 (SL-responsive mutant) were Shiokari ecotype [30], arf1 mutant was Dongjin (DJ) ecotype, and CRISPR-edited PIN2 knockout mutant lines (pin2) were Nipponbare ecotype. The arf1 was obtained from Kyung Hee University, Korea (Supplementary Figure S1).

4.2. Plant Growth

Plants were grown in a greenhouse under natural light at day/night temperatures of 30 °C/18 °C. Germinated seeds of uniform size were transplanted into holes in a PCR tube rack for 14 d. PCR tubes receiving nitrogen treatments were filled with 1.25 (NH$_4$)$_2$SO$_4$ and/or Ca(NO$_3$)$_2$. Other chemical compositions of International Rice Research Institute (IRRI) nutrient solution were (mM): 1.25 (NH$_4$)$_2$SO$_4$ and/or Ca(NO$_3$)$_2$, 0.3 KH$_2$PO$_4$, 0.35
K₂SO₄, 1.0 CaCl₂, 1.0 MgSO₄·7H₂O, 0.5 Na₂SiO₃; and (µM) 9.0 MnCl₂, 0.39 (NH₄)₆Mo₇O₂₄, 20.0 H₃BO₃, 0.77 ZnSO₄, and 0.32 CuSO₄ (pH 5.5) as previously described [30].

The treatments applied were as follows: 10 nM 1-naphthylacetic acid (NAA), 2.5 µM GR24 (an SL analogue) [30,39].

4.3. Root System Architecture

The fibrous root system of rice includes seminal root, adventitious roots, and lateral root (LR) grown from seminal and adventitious roots. The preliminary experiments suggested that the response of LRs on seminal root to two N forms was similar to that on adventitious roots. Therefore, the numbers of LRs on SRs were chosen to evaluate the effects of NH₄⁺ and NO₃⁻ on LR growth. LRs were enumerated visually. The LR density was calculated as LR number divided by the length of the SR.

4.4. Determination of IAA Content

The plant tissues were ground with quartz sand and butylated hydroxytoluene (BHT) in liquid N₂ and lixiviated in 80% methanol (20 mL) for 12 h. The extracted fluid was collected and concentrated by a rotary evaporator to 10 mL at 40 °C, and then the concentrated fluid was extracted with petroleum ether of the same volume. Underlayer liquid was adjusted to pH 8.5 and added 0.2 g polyvinylpyrrolidone (PVP) then vibrated for 30 min, and then filtered through a 0.45 µm filter. The cartridge was initially washed with 0.1 M acetic acid, eluted with 4 mL of a mixture of 25% (v/v) methanol and 0.1 M acetic acid, and eventually with 70% (v/v) methanol only. After vacuum evaporation, the purified samples were metered volume to 1 mL with mobile phase and then loaded on a reverse-phase HPLC column. Standard auxin samples were from Sigma-Aldrich (St. Louis, MO, USA), and chromatographic conditions were described as: Waters 600–2487; Hibar column RT 250 mm × 4.6 mm; Purospher STAR RP-18 (5 µm); column temperature 45 °C; fluid phase: methanol: 1% acetic acid (v/v), isocratic elution; fluid rate: 0.6 mL min⁻¹; UV detector, λ = 269 nm; injection volume 20 µL. A 0.22 µm filter was used for filtration of both the buffer and the samples before HPLC analysis as previously described [40].

To assess auxin distribution, rice plants were transformed with the pDR5::GUS constructs using Agrobacterium tumefaciens (strain EHA105). DR5::GUS, a specific reporter that contains seven repeats of a highly active synthetic auxin-response element and can reflect the in vivo auxin level [41]. The roots were subjected to GUS staining. Stained plant tissues were photographed using a stereomicroscope (Stemi 508; Zeiss, Gottingen, Germany) equipped with a colour CCD camera. All experiments included eight replicates.

4.5. qRT-PCR Analysis

Total RNA was isolated from the roots of rice plants under NH₄⁺ or NO₃⁻ supply. The RNA extraction, reverse transcription, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) methods were as previously described [42]. All experiments were with three replicates. The primer sets for ARF1 and PIN2 are listed in Supplemental Table S1.

4.6. 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–Einot–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, USA). All experiments included three independent biological replicates.

5. Conclusions

NO₃⁻ stimulated LR formation within 7 d, but the stimulatory effect disappeared after 7 d, in parallel with the auxin response and transport in roots. ARF1 was involved in the short-term NO₃⁻-induced LR formation. SL production was increased under NO₃⁻ treatment. The application of SLs and NO₃⁻ inhibited PIN2 transcription. PIN2 mutation
inhibited the sensitivity of the response of LR formation to NO$_3^-$ application. These results demonstrate that NO$_3^-$ modulated LR formation by affecting the auxin response and transport in rice roots, with SL involvement in the long term.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/genes12060850/s1, Supplementary Table S1. The primers for qRT-PCR of ARF1 and PIN2 genes. Supplementary Figure S1. Identification of T-DNA insertion arf1 mutant. Supplementary Figure S2. Sequencing verification of CRISPR-edited PIN2 knockout mutants.

Author Contributions: B.W. and X.G. performed experiments; X.Z., X.Q., and F.F. assisted the experiment; Q.Z. and Y.Z. analysed data; D.H. and H.S. designed the experiment and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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