Inhibition of nitric oxide production under alkaline condition regulates iron homeostasis

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July 13, 2020

Abstract

Rice is one of the most susceptible plant to iron (Fe) deficiency under neutral and alkaline conditions. Alkaline stress induces H2O2 production and increases the deposition of Fe on roots surface, which causes leaf chlorosis and Fe deficiency in rice. Gene chip and qRT-PCR analysis indicated that the expression of nitrate reductase (NR) genes were down-regulated by alkaline treatment, which resulted in significantly decreased nitrate activities and nitric oxide (NO) production in epidermis and stele, where the H2O2 was accumulated. In contrast, treatment with sodium nitroprusside (SNP), a NO donor, strongly alleviates alkaline-induced Fe deficiency by limiting Fe plaque formation. Increasing the NO signal significantly reduces the accumulation of H2O2 and lignin barrier, but enhances phenolic acid secretion in root epidermis and stele under alkaline stress. The secreted phenolic acid effectively mobilized the apoplast Fe and increased Fe uptake in root, thus which alleviate the Fe deficiency response and down-regulate expression of Fe uptake genes under alkaline condition. In conclusion, alkaline stress inhibits the NR activity and NO production in roots of rice, which plays a vital role in mobilizing the apoplast Fe by regulation of H2O2 and phenolic acids concentrations.

Introduction

Iron (Fe) is one of the essential microelements for plant growth and development. Although Fe is the second most abundant metal in the earth's crust, the solubility of Fe is extremely low, especially in aerated neutral to alkaline soils. Plants require $10^{-6}$to 10^{-5} M of Fe for optimal growth, but the concentration of phytoavailable Fe in calcareous soils barely exceeds 10^{-10} M (Tsai & Schmidt, 2017a). Fe deficiency is a common phenomenon for crops grown on calcareous soils. To cope with the Fe deficiency stress, plants have evolved sophisticated mechanisms for acquiring Fe from the soil, which can be grouped into two strategies (Kobayashi & Nishizawa, 2012).

Strategy I Fe uptake mechanism has been observed in most investigated species, but is found to be absent in grasses (Kobayashi & Nishizawa, 2012). It is characterized by pumping protons to acidify the rhizosphere, increasing Fe^{3+} reduction capacity and Fe^{2+} uptake system. Under Fe deficiency condition, the expressions of *H^+-ATPase* (*AHA1*),*FERRIC REDUCTASE OXIDASE 2* (*FRO2*) and *IRON REGULATED TRANSPORTER 1* (*IRT1*) are induced in Arabidopsis, which are responsible for the pumping of protons, reduction of Fe^{3+} and uptake of Fe^{2+} in strategy I plants, respectively (Kobayashi & Nishizawa, 2012). Instead of reducing Fe^{3+} by strategy I system, grasses employ strategy II system that is characterized as the synthesis and release of mugineic acid (MAs) family phytosiderophores (PS) from their roots to solubilize the Fe^{3+} sources in soil (Romheld & Marschner, 1986; Kobayashi & Nishizawa, 2012). The Fe^{3+}-MA complex is then taken up through yellow strip (YS) or yellow strip like (YSL) transporters (Curie *et al.* , 2001; Inoue *et*

al. , 2009). Although the PS-based Fe uptake ability decreased with increasing pH, the reduction-based system is much more sensitive to alkaline condition owing to a strongly compromised reduction of Fe^{3+} under such conditions (Tsai & Schmidt, 2017a). In addition to the reported two strategies for Fe uptake, recent studies show that traditionally defined strategy I plants secrete phenolic compounds to bind and possibly reduce Fe^{3+} under neutral and alkaline conditions, which increased the resistance to Fe deficiency in plants (Rodríguez-Celma & Schmidt, 2013; Schmid *et al.* , 2014; Tsai & Schmidt, 2017a).

As in other graminaceous plants, rice induces the synthesis and secretion of MAs for Fe absorption (Wu *et al.* , 2011). In addition, rice has been evolved a strategy I like system to uptake Fe. It is reported that rice can directly uptake Fe^{2+} by inducing the expression of *OsIRT1* and *OsIRT2* under Fe deficiency (Ishimaru *et al.* , 2006). Moreover, rice secretes phenolic compounds by efflux transporters, *OsPEZ1* and*OsPEZ2* , which are involved in apoplasmic precipitated Fe solubilizing and uptake (Bashir *et al.* , 2011; Ishimaru *et al.* , 2011). Although different Fe uptake systems exist in rice, it is one of the most sensitive plant species to Fe deficiency under alkaline condition (Römheld & Marschner, 1990). High external pH induces a large number of aerenchymas in the roots of rice, which causes the formation of Fe plaque on the root surface and decreases the available Fe concentration around the roots (Chen *et al.* , 2018). Alkaline stress also induces the expression of several peroxidase genes and H_2O_2 accumulation in the roots of rice seedlings, which are stimulated by the biosynthesis of lignin in the cell walls (Chen *et al.* , 2018). As a result, the concentration of phenolic compounds, which share the same precursors with lignin, significantly decreases in the cell walls. Low concentration of phenolic compounds caused Fe deposition in apoplast and Fe deficiency response under alkaline condition in rice (Araki *et al.* , 2015; Chen *et al.* , 2018).

It is reported that Nitric oxide (NO), an important signaling molecule, plays a crucial role in regulation of the Fe-starvation response in plants. Fe deficiency significantly increases NO contents in both strategy I and strategy II plants (Magdalena & Lorenzo, 2007; Chen*et al.* , 2010; Jin *et al.* , 2011; Ye *et al.* , 2015; Sun *et al.* , 2017; Zhu *et al.* , 2018). In Arabidopsis, the mobile reservoir of NO, S-nitrosoglutathione (GSNO), is involved in regulating expressions of the central transcription factors,*FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR 1*(*AtFIT1*) and subgroup Ib basic helix-loop-helix genes bHLH38/39/100/101 (Chen *et al.* , 2010; Kailasam *et al.* , 2018). Therefore, NO significantly increases the expressions of Fe acquisition genes and soluble Fe content in both roots and shoots. In addition to regulation of the Fe uptake system, NO also participates in utilization of the cell wall bound Fe reservoir (Ye *et al.* , 2015; Zhu *et al.* , 2016; Tsai & Schmidt, 2017b). Fe deficiency induces the synthesis of putrescine, which can induce cell wall Fe remobilization and correct Fe deficiency symptoms in Arabidopsis. However, the alleviation of Fe deficiency effect by putrescine is lost in the NO synthase mutant *noa1* and nitrate reductase mutant*nia1nia2* , in which the endogenous NO level is significantly reduced (Zhu *et al.* , 2016).

Nitrate Reductase (NR) and NO synthase-like (NOS) are two potential enzymatic sources of NO production in plants (Besson-Bard *et al.* , 2008). More and more evidences suggest that NR is one of the most important enzymes in NO production in plants (Chamizo-Ampudia *et al.* , 2017). After nitrate is absorbed into plant cells, it is firstly reduced to nitrite by the cytosolic enzyme NR. The generated nitrite is further reduced to ammonium or NO (Chamizo-Ampudia *et al.* , 2017). The NR-deficient double knock-out mutant *nia1nia2* exhibited reduced levels of NO in Arabidopsis (Chen *et al.* , 2010). Similarly, a nitrate reductase deficient knock mutant *nia1*decreases the production of NO in rice (Sun *et al.* , 2016). In contrast to the detailed studies in NR pathway, it is proved that the plant did not possess NO synthase activity and the *AtNOS1* is renamed as NO-associated enzyme (*AtNOA1*) (Moreau *et al.* , 2008; Gas *et al.* , 2009).

Significant differences exist in Fe efficiency adaptation between graminaceous species, which is related to the amount and type of the phytosiderophores released under Fe deficiency (Römheld & Marschner, 1990). Although most of the grasses are well adapted to calcareous soils, rice is one of the most susceptible plant to Fe deficiency under neutral and alkaline conditions. The reason of the phenomenon is generally considered as rice secreting very low amounts of MAs compared with other graminaceous plants (Römheld & Marschner, 1990). However, the signal transduction and physiological mechanisms of the phenomenon were largely unknown for the high pH inhibited Fe uptake in rice. In this study, we showed that NR mediated nitric oxide

production plays a key role in alkaline-induced Fe deficiency response in rice. A working model was proposed by a combination of physiological and biochemical analyses in rice under alkaline condition.

Materials and Methods

Plant materials and growth conditions

The rice (*Oryza sativa L.* cv. *ZH11*) was used in this study. Seeds soaked in 1% nitric acid for 12 hours at 30 °C, then washed with tap water and germinated in tap water for 2 days at 30 °C in the dark. Germinated seeds were transferred to a net floating on a culture solution with pH 5.5, which consisted of 1.425 mM NH_4NO_3, 0.323 mM NaH_2PO_4, 0.513 mM K_2SO_4, 0.998 mM $CaCl_2$, 1.643 mM $MgSO_4$, 0.25 mM Na_2SiO_3, 9.5 μM $MnCl_2$, 0.075 μM $(NH_4)_6Mo_7O_{24}$, 0.019 mM H_3BO_3, 0.155 μM $CuSO_4$, 0.152 μM $ZnSO_4$ and 0.125 mM EDTA-Fe(II). The plants were grown in a greenhouse at dark 28 °C /day 30 °C under natural light. 10-day-old plants were transferred to nutrition solutions with different pH. For the Sodium Nitroprusside (SNP) or nitrite treatments, nutrient solutions were applied with 50 μM SNP or 1 mM nitrite, respectively. The pH of the nutrient solution was adjusted every day with 1 M HCl or NaOH. All the nutrient solutions were renewed every 4 days.

Measurement of Chlorophyll Content

Soil–plant analyzer development (SPAD) values were determined with a portable chlorophyll meter (SPAD-502, Minolta Sensing) on the young leaves of seedlings under different treatment conditions.

Measurement of the total Fe concentration

Leaves were sampled and killed at 105 °C for two hours, then dried at 80 °C to constant weight. Dried leaves (˜0.1 g) were digested with HNO_3 and $HClO_4$ (4:1, v/v) at 120 °C for 12 hours. The digested solutions were cooled to room temperature and then diluted to 20 ml. Fe concentrations were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Perkin Elmer, ELAN DRC-e, USA).

Quantitative Real-Time PCR (qRT-PCR)

The total RNA was extracted from the roots using OmniPlant RNA Kit (DNase I) reagent following the manufacturer's instructions (CWBIO, NO. CW2598S, China). Samples of 1 μg of total RNA were used to synthesize cDNA using a High-Capacity cDNA reverse-transcription kit according to the instructions (HiFiscript gDNA Removal cDNA Synthesis kit, CWBIO, China). qRT-PCR assays were performed with SYBR Green FAST qPCR Kit (Kapa Biosystems) on an Applied Biosystems 7500 Real-Time PCR System. The rice *ACTIN* gene (*LOC_Os03g13170*) was used as the internal reference. Relative expression levels were calculated using the $2^{-\Delta\Delta\tilde{}\tau}$method. The sequences of the primers used in this research were listed in the Table. S2.

H_2O_2 staining and quantification

For histochemical visualization of H_2O_2, root apexes were incubated with 50 mM Tris-HCl (pH 3.8) containing 1 mg/ml diaminobenzidine (DAB) for 3 hours. The stained roots were observed by a JSZ6S stereomicroscope with a colour CCD camera (Bajiu, China). For a cross-section of root tips, the stained root samples were fixed in 2.5% agar. Sections (100 μm) were made using a vibration microtome and visualized under white light with a Nikon Eclipse E600 microscope (Nikon, Japan).

For quantitative H_2O_2 assay, root samples (˜0.1g) were collected and homogenized in 1 mL ice-cold acetone, and then centrifuged at 8000 g at 4 for 10 min. The supernatant was used to the H_2O_2 content assay according to the instruction of H_2O_2 detection kit (Solarbio, NO. BC3590, China). The absorbance of the reaction solution mixture was measured at 415 nm.

Phenolic acids fluorescence and lignin staining in roots

Root samples (5 cm from the root tip) were fixed in 2.5% agar and cross-sections (100 μm) were made using a vibration microtome. The fluorescence of phenolic acids in the cross-section was observed and photographed under ultraviolet light with a Nikon Eclipse E600 microscope (Nikon, Japan). For lignin staining, the cross-sections of roots were incubated with 2% resorcinol solution (0.5 g of resorcinol in 95% ethanol) for 5 min, and then transferred to 50% HCl for 10 minutes. The samples were observed and photographed under white light with a Nikon Eclipse E600 microscope (Nikon, Japan).

Nitrate reductase activity assay

Root samples (˜0.3 g) were collected and ground into fine powders in liquid nitrogen and homogenized in 2 mL ice-cold extraction buffer containing 25 mM phosphate (pH 8.7), 7 mM cysteine, and 1 mM EDTA. Samples were gently agitated on ice for 0.5 h and then centrifuged at 10000 g at 4 °C for 20 min, take supernatant to a new tube. The supernatant was protein extracts. The protein content was quantified using BCA Protein Assay Kit (CWBIO, NO. CW0014S, China). The activities of nitrate reductase were measured by adding 3.5 mg protein extracts to 1 mL 80 mM Phosphate buffer solution (pH 7.5) containing 60 mM KNO_3 and 0.6 mM NADH. The mixtures were incubated at 25°C for 30 min, and then 0.5 mL of 58 mM sulphanilamide and 7 mM N-(1-naphthyl) ethylenediamine in 12 M glacial acetic acid were added to the solutions. The absorbance of the mixtures was measured at 540 nm after 15 min. Nitrate reductase activity was expressed as nmol NO_2^- per milligram protein per h (nmol $NO_2^- mg^{-1}$ Pr h^{-1}).

Measurement of NO content in the Roots

Root apexes were incubated with 5 μM DAF-FM DA (Beyotime, NO. S0019, China) for 20 min at 37, and then washed three times in 20 mM HEPES-KOH buffer (pH 7.4). The stained roots were visualized with a Nikon Eclipse E600 microscope at EX 495 nm, EM 515 nm (Nikon, Japan). The cross-sections of roots were made as described above. The signal intensities of green fluorescence in the root images were determined by measuring the average pixel intensity with ImageJ software. Data were presented as the means of fluorescence intensity relative to that of control.

Results

Increased pH inhibited NO production and nitrate reductase activity in the roots

It is reported that reactive oxygen species (ROS) are involved in the alkaline induced Fe deficiency response in rice. Because ROS and NO signaling interacts with each other in plant, the level of NO was measured under different pH conditions by using a NO-specific fluorescent probe DAF-FM DA. A high NO signal was detected on the root under pH 4 condition and the signal intensity of NO decreased with increasing the pH values in the solution (Fig. 1a). Quantification of the fluorescence signal intensity showed the decreased accumulation of NO in the root tips by 29% and 39% under pH 6 and pH 8 conditions compared with pH 4, respectively (Fig. 1b). Cross section of the roots showed that pH 8 treatment mainly decreased NO concentration in the exodermis and steel (Fig. 1c). Interestingly, pH 8 induced Fe plaque formation is also in the exodermis, indicating a putative role of NO production in alkaline response in rice (Chen *et al.* , 2018).

It is reported that *NIA* and *NOA* genes were accounted for the NO production in plants. Therefore, the annotated *NIA* and*NOA* genes were selected for analysis according to a reported transcriptional profiling data, which were treated the rice under different pH 6 and pH 8 conditions. Interestingly, the expressions of*OsNIA1* and *OsNIA2* were significantly reduced under high pH condition compared with low pH condition. However, the expressions of*OsNOA1* genes were not changed under different pH conditions (Table. S1). To further confirm the RNA sequencing data, qRT-PCR was used to measure the transcripts levels of *OsNIA1* and*OsNIA2* under different pH treatments. The expressions of*OsNIA1* and *OsNIA2* were significantly decreased under pH 8 condition compared with that of pH 6 condition, which was in accordance with the RNA sequencing data (Fig. 2a). Moreover, the expression of*OsNIA1* and *OsNIA2* were significantly lower

under pH 6 condition compared with that of pH 4 condition (Fig. 2a). Then the nitrate reductase activity was measured under different pH conditions. It was shown that the nitrate reductase activities of roots decreased 50% and 77% under pH 6 and pH 8 conditions, respectively, compared with that under pH 4 condition (Fig. 2b). Therefore, increasing the pH values of solution simultaneously inhibited the nitrate reductase activity and NO production, which showed a significantly negative correlation with medium pH values (Fig. S1).

The NO donor SNP relieved alkaline induced Fe deficiency in rice

To further evaluate the roles of NO in alkaline condition, sodium nitroprusside (SNP), which is a commonly used NO donor, was used to mimic the NO production. Rice seedlings were grown in normal hydroponic nutrient solution for 10 days, then transferred to nutrient solutions with pH 5.5 and pH 7.5, respectively. pH 7.5 treatment, which represented a slight alkaline condition, suppressed the growth of rice seedlings and induced the formation of Fe plaque on the roots (Fig. 3a, c). Plant height and weight were decreased 16% and 34% at pH 7.5 condition compared with that at control pH condition (Fig. 3d, e). In addition, pH 7.5 treatment induced chlorosis on young leaves, which resulted in a significantly decreased SPAD values on the 1^{st} to 3^{rd} newly emerged leaves (Fig. 3b, f). It was reported that growth inhibition and chlorosis of young leaves under alkaline conditions were caused by the decreased Fe uptake in rice. Therefore, the Fe concentrations were measured in leaves. Fe concentrations were decreased 48%, 25% and 15% from the 1^{st} to 3^{rd} newly emerged leaves, respectively, which was positively correlated with SPAD values of the leaves (Fig. 3f, g).

SNP treatment significantly alleviated the growth inhibition of rice seedlings under pH 7.5 condition (Fig. 3a, d, e). Moreover, the formation of Fe plaque on roots and chlorosis on leaves was suppressed by SNP treatment under pH 7.5 condition (Fig. 3a, b, c). Accordingly, SNP treatment retuned the SPAD values of young leaves under pH 7.5 condition to similar levels of pH 5.5 condition (Fig. 3f). Interestingly, SNP stimulated Fe accumulation in all leaves at standard pH (5.5), whilst significantly increased Fe concentrations in the 1^{st} and 2^{nd} new leaves under alkaline pH (Fig. 3g).

SNP inhibited the expression of Fe deficiency induced genes

To explore whether SNP stimulated Fe uptake was mediated by up-regulation of Fe transport systems, the expression of genes that was involved in Fe uptake and translocation were measured under different pH and SNP treatment conditions. *OsIRO2* , *OsNAS1* , *OsNAS2* , *OsYSL15*and *OsIRT1* were responsible for Fe^{3+} and Fe^{2+} uptake in rice, respectively (Ishimaru *et al.* , 2006; Wang *et al.* , 2020). The expressions of *OsIRO2* ,*OsNAS1* , *OsNAS2* , *OsYSL15* and *OsIRT1* were significantly induced under high pH condition (Fig. 4), indicating the rice seedlings suffered with Fe starvation. However, the expressions of these genes were significantly suppressed by SNP treatment under both pH 5.5 and pH 7.5 conditions (Fig. 4). *OsFRDL1* was a citrate transporter and regulated the Fe translocation from root to shoot (Yokosho *et al.* , 2009). *OsPEZ1* and *OsPEZ2* encoded the phenolics efflux transporters and stimulated Fe uptake (Bashir*et al.* , 2011; Ishimaru *et al.* , 2011). Although the expressions of *OsFRDL1* , *OsPEZ1* and *OsPEZ2* were induced by SNP under pH 5.5 condition, a significantly decreased or unchanged expression levels of these genes were observed by SNP treatment compared with that of control plants under pH 7.5 condition (Fig. 4). Therefore, enhanced Fe uptake and accumulation by SNP treatment unlikely caused by the regulation of the expressions of genes related to Fe transport and translocation.

SNP reduced the alkaline induced accumulation of H_2O_2 and lignin barriers in exodermis and steel of roots

Alkaline stress induced the accumulation of H_2O_2 and synthesis of lignin in roots, which resulted in decreased concentration of phenolic acids and Fe deposition in the apoplast (Chen *et al.* , 2018). To determine whether SNP affected these processes, H_2O_2 concentrations were measured under different pH and SNP treatments. It is showed that H_2O_2 concentration significantly enhanced in the roots by pH 7.5 treatment compared with that at pH 5.5 condition (Fig. 5a, b). Interestingly, SNP treatment significantly decreased the H_2O_2 concentration under pH 7.5 condition (Fig. 5a, b), which is in accordance with the scavenging effect of NO on H_2O_2 (Kolbert & Feigl, 2017). A cross section of the root tips further indicated that the alkaline induced

H_2O_2 mainly restricted in the exodermis and steel, where the NO production was significantly decreased (Fig. 1c). SNP treatment significant decreased the H_2O_2 concentration in these tissues (Fig. 5c). Because the synthesis of lignin was stimulated by the H_2O_2 concentration, the changed H_2O_2 concentration have a pronounced effect on the lignin concentrations in roots. Lignin concentration was accumulated under pH 7.5 condition while SNP treatment decreased the lignin concentration (Fig. 6a). In contrast, the phenolic acids, which use the same precursor of lignin and important for solubilizing apoplasmic Fe, were inhibited by pH 7.5 treatment but stimulated by SNP treatment (Fig. 6b).

Nitrite treatment induced NO production in roots

It is reported that NO was generated by reduction of the nitrite, which was the product NO_3^- reduction by NR (Desikan *et al.* , 2002). Therefore, the nitrite was used to treat the rice plants under pH 5.5 and pH 7.5 conditions. However, nitrite is toxic to plant and inhibits rice growth after a prolonged treatment (data not show). Then a short-term treatment was employed to avoid the toxicity symptoms by nitrite in plants. As expected, nitrite treatment significantly increased NO production in rice under both pH 5.5 and pH 7.5 conditions (Fig. 7a, b). Although NO concentration significantly decreased under pH 7.5 condition, nitrite treatment increased the NO concentration similar to that of pH 5.5 condition (Fig. 7b). 3-day nitrite treatment did not change the growth of rice seedlings, however, the induced Fe plaque and H_2O_2accumulation by pH 7.5 treatment were inhibited in roots by nitrite treatment (Fig. 7c, d, e).

Discussion

Alkaline treatment significantly inhibited NR mediated NO production

To figure out whether NO is directly involved in the regulation of alkaline induced Fe deficiency, the NO concentrations were measured under three different pH conditions. It showed that NO concentrations of roots decreased with the increase of pH in the solutions (Fig. 1). More precisely, the high pH inhibited NO production mainly restricted in the exodermis and steel, where the H_2O_2 and lignin accumulated (Fig. 1c, Fig. 5c and 6a). In plants, NR and NOS-like are two potential pathways in NO production. Although the NOS-like pathway did not affect NOS activity, it proved to produce NO and renamed to NO-associated enzyme (Moreau *et al.* , 2008; Gas *et al.* , 2009). There are two NR genes (*OsNIA1* and *OsNIA2*) and one*NOA* gene (*OsNOA1*) in rice genome. According to a transcript profiling data, high pH down-regulated the expression of*OsNIA1* and *OsNIA2* other than the *OsNOA1* genes (Tab S1). qRT-PCR analysis confirmed the high pH inhibited expressions of*OsNIA1* and *OsNIA2* genes (Fig. 2a). Consistence with the expression data, NR activity significantly decreased when the pH of the solution increased (Fig. 2b). Therefore, alkaline inhibited NO production should be controlled by the NR pathway, which is supported by the positive correlation between NR activity and NO production under different pH conditions (Fig. S1).

To further prove whether the alkaline stress induced Fe deficiency was caused by the decreased NR activity and NO production, the major NR gene in roots, *OsNIA1* , was overexpressed in rice. Although overexpression of *OsNIA1* slightly increased Fe concentration compared with WT in rice, it could not restore the growth and leaf chlorosis responses under pH 7.5 (data not show). It is reported that alkaline stress caused the reduction of NO_3^- content in the roots of rice (Wang *et al.* , 2012). Therefore, only overexpression of*OsNIA1* was not enough to stimulate the synthesis of NO and restore the growth of rice under pH 7.5 because of decreased NO_3^- concentration. Consistence with the assumption, our transcripts profiling data indicated that the expressions of *OsNRT1.1B* , *OsNPF2.2* and *OsNAR2.1*were significantly decreased under alkaline condition compared with the acidic condition (Tab. S1). Although the relation between NR and NO production has accumulated steadily in plants, the efficiency of NO production by NR is very low at physiological conditions (Rockel*et al.* , 2002). It is proposed that NO-forming nitrite reductases (ARC proteins) are responsible for the reduction of nitrite to NO in plants (Chamizo-Ampudia *et al.* , 2016; Chamizo-Ampudia *et al.* , 2017). In rice, there are three ARC genes and their expressions are not affected under different pH conditions (Tab. S1). This may explain the phenomenon that NO_2^-, the direct products of NR, significantly

increased the NO production and inhibited the H_2O_2 accumulation and Fe plaque in roots under pH 7.5 (Fig. 7).

Alkaline stress induces apoplast Fe deposition and Fe deficiency responses in rice

Alkaline stress has a significantly negative effect on plant growth and reduced effectiveness of micronutrients such as Fe. The chlorophyll and Fe concentrations are significantly lower in shoots of Arabidopsis and rice under alkaline condition (Rodríguez-Celma & Schmidt, 2013; Schmid*et al.* , 2014; Araki *et al.* , 2015; Chen *et al.* , 2018). However, supplying the chelated Fe(III) or spaying soluble Fe can recover the chlorosis of leaves and moderately improve the growth of plants under alkaline condition (Schmid *et al.* , 2014; Chen *et al.* , 2018). These phenomena indicate that alkaline stress inhibited plant growth was mainly caused by Fe deficiency. Rice is one of the most sensitive crops to alkaline condition, which exhibited Fe deficiency responses under pH 7.5 condition (Fig. 3). Transcripts profiling data showed that high pH induced the expression of a number of Fe-regulated gene expressions, including Fe uptake genes involved in strategy I-like and strategy II systems (Chen *et al.* , 2018). In this study, qRT-PCR confirmed that the expressions of *OsIRO2* , *OsNAS1* , *OsNAS2* ,*OsYSL15* and *OsIRT1* were significantly induced under pH 7.5 condition (Fig. 4). Moreover, the expressions of Fe translocation gene *OsFRDL1* and phenolic efflux transporters, *OsPEZ1* and *OsPEZ2* , were induced or not affected under pH 7.5 condition (Fig. 4). Therefore, high pH induced Fe deficiency responses were not caused by the down-regulation of Fe uptake systems.

Although the concentrations of solubility Fe^{2+} are high in the flooded paddy field, rice suffered Fe deficiency under calcareous soils (Ishimaru *et al.* , 2007). This is because alkaline stress increases the radial oxygen loss by inducing the formation of aerenchyma in rice, which oxidizes the rhizosphere Fe^{2+} into $Fe(OH)_3$. The solubility of $Fe(OH)_3$ is extremely low and deposits in the apoplast of root, finally forming the Fe plaques on the roots of rice (Chen *et al.* , 2018). Therefore, the deposition of $Fe(OH)_3$ in the roots decreased phytoavailable Fe and resulted in Fe deficiency under high pH condition in rice. Accordance with the assumption, adding 3-30 μM 2'-deoxymugineic acid in solution increased the solubility of Fe^{3+} and almost complete restoration in shoot height and SPAD values under high pH condition in rice (Araki *et al.* , 2015).

NO stimulates apoplast Fe mobilizing by regulating the H_2O_2 accumulation in rice

NO is reported to be involved in regulating Fe absorption in various plants. In Arabidopsis, NO positively regulates the high-affinity Fe-uptake genes in roots, such as *AtFRO2* , *AtFIT* and*AtIRT1* (Chen *et al.* , 2010). In this study, it is shown that the SNP not only restored the leaves chlorosis, plant growth and Fe concentration under pH 7.5 condition, but also stimulated the Fe uptake under pH 5.5 condition (Fig. 3). To elucidated whether NO influenced the expression of Fe uptake systems, the expressions of Fe responsive marker genes involved in Fe absorption and translocation were examined.*OsIRO2* , *OsNAS1* , *OsNAS2* and *OsYSL15* are responsible for the Fe^{3+} uptake, while *OsIRT1*is the key transporter for Fe^{2+} uptake (Kobayashi & Nishizawa, 2012). Differ from the NO role in Fe uptake in Arabidopsis, all the expressions of these genes were down-regulated by SNP treatment under both pH 7.5 and pH 5.5 conditions (Fig. 4), excluding the possibility that SNP stimulated Fe absorption by inducing the expressions of Fe uptake genes in rice.

In addition to directly enhance Fe uptake, NO acts downstream of putrescine to reutilization of root cell-wall Fe in *Arabidopsis* , although the mechanism is still unknown. In this study, it was shown that the formation of Fe plaque on roots were suppressed by SNP and nitrite treatment under pH 7.5 condition (Fig. 3), indicating the role of NO in apoplast Fe solubilizing in rice. Both Arabidopsis and rice could secret phenolic acids to mobilize insoluble Fe^{3+}in cell walls and increase the Fe uptake ability (Ishimaru *et al.* , 2011; Fourcroy *et al.* , 2014). In this study, it is shown that the concentration of phenolic acids was inhibited by pH 7.5 treatment but stimulated by SNP treatment, which is negatively correlated with the Fe plaque formation on roots (Fig. 3c and Fig. 6b). Moreover, SNP treatment significantly decreased H_2O_2 accumulation in the roots under pH 7.5 condition (Fig. 5), which was in accordance with the antioxidation function of NO in plants. Because H_2O_2stimulates Fe plaque formation and lignin synthesis, the inhibition of H_2O_2 by SNP results in the decreased lignin synthesis and increased accumulation of phenolic compounds (Fig. 6), which

may help to mobilize and uptake the deposited Fe^{3+} in the roots. This assumption is supported by the decreased Fe plaque and increased Fe concentrations under pH 7.5 condition treated with SNP (Fig. 3c, g). In fact, overexpression of*OsPEZ1* , which is the phenolic compounds efflux transporter, also increased the growth performance in rice under calcareous soils (Ishimaru *et al.* , 2011). The increased Fe concentrations may feedback regulation the Fe uptake systems, which explained the suppressed expressions of Fe responsive genes by SNP (Fig. 4).

In conclusion, we proposed a working model to explain the NO production and apoplastic Fe utilization under alkaline condition in rice (Fig. 8). Alkaline stress inhibits the expressions of nitrate transporter genes and reduces the NO_3^- content in roots, which causes the down-regulation of NR activity and NO production in root epidermis and stele. Because the NO negatively regulates H_2O_2 concentration, decreasing the NO level will accumulate H_2O_2 in roots, which stimulates the synthesis of lignin and decreased the concentration of phenolic acids. Our previous study has proved that alkaline stress induces the oxidation of rhizosphere Fe^{2+} into Fe^{3+}, which results in $Fe(OH)_3$deposition and reduces the Fe availability in the apoplast of rice (Chen*et al.* , 2018). The decreased concentration of phenolic acids in cell walls prevented the $Fe(OH)_3$ solubilization, which exaggerates the Fe plaque formation and Fe deficiency under alkaline condition in rice.

Acknowledgments

The authors thank Dr. Bo Xu for the correction of the manuscript. This work was supported by the National Natural Science Foundation of China (31772378, 31801934) and Fundamental Research Funds for the Central University of China (2662019PY013, 2662017PY012 and 2016RC005).

Author Contribution

CW, HC and FX designed the research, and wrote the manuscript. HL, HC and SD performed the experiments. HC and LS assisted the experiments and discussion.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. Measurement of NO content in roots under different pH conditions. (a) NO staining in roots apexes under different pH conditions. Bars = 100 μm. (b) NO concentration of roots expressed as relative

fluorescence intensity (% of minimal concentration). Bars = 5 cm. Data were means of five biological replicates with error bars indicating SD. Columns with different letters are significantly different at P<0.05. (c) NO staining in root cross sections. 10-day-old seedlings were transferred to pH 5.5 or pH 7.5 nutrient solutions with or without of 50 μM SNP. Roots were sampled after treatment for 5 days.

Figure 2. The transcripts level and enzyme activity of nitrate reductase in roots of rice plants. (a) The relative expression of*OsNIA1* and *OsNIA2* . (b) Nitrate reductase activity in roots. 10-day-old seedlings were transferred to pH 5.5 or pH 7.5 nutrient solution in the absence and presence of SNP (0 and 50 μM SNP). After treatment for 5 days, roots were sampled for qRT-PCR and nitrate reductase activity assay. All data were means of three biological replicates with error bars indicating SD. Expression of *OsACTIN* was used as the internal control. Columns with different letters are significantly different at P<0.05.

Figure 3. The Phenotypes of rice plants growth under different pH conditions with or without of SNP treatments. (a -c) The morphology of the whole plants, the newest leaf and the roots of the rice plants. (d-e) The plant height and dry weight of rice. (f-g) The SPAD values and Fe concentrations of the newest 1^{st} to 3^{rd} leaves. 10-day-old seedlings were transferred to pH 5.5 or pH 7.5 nutrient solution with or without of 50 μM SNP. The phenotypes were recorded after treatment for 10 days. All data were means of five biological replicates with error bars indicating SD. Columns with different letters are significantly different at P < 0.05. Bars = 5 cm.

Figure 4. Expression of iron signaling regulation and uptake genes in rice roots. 10-day-old seedlings were transferred to pH 5.5 or pH 7.5 nutrient solution in the absence and presence of SNP (0 and 50 μM SNP). After treatment for 5 days, total RNA samples were extracted from the roots of seedlings and used for qRT-PCR. Expression of OsACTIN was used as the internal control. All data were means of three biological replicates with error bars indicating SD (* P value <0.05, ** P value <0.01, *** P value <0.001).

Figure 5. Measurement of H_2O_2in root under different pH conditions with or without of SNP treatment. (a) Histochemical visualization of $H_2O_2$by DAB staining in root apexes. Bars = 3mm. (b) Quantitative determination of H_2O_2 content in roots. (c) H_2O_2 staining in root cross sections. 10-day-old seedlings were transferred to pH 5.5 or pH 7.5 nutrient solution with or without of 50 μM SNP. H_2O_2 content were measured after treatment for 3 days.

Figure 6. Lignin staining (a) and phenolic acids fluorescence (b) in roots cross sections under different conditions. 10-day-old seedlings were treated for 5 days and roots were sampled for observation. pH 5.5, nutrient solution under pH 5.5 with no SNP; pH 5.5 + SNP, nutrient solution under pH 5.5 with 50 μM SNP; pH 7.5, nutrient solution under pH 7.5 with no SNP; pH 7.5 + SNP, nutrient solution under pH 7.5 with 50 μM SNP.

Figure 7. The Phenotypes of rice plants growth under different pH conditions with or without of nitrite treatment. (a-b) NO staining and relative fluorescence intensity in roots. (c) The morphology of the whole plants. Bar=5cm. (d)The iron plaque on the roots surface. (e) Histochemical visualization of H_2O_2 in the roots apexes. Bar = 2 mm. 10-day-old seedlings were transferred to pH 5.5 or pH 7.5 nutrient solution in the presence or absence of 1 mM nitrite for 3 days. All data were means of three biological replicates with error bars indicating SD. Columns with different letters are significantly different at P<0.05.

Figure 8. Working model of the NO production and iron uptake under alkaline stress condition in rice. Alkaline stress (AS) inhibits the expressions of nitrate transporter genes and reduces the NO3- content in roots, which causes the downregulation of NR activity and NO production. Because the NO negatively regulates H_2O_2 concentration, decreasing the NO level will accumulate H_2O_2 in roots, which stimulate the synthesis of lignin through consumption of phenolic compounds. Alkaline stress also induces the oxidation of rhizosphere Fe^{2+} into Fe^{3+}, which results in $Fe(OH)_3$ deposition and reduces the Fe availability in the apoplast of rice. The decreased concentration of phenolic compounds in cell wall reduces the Fe^{3+} mobilization, which exaggerates the Fe plaque formation and Fe deficiency under alkaline condition in rice. AS, alkaline stress; PM, plasma membrane; NRTs, nitrate transporters.

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Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 The correlation between NO content and NR activity in the roots of rice under different pH conditions.

Table S1 Expression levels of target genes under pH 6 and pH 8 conditions. Data was extracted from the published transcripts profiling data (Chen *et al.* , 2018).

Table S2 The qRT-PCR primers used in this research.

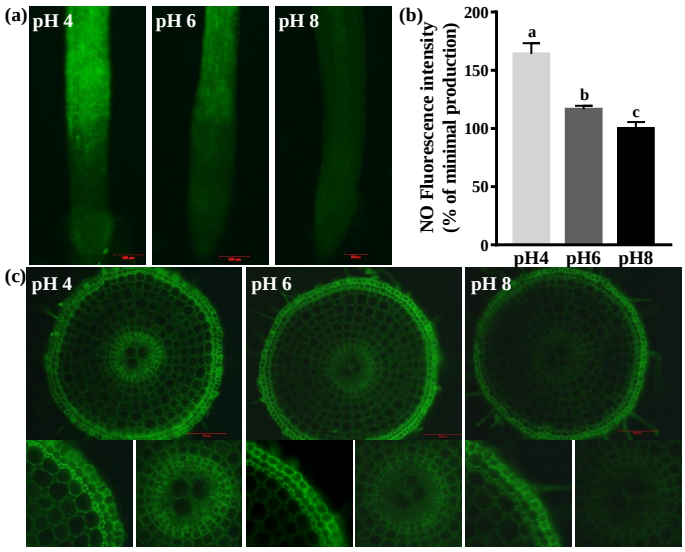

FIGURE 1. Measurement of NO content in roots under different pH conditions. (a) NO staining in roots apexes under different pH conditions. Bars = 100μm. (b) NO concentration of roots expressed as relative fluorescence intensity (% of minimal concentration). Data were means of five biological replicates with error bars indicating SD. Columns with different letters are significantly different at $P<0.05$. (c) NO staining in root cross sections. 10-day-old seedlings were transferred to pH 4, pH 6 or pH 8 nutrient solutions. Roots were sampled after treatment for 5 days.