RESEARCH PAPER

The ferroxidase LPR5 functions in the maintenance of phosphate homeostasis and is required for normal growth and development of rice

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Received 1 January 2020; Editorial decision 24 April 2020; Accepted 2 July 2020

Editor: Karl-Josef Dietz, Bielefeld University, Germany

Abstract

Members of the Low Phosphate Root (LPR) family have been identified in rice (Oryza sativa) and expression analyses have been conducted. Here, we investigated the functions of one of the five members in rice, LPR5. qRT-PCR and promoter–GUS reporter analyses indicated that under Pi-sufficient conditions OsLPR5 was highly expressed in the roots, and specific expression occurred in the leaf collars and nodes, and its expression was increased under Pi-deficient conditions. In vitro analysis of the purified OsLPR5 protein showed that it exhibited ferroxidase activity. Overexpression of OsLPR5 triggered higher ferroxidase activity, and elevated concentrations of Fe(III) in the xylem sap and of total Fe in the roots and shoots. Transient expression of OsLPR5 in Nicotiana benthamiana provided evidence of its subcellular localization to the cell wall and endoplasmic reticulum. Knockout mutation in OsLPR5 by means of CRISPR-Cas9 resulted in adverse effects on Pi translocation, on the relative expression of Cis-NATOsPHO1;2, and on several morphological traits, including root development and yield potential. Our results indicate that ferroxidase-dependent OsLPR5 has both a broad-spectrum influence on growth and development in rice as well as affecting a subset of physiological and molecular traits that govern Pi homeostasis.

Keywords: Agronomic traits, ferroxidase activity, growth and development, Oryza sativa, LPR5, phosphate homeostasis, rice.

Introduction

Rice (Oryza sativa) is a staple and main source of dietary energy supply for ~3.5 billion people in the world, and most (~90%) is consumed in Asia alone (www.irri.org/rice-today). Increasing the productivity of rice is therefore critical for global food security. Rice ecosystems, particularly acid upland soils, often experience severe deficiency of inorganic phosphate (Pi) due to high soil
P-fixation (Gamuyao et al., 2012). Pi, one of the major essential macronutrients, is a key structural component and is required for several metabolic pathways (López-Arredondo et al., 2014). Low Pi availability results in adverse effects on growth and development, and hence the yield potential, of agronomically important crop species (Veneklass et al., 2012). Pi deficiency triggers an array of morpho-physiological and adaptive molecular responses in rice (Torabi et al., 2009; Oono et al., 2013; Secco et al., 2013; Wu et al., 2013; Mehta et al., 2015; Negi et al., 2016; Xu et al., 2016).

The root system plays a critical role in the uptake of nutrients and exhibits extensive developmental plasticity in response to different nutrient deficiencies (Gruber et al., 2013; Kellermeier et al., 2014). In Arabidopsis, the cells of the primary root tip have an inherent ability to sense local Pi availability independent of the whole-plant Pi status (Linkohr et al., 2002), and inhibition of primary root growth is a hallmark of the Pi-deficiency response (Williamson et al., 2001; López-Bucio et al., 2002). Seedlings of transgenic Arabidopsis expressing the cell-cycle marker CycB1;1::uidA exhibit a progressive loss of its expression in the primary root tip when deprived of Pi, which triggers a shift from an indeterminate to an irreversible determinate developmental program (Sánchez-Calderón et al., 2005). Low Phosphate Root (LPR1; At1g23010), which is negatively regulated by Phosphate Deficiency Response 2 (PDR2), encodes multi-copper oxidase and is associated with the inhibition of primary root growth that is mediated by Pi deficiency (Reymond et al., 2006; Svistoonoff et al., 2007; Ticconi et al., 2009). It has been shown that root meristem–specific LPR1 in Arabidopsis encodes a cell wall–targeted ferroxidase, which is crucial for local Pi sensing (Müller et al., 2015). This study also demonstrated cell-specific expression of pLPR1::GUS, which was correlated with the patterns of iron (Fe) and callose deposition in the root tips of Pi-deprived wild-type seedlings. During Pi deficiency, the CLE14 peptide is expressed in the surrounding root apical meristem, a phenomenon known as root meristem exhaustion or determined growth (Gutiérrez–Alanís et al., 2017). However, a recent study has shown that it is actually the blue–light effect in the Petri dish that triggers the Fe-dependent Pi deficiency–mediated inhibition of primary root growth observed in Arabidopsis (Zheng et al., 2019).

Unlike Arabidopsis, the growth of roots in rice is either stimulated (Dai et al., 2012, 2016) or remains unaffected during Pi deficiency (Hu et al., 2011; Wang et al., 2014). Although several genes associated with the embryonic and post-embryonic development of rice roots have been identified (Mai et al., 2014; Wu and Cheng, 2014), the role of homologs of LPR1 remains elusive. In a previous study, we identified five homologs of LPR1 in rice (OsLPR1–5), of which OsLPR5 displayed strong tissue–specific induction during Pi deficiency (Cao et al., 2016). However, it is not known whether OsLPR5 confers ferroxidase activity and if it affects the responses of different root traits in seedlings during growth under different Pi conditions, and/or other morpho-physiological and molecular traits that govern the maintenance of Pi homeostasis.

Here, we show that Pi deficiency–induced OsLPR5 is a ferroxidase. Transient expression analysis indicated the subcellular localization of OsLPR5 to be the endoplasmic reticulum (ER) and the cell wall. In addition, overexpression and CRISPR–Cas9–mediated mutation of OsLPR5 provided empirical evidence for its broad-spectrum role in the developmental responses and maintenance of Pi homeostasis.

Materials and methods

Plant materials and growth conditions

Rice seeds (Oryza sativa L. sp. japonica cv. Nipponbare) were surface-sterilized for 30 min with diluted NaClO (1:3, v/v), followed by thorough rinsing for 30 min with deionized water. Seeds were germinated in the dark at 25 °C for 3 d. Hydroponic experiments were carried out in a growth room with a 14/10-h light/dark photoperiod at 30/22 °C at 200 μmol m⁻² s⁻¹, and the relative humidity was maintained at ~70%. The containers used for the hydroponic experiments were all painted black to exclude light from the roots. Seedlings at 10 d old were transferred to a nutrient medium consisting of 1.25 mM NH₄NO₃, 1 mM CaCl₂, 1 mM MgSO₄, 0.5 mM Na₂SiO₃, 0.35 mM K₂SO₄, 0.2 mM KH₂PO₄, 20 μM Fe-EDTA, 20 μM H₂BO₃, 9 μM MnCl₂, 0.77 μM ZnSO₄, 0.39 μM Na₂MoO₄ and 0.32 μM CuSO₄. Hereafter, this medium is referred to as +P (i.e. Pi-sufficient). For the Pi-deficient medium (−P), KH₂PO₄ was removed and replaced with 0.2 mM KCl. For pot experiments, the soil was obtained from an agricultural farm at Nanjing Agricultural University. Each pot was filled with 15 kg of air-dried soil and supplemented with 40 mg of Pi fertilizer kg⁻¹ soil. The plants were cultivated in a greenhouse at ambient temperatures and under natural daylight, as described previously (Jia et al., 2011).

Construction of promoter–GUS fusion and overexpression lines

A fragment (2289 bp) upstream of the coding sequence of OsLPR5 was amplified from the genomic DNA of the wild-type (Nipponbare) using OsLPR5–specific primers. The BamHI and Kpnl restriction sites were incorporated into these primers to facilitate directional cloning in the expression vector 1300GN. For overexpression, the coding sequence (1914 bp) of OsLPR5 was amplified from cDNA isolated from the wild-type using OsLPR5–specific primers. The PCR product was cloned downstream of the ubiquitin promoter of the pTCK303 vector, digested with BamHI and SpeI, and transformed into Agrobacterium tumefaciens strain EHA105 through electroporation. Both the constructs were transformed into the wild-type as described by Upadhyaya et al. (2000). The three independent overexpression lines generated are hereafter referred to as Os3, Os6, and Os7. The primers used for promoter–GUS fusion and overexpression are listed in Supplementary Table S1 at JXB online.

GUS histochemical analysis

Samples of panicles, nodes, and culms were collected from 17-week-old plants (grain-filling stage) of the wild-type and the CRISPR-Cas9–mediated mutation of OsLPR5. CRISPR-Cas9–mediated mutagenesis of OsLPR5 provided empirical evidence for its broad-spectrum role in the developmental responses and maintenance of Pi homeostasis.

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GUS histochemical analysis

Samples of panicles, nodes, and culms were collected from 17-week-old plants (grain-filling stage) of the wild-type and the overexpression lines and digested with EcoRI and BamHI overnight at 37 °C, before being separated on 0.8% (w/v) agarose gel and then transferred to a Hybond N+ nylon membrane (Amersham). Hybridization was carried out using a digoxigenin-labeled hygromycin-resistant gene as the
probe at 65 °C overnight. The blots were washed at 65 °C and examined using a phosphorimager (Typhoon 8600).

**Transient expression of OsLPR5**

*Agrobacterium tumefaciens*-mediated transient co-expression of 35S::35S::OsLPR5::EGBP and 35S::mCherry::HDEL in *Nicotiana benthamiana* plants was conducted as previously described by Bürstenbinder et al. (2013). Tobacco leaves were collected 3 d after infiltration, and EGBP/mCherry fluorescence was visualized using a confocal laser-scanning microscope (Leica SP8X) on the experimental platform at the College of Resources and Environmental Sciences, Nanjing Agricultural University, China.

**Protein expression, staining, and immunoblotting**

The coding sequence of OsLPR5 was cloned into a pGS-21a vector to generate the glutathione-S-transferase (GST) fusion protein. The recombinant constructs and an empty vector (control) were transformed into *E. coli* strain Rosetta (DE3). The *E. coli* cells were induced with 0.5 mM IPTG overnight at 16 °C and collected by centrifugation at 5500 g for 10 min at 4 °C. The fusion and control proteins were purified with Ni Sepharose. Equal amounts of purified proteins were separated by 12% (w/v) SDS-PAGE and stained with Coomassie Brilliant Blue. Western blotting was carried out to detect the fused protein. Purified proteins were electrophoresed and transferred to polyvinylidene fluoride membranes using a Trans-Blot Turbo transfer system (Bio-Rad). The membranes were blocked with TBST (150 mM NaCl, 10 mM Tris-Cl, and pH 7.6, and 100 mM CuSO4), 15 ml of the root protein extract, and the supernatant was collected. The supernatants collected from roots of the Nipponbare wild-type, as described previously (Upadhyaya et al., 2000; Mao et al., 2013).

**Ferroxidase assays**

Transformed tobacco leaves (0.5–1.0 g) were ground in ice-cold extraction buffer, centrifuged at 8500 g for 10 min at 4 °C, and the supernatant was collected as described by Yan et al. (2011). For the expression of OsLPR5 in yeast, its coding sequence (1.913 kb) was expressed in the pYES2 vector under the control of the galactose inducible promoter GAL1 and transformed into *Saccharomyces cerevisiae* strain BY4741. As a control, BY4741 was transformed with an empty vector. Fresh in-strain BY4741. As described by Hoopes and Dean (2004). Ferrozine complex, as described by Yan et al. 2000; Miao et al., 2013).

**Quantification of soluble Pi, Fe(II), and Fe(III) in the xylem sap**

The wild-type and the overexpression lines were grown hydroponically in +P medium for 5 weeks. Plants were then transferred to fresh +P medium. After 6 h, their stems were cut 2 cm above the stem–root junction using a sharp razor blade, and the xylem sap was collected from the cut ends for 1 h using a micropipette, as described by Yokosho et al. (2009). The soluble Pi in the sap was quantified as described by Nanamori et al. (2004), and Fe(II) and Fe(III) were determined using a QuantiChrom™ Fe assay kit (BioAssay Systems).

**Construction of the expression vectors for mutations in OsLPR5**

Using CRISPR–Cas9 gene editing technology to knockout OsLPR5, two guide RNAs (gRNA) were designed. These gRNAs were introduced to the intermediate vector pOs-sgRNA via BsaI and then constructed to the final expression vector pPH-Ubi-cas9-7 using Gateway recombination (Invitrogen). The constructs were transformed into *A. tumefaciens* (EHA105) for transformation through mature embryos of the Nipponbare wild-type, as described previously (Upadhyaya et al., 2000; Miao et al., 2013).

**Histological analysis**

Roots of the wild-type and the overexpression lines were washed thoroughly with water, then fixed with chloral hydrate solution (8 g chloral hydrate, 1 ml glycerol, and 3 ml water) for 20–40 min. Cells were observed under a differential interference contrast (DIC) microscope (Zeiss Axios Imager). Measurements were made of the development angle and width of the root cells using the ImageJ software.

**RT-PCR and qRT-PCR**

Total RNA (~1 μg) was isolated from ground tissues using Trizol reagent (Invitrogen) and treated with RNase-free DNase. First-strand cDNA was synthesized using oligo (dT)-18 primer and SuperScript II™ Reverse Transcriptase (Invitrogen). *Actin* was used as an internal control for both RT-PCR and qRT-PCR. The qRT-PCR analysis was performed in triplicate for each sample using SYBR green master mix (Vazyme Biotech Co., Ltd) in a StepOnePlus™ Real-time PCR System (Applied Biosystems). Relative expression levels of the genes were computed using the 2ΔΔCT method of relative quantification (Livak and Schmittgen, 2001). Gene-specific primers are listed in Supplementary Table S1.

**Quantification of soluble Pi and total Fe**

The wild-type and the overexpression lines were grown under +P and –P conditions for 3 weeks, harvested, and washed thoroughly with deionized water. The shoots and roots were separated at the hypocotyl–shoot junction and oven-dried at 80 °C overnight. For quantification of Pi, samples (500 mg) were frozen and then homogenized in 1 ml of 10% (w/v) perchloric acid. The homogenate was diluted 10 times with 5% (w/v) perchloric acid. The supernatant was collected. The supernatants collected from the transformed tobacco leaves and yeast were then used for assays of the ferroxidase activity as described by Müller et al. (2015). Briefly, the electron donor ferrous ammonium sulfate [Fe(NH4)2(SO4)2·6H2O] was used as the substrate and ferrozine [3-(2-pyridyl)-5,5-dimethyl-1,2,4-triazine], a specific Fe2+ chelator, was added to binding the substrate at the end of the reaction. The reaction was initiated by mixing 1050 μl buffer (450 mM Na-acetate, pH 5.8, and 100 mM CuSO4), 15 ml of the root protein extract, and 225 ml substrate containing 100 mM CuSO4. At regular intervals, 200-ml aliquots were transferred to microtiter plates, and the reaction was quenched by adding 14 ml of ferrozine (18 mM). In the control, the substrate was not added to the reaction mixture. The rate of Fe2+ oxidation was calculated from the reduction in the absorbance at 560 nm using a molar absorptivity of ε560=25400 M⁻¹ cm⁻¹ for the Fe2+-ferrozine complex, as described by Hoopes and Dean (2004).

**35Pi uptake assays**

Seeds (7-d-old) of the wild-type, the *Oslpr5* mutant generated using CRISPR–Cas9, and the overexpression lines *Ox3* and *Ox6* were grown hydroponically under +P conditions for 7 d. The seedlings were then grown for different time periods (3–24 h for the mutant and 1–7.5 h for *Ox3* and *Ox6*) in 200 ml of +P solution labeled with 35Pi (8 μCi
of KH$_2$PO$_4$; Perkin–Elmer). The apoplastic $^{32}$Pi was then removed by incubating the roots of the seedlings in ice-cold desorption solution (2 mM MES, 0.5 mM CaCl$_2$, and 0.1 mM NaH$_2$PO$_4$, pH 5.5) for 10 min. The seedlings were then blotted dry, and the roots and shoots were harvested separately, with their fresh weights being determined. The tissues were digested in a mixture of HClO$_4$ and 30% (v/v) H$_2$O$_2$ at 28 °C for 8–12 h. A scintillation cocktail (3 ml) was added to the digested tissue and $^{32}$Pi activity was determined by using a liquid scintillation counter (Tri-Carb 2100, Packard). The $^{32}$Pi counts in the roots and shoots were used to determine the uptake rates and the shoot/root distribution ratio.

Statistical analysis

Data were analysed by multiple comparisons of one-way ANOVA using Duncan’s test in the SPSS 20 software ($P<0.05$).

Results

OsLPR5 exhibits higher expression in the roots and collar, and is induced during Pi deficiency

qRT-PCR analysis was used to determine the expression patterns of OsLRP5, and it was found that expression was far greater in the roots than in the other tissues (Fig. 1A). Expression in the collar was significantly higher than the leaf sheath and blade. To further assess tissue-specific expression, the promoter of OsLPR5 was fused with the GUS reporter gene and transformed into the Nipponbare wild-type. GUS activity was then assayed histochemically in the tissues of plants grown in soil at the grain-filling stage (Fig. 1B). The activity was observed to be weak in the whole-sections of the panicle nodes and in their longitudinal and transverse sections (Fig. 1Bi, v, ix). In contrast, strong activity was detected in the whole-sections of nodes I–III (Fig. 1Bi–iv). Longitudinal and transverse sections revealed higher GUS activity in the xylem vessels and sieve tubes compared with the other tissues (Fig. 1Bvi–vii, x–xii). High GUS activity was also specific in the collars I–III (Fig. 1Bxiii–xv), suggesting a likely role of OsLPR5 in the developmental responses of the leaf. Similar patterns of GUS activity driven by the OsLPR5 promoter were also observed in whole-tissue and cross-sections of nodes I–III, and in collars I–III of two independently generated transgenic lines, #6 and #33 (Supplementary Fig. S1). Notably, GUS activity could not be detected in the roots despite an elevated relative expression level of OsLPR5 (data not shown). This anomaly could have been due to the fact that the intergenic region (−3384 to −2290 bp) of the GUS reporter gene was not included. To determine the enrichment of cis-elements that might influence expression in the roots, this region was annotated by using the PLACE database (http://www.dna.affrc.go.jp/PLACE). This analysis revealed the presence of seven ‘ROOTMOTIFTAPOX1’ (ATATT) motifs in this region, which have been implicated in conferring rootspecific expression (Elmayan and Tepfer, 1995).

To determine the effect of Pi deficiency on the relative expression level of OsLPR5, wild-type seedlings were grown hydroponically under +P or −P conditions for 3 weeks. Pi deficiency triggered significant induction in the relative expression of OsLPR5 in the roots (Fig. 1C), consistent with a previous study that reported that Pi deficiency mediated the

![Fig. 1](image-url) Relative expression levels of OsLPR5 in different tissues of rice and its tissue specificity. (A, C) Wild-type (WT) plants were grown hydroponically in a nutrient-rich medium for 5 weeks (A), or under Pi-sufficient (+P) or Pi-deficient (−P) conditions for 3 weeks. qRT-PCR was used to determine the relative expression levels of OsLPR5 in (A) different tissues, and (C) in the roots. OsRAC1 (LOC_Os03g50885) was used as the internal control. Data are means (±SE), $n=3$. Different letters indicate significant differences between means as determined using ANOVA followed by Duncan’s test ($P<0.05$). (B) Transgenic plants with GUS driven by the OsLPR5 promoter were grown in soil for 17 weeks (grain-filling stage). GUS activity was then assayed in the panicle nodes (i, v, ix), in node I (ii, vi, x), node II (iii, vii, xi), and node III (iv, viii, xii), and in collar I (xiii), collar II (xiv), and collar III (xv). From left to right, the columns of images show the whole tissues, and their longitudinal and cross-sections. Scale bars are 2 mm.
elevated expression of OsLPR5 and its homolog OsLPR3 (Cao et al., 2016).

**Augmented ferroxidase activity in OsLPR5-overexpression lines mimics the Pi-deficiency response**

Bioinformatic analysis was employed to determine whether OsLPR5 has a potential ferroxidase activity (Supplementary Fig. S2). The MAFFT algorithm (www.ebi.ac.uk/Tools/msa/mafft/) was used for multiple amino-acid sequence alignment of OsLPR5 with Atlpr1/2 and Fet3p from yeast (Supplementary Fig. S2A) and indicated that the Cu sites are organized into three domains, namely a monocluster Cu site (T1), a trinuclear Cu cluster with three amino acid residues (E185, D283, and D409; T2/T3), and a Fe$^{2+}$ substrate-binding site. The T2/T3 site is located in the vicinity of a T1 site, which is critical for binding Fe$^{2+}$ and for electron transfer. The annotation revealed that the amino acid sequences in these regions are largely conserved. In addition, the Phyre2 software (www.sbg.bio.ic.ac.uk/phyre2/) was used to predict the 3D structural model of OsLPR5 (Supplementary Fig. S2B).

To investigate whether OsLPR5 encodes ferroxidase, the OsLPR5 protein was fused with a GST tag, and the purified fusion protein was then heterologously expressed in E. coli strain BL21 (DE3) and assayed by SDS-PAGE (Fig. 2A), and immunoblotted using the GST antibody (Fig. 2B). These assays revealed the successful expression of the fusion protein (pGS::GST::OsLPR5) compared with the control (pGS::GST), and the ferroxidase activity was significantly higher in BL21 expressing the fusion protein compared with the control (Fig. 2C). To determine whether OsLPR5 had ferroxidase activity in vivo, transgenic OsLPR5-overexpression lines were generated (Ox3, Ox6, and Ox7). RT-PCR and qRT-PCR analyses respectively showed significant increases in transcript abundance and relative expression levels of OsLPR5 in Ox3, 6, and 7 compared with the wild-type (Supplementary Fig. S3A, B). Southern blotting confirmed the fidelity of the overexpressing lines (Supplementary Fig. S3C).

Assays for ferroxidase in the wild-type indicated that roots of Pi-deprived plants had significantly higher activities (~3-fold) than in Pi-sufficient plants (Fig. 2C). In contrast, the overexpressing lines all had elevated ferroxidase activities (~4-fold) irrespective of the Pi treatment. These results thus revealed the mimicking of the Pi-deficiency response by the transgenic lines overexpressing OsLPR5.

In higher plants, Fe is essential for the synthesis of ferredoxins and other redox-related proteins, and in grasses, the chelation-based approach (Strategy II) is used for its acquisition by the roots and subsequent mobilization through the xylem to the aerial parts (Palmer and Guerinot, 2009). Xylem sap is conventionally used for determining the concentrations of different chemical forms of Fe in graminaceous plants (Kawai et al., 2001; Yokosho et al., 2009; Ariga et al., 2014), and hence we took samples of sap to determine the concentrations of Fe(II) and Fe(III). The concentrations of Fe(II) in Ox6 and 7 were significantly lower (~28–52%) compared with the wild-type, whilst those of Fe(III) in Ox3, 6, and 7 were significantly higher (~1.5–2.0-fold) (Fig. 2D). The total concentration of Fe was also significantly higher in the roots (~64–83%) and shoots (~46–66%) of the OsLPR5-overexpression plants compared with the wild-type (Fig. 2E). These results were in agreement with the elevated ferroxidase activity observed in the overexpressors under the different Pi conditions (Fig. 2C).

The growth of the primary root in the Atlpr1 mutant becomes insensitive to Pi deficiency due to the loss of ferroxidase function (Müller et al., 2015). We therefore hypothesized that overexpressing OsLPR5 in the Atlpr1 background would result in the complementation of this growth response in Pi-deprived plants. Hence, we transformed the p35S::OsLPR5 construct into Atlpr1, and three independent highly expressing lines were obtained (OsLPR5/atlpr1 #1, #2, and #3; Supplementary Fig. S4A). Under +P conditions, the primary root growth of Atlpr1 and the OsLPR5/atlpr1 lines was comparable with the wild-type (Supplementary Fig. S4B, C) however, under −P conditions, the root length in the wild-type was significantly shorter, which was consistent with previous studies (Williamson et al., 2001; López-Bucio et al., 2002; Jain et al., 2007). Meanwhile, the primary root length of the Atlpr1 mutant was comparable between the two Pi treatments. The roots of the OsLPR5/atlpr1 lines under −P conditions were significantly longer than those of the wild-type, but shorter than those of the Atlpr1 mutant.

**OsLPR5 is localized to both the endoplasmic reticulum and the cell wall**

The endoplasmic reticulum (ER) acts as a reservoir for proteins destined either for the ER itself or for other organelles (Crofts et al., 2004). A signal peptide at the N-terminal end of the protein is necessary to facilitate its retention in the ER (Gomord et al., 1997; Pagny et al., 1999). Use of SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP; Petersen et al., 2011) enabled us to identify a putative ER-specific signal peptide in OsLPR5, which was similar to Arabidopsis LPR1 (Ticconi et al., 2009). Ferroxidases typically reside in the extracellular matrix, and LPR1 has been shown to function in the apoplast (Müller et al., 2015). Therefore, to examine the subcellular localization of OsLPR5 as a putative ER–targeted protein, we used mCherry (Nelson et al., 2007). Accordingly, p35S::SIgOsLPR5::EGFP::OsLPR5 and p35S::mCherry::HDEL were transiently co-expressed in N. benthamiana by Agro-infiltration and confocal microscopy revealed the localization of OsLPR5 to the ER (Fig. 3A). To further determine whether OsLPR5 was also targeted to the cell wall, epidermal leaf cells of the transgenic N. benthamiana were plasmolysed with 0.8 M mannitol. Although there was distinct plasmolysis in cells expressing 35S::EGFP, GFP fluorescence could not be detected in the cell wall (Fig. 3B). In contrast, distinct GFP fluorescence was observed in the epidermal cell walls of plants expressing 35S::SIgOsLPR5::EGFP::OsLPR5 (Fig. 3C).

**Mutation and overexpression of OsLPR5 affects translocation of Pi from the root to shoot**

To determine the role of OsLPR5 in the maintenance of Pi homeostasis in rice, CRISPR/Cas9 gene editing was used to
generate homozygous OsLPR5 knockout mutants, of which three independent lines (OsLPR5-1, 5-2, and 5-3) were selected for further study (Supplementary Fig. S5). Since OsLPR5 was induced during Pi deficiency (Fig. 1B), the effects of the mutation on Pi homeostasis were investigated (Fig. 4). The wild-type and mutants were grown hydroponically under +P and –P conditions for 2 weeks, and were then treated with 32P for different time periods. Under +P conditions, the 32P uptake rate was consistently comparable between the wild-type and the mutants (Fig. 4A); in contrast, under –P conditions the uptake was significantly higher in the mutants. Meanwhile, the shoot/root distribution ratio of 32P distribution was significantly lower in the mutants compared with the wild-type under both P conditions and at all the time-points (Fig. 4B). In addition, there was a significant increase in the concentration of total P in the mutants compared with the wild-type...
Fig. 3. Subcellular localization of OsLPR5 in epidermal leaf cells of transformed *Nicotiana benthamiana* as determined using confocal microscopy. (A) Transient expression of 35S::SPoLPR5::EGFP::OsLPR5 and 35S::mCherry::HDEL. Green fluorescent protein (GFP) and mCherry (red) together with the merged images indicate the localization of OsLPR5 to the endoplasmic reticulum. The insets show magnified images of the nucleus. (B, C) After transient expression of (B) 35S::EGFP and 35S::mCherry::HDEL, and (C) 35S::SPoLPR5::EGFP::OsLPR5 and 35S::mCherry::HDEL, the epidermal leaf cells were plasmolysed by treating with 0.8 M mannitol. The fluorescence and bright-field images demonstrate the localization of OsLPR5 to the cell wall. The areas of the cell walls indicated by the boxes are shown in magnified view in the images below. White arrowheads indicate the cell wall and yellow arrowheads indicate the protoplast membrane. Scale bars are 25 µm in the main images, and 5 µm in the inset in (A) and in the magnified views in (B, C).
Mutation of OsLPR5 affects Pi homeostasis in rice. The wild-type (WT) and the OsLPR5 mutants oslpr5-1, oslpr5-2, and oslpr5-3 were grown hydroponically under Pi-sufficient (+P) or Pi-deficient (–P) conditions for 3 weeks. The WT, oslpr5-1, and oslpr5-2 were then fed with $^{32}$Pi for 10 min.

(A) Total $^{32}$P uptake, (B) shoot/root distribution ratio of $^{32}$P, and (C) the concentration of total P in the roots and shoots. (D) Relative expression levels of OsPHO1;2 and cis-NAT OsPHO1;2 as determined by qRT-PCR in the roots of the WT and OsLPR5 mutants grown under +P and –P conditions for 3 weeks. OsRAC1 was used as the internal control. Data are means (±SE), n=3. Different letters indicate significant differences between means as determined using ANOVA followed by Duncan’s test (P<0.05).

(+P roots for oslpr5-1, 5-2, and 5-3 Fig. 4C). These data suggested an inhibitory effect of the mutation in OsLPR5 on the translocation of Pi from the root to shoot. No significant difference could be detected in the concentration of total Fe between the wild-type and the mutants (data not shown). Compared with the wild-type, a significant increase in $^{32}$Pi uptake by the overexpressor lines Osx3 and Osx6 could be observed by as early as 3 h under +P conditions, and this trend continued through to the end of the experiment at 7.5 h (Supplementary Fig. S6A). In contrast, under –P conditions there were no differences between the wild-type and the overexpressing lines. The concentration of Pi in the xylem sap was significantly higher in the two overexpressor lines than in the wild-type under both +P and –P conditions (Supplementary Fig. S6B). Irrespective of the Pi conditions, there were no significant differences in the shoot/root distribution ratio of $^{32}$Pi between the wild-type and the overexpressor lines (Supplementary Fig. S6C). However, the shoot Pi concentration in the overexpressor lines was significantly higher under both +P and –P conditions compared with the wild-type (Supplementary Fig. S6D).

OsPHO1;2 is involved in Pi loading into the xylem and thus facilitates its mobilization from the roots to shoots in rice (Secco et al., 2010). Natural antisense transcripts (NATs) are long RNAs comprising sequences that are complementary to sense mRNAs, and they are categorized into cis and trans forms (Lapidot and Pilpel, 2006). cis-NATs are prevalent in plants and are often associated with the down-regulation of their associated sense genes (Swiezewski et al., 2007; Held et al., 2008; Ron et al., 2010). Since the Pi concentration in the shoots were consistently higher in the overexpressor lines (Supplementary Fig. S6D), we examined the effects of mutation in OsLPR5 on the relative expression levels of OsPHO1;2 and cis-NAT OsPHO1;2. Under +P conditions the relative expression level of OsPHO1;2 in the mutants was significantly lower compared with the wild-type (Fig. 4D), whilst it was
comparable under –P conditions. In contrast, Pi deficiency triggered a significant reduction in the relative expression of *cis*-NAT_{OsPHO1;2} in the mutants compared with the wild-type.

### Mutation and/or overexpression of OsLPR5 affects root development and other morphological traits

The root system exhibits extensive developmental plasticity in response to various environmental cues (Malamy and Ryan, 2001). In rice, the primary and lateral roots constitute a fibrous network and the latter contribute towards branching, which is crucial for anchorage and the acquisition of nutrients (Hochholdinger and Zimmermann, 2008; Atkinson et al., 2014). We therefore examined the effects of mutation of OsLPR5 on different root traits during growth in a hydroponic system under different Pi conditions. The wild-type and the oslpr5-1, oslpr5-2, and oslpr5-3 mutants were grown hydroponically under +P or –P conditions for 4 d or 7 d. There was a significant reduction in the primary root length of the mutants compared with the wild-type after 4 d in both the +P and –P treatments (Fig. 5A, B). However, irrespective of the Pi conditions, there were no effects of the on the mean length of the three longest adventitious roots (Fig. 5C), or on the mean length of the lateral roots (Fig. 5D).

To further examine whether the overexpression of OsLPR5 exerted any influence on the developmental responses of different root traits, the wild-type and the overexpressing lines were grown hydroponically in +P medium for 7 d. The laterals on the primary roots were then spread gently to reveal their architectural details, and different traits were quantified by using the ImageJ software (https://imagej.nih.gov/ij/; Collins, 2007). The primary root length of the overexpressing

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**Fig. 5.** Effects of mutation of OsLPR5 on the growth of roots in rice grown under different Pi conditions. Seedlings (4 d old) of the wild-type (WT) and the OsLPR5 mutants oslpr5-1, oslpr5-2, and oslpr5-3 were grown hydroponically under Pi-sufficient (+P) or Pi-deficient (–P) conditions. (A) Images of the roots after 7 d. (B) Length of the primary root after 4 d and 7 d, (C) mean length of the three longest adventitious roots after 7 d, and (D) length of lateral roots after 7 d. Data are means (±SE), n=6. Different letters indicate significant differences between means as determined using ANOVA followed by Duncan’s test (P<0.05). (This figure is available in color at JXB online.)
Overexpression of OsLPR5 reduces lateral root elongation in rice. Seedlings (4 d old) of the wild-type (WT) and the OsLPR5-overexpression lines Ox3, Ox6, and Ox7 were grown hydroponically in Pi-sufficient medium for 7 d. (A) Images showing lateral root development on the primary roots. The scale bar is 1 cm. (B) Length of lateral roots, (C) cell development angle (see below), and (D) width of epidermal cells. Data are means (±SE), n=6. Different letters indicate significant differences between means as determined using ANOVA followed by Duncan's test (P<0.05). (E) Sections of the lateral root tip (top row) together with schematic representations (middle row). Blue indicates the epidermis, green is the cortex, purple is the quiescent center, and yellow indicates stem cells. Magnified views of the areas within the boxes are shown in the bottom row of images, and illustrate the cell development angle, i.e. the angle between the axis of the direction of growth of the root and the lower cortical cells. Scale bars are 20 μm.
was comparable between the wild-type and the overexpressing lines (data not shown). To determine whether overexpression of OsLPR5 caused any perturbations in the spatial arrangements of the quiescent center and the surrounding stem, cortical, and epidermal cells of the lateral root tip, we observed sections under a stereomicroscope, and found that there was a significant reduction in the width of the cells in the overexpressing lines compared with the wild-type (Fig. 6E), and also in the angle between the axis of growth and the lower cortical cells. Finally, the effects of OsLPR5 mutation on different morphological traits were examined at the grain-filling stage in plants grown in soil supplemented with Pi fertilizer. The Oslpr5 mutants appeared relatively stunted compared with the wild-type (Fig. 7A) due to a significant reduction in their height (Fig. 7C). There were no observable effects of the mutation in the panicles (Fig. 7B) or on the effective number of tillers per plant (Fig. 7D); However, there were significant reductions in the seed-setting rate (Fig. 7E), the

![Fig. 7](image-url)
1000-grain weight (Fig. 7F), and in the grain yield per plant (Fig. 7G).

**Discussion**

**OsLPR5 is expressed in the roots and the collar, and is responsive to Pi deficiency**

The expression of *OsLPR5* was significantly higher in the roots compared with the other tissues (Fig. 1A). A previous study also reported higher expression levels of *OsLPR1*, 3, 4, and 5 in the roots, with the notable exception of *OsLPR2* (Cao et al., 2016), suggesting a potential functional overlap amongst the members of the *OsLPR* family. Strong GUS activity driven by the *OsLPR5* promotor was observed in the xylem vessels and sieve tubes of the nodes I–III (Fig. 1B). Since nodes are junctions of vasculatures that connect leaves, stems, and panicles, and act as hubs in redirecting essential mineral elements taken up by the roots to different organs (Yamaji and Ma, 2014), a role of *OsLPR5* in the mobilization of minerals can thus be envisaged. Although the expression of *OsLPR5* in the collar was relatively lower than in the roots, it was significantly higher compared with the leaf sheath and blade (Fig. 1A). In addition, the GUS activity was high in collars I–III (Fig. 1B). Cell proliferation in the leaf collar (also referred to as the lamina joint) is closely related to leaf erectness in rice (Sun et al., 2020), suggesting a potential functional overlap amongst the members of the *OsLPR* family. Strong GUS activity driven by the *OsLPR5* promotor was observed in the xylem vessels and sieve tubes of the nodes I–III (Fig. 1B). Since nodes are junctions of vasculatures that connect leaves, stems, and panicles, and act as hubs in redirecting essential mineral elements taken up by the roots to different organs (Yamaji and Ma, 2014), a role of *OsLPR5* in the mobilization of minerals can thus be envisaged. Although the expression of *OsLPR5* in the collar was relatively lower than in the roots, it was significantly higher compared with the leaf sheath and blade (Fig. 1A). In addition, the GUS activity was high in collars I–III (Fig. 1B). Cell proliferation in the leaf collar (also referred to as the lamina joint) is closely related to leaf erectness in rice (Sun et al., 2015; Ruan et al., 2018). Ruan et al. (2018) found that Regulator of Leaf Inclination1 (RLI1) shows strong expression in the collar and positively regulates leaf inclination in rice by influencing cell elongation in the lamina joint. However, during Pi deficiency, the proteins SPX1 and SPX2 are induced and interact with RLI1, thus repressing its activity. Hence, RLI1 and SPX form a module to regulate leaf inclination in response to Pi availability in rice. The expression of *OsLPR5* in the collar does suggest it has a potential role in the regulation mechanism for leaf inclination; however, further studies will be required to examine this more closely.

Overall, the spatial expression pattern of *OsLPR5* was consistent with the findings of Cao et al. (2016), who determined expression of *OsLPR* homologs not only in the roots, but also in leaf blades, leaf sheaths, and other tissues. Variable expression levels in different tissues of several functionally diverse genes have often been correlated with their diverse roles, for example OsPT2 and OsPT4 (encoding Pi transporters), OsWRKY74 (encoding a transcription factor), and OsLTN1 (an ortholog of Arabidopsis PHO2) (Ai et al., 2009; Hu et al., 2011; Zhang et al., 2015; Dai et al., 2016). Multiple tissue-specific roles of *OsLPR5* may thus be assumed. Pi deficiency in Arabidopsis does not exert any significant effect on the transcription of *AtLPR1* (Svistoonoff et al., 2007; Müller et al., 2015). In contrast, in our study a significant increase in the relative expression level of *OsLPR5* in Pi-deprived roots was observed (Fig. 1C) and this was consistent with an earlier study that reported elevated expression levels of *OsLPR5* and its homolog *OsLPR3* in response to Pi deficiency (Cao et al., 2016).

**Overexpression of OsLPR5 triggers elevated ferroxidase activity and mimics the Pi deficiency response**

Several studies in Arabidopsis have demonstrated a significant role of the antagonistic interaction between Pi and Fe in maintaining nutrient homeostasis (Misson et al., 2005; Hirsch et al., 2006; Ward et al., 2008; Bournier et al., 2013; Müller et al., 2015; Dong et al., 2017; Gutiérrez-Alanis et al., 2017; Wang et al., 2019). *AtLPR1* plays a pivotal role in Pi deficiency-mediated developmental responses of the root system architecture in a Fe-dependent manner (Müller et al., 2015). In rice, physiological and transcriptome analyses of the interaction between Pi and Fe has shown that the presence of the former can affect the availability of the latter, and can consequently influence the regulation of Fe-responsive genes (Zheng et al., 2009). Secco et al. (2013) used next-generation RNA-seq in conjunction with a comprehensive time-course experiment in order to decipher the spatiotemporal molecular responses of the rice transcriptome and small RNA-ome in response to Pi deprivation and resupply. As well as identifying several key potential regulators of Pi homeostasis, they also found up-regulation of Fe-responsive genes in the roots (two putative vacuolar iron transporters) and shoots (two ferritins and one Fe transporter) in response to short-term (6–h) Pi deprivation.

Multicopper oxidases (MCOs) are a varied group of enzymes that couple the oxidation of an array of substrates to the reduction of dioxygen, and include members such as Fet3p, ascorbate oxidase, laccases, and ceruloplasmin (Solomon et al., 1996). *LPR1/2* and *OsLPR5* encode MCOs in Arabidopsis and rice, respectively (Svistoonoff et al., 2007; Cao et al., 2016). In *Saccharomyces cerevisiae*, the high-affinity Fe-uptake complex in the plasma membrane consists of Fet3p, which is involved in the ferroxidase reaction that catalyses the oxidation of Fe(II) to Fe(III) using O2 as a substrate (Van Ho et al., 2002; Singh et al., 2006). Multiple amino-acid sequence alignment of *OsLPR5* with *AtLPR1/2* and Fet3p revealed the presence of three conserved Cu sites (T1, T2/T3, and a Fe2+ substrate binding site) that are crucial for the ferroxidase activity (Supplementary Fig. S2A), and the predicted 3D structural model of *OsLPR5* (Supplementary Fig. S2B) was also comparable with those of *AtLPR1/2* and Fet3p (Müller et al., 2015). To demonstrate empirically that *OsLPR5* encodes a ferroxidase, we heterologously expressed the purified pGS::GST::OsLPR5 fusion protein (Fig. 2A, B). The ferroxidase activity was significantly higher in the *E. coli* strain BL21 expressing the fusion protein compared with the control (pGS::GST) (Fig. 2C). In addition, Pi deficiency triggered a significant increase in the ferroxidase activity in the roots of the wild-type (Fig. 2C). Interestingly, *OsLPR5*-overexpressing lines grown hydroponically for 3 weeks under +P and −P conditions both had significantly higher ferroxidase activity compared with the Pi-deprived wild-type. In Arabidopsis, the concentration of Fe has been shown to increase during Pi deficiency (Misson et al., 2005). Consistent with this, the concentrations of Fe(III) in the xylem sap and total Fe in the roots and shoots of *OsLPR5*-overexpressing lines were significantly higher than...
those of the wild-type (Fig. 2D, E). Our results thus demonstrated that the overexpression of OsLPR5 mimicked the Pi-deficiency response. Furthermore, the partial complementation of the response of the primary root of the Atlpr1 mutant of Arabidopsis to Pi deficiency that resulted from overexpressing OsLPR5 in the Atlpr1 background (Supplementary Fig. S4) demonstrated the ferroxidase activity of OsLPR5. Our results thus suggest a pivotal role of OsLPR5 in mediating cross-talk between Pi and Fe in rice. In addition, transient expression in N. benthamiana revealed the subcellular localization of OsLPR5 to both the ER and the cell wall (Fig. 3). These results are consistent with earlier studies on Arabidopsis LPR1 that have demonstrated its targeting to the ER (Ticconi et al., 2009), and to the cell wall, which is crucial for the response of the primary root to local Pi availability (Müller et al., 2015).

OsLPR5 plays a role in the maintenance of Pi homeostasis and in developmental responses

To determine whether OsLPR5 plays any role in the maintenance of Pi homeostasis in rice, knockout mutants were generated (Supplementary Fig. S5). $^{32}$Pi uptake rates at different time-points were significantly higher in the mutants compared with the wild-type under –P conditions (Fig. 4A), whilst the shoot/root distribution ratio of $^{32}$P was significantly lower in the mutants under both +P and –P conditions (Fig. 4B). The mutation in OsLPR5 also resulted in a higher concentration of total P in the roots under +P conditions and in both the roots and shoots under –P conditions compared with the wild-type (Fig. 4C). In the OsLPR5-overexpressing lines there was a significant increase in $^{32}$Pi uptake under +P conditions, and higher concentrations of Pi in the xylem sap and in the shoots Pi under both +P and –P conditions compared with the wild-type (Supplementary Fig. S6). Overall, these results suggest a role for OsLPR5 in the translocation of Pi from the root to shoot. This was further corroborated by reduced expression of cis-NAT_OsPHO1,2 in the mutants compared with the wild-type in response to Pi deficiency (Fig. 4D). cis-NAT_OsPHO1,2 is essential for maintaining OsPHO1,2 under Pi-deficient conditions (Jabnoune et al., 2013). Thus, the reduced expression of cis-NAT_OsPHO1,2 in the OsLPR5 mutants would have caused lower expression of the OsPHO1,2 protein, thereby resulting in reduced translocation of Pi from the root to shoot. However, it is not clear how OsLPR5 would have negatively regulated cis-NAT_OsPHO1,2 and hence this merits further investigation.

OsLPR5 also played an important role in the developmental responses of ontogenetically distinct root traits. For example, the primary root of the mutants were significantly shorter under both +P and –P conditions compared with the wild-type (Fig. 5A, B), thus highlighting the role of OsLPR5 in the developmental response of embryonically developed primary roots through both Pi-dependent and Pi-independent pathways. In contrast, the OsLPR5-overexpression lines exhibited defects in lateral root development compared with the wild-type (Fig. 6A), as indicated by significant reductions in the mean length of lateral roots (Fig. 6B), in the cell development angle (Fig. 6C), and in the mean width of the epidermal cells (Fig. 6D). Sections of the lateral roots of the OsLPR5-overexpression lines revealed defects in the spatial arrangements of the quiescent center and the surrounding stem, cortical, and epidermal cells (Fig. 6E). We assume that these defects in the root tips of the overexpressing lines could have contributed to the reduced lateral root growth. LPR1 in Arabidopsis has been implicated in adjusting the meristem activity of the primary root upon sensing changes in external Pi (Svistoonoff et al., 2007; Ticconi et al., 2009; Müller et al., 2015); however, LPR1 has not been reported to exert any apparent influence on the developmental responses of the lateral roots. Our study has thus highlighted a functional divergence between LPR1 in Arabidopsis and OsLPR5 in rice on their influence on the ontogenetically distinct embryonically developed primary roots and the post-embryonically developed lateral roots. In Arabidopsis, it has been shown that the blue-light effect in Petri dishes is the actual cause of the Fe-dependent Pi deficiency-mediated inhibition of primary root growth (Zheng et al., 2019). For this reason, in all our experiments seedlings were grown in a hydroponic system with the container painted black to ensure complete exclusion of light from the growing root system. The adverse effects of the mutation in OsLPR5 were also evident in various morphological traits of plants grown to maturity in soil supplemented with Pi fertilizer. The height of the mutants was significantly reduced compared with the wild-type (Fig. 7A, C), and they also exhibited significant reductions in seed-setting rate (Fig. 7E), 1000-grain weight (Fig. 7F), and grain yield per plant (Fig. 7G). These results therefore suggest that OsLPR5 has a broad-spectrum influence on the agronomic traits of rice.

Conclusions

Our study provides evidence that OsLPR5 is a ferroxidase, and that it plays a broad-spectrum role in influencing a subset of the traits that govern Pi homeostasis. It also influences the developmental responses of different root traits and agronomic traits. We have previously shown that OsPDR2 (homolog of AtPDR2) plays important roles in the development responses and maintenance of Pi homeostasis in rice (Cao et al., 2020). In future studies it would be interesting to determine whether OsLPR5 and OsPDR2 interact genetically to regulate Pi and/or Fe homeostasis and/or developmental responses under different Pi and Fe regimes.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Histochemical analysis of GUS activity driven by OsLPR5 promoter.

Fig. S2. Sequence alignment of OsLPR5 with Fet3p, AtLPR1, and AtLPR2, and 3D structural model of OsLPR5.

Fig. S3. Expression analysis and Southern blotting of the OsLPR5-overexpression lines.

Fig. S4. Complementation of the Atlpr1 phenotype by the overexpression of OsLPR5.

Fig. S5. Identification of the Oslpr5 mutant lines.
Fig.S6. Effects of OsLPR5 overexpression on Pi homeostasis. Table S1. The primers used for promoter–GUS fusion, overexpression, RT-PCR, and qRT-PCR.

Table S2. The primers used for the generation and identification of the mutation of OsLPR5 by CRISPR/Cas9.

Acknowledgements
This work was supported by the National Program on R&D of Transgenic Plants (2016ZX08009-003-005), the Chinese National Natural Science Foundation (31672226), the National Key Research and Development Program of China (2016YFD0100700 and 2019YFD0900702), an International Workshop on the nexus of food-energy-water (FEWS) systems: US-China (2019J1006), the Innovative Research Team Development Plan of the Ministry of Education (IRT_17R56; KYT201802), the 111 Project (number 12009), and the Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX17_0592 and KYCX17_0594). We thank Prof. Yang Chengwei at South China Normal University for insightful suggestions during this study.

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