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Strigolactones are required for nitric oxide to induce root elongation in response to nitrogen- and phosphate-deficiency in rice

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ABSTRACT

The response of the root system architecture to nutrient deficiencies is critical for sustainable agriculture. Nitric oxide (NO) is considered a key regulator of root growth, although the mechanisms remain unknown. Phenotypic, cellular, and genetic analyses were undertaken in rice to explore the role of NO in regulating root growth and strigolactone signaling under nitrogen- and phosphate-deficient conditions (LN and LP). LN- and LP-induced seminal root elongation paralleled NO production in root tips. NO played an important role in a shared pathway of LN- and LP-induced root elongation via increased meristem activity. Interestingly, no responses of root elongation were observed in strigolactone d mutants compared to wild-type plants, although similar NO accumulation was induced by sodium nitroprusside (SNP) application. Application of Abamine (the strigolactone inhibitor) reduced seminal root length and pCYCB1;1::GUS expression induced by SNP application in wild-type; furthermore, comparison with wild-type showed lower strigolactone-signaling genes in nia2 mutants under control and LN treatments and similar under SNP application. Western blot analysis revealed that NO, similar to strigolactone, triggered proteasome-mediated degradation of D53 protein levels. Therefore, we presented a novel signaling pathway in which NO activated seminal root elongation.
under LN and LP conditions, with the involvement of SLs.

Key-words: Nitrogen; nitric oxide; phosphate; rice; root; strigolactone

INTRODUCTION

Nitrogen (N) and phosphate (P) are major nutrients required for plant growth and development, and deficiencies dramatically affect plant development and crop productivity (Forde & Lorenzo, 2001; López-Bucio et al. 2003). The response of the root system architecture in nutrient-limited conditions is an essential mechanism for plants to optimize growth and productivity (Forde & Lorenzo 2001; López-Bucio et al. 2003). Increases in the root-to-shoot ratio and root surface area induced by deficiencies of N and P have been reported in several plant species (López-Bucio et al. 2003; Chun et al. 2005; Gruber et al. 2013). Changes in the root morphology under nutrient deficiency are complex and vary according to the experimental conditions and plant species. A deficiency in P dramatically inhibited Arabidopsis (Arabidopsis thaliana) primary root growth (Williamson et al. 2001; Linkohr et al. 2002; Chevalier et al. 2003; López-Bucio et al. 2003; Pérez-Torres et al. 2008; for review, see Abel, 2011; Niu et al. 2013; Giehl et al. 2014; Gruber et al. 2013; Kellermeier et al. 2014). This growth arrest was caused by reduced cell elongation and progressive cessation of cell proliferation in the root meristem that ultimately exhausted the primary root (PR) stem cell niche (Péret et al. 2014). In contrast to Arabidopsis, in other plant species such as rice (Oryza sativa L.), elongation of the PRs occurs as a typical response to P deficiency (Yi et al. 2005; Niu et al. 2013; Sun et al. 2014). Interestingly, PR
elongation also represents a typical response to N deprivation in rice and maize (*Zea mays*) (Chun et al. 2005; Tian et al. 2008; Zhang et al. 2012; Sun et al. 2014), while the inconsistent response in *Arabidopsis* to N deprivation largely depends on plant age and the concentration of N (Zhang et al. 1998; Linkohr et al. 2002; Gruber et al. 2013). Although root plasticity has been well documented in N- and P-deficient roots of different plant species (Zhang et al. 1998; Linkohr et al. 2002; López-Bucio et al. 2003; Yi et al. 2005; Niu et al. 2013; Sun et al. 2014; Gruber et al. 2013; Péret et al. 2014), the signals involved in the regulatory cascade leading to changes in root growth are still not fully understood.

Root growth is regulated by environmental conditions and intrinsic factors (e.g. plant hormones). Strigolactones (SLs) have been identified as novel phytohormones that regulate root development (Kapulnik et al. 2011; Ruyter-Spira et al. 2011; Koltai 2011; Arite et al. 2012; Mayzlish-Gati et al. 2012; Rasmussen et al. 2012; Sun et al. 2014; Waldie et al. 2014; Sun et al. 2015b; De Cuper et al. 2015). Several lines of evidence suggest that the SL pathway is involved in rice root growth under low-N and -P conditions (Arite et al. 2012; Sun et al. 2014). Under conditions of N- and P-deficiency, elevated SL mirrored changes in the root architecture in rice plants; however, application of GR24 under nutrient-sufficient conditions corresponded to reduced LR density and increased primary root lengths in WT plants, to similar level as under nutrient-deficient conditions. Moreover, application of GR24 led to a recovery of the root phenotype induced by nutrient-limiting conditions in SL synthetic mutants (*d10* and *d27*), but not in a signaling mutant (*d3*), further suggesting that
elevated SL levels under low-nutrient conditions may lead to a SL signaling-dependent reduction in lateral root density and induction of seminal root length. Taken together, this suggested that a similar regulatory pathway was involved in root development regulated by N- and P-deficiency.

In addition to SLs, nitric oxide (NO), another signaling molecule, plays a pivotal role in root growth modulation (adventitious root formation, Pangnussat et al. 2003; lateral root development, Correa-Aragunde et al. 2004; Sun et al. 2015a; root hair formation, Lombardo et al. 2006; primary root elongation, Zhao et al. 2007; Fernández-Marcos et al. 2011; Bai et al. 2014; Manoli et al. 2014). Fernández-Marcos et al. (2011) suggested that higher levels of NO reduced root meristem activity. Conversely, Sanz et al. (2014) reported that depletion of NO reduced primary root elongation, and NO-deficient mutant roots had small root meristems, suggesting an important role for NO in the regulation of stem cell decisions. Studies have shown the involvement of NO in root development, modulated by nutrient deficiency (Zhao et al. 2007; Wang et al. 2010; Meng et al. 2012; Chen et al. 2010; Trevisan et al. 2014). For example, higher endogenous NO in N-deficient roots was similar to the enhancement of primary root lengths in maize (Zhao et al. 2007). Similarly, endogenous NO concentration in P-deficient roots was positively correlated with the development of cluster roots and citrate exudation in the white lupin (Wang et al. 2010; Meng et al. 2012). Interestingly, NO is important in the shared signaling pathway of the P- and Fe-deficiency-induced formation of cluster roots in the white lupin. Therefore, it could be...
hypothesized that NO was also involved in a similar signaling pathway in N- and P-deficiency-induced root growth. The physiological basis of these processes and the regulatory mechanisms are unknown and warrant further investigation.

Since SLs are involved in root growth and are modulated by N- and P-deficiency in rice plants, further research is needed to understand possible links between SLs and NO in the control of root development in response to low N and P. In this study, NO was reported to have a close link with SLs under N- and P-deficiency that led to elongation of seminal roots via increasing meristem activity.

MATERIALS AND METHODS

Plant materials

The T-DNA insertion nia2 mutant lines (nia2-1 and nia2-2) with the japonica cv. Dongjin ecotype were obtained from RiceGE, the Rice Functional Genomics Express Database, in Pohang city, Korea. The SL-deficient mutants (d10) and the SL signaling mutants (d3) with the Shiokari ecotype were provided by Shinjiro Yamaguchi of the RIKEN Plant Science Center. The d53 mutant with the Nipponbare ecotype was provided by Academician Jiayang Li and Professor Yonghong Wang at Chinese Academy of Sciences, Beijing, China.

Plant growth

Plants were grown in a greenhouse under natural light at day/night temperatures of 30/18°C.
Seven-day-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 varying from one-quarter to full-strength strength were applied for one week, followed by full-strength nutrient solution for a further week. Pots receiving normal nutrition (Control) were filled with 2.5 mM N (NH$_4$NO$_3$) and 300 µM P (KH$_2$PO$_4$), and those receiving N- and P-deficient nutrition were filled with 0.02 mM N (LN) and 2 µM P (LP). To exclude any potential effects of potassium (K$^+$) on the treatments, the low-P treatment solutions were supplemented with K$^+$ to the same levels as those under sufficient P conditions (300 µM) using K$_2$SO$_4$. The full chemical composition of the International Rice Research Institute (IRRI) nutrient solution was (mM): 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) 20.0 Fe-EDTA, 9.0 MnCl$_2$, 0.39 (NH$_4$)$_6$Mo$_7$O$_{24}$, 20.0 H$_3$BO$_3$, 0.77 ZnSO$_4$, and 0.32 CuSO$_4$, pH 5.5. The nutrient solution was replaced with fresh solution daily. Each treatment consisted of four replicates arranged in a completely randomized design to avoid edge effects. In addition, all experiments included three independent biological replicates.

The pharmacological treatments of sodium nitroprusside (SNP) varied from 0–20 µM, 80 µM 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxie (cPTIO), 25 µM Tungstate (Tu), 100 µM N$^G$-Nitro-L-arginine methyl ester (L-NAME), 2.5 µM GR24 and 100 µM Abamine were applied to the hydroponic media.
Measurement of root system architecture and histological observation

Seminal roots were significantly longer than adventitious roots under our experimental conditions. Our preliminary experiment showed similar responses of seminal and adventitious roots to low N and P treatments, and the number of adventitious root did not change significantly under the different nutrient conditions during the experimental period (Sun et al. 2014). Therefore, seminal roots were chosen as representative organs to study the effects of LN and LP on the rice root system. The seminal root lengths were measured with a ruler.

To analyze the length of the epidermal cells in the maturity zone and cell number in the meristem zone, the root tips of the seminal roots were treated for 1 h with 1.8 M KOH solution heated to 90°C to clear the tissues, and then treated with 1% HCl (v/v) solution for 5 min (Chen et al. 2011). The epidermal cells of the root tips were observed under a microscope using a color charge-coupled device (CCD) camera (Olympus Optical Co. Ltd, Tokyo, Japan). The lengths of mature cells were measured and cell numbers of the root meristem were determined by counting the cortical cells in files extending from the quiescent center to the first elongating cell.

The pCYCB1;1::GUS construct

The pCYCB1;1::GUS fusion construct was transformed into WT plants (Kasalath) via A. tumefaciens-mediated transformation (Chen et al. 2013). The construct was kindly provided
by Professor Chuanzao Mao from Zhejiang University, Hangzhou, China. The root tips were used in histochemical GUS staining analyses. The stained tissues were photographed using an Olympus SZX2-ILLK microscope with a color CCD camera (Olympus) (Chen et al. 2011).

Measurements of NO in the root tips

Nitric oxide was imaged by DAF-FM DA (diaminofluorescein-FM diacetate) and epifluorescence microscopy. The root tips were loaded with 10 µM DAF-FM DA in 20 mM HEPES-NaOH buffer (pH 7.5). After incubating in darkness for 30 min, the root tips were washed three times in fresh buffer and immediately visualized using a stereomicroscope, with a color CCD camera, excitation 488 nm, emission 495–575 nm (OLYMPUS MVX10). Signal intensities of green fluorescence in the images were quantified according to Guo & Crawford, (2005) using Photoshop software (Adobe Systems, San Jose, CA, USA). Data were presented as the means of the fluorescence intensity.

Measurements of strigolactones

After rice plants were treated for two weeks, root exudates (approximately 500 mL) were collected at 24-h intervals as described previously (Sun et al. 2014). Root exudates adsorbed on charcoal were eluted with acetone. After evaporation of the acetone in vacuo, the residue was dissolved in 50 mL of water and extracted three times with 50 mL of ethyl acetate. The ethyl acetate extracts were combined, washed with 0.2 M K₂HPO₄ (pH 8.3), dried over...
anhydrous MgSO₄, and concentrated in vacuo. These crude extracts were stored in sealed glass vials at 4°C until use.

The concentrations of SLs in the root exudates were determined by LC–MS/MS as described previously (Xie et al. 2013). Data acquisition and analysis were performed using MassLynx software (ver. 4.1; Waters, Milford, MA, USA).

**qRT-PCR analysis**

Total RNA was isolated from the roots of the rice seedlings. RNA extraction, reverse transcription, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) were performed as described previously (Chen et al. 2012). Primer sets for the NOA, D and *CYCB1;1* genes are listed in Supplemental Table S1, online.

**Western blotting**

Two-week-old rice seedlings were treated with LN and LP, GR24 (2.5 μM) and SNP (10 μM) with or without MG132 (50 μM) for 24 hours. 1-cm root apices of WT and *d14* mutant were collected and frozen at -80°C. The OsD53 antibody was kindly provided by the Academician Jiayang Li and Professor Yonghong Wang at Chinese Academy of Sciences, Beijing, China. Total protein from 1-cm root apices was extracted and denatured in SDS sample buffer containing 5% β-mercaptoethanol (β-ME) at 95°C for 10 min, transferred to a polyvinylidene fluoride membrane (Whatman), and hybridized with the OsD53-specific antibody (dilution 1:3000) and an OsActin-specific antibody (1:1000).
Data analysis

Data from the experiments were pooled for calculation of the means and standard errors (SE) and analyzed by one-way ANOVA followed by an LSD test at $P \leq 0.05$ to determine the statistical significance of the differences between individual treatments. All statistical evaluations were conducted using SPSS (version 11.0) statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

NO is involved in seminal root elongation

Seminal root lengths increased significantly in WT plants (Shiokari) under LN and LP conditions compared with normal nutrition (Control, Fig. 1A). To determine the effects of N- and P-deficiency on seminal root lengths, its development was examined over 16 days under nutrient conditions (Fig. 1B). Seminal root length markedly increased from day 6 in response to LN and LP relative to normal nutrition. At day 10, LN- and LP-induced seminal root length was ~18 and 24% longer, respectively and this increased to 25 and 34%, at day 16, compared with the control treatment.

The NO-associated green fluorescence in the root tip was visibly increased by LN and LP even at day 1, compared with normal nutrition (Figs. 1C-D). Quantification of the fluorescence signal intensity showed increased accumulation of NO in the root tips by 71
and 107% under LN and LP at day 1, and by 114 and 135% at day 14. Root morphology in response to the application of the NO donor (SNP) was examined to assess whether rice root morphology regulated by LN and LP was mediated by NO. The application of SNP at concentrations ranging from 2.5–20 µM significantly increased NO-associated green fluorescence in the root tips, and SNP application at 10 µM induced the seminal root length to the same extent as LP and LN (Supplemental Fig. S1-2; Fig. 2). Application of the NO scavenger (cPTIO) under LN or LP decreased NO-associated green fluorescence in the root tips and also decreased the seminal root length to a similar level to the control treatment (Fig. 2A-C). These results indicated that NO production was enhanced by LN and LP in the rice root tip, which was involved in the elongation of seminal roots induced by LN and LP.

**NO results from the NO synthase and/or nitrate reductase pathways**

Nitrate reductase (NR) and NO synthase (NOS) are major enzymatic pathways of NO production in plants (Yamasaki et al. 1999; Meyer et al. 2005). Relative NR activity was assessed in the shoots and roots of rice plants at days 1, 7, and 14 (Supplemental Fig. S3; Fig. 2D). Compared to normal nutrition, LN significantly reduced NR activity in rice shoots by 20% at day 1, 37% at day 7 and 50% at day 14 (Supplemental Fig. S3), consistent with the results of Fan et al. (2007). However, a different time-course development of NR activity was observed in rice roots (Fig. 2D). Compared with the control treatment, NR activity in rice roots was induced by 42% by LN treatment at day 1 and then decreased over time. A similar
NR activity at day 7 and reduced NR activity at day 14 were recorded in LN rice roots. Meanwhile, compared with the control, similar transcription levels of the *NO-associated* (NOA) gene, a homolog of *NOA1* in Arabidopsis, was observed in rice roots at day 1, followed by a 35% increase in the NOA transcription levels at day 7 and 50% at day 14, under LN conditions (Fig. 2E). Interestingly, LP increased NOA transcription levels and had no effect on NR activity throughout the experimental period compared to the control treatment. This suggested that LN-induced NO was generated via the NR pathway at the start of treatment, while LP-induced NO was excluded from the NR pathway.

After treatment of rice plants with the NOS inhibitor L-NAME (100 µM) under LN or LP conditions, NO-associated green fluorescence in the root tips and the lengths of the seminal roots markedly decreased to the levels of those with sufficient nutrition (Figs. 2A-C). These results suggested that NO production from the NOS pathway was involved in elongation of the seminal root, induced by LN or LP. Meanwhile, application of the NR inhibitor tungstate (Tu, 20µM) under LN decreased NO-associated green fluorescence in the root tips and the length of the seminal root to levels similar to those under normal nutrition conditions. These results further suggested that NO produced via the NR pathway was involved in seminal root elongation, and was regulated solely by LN.

**nia2 mutants are less sensitive to low N rather than low P**

In plants, the transcription level of *NIA2* was markedly higher than that of *NIA1* (Wilkinson
Two lines of nia2 mutants (nia2-1 and nia2-2) and the WT (Dongjin) were used to assess whether seminal root length and the accumulation of NO in the nia2 mutant were sensitive to nutrient deficiencies. Molecular characterization of the two homozygous T-DNA insertion nia2 mutants revealed that the T-DNA was inserted into the second intron. Compared with the WT plant, the expression level of NIA2 was almost completely suppressed in both nia2 lines, and NR activity in the shoots and roots decreased to ~6% of that of the WT in nia2-1 and ~23% of that of the WT in nia2-2 (Supplemental Fig. S4).

In contrast to the WT plants, NO accumulation in the root tips and the length of the seminal roots of nia2-1 and nia2-2 were less responsive to LN rather than LP (Figs. 3A-D). Furthermore, the application of SNP significantly increased the seminal root length of both mutant lines to the same extent as that of WT plants under LN conditions. Application of tungstate decreased the seminal root length in the WT plants to a level similar to in the nia2 mutants (Fig. 3E). The results using nia2, containing a mutant of NR gene, confirmed that NO production induced by LN rather than by LP resulted from NIA2-dependent NR enzymatic sources, which was also involved in modulation of the seminal root length under LN conditions.

**Cross talk between NO and SLs**

SLs have been shown to participate in seminal root elongation induced by LN and LP, and the
seminal roots of d10 and d3 mutants were less responsive to LN and LP than WT rice plants (Sun et al. 2014). Surprisingly, similar NO accumulation was observed in the root tips of WT, d10 and d3 mutants regardless of the nutrient conditions (Fig. 4) or SNP applications (supplemental Fig. S5). However, no response of seminal root length to SNP application was observed in two d mutants (supplemental Fig. S5 and Fig. 4C). Interestingly, application of an SL inhibitor (Abamine, a CCD inhibitor, Nakamura et al. 2014) prevented the LN, LP and SNP-induced seminal root length increase in WT plants (Fig. 4C). More importantly, compared with WT plants, expression level of SL signaling genes (D14 and D53) decreased under control and LN treatments while kept no difference under SNP application in two nia2 mutants (supplemental Fig. S6A). At the same time, even if GR24 was applied to rice plants treated with control and LN treatments, no response of seminal root length was observed in two nia2 mutants relative to the WT plants (supplemental Fig. S6B); SNP application rescued the root phenotype in two nia2 mutants (Fig. 3E). Our observation suggested that the link between NO and SL in inducing of rice root length.

**NO decreases D53 protein level**

To clarify a possible role of SLs in NO-induced root elongation in response to SNP application on three SL fractions (2’-epi-5-deoxystrigol, orobanchol and orobanchyl acetate) secreted by WT rice plants, and the SL synthetic genes, were investigated (Fig. 5). No difference was recorded in the level of SL fractions and of three synthetic genes with or
without the application of SNP (Figs. 5A-B). However, the expression levels of signaling genes \textit{D14} and \textit{D53} were stronger under SNP application relative to control treatment. Consequently we analyzed the transcript level of \textit{D53} in response to LN, LP and the application of GR24 and SNP (supplemental Fig. S7). Compared with the control treatment, the transcript level of \textit{D53} was induced by the four treatments. However, western blot analysis revealed that the protein level of D53 in 1-cm rice apices was reduced by the four treatments (Fig. 6A). It has been reported that the \textit{D53} transcript level was induced by the application of GR24, while the protein level was markedly reduced via proteasome degradation in a manner that required D14 and the SCF\textsuperscript{D3} ubiquitin ligase (Zhou et al. 2013; Jiang et al. 2013). Therefore, proteasome inhibitor (MG132) experiments were conducted to ascertain the mechanism of the NO-dependent reduction of the D53 protein levels. Interestingly, the SNP-mediated degradation of D53 could be prevented by an inhibitor (MG132) of proteasome-dependent protein degradation, suggesting that NO, like SLs, triggered the degradation of D53 protein levels by the proteasome (Fig. 6A). Meanwhile, no difference in protein level of D53 was found in \textit{d14} mutant among control, LN, LP, and SNP application (Fig. 6B), supporting the D14–SCF\textsuperscript{D3}-mediated degradation of D53 protein reported by Zhou et al. (2013) and Jiang et al. (2013). No difference were observed in seminal root length of \textit{d53} mutants compared to WT plants, under LN, LP, application of GR24 and SNP (Fig. 6C). This suggested that NO, just like SL, degrades D53 protein level in a proteasome-dependent manner.
NO and SLs increase root meristem activity but not cell elongation

Root growth depends on two basal developmental processes: cell division in the root apical meristem and elongation of cells that leave the root meristem (Scheres et al. 2002). To verify NO- and/or SL-induced root elongation regulated by nutrient deficiency, the length of epidermal cells in the maturity zone were analyzed (Figs. 7A-B). No differences were recorded in the arrangement and morphology of the epidermal cells, including lengths under nutrient deficiency and after application of SNP and GR24, relative to the control. These results indicated that NO- and/or SL-induced root elongation was not due to the changes in cell elongation.

The meristematic activity of cells within the root meristem affects root growth (Blilou et al. 2005). Transgenic plants expressing the \( pCYCB1;1::GUS \) construct were used in the assessment of the cell cycle activity within the root meristem. The activity of \( CYCB1;1::GUS \) and the expression of \( CYCB1;1 \) gene were stronger under N- and P-deficiency compared to the control (Figs. 7C-D). Significant induction of \( pCYCB1;1::GUS \) and \( CYCB1;1 \) gene expression levels were observed in rice seedlings with SNP or GR24 application relative to the control, and a marked inhibition after application of Abamine under nutrient deficient conditions, to the same level as the control. Interestingly, the application of SNP and Abamine markedly decreased \( CYCB1;1::GUS \) and \( CYCB1;1 \) gene expression levels, suggesting that NO functioned upstream of SLs to participate in the cell cycle activity of the meristem of rice roots. Furthermore, cell number in meristem zone,
showed in Fig. 7E, had the similar pattern as that of pCYCB1;1::GUS. Taken together, these data showed that NO affected root elongation primarily by regulation of root meristem activity rather than by elongation of cells that leave the root meristem, with the involvement of SLs.

DISCUSSION

LN- and LP-induced NO accumulation is needed for seminal root elongation

NO has been identified as a signaling molecule in the regulation of root development (Pagnussat et al. 2002; Pangnussat et al. 2003; Correa-Aragunde et al. 2004; Lombardo et al. 2006; Zhao et al. 2007; Fernández-Marcos et al. 2011; Bai et al. 2014; Manoli et al. 2014). The promotion or inhibition of root elongation most likely depends on NO concentrations and the experimental conditions. Results showing NO inhibition of primary root elongation are mostly derived from experiments of the exogenous application of NO donors (Tomato, Correa-Aragunde et al. 2004; Arabidopsis, Fernández-Marcos et al. 2011; Bai et al. 2014; Méndez-Bravo et al. 2010) and NO-overproducing mutants (Arabidopsis, He et al. 2004). Results showing a positive link between endogenous NO and root growth, including primary root elongation were mostly derived from stress experiments (Maize, Zhao et al. 2007; Manoli et al. 2014; Trevisan et al. 2014; Rice, Xiong et al. 2009; White lupin, Wang et al. 2010; Meng et al. 2012) and mutant plants exhibiting lower NO levels (Arabidopsis,
Lozano-Juste & León, 2010a, 2010b; Sanz et al. 2014). In this study, although NO accumulation was induced by increased SNP concentrations of 0-20 μM, root elongation increased after 5 and 10 μM of SNP were applied, but decreased with 20 μM (Supplemental Fig. S1), confirming that the appropriate increment of NO could induce elongation of seminal roots. Interestingly, NO accumulation and seminal root lengths were increased by the application of 10 μM SNP, to levels similar to those under LN and LP, and were considerably inhibited by the application of cPTIO under LN and LP to the same levels as the control. This suggested that NO induced by N- and P-deficiency was positively correlated with elongation of the seminal roots.

Three local sites of NO synthesis were detected in Arabidopsis roots: one at the root cap statocytes, another at the QC and distal portion of the meristem, and the third at the distal part of the transition zone (Illés et al. 2006). The different localization of NO possibly reflects its diverse effects on plant growth and development. Fernández-Marcos et al. (2011) and Sanz et al. (2014) confirmed that organization of the primary root meristem was sensitive to changes in NO levels. Our data also showed that endogenous NO mainly accumulated in the root tip during seminal root development under LN and LP conditions. Furthermore, increased NO due to LN, LP and SNP application paralleled the increased levels of pCYCB1;1::GUS in the root meristem, suggesting that LN- and LP-induced NO affected seminal root elongation by inducing root meristem activity. These were consistent with the primary root-modulating properties of NO in Arabidopsis (Fernández-Marcos et al.
Our results defined the synthesis of NO induced by LN and LP in sites where a physiological effect was observed (i.e., the meristem of the seminal roots), and confirming the importance of NO in the shared signaling pathway of N- and P-deficiency-induced elongation of seminal roots by increasing the meristem activity in the root tips.

**NR- and NOS-dependent NO is induced by LN and NOS-dependent NO by LP**

Nitrate reductase (NR) and a putative NOS enzyme represent potential enzymatic sources of NO production in plants (Wilson et al. 2008). Plant NOS has not been identified to date (Crawford et al. 2006; Moreau et al. 2008; Gas et al. 2009; Moreau et al. 2010; Gupta et al. 2011), although experiments using inhibitors of the animal NOS enzyme have provided some evidence for the role of the L-arginine pathway in NO production (Zhao et al. 2007; Jin et al. 2011). Moreau et al. (2008) demonstrated that the Arabidopsis AtNOS1 did not possess NOS activity, as it was a GTPase, and thus was renamed as an NO-associated enzyme (AtNOA1). Despite questions on the role of AtNOS, noa1 mutants (formerly Atnos1) contained lower NO levels in the roots compared to WT plants (Guo & Crawford, 2005; Schlicht et al. 2013). Besides the NOA1-dependent pathway, NR is also involved in NO production in response to biotic and abiotic stresses (Modolo et al. 2005; Srivastava et al. 2009; Zhao et al. 2009; Chen et al. 2010; Freschi et al. 2010; Kolbert et al. 2010). As NIA1 was implicated in NO production in the guard cells (Bright et al. 2006) and in response to cold acclimation (Zhao et al. 2009), this supported the idea that NIA1 was a key component...
of NR-mediated NO production. Despite research showing that NOS rather than NR was involved in LN- or LP-induced NO production in several upland plants (Zhao et al. 2007; Mi et al. 2008; Trevisan et al. 2014; Wang et al. 2010), the mechanism by which LN and LP modulate NO levels in rice roots remains unclear.

In this study, NO was shown to be generated from a NIA2-dependent NR pathway, initially under LN conditions. Significant LN-induced NR activity in the roots was observed at day 1 relative to the control (Fig. 2D), while NR activity in the shoots dramatically decreased under LN (Supplemental Fig. S3). This suggested that initially under N deficiency, nitrate mainly accumulated in rice roots rather than being transported from the roots to the shoots. It can be speculated that NR activity in the roots decreased during the experimental period due to insufficient substrate for NR (Fig. 2D). In rice plants, transcription levels of NIA2 were considerably higher than NIA1 (Fan et al. 2007; Cao et al. 2008), consistent with the results obtained in Arabidopsis (Wilkinson & Crawford, 1991). In two nia2 mutants, NO accumulation and seminal root length were less sensitive to LN compared with the WT (Dongjin). Meanwhile, in WT plants NO accumulation and seminal root length were reduced after the application of tungstate to similar levels as those in nia2 mutants, and considerably induced by the application of SNP in nia2 mutants to the levels of WT plants under LN. Based on these results, NiA2-dependent NR was confirmed to be involved in LN-induced NO generation in rice plants.

In this study, there was a possible involvement of NOA and/ or NOS in LN- and
LP-induced NO production. No difference of NR activity, NO accumulation and seminal root lengths was observed in WT and \textit{nia2} mutants between control and LP treatments, confirming that LP-induced NO was not connected to the NR pathway. Furthermore, LN- and LP-induced \textit{NOA} transcription levels in rice roots, and L-NAME inhibited NO accumulation and root elongation under LN and LP conditions similar to those in the control group, indicated that the NOS pathway was likely involved in LN- and LP-induced NO generation.

**SLs are involved in NO-induced seminal root elongation**

The results above indicated that NO is considered a key regulator of rice seminal root elongation under LN and LP conditions. Previous studies have described its interaction with auxin and cytokinin in modulating root growth and developmental processes under nutrient deficiency (Fernández et al. 2011; Chen et al. 2010; Jin et al. 2011; Feng et al. 2012; Niu et al. 2013 and references therein; Shen et al. 2013). More recently, SLs have been shown to be involved in LN- and LP-induced seminal root elongation (Sun et al. 2014). The mechanisms of NO and SLs in modulating the perception and transduction of N- and P-deficiency, leading to root elongation, are of interest.

In this study, evidence suggested that SLs were involved in NO induction of root elongation in response to N- and P-deficiency in rice. First, similar NO accumulation was observed in the root tips of WT, \textit{d10} and \textit{d3} mutants in response to LN, LP and SNP.
application, indicating that deletion of SL synthesis and signaling did not affect NO accumulation (Fig. 4; Supplemental Fig. S5). Secondly, no response in terms of root elongation to LN, LP and SNP application was observed in \textit{d} mutants compared to WT plants. Thirdly, the application of SL inhibitor Abamine in addition with LN, LP and SNP application, decreased seminal root lengths by reducing the expression level of \textit{pCYCB1;1::GUS} and \textit{CYCB1;1} gene and cell number in meristem zone in WT plants to similar levels as the control group (Fig. 4C, Fig. 7). In addition, comparison with WT plants showed that lower expression level of \textit{D14} and \textit{D53} was observed in two \textit{nia2} mutants under both control and LN treatments and similar expression level was observed under SNP application, further indicating the link between NO and SL in regulating seminal root elongation.

NO action is dependent on its redox state, enabling it to interact with thiol groups, resulting in S-nitrosylation of proteins and thereby changes the protein functions (Palmieri et al. 2008). Several results provide support for the S-nitrosylation of key proteins via the ubiquitin-proteasome pathway (Kim et al. 2004; Romero-Puertas et al. 2007). In this study, although transcription levels of \textit{D14} and \textit{D53} were induced by SNP application in rice roots, only \textit{D53} had stronger transcript level induced by LN, LP, GR24 and SNP application relative to the control treatment (Fig. 5B and Supplemental Fig. S7). Meanwhile, results from western blot indicated that D53 protein level was reduced by the four treatments. D53-promoter-driven GUS reporter gene (\textit{pD53::GUS}) assay showed GUS staining in the
roots (Zhou et al. 2013), which matched the localization of NO. Furthermore, the SNP-mediated degradation of D53 could be prevented by an inhibitor (MG132) of proteasome-dependent protein degradation; similar protein level of D53 in d14 mutant among control, LN, LP, and SNP application supported the D14–SCF<sup>D3</sup>-mediated degradation of D53 protein reported by Zhou et al. (2013) and Jiang et al (2013). These suggested that NO, similar to SLs, triggered proteasome-mediated degradation of D53 and the D53 protein was the possible target of the NO action.

In this study, using NO-related and SL synthetic and signaling rice mutants, NO was shown to have a crosstalk with the SLs under N- and P-deficiency that led to the elongation of seminal roots via induction of meristem cell activity. Results from western blot suggested that D53, a repressor of SLs, was a target of NO signaling.

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Fig. 1. The length of seminal roots and the accumulation of nitric oxide (NO) in wild-type (WT) rice seedlings (Shiokari) in response to N- and P-deficiency. Seedlings were grown in a hydroponic media containing normal nutrition (Control; 2.5 mM N, 300 μM P), or under N- and P deficiency (LN, 0.02 mM N; LP, 2 μM P) for 16 days. A, The morphology of the rice plants; B, Seminal root lengths over time; C, NO production shown as green fluorescence in the root tips; D, NO production in the root tips expressed as fluorescence intensity. Bar = 1mm. Data are means ± SE. *, P < 0.05 (ANOVA) comparing the control and nutrient deficiencies at the same experimental period. d = days.
Fig. 2. qRT-PCR analysis of *NO-associated* (*NOA*) gene, relative nitrate reductase (NR) activity, accumulation of nitric oxide (NO) and seminal root lengths in the wild-type (WT) rice seedlings (Shiokari). Seedlings were grown in hydroponic medium containing normal nutrition (Control; 2.5 mM N, 300 μM P), N- and P-deficiency (LN, 0.02 mM; LP, 2 μM P) in addition to SNP (10 μM), cPTIO (80 μM), and L-NAME (100 μM) for 14 days. A-B, photographs of NO production shown as green fluorescence in the root tips (A), and NO production expressed as fluorescence intensity relative to the root tips (B). C, Seminal root lengths. D, Relative nitrate reductase (NR) activity in the roots. E, Relative expression of *NOA* at days 1, 7 and 14. Bar = 1mm. Data are means of 12 replications ± SE. *, P < 0.05 (ANOVA) comparing the control and other treatments at the same experimental period. d = days.
Fig. 3. Root morphology and the accumulation of nitric oxide (NO) in nia2 mutants and wild-type (WT) rice seedlings (Dongjin). Seedlings were grown in hydroponic medium containing normal nutrition (Control; 2.5 mM N, 300 μM P), N- and P-deficiency (LN, 0.02 mM N; LP, 2 μM P) in addition to SNP (10 μM) and tungstate (Tu; 25 μM) for 14 days. A, The morphology of rice plants under LN and LP for 14 days. B, Seminal root lengths. C, NO production shown as green fluorescence in the root tips; D, NO production in the root tips expressed as fluorescence intensity. E, Seminal root lengths in response to LN, LP, SNP and Tu. Bar = 1mm. *, P < 0.05 (ANOVA) comparing WT plants under the control treatment and other treatments.
Fig. 4. Accumulation of nitric oxide (NO) and seminal root length. Seedlings were grown in hydroponic media containing normal nutrition (Control; 2.5 mM N, 300 μM P), N- and P-deficiency (LN, 0.02 mM N; LP, 2 μM P) for 14 days. A, NO production shown as green fluorescence in the root tips; B, NO production in the root tips expressed as fluorescence intensity; C, Seminal root lengths in strigolactone-synthesis (d10) and -signaling (d3) mutants and wild-type (WT, Shiokari) rice seedlings in response to LN, LP, SNP (10 μM), GR24 (2.5 μM) and Abamine (100 μM); Bar = 1 mm. *, P < 0.05 (ANOVA) comparing WT plants under the control treatment and other treatments.
Fig. 5. Levels of three strigolactone fractions exuded by wild-type (WT) rice plants (Shiokari), qRT-PCR analysis of strigolactone-synthesis and -signaling genes in WT rice plants. Seedlings were grown in hydroponic medium containing normal nutrition (Control; 2.5 mM N, 300 μM P), N- and P-deficiency (LN, 0.02 mM N; LP, 2 μM P) in addition to SNP (10 μM) for 14 days. A, Levels of three strigolactone fractions exuded. B, Gene expression. *, P < 0.05 (ANOVA) comparing the control treatment and the application of SNP.
Fig. 6. Protein levels of OsD53 in 1-cm root apices of wild-type (WT, Shiokari) rice plants (A) and d14 mutant (B), and seminal root length in d53 (C). Seedlings were grown in hydroponic medium containing normal nutrition (Control; 2.5 mM N, 300 μM P), N- and P-deficiency (LN, 0.02 mM N; LP, 2 μM P) in addition to GR24 (2.5 μM) and SNP (10 μM) with or without MG132 (50 μM) for 24 hours (A, B) and for 14 days (C). Protein levels of D53 were determined by immunoblotting with anti-D53 antibodies. *, P < 0.05 (ANOVA) comparing WT plants under the control treatment and other treatments.
Fig. 7. Epidermal cell lengths in the maturity zone, expression levels of pCYCB1;1::GUS, OsCYCB1;1 and cell number in the meristem zone. Seedlings were grown in hydroponic media containing normal nutrition (Control; 2.5 mM N, 300 μM P), N- and P-deficiency (LN, 0.02 mM N; LP, 2 μM P) in addition to SNP (10 μM), GR24 (2.5 μM) and Abamine (100 μM) for 14 days. A-B, Epidermal cell length. Bar = 100 μm. C, Cell cycle activity of the root meristem, as monitored by the pCYCB1;1::GUS reporter. Bar = 500 μm. D, The expression of CYCB1;1 gene. E, Cell number of meristem zone. Data are means ± SE. *, P < 0.05 (ANOVA) comparing WT plants under the control treatment and other treatments.