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Effects of propiconazole on rice growth and gene expression in response to nitrogen and phosphorus deficiencies

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ABSTRACT: Nitrogen (N) and phosphorus (P) are essential macronutrients required for plant growth and development. Under N and inorganic phosphate (Pi) deficiencies, plants undergo adaptation at physiological, morphological, and transcriptional levels via modulation of endogenous signals, such as phytohormones, in an attempt to increase nutrient acquisition from the environment. Biosynthesis of brassinosteroid (BR), a class of plant hormones, has been shown to be crucial in nutrient deficiency responses in the plant model Arabidopsis. In this study, the responses of rice (*Oryza sativa* L.) to N and Pi deficiencies were investigated using rice seedlings grown in the presence and absence of a BR biosynthesis inhibitor, propiconazole (PPZ). Transcript levels of BR biosynthesis genes were induced by N and Pi deficiencies. PPZ-treated plants showed retarded growth in both sufficient and deficient conditions. Besides, gene expression of N- and Pi-deficiency-responsive genes was also attenuated by PPZ treatments. These results suggest that inhibition of BR biosynthesis by PPZ could restrain plant growth and adaptation in response to both N and Pi deficiencies.

KEYWORDS: nitrogen deficiency, phosphorus deficiency, propiconazole, rice

INTRODUCTION

Nitrogen (N) and phosphorus (P) are two macronutrients required for plant growth and development, and often the major limiting factors in most agricultural system. Deficiencies in N and inorganic phosphate (Pi) severely affect many physiological processes, including photosynthesis, carbon and nitrogen metabolism, and plant hormone metabolism, leading to reduction in growth, biomass, and yields [1, 2]. Plants have evolved diverse mechanisms to sense the availability of nutrients in the soil and undergo adaptation at morphological, physiological, and transcriptional levels to cope with the unfavorable conditions [3]. N and Pi deficiencies have been reported to increase root-shoot biomass ratio and alter root system architecture, allowing plants to explore their surrounding soil and increase nutrient uptake efficiency [4, 5].

Plant hormones serve as an important endogenous signal to control plant growth and development in response to nutrient availability and facilitate communication of nutrient status at systemic levels [6]. Brassinosteroid (BR) is a class of growth-promoting plant hormones that regulates diverse physiological processes, including photomorphogenesis, root growth and development, plant defense, and reproductive development [7]. Recent studies have revealed roles of BR in abiotic stresses, such as salinity, drought, and N and Pi deficiencies [8, 9]. Under mild N deficiency, expression of BR biosynthesis genes is up-regulated on Arabidopsis roots, contributing to low N-mediated root elongation as a foraging adaptation to explore more soil volume [10]. Overexpression of a BR biosynthesis enzyme DWARF1 (DWF1) improves plant growth and overall N accumulation in Arabidopsis [10]. A proteomic study in rapeseed roots also found that DWF1 is enriched by N deficiency, suggesting that the roles of BR biosynthesis in N deficiency responses are likely conserved across plant species [11]. Under Pi deficiency, BR biosynthesis has been shown to be down-regulated on root elongation in Arabidopsis [12]. Recently, several works

have shown roles of BR biosynthesis in N and Pi deficiencies in rice. NH_4^+ promotes BR biosynthesis via miR444-MADS box-OsBRD1 signaling cascade, leading to NH_4^+-triggered root elongation inhibition [13]. BR biosynthesis or signaling mutants attenuated Pi deficiency-induced leaf inclination by compromising expression of *BU1*, a BR-induced gene regulating cell elongation of lamina joint in rice [14].

The use of chemical inhibitors to inhibit plant hormone biosynthesis has played significant roles in the advancement of plant hormone research, especially when responsible mutants are not available. Several triazole compounds have been shown to inhibit cytochrome P450 monooxygenase enzymes that catalyze biosynthesis pathways of plant hormones. Paclobutrazole and uniconazole are well-known plant growth regulators that inhibit gibberellin biosynthesis and are used to alleviate plant abiotic stress [15]. Propiconazole (PPZ) is a specific BR biosynthesis inhibitor that binds CYP90D1 enzyme in the BR biosynthesis pathway [16], resulting in reduced endogenous BR levels and signaling activities [17]. PPZ treatment has been shown to reduce plant height, root length, and biomass, similar to BR mutants, in several plant species including Arabidopsis, soybean, maize, and Brachypodium [17–20].

In agriculture, PPZ application is one of the most widely used fungicides in many crop plants [21]. Highly repeated applications lead to PPZ contaminations, which have been reported in soils and water sources [22], as well as topsoil of paddy rice field [23]. In this study, we investigated effects of N and Pi deficiency stresses on expression of BR biosynthesis genes in rice seedlings and effects of PPZ application on N and Pi deficiency responses by evaluating plant growth, mineral concentrations, as well as expression of genes known to be regulated by N and Pi deficiencies.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of rice (*Oryza sativa* L.) cultivar Zhonghua 11 were sterilized with 3% H_2O_2 overnight and rinsed three times with distilled water. The sterilized seeds were soaked in distilled water and kept in the dark at 30 °C for 2 days before transferring to a hydroponic condition using Yoshida's nutrient solution (full-strength under sufficient conditions: 1.427 mM NH_4NO_3, 0.323 mM NaH_2PO_4, 0.512 mM K_2SO_4, 0.998 mM $CaCl_2$, 1.643 mM $MgSO_4$, 0.009 mM $MnCl_2$, 0.075 μM $(NH_4)_6Mo_7O_{24}$, 0.019 mM H_3BO_3, 0.152 μM $ZnSO_4$, 0.155 μM $CuSO_4$, and 0.036 μM Fe-EDTA) [24]. Germinated seeds were transferred to a net floating on half-strength Yoshida's nutrient solution in the absence and presence of 10 μM PPZ for 4 days. Then, uniform seedlings were selected and transferred to full-strength Yoshida's nutrient solution under sufficient conditions and N-deficient (0.014 mM NH_4NO_3) or Pi-deficient (0.003 mM NaH_2PO_4) conditions in the absence and presence of 10 μM PPZ. The nutrient solutions were renewed every 2 days. All seedlings were grown in a growth room controlled at 30 °C under 16 h light and 8 h dark cycle.

After 10 days of treatments, plant samples were harvested for biomass, N and P contents, and gene expression analyses. Three biological replicates were used in all experiments.

Measurements of biomass and total N and P contents

Plant biomass was measured in terms of dry weight and plant height. To measure plant dry weight, root and shoot samples were dried at 70 °C until a constant weight was recorded. To determine total N and P contents, dry root and shoot samples were digested with concentrated H_2SO_4, followed by addition of H_2O_2 in block digestion machine. The digested solution was completed volume with deionized water. N and P concentrations were determined by continuous flow analyzer (Skalar Analytic B.V., Netherlands).

Gene expression analysis

After treatment, roots and shoots were separated and immediately frozen in liquid nitrogen. Total RNA was extracted from whole root and whole shoots tissues using RNAprep Pure Kit (For Plant) (TIANGEN, China). For each sample, 1 μg of total RNA was treated with DNase to eliminate genomic DNA contamination, and first-strand cDNA was synthesized using PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, China) according to the manufacturer's protocol. The cDNA templates were used to quantify target gene expression level by quantitative RT-PCR (qRT-PCR) analysis using gene specific primers listed in Table S1. qRT-PCR was performed on QuantStudio™ 6 Flex Real-Time PCR System using SYBR® Premix Ex Tag™ (TaKaRa, China). The following thermal profile was used for PCR amplification: initial denaturation at 95 °C for 30 s, followed by 40 cycles of PCR at 95 °C for 5 s and at 60 °C for 34 s. Melt curve analysis was performed

to confirm the specificity of the reactions. *OsACTIN1* was used as internal control for normalizing gene expression. The relative expression level was calculated by $2^{-\Delta\Delta Ct}$ method. The analysis includes three biological replicates, 12 plants per replicate.

STATISTICAL ANALYSIS

Means and standard errors (SE) were calculated and analyzed by one-way analysis of variance (ANOVA). Mean comparison was calculated according to Duncan's multiple range test (DMRT) using IBM SPSS statistics 20.

RESULTS AND DISCUSSION

Expression analysis of BR biosynthesis genes

To determine whether N and Pi deficiencies affect expression levels of BR biosynthesis genes (*OsDWF4*, *OsD2*, and *OsCYP85A1*), total RNAs from shoots and roots of rice seedlings grown under N-deficient, Pi-deficient, or sufficient (control) conditions were harvested and determined transcript levels by quantitative RT-PCR. The results showed that N deficiency strongly induced *OsDWF4* in the roots (∼6 fold compared with the control) (Fig. 1a), consistent with a previous report in Arabidopsis roots [9]. In addition, *OsDWF4* and *OsD2* were also induced by N deficiency in the shoots (Fig. 1b). Pi deficiency promoted the expressions of *OsDWF4*, *OsD2*, and *OsCYP85A1* in the shoots, but not in the roots (Fig. 1ab). These results suggest that both N and Pi deficiencies induced expressions of BR biosynthesis genes, but different BR biosynthesis genes might show varied tissue-specific regulation by nutrient status.

Effect of PPZ on plant growth under N and Pi deficiencies

To investigate whether inhibition of BR biosynthesis by exogenous PPZ treatment affects N and Pi deficiency responses in rice, growth phenotypes of rice seedlings grown in the presence or absence of PPZ under nutrient-sufficient conditions were evaluated. Compared with PPZ-untreated controls, PPZ-treated plants showed significant reduction in root biomass, shoot biomass, and plant height by 49.4%, 68.1%, and 76.3%, respectively (Fig. 2). Moreover, the PPZ-treated plants displayed several phenotypes common to BR-deficient or signaling mutants, including dwarf shoots, short roots, and dark green leaves [18, 25, 26]. The reduction in growth observed in this study is likely due to reduced endogenous BR levels by PPZ inhibiting the BR biosynthesis enzymes [18, 27]. Transcript levels of the BR biosynthesis genes (*OsDWF4*, *OsD2*, and *OsCYP85A1*), which are feedback-regulated by the BR signaling pathway [28], showed significant upregulation (Fig. 1cd). In spite of the upregulation of the transcripts, endogenous BR levels remain low because PPZ binds to CYP90D1 enzyme in the BR biosynthesis pathway and blocks the activities [16]. Thus, the upregulation of the BR biosynthesis genes supported that endogenous BR levels in the PPZ-treated plants are blocked, which is consistent with previous reports [29].

In the absence of PPZ, N deficiency reduced root biomass (−31.4%), shoot biomass (−59.2%), and plant height (−28.3%), while Pi deficiency reduced shoot biomass (−5.6%) and plant height (−10.0%) but increased root biomass (+17.6%) when compared with sufficient condition (Fig. 2). Moreover, when plants were N-deficient, PPZ treatment reduced root biomass, shoot biomass, and plant height (−26.6%, −55.7%, and −72.5%, respectively), when compared with PPZ-untreated plants. Similarly, when plants were Pi-deficient, PPZ treatment reduced root biomass, shoot biomass, and plant height (−57.3%, −68.0%, and −72.9%, respectively). Thus, the results showed that PPZ-treated plants showed similar growth retardation under sufficient, N-deficient, and Pi-deficient conditions, except reduced degrees of root biomass reduction was found under N-deficient condition. In N-deficient plants, PPZ treatment did not reduce root growth much further than the effect of N deficiency.

Our results demonstrated that PPZ treatment reduced root adaptation to N and Pi deficiencies. Previous studies have shown that application of BR biosynthesis inhibitor, brassinazole, suppressed root elongation in low N-treated Arabidopsis [10] and inhibited root formation in low Pi-treated lupin (*Lupinus albus* L.) [30].

Effects of PPZ on total N and P contents in tissues

Effects of PPZ application on N and Pi acquisition or accumulations in plant tissues were investigated by measuring total N and P contents in rice seedlings. N and Pi deficiency treatments similarly reduced total N and P contents in the PPZ-treated and the PPZ-untreated plants. Interestingly, the PPZ-treated plants had higher total N and P contents than the PPZ-untreated plants under both sufficient and deficient conditions (Fig. 3). This may be a consequence of smaller cell size and greater cell density of PPZ-treated plants. In agreement with a

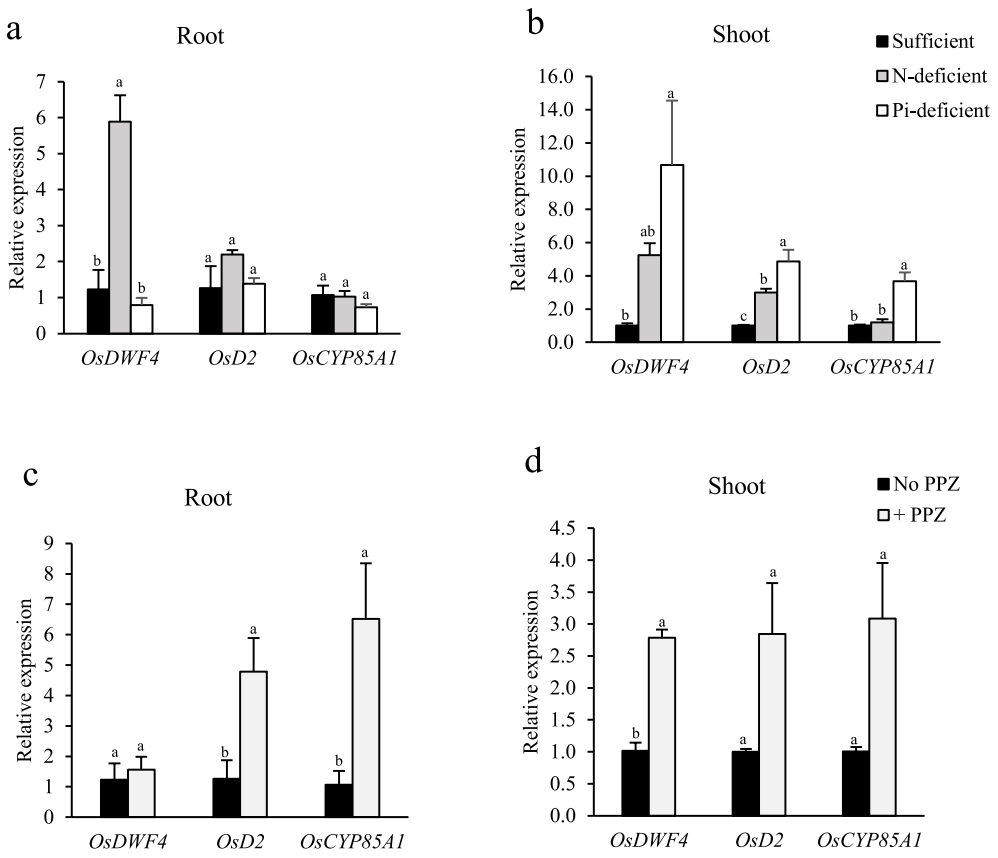

Fig. 1 Gene expression analyses of BR biosynthesis genes in roots (a,c) and shoots (b,d) of rice seedlings. Effects of nutrient deficiency (a,b): seedlings were grown under sufficient or N-deficient or Pi-deficient conditions. Effects of PPZ treatments (c,d): seedlings were grown in nutrient sufficient conditions in the absence or presence of PPZ treatment. Relative expression levels were normalized with *OsACTIN*. Data are means ± SE ($n = 3$). Different letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test (DMRT).

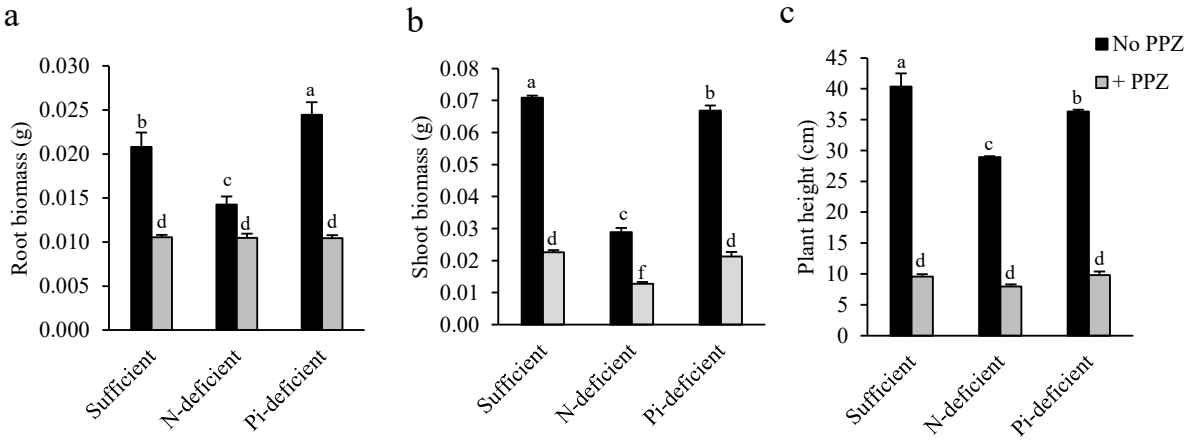

Fig. 2 Effects of PPZ on growth of rice seedlings grown under sufficient, N-deficient, or Pi-deficient condition showing quantification of root biomass (a), shoot biomass (b), and plant height (c). Data are means ± SE ($n = 3$). Different letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test (DMRT).

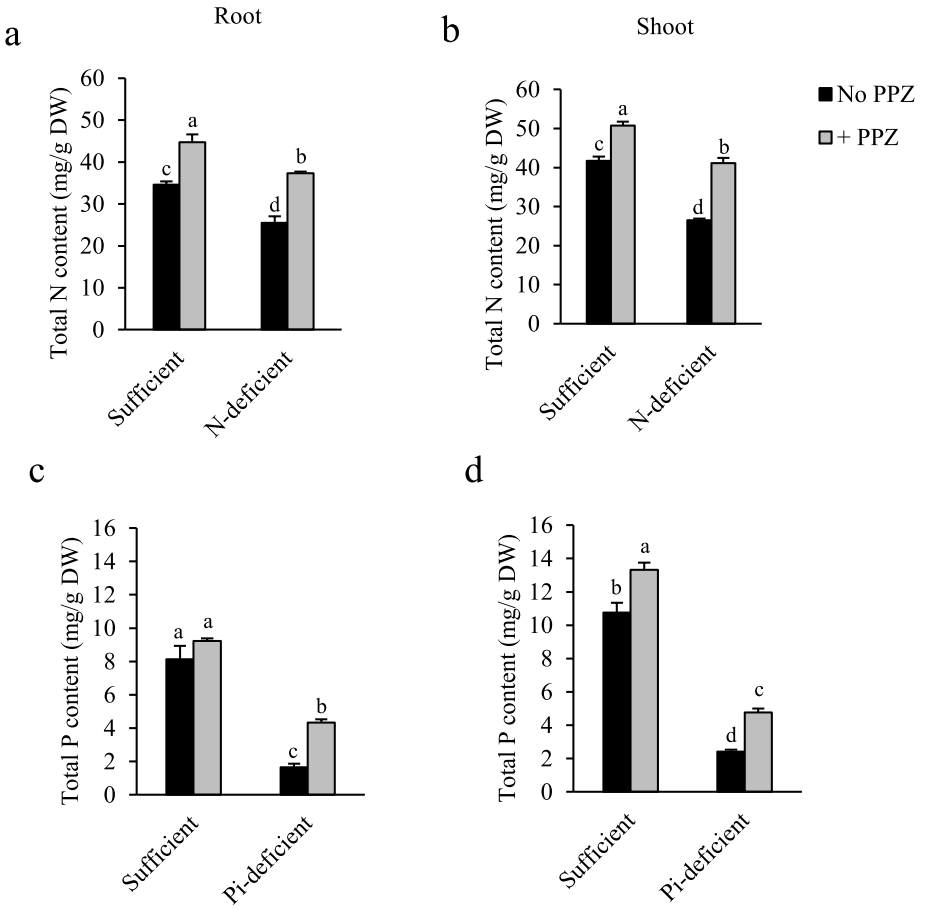

Fig. 3 Effects of PPZ on total N and P contents of rice seedlings grown under sufficient or deficient condition. Total N contents (mg/g dry weight): in roots (a) and shoots (b) of rice seedlings grown in the sufficient or N-deficient condition. Total P contents (mg/g dry weight): in roots (c) and shoots (d) of rice seedlings grown in the sufficient or Pi-deficient condition. Data are means ± SE ($n = 3$). Different letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test (DMRT).

previous study, PPZ-treated plants increased chlorophyll content in leaves, which is likely due to the specific inhibition of cell elongation [31]. However, when total N or P accumulations per plant were considered instead of per g dry weight, PPZ-treated plants had lower total N and P contents due to the small shoot biomass (data not shown), similar to previous reports in BR mutants [10].

PPZ treatment reduced growth rates of seedlings (Fig. 2), which may lead to a reduction in nutrient demand compared with the untreated plants. However, both N and Pi deficiencies led to similar reduction of total N and P contents when compared with sufficient conditions (Fig. 3), indicating that the PPZ-treated plants also encountered similar nutrient starvation. Regarding mineral contents in growth-retarded plants, a previous study has shown that under Fe-sufficient conditions, the BR biosynthesis rice mutant (*d2-1*) showed increased Fe concentrations, but no differences in Mg and K concentrations were found when compared with wild-type plants, suggesting that the effect of plant hormone deficiency is specific to Fe and not due to reduced nutrient demand of growth-retarded plants [32].

Expression analyses of genes related to N and Pi deficiencies

Under N-deficient conditions, N signaling has been shown to induce expressions of many genes related to N uptake and metabolism, such as nitrate transporters *OsNRT2.1* and *OsNRT1.1*, an ammonium transporter *OsAMT1.1*, and N-assimilating enzymes *OsGS1.1* and *OsNADH-GOGAT1* [33]. To determine

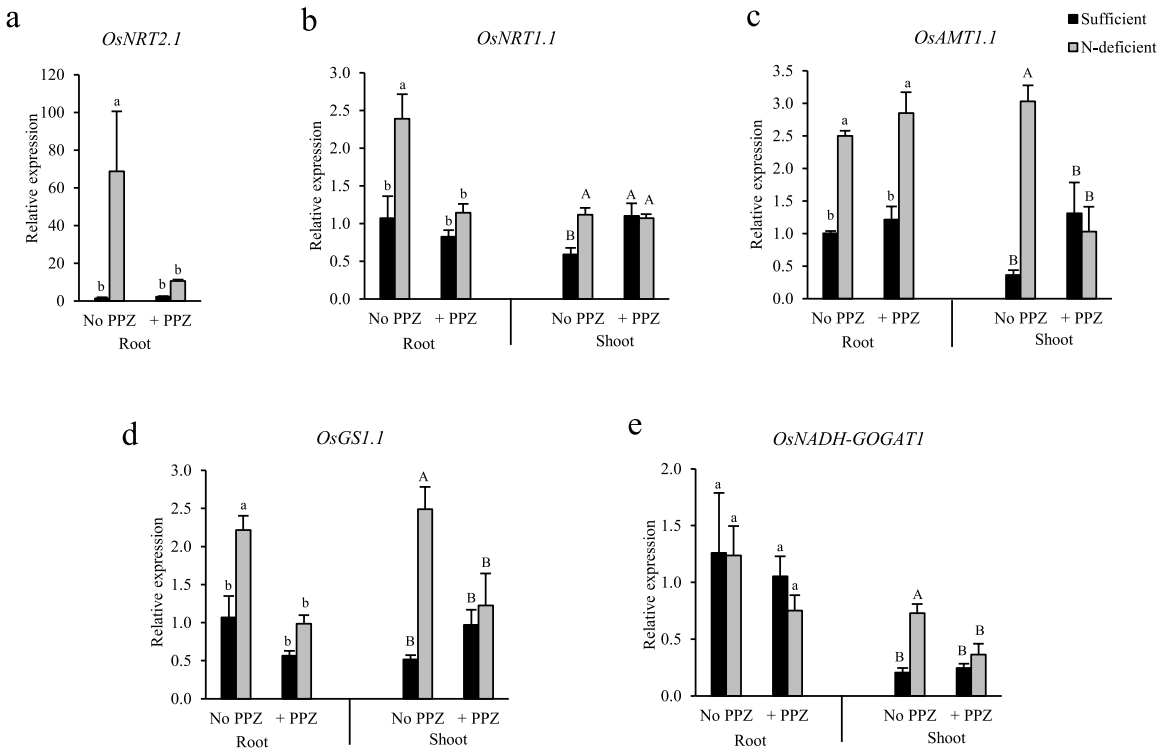

Fig. 4 Gene expression analyses of N deficiency-responsive genes in roots and shoots of rice seedlings grown in the absence and presence of PPZ and subjected to sufficient or N-deficient condition. Relative expression levels were normalized with *OsACTIN*. Data are means ± SE ($n = 3$). Different letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test (DMRT) (lowercase for root data; uppercase for shoot data).

whether PPZ treatment affects expression of these genes under N deficiency, transcript levels in shoots and roots were measured by quantitative RT-PCR. It was found that expressions of these genes in PPZ-untreated plants were significantly upregulated under N-deficient condition. However, the degrees of upregulation were attenuated by PPZ treatment (Fig. 4). In particular, when PPZ was not applied, *OsNRT2.1* in roots was dramatically induced by N deficiency (Fig. 4a), but its expression in shoots was not detectable, which is consistent with previous reports that this gene is strongly inducible in roots by N deficiency [34]. These results suggest that PPZ treatment could attenuate the N-deficiency-induced transcriptional response. In contrast, the attenuation effect was not observed in N-deficiency-induced *OsAMT1.1* expression in the root (Fig. 4c). This might be due to distinct expression patterns of different members of *AMT1* gene family and their regulation by BR, as suggested by works in Arabidopsis that *AMT1* expression is controlled by a complex interaction between nutrient and hormone signaling in plants [35].

Upon Pi deficiency, a MYB transcription factor PHOSPHATE STARVATION RESPONSE (PHR), which is the central regulator of the Pi signaling pathway, activates expression of the Pi starvation-induced (PSI) genes, such as *INDUCED BY PHOSPHATE STARVATION 1 (IPS1)* and *SPX1*, in response to Pi deficiency [36]. To determine whether PPZ treatment affects Pi signaling and the expression of PSI genes, expressions of five PSI genes: *OsIPS1*, *OsSPX1*, *OsSQD2* (*SULFOQUINOVOSYLDIACYLGLYCEROL* 2), *OsPAP10 (PURPLE ACID PHOSPHATASE 10), and OsPT8 (PHOSPHATE TRANSPORTER 8), were investigated* in rice seedlings grown under Pi-sufficient and Pi-deficient conditions in the presence and absence of PPZ. Their expression in roots showed dramatic upregulation by Pi deficiency, and such response was suppressed by PPZ treatment

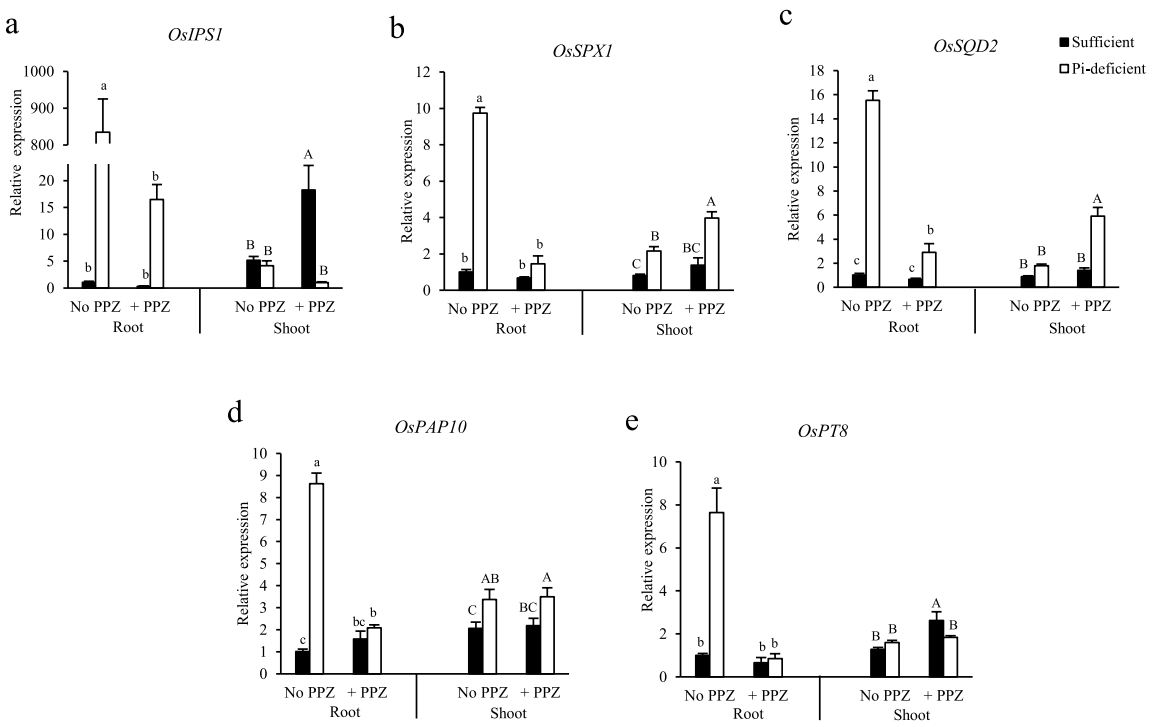

Fig. 5 Gene expression analyses of Pi deficiency-responsive genes in roots and shoots of rice seedlings grown in the absence and presence of PPZ and subjected to sufficient or Pi-deficient condition. Relative expression levels were normalized with *OsACTIN*. Data are means ± SE ($n = 3$). Different letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test (DMRT) (lowercase for root data; uppercase for shoot data).

(Fig. 5). PPZ treatment, however, enhanced responses of *OsSQD2* expression in shoots in response to Pi deficiency (Fig. 5c). We also found that expressions of *OsSPX1* and *OsPAP10* in shoots was moderately induced by Pi deficiency under both PPZ-treated and untreated conditions (Fig. 5bd).

Under Pi-sufficient conditions, PPZ treatment did not significantly alter expression of the PSI genes with the exception of *OsIPS1* and *OsPT8* expression in shoots that showed significant upregulation by PPZ (Fig. 5a-e). Interestingly, PPZ treatment significantly reduced expression of *OsIPS1* in shoots under Pi-deficient conditions (Fig. 5a). Together, these expression analyses demonstrated an interesting trend that PPZ treatment suppressed upregulation of PSI genes, especially in roots.

Our gene expression analysis results showed that PPZ treatment reduced levels of N and Pi deficiency-responsive gene expressions (Figs. 4 and 5). Previous studies have shown that PPZ treatments could block nuclear localization of the BR-regulated transcription factors BRASSINAZOLE RESISTANT1 (BZR1) in Arabidopsis, and thus inhibiting the BR-regulated transcriptional regulation [17]. Furthermore, direct targets of BZR1 and BES1/BZR2 transcription factors in Arabidopsis have been determined by chromatin immunoprecipitation assays (ChIP) followed by microarray (ChIP-chip) or sequencing (ChIP-seq), and the results revealed thousands of target genes involved in various physiological responses [37–39]. These include genes encoding nitrate transporters (*AtNRT1.1*, *AtNRT1.2*, *AtNRT1.7*, *AtNRT1.8*, *AtNRT2.2*, *AtNRT2.6*, and *AtNRT2.7*); ammonium transporters (*AtAMT1;1*, *AtAMT1;2*, and *AtAMT2*); phosphate transporters (*AtPHT4;1* and *AtPHT4;2*); purple acid phosphatases (*AtPAP19*, *AtPAP28* and *AtPAP29*); and SPX proteins (*AtSPX1* and *AtSPX2*). Such findings suggest that expression of target genes might be BR dependent, and the PPZ treatment could interfere their transcriptional regulation by nutrient deficiency. In agreement, recent studies found

that brassinazole treatment reduced expression of several *NRT1* genes in cucumber under suboptimal root zone temperature when compared with 24-epibrassinolide treatments [40].

Our results showed that expression of N and Pi deficiency-responsive genes was attenuated by PPZ, although PPZ-treated plants did not have lower total N and P contents when compared to the untreated plants (Fig. 3). This may be due to the experimental conditions, which were performed in closed hydroponic containers. In such limited conditions, plants cannot acquire more of the nutrients despite up-regulating expression of relevant transporters and enzymes. Future experiments conducted in soil condition would be necessary for a better understanding on plant adaptation to nutrient deficiency.

CONCLUSION

This study demonstrated that N and Pi deficiencies induced expression of BR biosynthesis genes. Direct quantification of endogenous BR will be needed to confirm whether N- and Pi-deficient plants produced more endogenous BR. Our studies supported putative roles of BR on nutrient deficiency stress in rice and illustrated the impact of BR biosynthesis inhibition in attenuating plant responses to nutrient deficiency. Hence, heavy repeated application of PPZ to control fungal diseases in rice paddy field could perhaps interfere plant adaptation in nutrient-limited environment.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2021.S011.

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Appendix A. Supplementary data

Table S1 Gene specific primers used in this study.

Gene	Sequence	Reference
OsNRT1.1	F: CCTCGCAAGTGACCCTTGAAT R: CGATGGCTAATGAGGAACCCTT	[1]
OsNRT2.1	F: TTCGCGAACCCGCATATGA R: GTTGAGGTTGTCGCGGATGAT	[1]
OsAMT1.1	F: GGTTTCTCTCCCTCTCCGAT R: CCACCTTCACACCACACATT	[2]
OsGS1.1	F: GAGTCGTCGTCTCATTTGACCC R: GTAGCCACCATCGTTCCTCATC	[1]
OsNADH-GOGAT1	F: TGCTTGAGAGAATGGCGCA R: AACCCAGCATCCTTTGTCACC	[1]
OsIPS1	F: AAGGGCAGGGCACACTCCACATTA R: ATTAGAGCAAGGACCGAAACACA	[3]
OsSPX1	F: GACCAGCTTCTACCATCAAACG R: AGTTCCTGCTGCTCCTCTGG	[4]
OsSQD2	F: CTGAAAACGGTAATGGATAGG R: AACAACAACAGCACGAGC	[5]
OsPAP10	F: ATACTGGCAGCCGACGGATGA R: GAGGGAGCTGGAGCGGAGAA	[5]
OsPT8	F: AGAAGGCAAAAGAAATGTGTGTTAAAT R: AAAATGTATTCGTGCCAAATTGCT	[6]
OsCYP85A1	F: TGATCCATTCCTGTACCCTG R: TACCTTCTTCCTCCCATCTG	[7]
OsDWF4	F: AGTCGCGTGCTGCCATTCTCGGAG R: AGCAAGCTCAGCAAGAGGTCCAGG	[8]
OsD2	F: AGCTGCCTGGCACTAGGCTCTACAGATCAC R: ATGTTGTCGGAGATGAGCTCGTCGGTGAGC	[9]
OsACTIN1	F: TGCTATGTACGTCGCCATCCAG R: AATGAGTAACCACGCTCCGTCA	[10]

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