OsPDR2 Mediates the Regulation on the Development Response and Maintenance of Pi Homeostasis in Rice

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Research article

Keywords: OsPDR2 • Phosphate • Homeostasis • Growth and development • Rice

Posted Date: August 19th, 2019

DOI: <https://doi.org/10.21203/rs.2.13126/v1>

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Version of Record: A version of this preprint was published at Plant Physiology and Biochemistry on April 1st, 2020. See the published version at <https://doi.org/10.1016/j.plaphy.2019.12.037>.

Abstract

Background: Phosphate (Pi), is the least accessible macronutrient in many natural and agricultural ecosystems and its low availability often limits plant growth and productivity. In *Arabidopsis thaliana* (*Arabidopsis*), Phosphate Deficiency Response2 (AtPDR2), interacts genetically with Low Phosphate Root1 (AtLPR1) in the endoplasmic reticulum (ER), play a key role in Pi deficiency-mediated inhibition of the primary root growth and remodeling of the root system architecture (RSA). However, the role of OsPDR2, the homolog of AtPDR2, either in roots response to Pi deficiency and/or in growth and development has not been elucidated as yet. **Results:** Therefore, we investigated the spatiotemporal effects and the availability of Pi on the relative expression levels of OsPDR2 by employing qRT-PCR. OsPDR2 showed variable levels of relative expression pattern in vegetative and/or reproductive tissues analyzed at different stages of growth and development (5-17 weeks). Transient expression analysis revealed its subcellular localization to the ER. Further, the reverse genetics approach was employed for determining the function of OsPDR2 by RNAi-mediated suppression. Three independently generated RNAi lines (Ri2, Ri9, and Ri18) were compared with the wild-type (WT) for various vegetative and reproductive traits. The study revealed significant inhibitory effects of RNAi-mediated suppression of OsPDR2 on the root and male reproductive traits, and yield. Moreover, ³²P isotope labeling and split-root experiments under different Pi regime with RNAi lines and the WT revealed the role of OsPDR2 in the maintenance of Pi homeostasis. **Conclusions:** The results from this study revealed the key role of OsPDR2 in the growth and development of rice and maintenance of phosphate homeostasis.

Background

Phosphorus (P) is one of the essential macronutrients required for plant growth and development (Raghothama 1999). In addition to being a constituent of key molecules such as nucleic acids, ATP, and membrane phospholipids, P plays crucial roles in signal transduction, energy transfer, photosynthesis and respiration (Plaxton and Tran 2011). Inorganic orthophosphate (Pi) is the major form of P available to plants for acquisition by roots and its subsequent assimilation (Nussaume et al. 2011, Gu et al. 2016). However, the mobility of Pi in soils is poor and/or has the tendency to form sparingly soluble salts with oxides/hydroxides of aluminum and iron in acidic soils, and with calcium and magnesium in alkaline soils (Marschner 1995). Therefore, availability of Pi is often limited in many natural and agricultural ecosystems and thus has an adverse effect on the growth and development of plants (Chiou and Lin 2011, [López-Arredondo et al. 2014](#)).

Arabidopsis thaliana (*Arabidopsis*), the first plant whose complete genome was sequenced, has been a favored model plant due to its small genome size, publicly available T-DNA insertion lines, and short generation time (The Arabidopsis Genome Initiative, 2000, Koorneef and Meinke 2010, O'Malley and Ecker 2010, Provart et al. 2015). Therefore, *Arabidopsis* has been extensively used to study the spatiotemporal effects of Pi deficiency on an array of morphophysiological and molecular adaptive responses (Rouached et al. 2010, Chiou and Lin 2011, [López-Arredondo et al. 2014](#)). In *Arabidopsis*, among the morphological traits, root system has been the focus of many studies since it not only plays a key role in

the acquisition of Pi but also show explicit developmental plasticity upon encountering deprivation of Pi and/or other nutrients (Gruber et al. 2013, Kellermeier et al. 2014, Gutiérrez-Alanís et al. 2018). Divided root study revealed that the expression of Pi-starvation-inducible (PSI) could be suppressed by the application of Pi to only a part of the Pi-deprived root system (Burleigh and Harrison 1999). The study provided conclusive evidence towards the role of systemic (long-distance) response in integrating global Pi status of the plant. Furthermore, Arabidopsis primary root tip cells have an innate ability to sense the local availability of Pi in the medium independent of whole plant Pi status (Linkohr et al. 2002). The growth of the primary root is thus inhibited upon encountering local Pi deficiency (Williamson et al. 2001; López-Bucio et al. 2002; Jain et al. 2007). Pi deficiency-mediated inhibition of primary root growth is caused by the progressive exhaustion of the root apical meristem resulting in an irreversible determinate growth (Sánchez-Calderón et al. 2005). Modulation of the root system architecture (RSA) in Pi-deprived Arabidopsis is an adaptive response to augment top soil foraging where Pi tends to accumulate (Péret et al. 2011). Local and systemic sensing thus acts in concert in regulating Pi homeostasis in Arabidopsis (Ticconi and Abel 2004, Abel 2011, Péret et al. 2011, Thebaud et al. 2010).

To decipher the molecular components that monitor external Pi status and interpret the nutritional signal, the forward genetics approach was employed by screening the Arabidopsis mutant seedlings, which could sustain the growth of the primary root under Pi deficiency (Péret et al. 2011). In this context, a complementary methodology was developed for screening and isolating constitutive and insensitive Pi starvation response Arabidopsis mutants based on their facultative ability to utilize organophosphates such as nucleic acids under Pi-limiting condition (Chen et al. 2000; Ticconi et al. 2001). This led to the identification of *phosphate deficiency response (pdr2)* mutant, which exhibited Pi concentration-dependent attenuation of cell division in developing root meristems (Ticconi et al. 2004). The hypersensitive response of *pdr2* mutant exhibited a likely disruption in Pi sensing pathway, which is pivotal for root system adaptation to low Pi availability. Based on map-based strategy, *PHOSPHATE DEFICIENCY RESPONSE2 (PDR2)* was found to encode the single P5-type ATPase (At5g23630) (Ticconi et al. 2009). Endoplasmic reticulum (ER) targeted PDR2 is essential for the appropriate expression of the *SCARECROW (At3g54220)*, which is an important regulator of stem cell maintenance, and patterning of Pi-deprived roots (Ticconi et al. 2009). The authors further demonstrated the epistatic interaction between *PDR2* and another ER-resident multicopper oxidase encoded by *LOW PHOSPHATE ROOT1 (LPR1; At1g23010)* (López-Bucio et al. 2005; Reymond et al. 2006; Sánchez-Calderón et al. 2006; Svistoonoff et al. 2007; Ticconi et al. 2009). Furthermore, the role of SENSITIVE TO PROTON RHIZOTOXICITY (STOP1), and its target ALUMINUM ACTIVATED MALATE TRANSPORTER 1 (ALMT1), which releases malate, an organic acid believed to chelate cations and in this way makes more Pi available for absorption via root hairs, were identified. The LPR1–PDR2 module interacts with an unknown pathway parallel to STOP1–ALMT1 to inhibit cell division in the SCN by a similar mechanism involving accumulation of Fe and callose, peroxidase-activity, and cell wall thickening (Balzergue et al., 2017; Mora-Macías et al., 2017). And MPK6 kinase also play a role in root architectural adjustments to low Pi-Fe imbalances responsible for determinate primary root growth in Arabidopsis (López-Bucio et al., 2019). In an earlier study, At5g23630 was also annotated as *MALE GAMETOGENESIS IMPAIRED ANTHERS (MIA)* and shown to

play a key role in pollen development and fertilization (Jakobsen et al. 2005). This suggested a wide-spectrum role of *PDR2* not only during Pi deficiency but also during the reproductive phase.

Rice (*Oryza sativa*) is the staple food and major source of dietary energy supply for more than half of the world's population (www.irri.org/rice-today). In 2016/2017, ~475 million metric tons of rice were consumed worldwide of which China was the leading consumer amounting to ~30.0% (www.statista.com/Agriculture/Farming). It is anticipated that rice consumption around the world will continue to grow gradually (www.graincentral.com/cropping/global-rice-consumption). However, high soil P-fixation particularly in acid upland ecosystems renders low availability of Pi to rice and adversely affects its growth and yield potential (Gamuyao et al. 2012). Therefore, there is a need to identify genes that are amenable for manipulation for generating rice that could sustain growth in soil poor in available Pi (Veneklaas et al. 2012). Rice was the first crop whose genome was sequenced and thus paved the way for significant advances in molecular genetics and breeding (Jackson 2016). Furthermore, genome sequencing of rice facilitated in deciphering the function of an array of genes involved in the sensing and signaling cascades governing Pi homeostasis (Wu et al. 2013), and those involved in the embryonic and postembryonic development of the root system (Mai et al. 2013). It is intriguing that unlike Pi deficiency-mediated inhibitory effect on primary root growth in Arabidopsis, in rice the growth of root remains either unaffected (Hu et al. 2011, Wang et al. 2014a) or stimulated (Dai et al. 2012, 2016) during Pi deficiency. However, the role of *OsPDR2*, a homolog of Arabidopsis *PDR2*, either in roots response to Pi deficiency and/or in growth and development has not been elucidated as yet. Therefore, in this study, we investigated the role of *OsPDR2* in the maintenance of Pi homeostasis and /or growth and developmental responses.

Methods

Plant materials and growth conditions

Rice seeds (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) were surface-sterilized for 30 min with diluted (1:3, v/v) NaClO, followed by thorough rinsing for 30 min with deionized water. Seeds were germinated in dark at 25°C for 3 d. The hydroponic experiments were carried out in a growth room with a 14-h-light (30°C)/10-h-dark (22°C) photoperiod, and the relative humidity was maintained at ~70%. Rice seedlings (10-d-old) were transferred to +P medium (1,250 μM NH₄NO₃, 1000 μM each of CaCl₂ and MgSO₄, 500 μM Na₂SiO₃, 350 μM K₂SO₄, 300 μM KH₂PO₄, 20 μM each of Fe-EDTA and H₃BO₃, 9.0 μM MnCl₂, 0.77 μM ZnSO₄, 0.39 μM Na₂MoO₄, and 0.32 μM CuSO₄). For -P medium, 300 μM KH₂PO₄ was replaced with 300 μM KCl. For the split-root experiment, the roots of the uniformly grown seedlings (10-d-old) in the +P medium were separated into two equal parts, and transferred to a split-root container with each half immersed in +P (300 μM Pi) and -P (10 μM Pi) media. The pH of the nutrient medium was adjusted to 5.0. The nutrient medium in the hydroponic set up was refreshed every 3rd d. For the pot experiment, the soil was obtained from an experimental farm at Nanjing Agricultural University. Each pot was filled with 15 kg

of air-dried soil. The pot was supplemented with fertilizer Pi (40 mg) kg⁻¹ soil and standard cultural practices recommended for rice were followed as described (Jia et al. 2011).

Database search, sequence alignment, and phylogenetic analysis

Complete genomic sequence and transcript of *OsPDR2* were retrieved from Michigan State University (<http://rice.plantbiology.msu.edu/>) and The Rice Annotation Project Database assembly (v7) (<https://academic.oup.com/>). tBLASTn program and PLAZA1.0 software (<http://bioinformatics.psb.ugent.be/plaza/>) were employed for identifying *PDR* homologs in dicots (*Arabidopsis thaliana*, *Carica papaya*, *Fragaria vesca*, *Glycine max*, *Malus domestica*, *Manihot esculenta*, *Populus trichocarpa*, *Prunus persica*, *Ricinus communis*, *Theobroma cacao*, and *Vitis vinifera*), monocots (*Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor*, and *Zea mays*), gymnosperms (*Selaginella moellendorffii*), bryophyte (*Physcomitrella patens*) algae (*Chlamydomonas reinhardtii*, *Micromonas sp. RCC299*, *Ostreococcus lucimarinus*, *Ostreococcus tauri*, and *Volvox carteri*). The unrooted phylogenetic tree of *PDR* homologs was made using the neighbor-joining method and displayed using the MEGA4.0 program.

Generation of *OsPDR2*-RNAi transgenics.

The knockdown transgenic lines of *OsPDR2* (*OsPDR2*-Ri) in rice (*Oryza sativa* ssp. japonica 'Nipponbare') were obtained by the method of RNA interfering (Ri). *OsPDR2* coding sequence-specific 267 bp fragment (1702–1968 bp downstream of the ATG) was amplified by PCR. To facilitate subsequent cloning of the PCR product into the binary vector pTCK303, *Bam*HI and *Kpn*I sites were incorporated into the 5' end of both forward primer AS-F (5'- CGCGGATCCATCCCTTTCCAACTTCT -3') and reverse primer AS-R (5'- CGGGGTACCTCTGGTGGTCAACCTGTA -3'). The purified PCR product was ligated to the pEASY cloning vector (Transgen). The PCR-amplified fragment and pTCK303 were digested with *Bam*HI and *Kpn*I and cloned in the sense orientation as described (Wang et al. 2004). *OsPDR2*-specific 267 bp fragment was then amplified using forward primer S-F (5'- CGAGCTCATCCCTTTCCAACTTCT -3') and reverse primer S-R (5'- GGACTAGTTCTGGTGGTCAACCTGTA -3') with *Sac*I and *Spe*I sites incorporated into their 5' end. The PCR-amplified fragment was cloned in the antisense orientation in pTCK303 using a similar strategy. The presence of the two inserts in the desired orientation in the plasmid was confirmed by sequencing. The plasmid was transferred to *Agrobacterium tumefaciens* strain EHA105 by electroporation and then transformed into embryonic calli of Nipponbare as described (Upadhyaya et al. 2000).

RT-PCR and qRT-PCR

Total RNA (~1 µg) was isolated from the ground tissues using Trizol reagent (Invitrogen) and treated with RNase-free DNase. First-strand cDNA was synthesized using oligo (dT)-18 primer and reverse transcribed

using Superscript II™ Reverse Transcriptase (Invitrogen). *OsActin* (LOC_Os03g50885) 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) in *StepOnePlus™* Real-Time PCR System (Applied Biosystems). Relative expression levels of the genes were computed by the $2^{-\Delta\Delta C_T}$ method of relative quantification (Livak and Schmittgen 2001). All the gene-specific primers used are listed in Supplementary Table S1.

Transient expression of OsPDR2 in tobacco leaves for subcellular localization

Agrobacterium-mediated transformation was employed for the transient co-expression of *35S::EGFP::OsPDR2* and *35S::mCherry::HDEL* in the epidermal leaf cells of tobacco as described (Bürstenbinder et al. 2013). Tobacco leaves were collected 2-3 d after infiltration. A 488 nm (EGFP) and 561 nm (mCherry) diode laser were used for the excitation and EGFP/mCherry fluorescence was visualized using a confocal laser scanning microscope (Leica).

Pollen fertility test

Anthers from the spikelet of the wild-type and *OsPDR2*-RNAi lines were collected just before anthesis. Anthers were crushed in *Lugol's* (I_2 -KI) solution and observed under a stereomicroscope as described (Shivanna and Rangaswamy 1992). Fertile and sterile pollens were stained and unstained, respectively and their images were captured.

Quantification of Pi and total P

Concentrations of Pi and total P were quantified as described (Ames 1966).

³²Pi uptake assay

Seedlings (10-d-old) of the wild-type and the *OsPDR2*-RNAi line (Ri9) were grown hydroponically under +P condition for 10 d. Subsequently, these seedlings were grown for 7.5 h in +P uptake solution (200 ml) labeled with 8 μ Ci of ³²Pi (KH₂PO₄, Perkin-Elmer). After the uptake, apoplastic ³²Pi was removed by incubating the roots of the seedlings in ice-cold desorption solution (2 mM MES [pH 5.5], 0.5 mM CaCl₂, and 0.1 mM NaH₂PO₄) for 10 min. Seedlings were then blotted-dry, roots and shoots were harvested separately and their fresh weights were documented. Tissues were digested in HClO₄ and 30% (v/v) H₂O₂ mixture at 28°C for 8-12 h. Scintillation cocktail (3 ml) was added to the digested tissue and ³²Pi activity was determined by using a liquid scintillation counter (Tri-Carb 2100, Packard). ³²Pi counts in the root and shoot were used for determining the per cent shoot/root distribution.

Statistical analysis

Data were analyzed for significant differences using IBM SPSS Statistics 20 program.

(<http://www-01.ibm.com/software/analytics/spss/>).

Results And Discussion

Effects of different growth stages and Pi availability on the tissue-specific expression profile of *OsPDR2*

OsPDR2 gene contains 21 exons and 20 introns and encodes a protein with 1175 amino acids (**Supplementary Fig. S1A**). To determine the phylogenetic relationship of *OsPDR2* with *AtPDR2* and P₅ type ATPases across lower and higher plant species, Clustal X program and the neighbor-joining method was employed (**Supplementary Fig. S1B**). *OsPDR2* homologs were identified in dicots (15), monocots (6), gymnosperms (1), bryophytes (1), and algae (6). Number of *OsPDR2* homologs ranged from 3 each in *Zea mays* (maize) and *Malus domestica* (apple) to 1-2 in other higher and lower plant species. The analysis revealed a close phylogenetic relationship of *OsPDR2* with maize (*ZM08G16190* and *ZM06G23900*), *Sorghum bicolor* (sorghum; *SB09G019760*), and *Sargassum mongolicus* (*BD2G25857*). Further, TMHMM and Topo2 software were used to determine the predicted transmembrane topology profile of *OsPDR2* protein, which revealed eight transmembrane domains in *OsPDR2* (**Supplementary Fig. S1C**). A heavy metal P-type ATPase *OsHMA4* also has eight transmembrane domains, and is involved in preventing the accumulation of copper (Cu) in rice grain (Huang et al. 2016). By aligning the part of the corresponding domains of *OsPDR2* with *AtPDR2* and *SPF1* from yeast, revealed the presence of P5 type ATPase-specific two motifs i.e., PPXXP and DKTGTLT (**Supplementary Fig. S1D**). This suggested that *OsPDR2* potentially encodes a P5 type ATPase containing a conserved PP (E/D)xPx (E/D) sequence motif with two conserved negatively charged residues and is largely associated with the endoplasmic reticulum (ER), which is characteristic of the eukaryotic cells (Sørensen et al. 2015).

To determine the spatiotemporal expression patterns of *OsPDR2* in the wild-type (WT) grown in the field, different tissues (leaf blade [I, II, and III], sheath [I and II], basal region, root zone [I and II], culm, nodes, panicle axis, anther, ovary, and glume) were harvested at 5 weeks, 10 weeks (tilling), 15 weeks (flowering), and 17 weeks (grain filling) of growth stages for quantitative real-time PCR (qRT-PCR) assay (**Fig. 1A**). *OsPDR2* showed constitutive expression in different tissues collected at different growth stages. The relative expression levels of *OsPDR2* were comparatively higher in the leaf blade, sheath and reproductive organs, and also it was relatively higher at tilling and flowering stages compared with seeding (5 weeks) and grain filling stages. To determine the effect of Pi deficiency on the relative expression levels of *OsPDR2*, WT seedlings (14-d-old) were grown hydroponically under +P and -P conditions for 3 d and 21 d. After 21 d of the treatment, half of the -P plants were replenished with +P for 1 d. The relative expression levels of *OsPDR2* were assayed in the root and leaf blade by qRT-PCR (**Fig. 1B**). The relative expression levels of *OsPDR2* were comparable in both the root and leaf blade of the 3d

+P and -P seedlings. However, Pi deficiency-mediated tissue-specific differential expression pattern of *OsPDR2* was revealed when the treatment was extended up to 21d. For instance, the relative expression levels of *OsPDR2* were significantly lower (~35%) in the root and ~2.5 times higher in the leaf blade of Pi-deprived seedlings compared with +P seedlings. The results suggested tissue-specific differential role of *OsPDR2* under different Pi regime. Although *PDR2* in Arabidopsis plays a pivotal role in Pi-deficiency-mediated root response (Ticconi et al. 2009), its expression was not reported to be induced or suppressed by Pi deficiency during a genome-wide spatiotemporal transcriptional analysis of Pi deficiency responses using Affymetrix gene chips (Misson et al. 2005). It is not surprising to anticipate the potential functional divergence between *PDR2* and *OsPDR2* in taxonomically diverse Arabidopsis and rice, respectively. The relative expression levels of *OsPDR2* in both the root and leaf blade of 21d Pi-deprived seedlings became comparable with the corresponding tissues of 21d +P seedlings upon replenishment with +P for 1 d (**Fig. 1B**). The study thus highlighted the pivotal role of Pi availability in the transcriptional regulation of *OsPDR2*.

OsPDR2 is localized to the ER

ER is the port of entry for the membrane and secretory proteins into the central vacuolar system of all the eukaryotic cells (Rapoport et al. 1996). Proteins destined for different organelles are folded and assembled in the ER (Gaut and Hendershot 1993). The web-based Softberry (www.linux1.softberry.com) was employed for the identification of a putative ER-resident OsPDR2. Red fluorescent protein (mCherry) is potent *in vivo* marker for the localization of the putative ER-resident proteins in diverse plant species (Nelson et al. 2007). To provide empirical evidence towards the localization of OsPDR2 to the ER, the reporter gene (EGFP) was fused in frame with the ORF of OsPDR2 at the C-terminal, and was driven by a CaMV35S promoter (*35S::EGFP::OsPDR2*). Transgenic *Nicotiana benthamiana* expressing *35S::mCherry::HDEL* exhibits characteristic red fluorescence in the ER (Feng et al. 2013). Therefore, *35S::EGFP::OsPDR2* and *35S::mCherry::HDEL* were transiently co-expressed in the leaf epidermal cells of *N. benthamiana*. Confocal microscopy was used for capturing their green (EGFP) and red (mCherry) fluorescence images and their subsequent merge revealed the localization of OsPDR2 to the ER (**Fig. 2**). The result was consistent with the localization of *PDR2* to the ER in Arabidopsis (Ticconi et al. 2009).

RNAi-mediated suppression of *OsPDR2* affects vegetative growth and root length

In Arabidopsis, ER-resident *PDR2* plays a pivotal role in Pi deficiency-mediated root patterning (Ticconi et al. 2009), pollen development and fertilization (Jakobsen et al. 2005). Therefore, the plausible role of *OsPDR2* in exerting influence on a subset of traits involved in sensing and signaling cascades governing Pi homeostasis and/or developmental responses was investigated. RNAi-mediated gene-silencing provides an attractive paradigm for generating mutants for the specifically targeted genes (Wilson and Doudna 2013). RNAi-mediated gene-silencing has thus been conventionally and successfully used for

determining in planta function of the several genes encoding Pi transporters and transcription factors that play pivotal roles in the maintenance of Pi homeostasis (Zhou et al. 2008, Ai et al. 2009, Sun et al. 2012, Wang et al. 2014b, Ruan et al. 2017). To determine in planta functions of *OsPDR2*, transgenic rice plants were generated with RNAi-mediated suppression in the expression of this gene (**Supplementary Fig. S2**). The level of suppression in the expression of *OsPDR2* was assayed in the independently generated 13 RNAi lines (designated as Ri1-Ri4, Ri6, Ri8-Ri11, Ri18, Ri20, Ri21, and Ri23) by employing semi-quantitative RT-PCR (**Supplementary Fig. S2A**). The analysis revealed notable reductions in the *OsPDR2* transcripts in 11 RNAi lines (Ri1-Ri4, Ri8-Ri11, Ri18, Ri20, and Ri21) except Ri6 and Ri23 compared with the WT. Ri11 did not show proper growth and eventually failed to survive and thus could not be used for further analysis. For the quantitative assessment of the level of suppression in the relative expression level of *OsPDR2* in these 12 RNAi lines compared with the WT, qRT-PCR was analysed in the roots of the WT and RNAi lines (**Supplementary Fig. S2B**). Consistent with the semi-quantitative RT-PCR, except Ri6 and Ri23, all the other Ri lines revealed variable but significant reductions in the relative expression level of *OsPDR2* compared with the WT. Among these Ri lines, those showed significant suppression (Ri2, Ri8, Ri9, and Ri18) and no significant suppression (Ri6) were selected for the Southern-blot analysis, which clearly showed that these Ri lines were generated independently and are suitable for further morphophysiological and molecular analyses (**Supplementary Fig. S2C**). Ri2, Ri9, and Ri18 were selected for subsequent analyses.

To determine the effects of the attenuated expression of *OsPDR2* in Ri lines (Ri2, Ri9, and Ri18) on biomass and different root traits, seedlings (10-d-old) of the WT and Ri lines were grown hydroponically under +P and -P conditions for 21 d (**Fig. 3**). Compared with the WT, Ri lines exhibited stunted growth irrespective of Pi regime (**Fig. 3A**). Further, the biomass of the root and leaf (blade and sheath) of the WT and Ri lines grown under +P and -P conditions were quantified (**Fig. 3B**). Barring -P leaf sheath of Ri18, all the other tissues of +P and -P Ri lines showed significantly lower biomass of the root and leaf (blade and sheath) compared with their respective WT. Biomass production is a complex quantitative trait with polygenic inheritance. The results thus suggested wide-spectrum effects of the mutation in *OsPDR2*, which resulted in the reduced biomass. In Arabidopsis, *PDR2* plays a key role in triggering Pi deficiency-mediated inhibition of the primary root growth, which is a typical adaptive response (Williamson et al. 2001, Linkohr et al. 2002, López-Bucio et al. 2002, Sánchez-Calderón et al. 2005, Jain et al. 2007, Ticconi et al. 2009). In rice, unlike Arabidopsis, root system comprises a fibrous network facilitating anchorage and acquisition of nutrients (Hochholdinger and Zimmermann 2008, Atkinson et al. 2014). Furthermore, contrary to Arabidopsis, Pi deficiency stimulates embryonically developed primary root growth (Shimizu et al. 2004, Torabi et al. 2009, Zheng et al. 2009, Dai et al. 2012, 2016). Coherent with these reports, in the present study also primary root length of the WT was significantly higher under -P condition compared with +P condition (**Fig. 3C**). However, irrespective of the Pi regime, primary root length was significantly lower in Ri lines compared with their respective WT (**Fig. 3C**). Post-embryonically developed adventitious from non-root tissues constitute the bulk of the functional root system in the mature plants (Hochholdinger et al. 2004, Hochholdinger and Zimmermann 2008, Steffens and Rasmussen 2016). To determine the effect of Pi deficiency on the developmental response of the

adventitious roots of the WT and Ri lines, length of their three longest ones were documented (**Fig. 3D**). Pi deficiency triggered a significant increase in the length of the three longest adventitious roots of the WT. The result was consistent with earlier studies reporting the stimulatory effect of Pi deficiency on the length of adventitious roots in the WT rice (Zhou et al. 2008, Hu et al. 2011, Wang et al. 2015). However, similar to the response of primary root length, length of three longest adventitious roots of +P and -P Ri lines (except -P Ri9) was significantly attenuated compared with their corresponding WT. Together, the study highlighted the Pi deficiency-mediated alteration in the root system architecture (RSA) by stimulating the growth of primary and adventitious roots of the WT and positively regulated by *OsPDR2*.

To further determine the effects of RNAi-mediated suppression in the expression of *OsPDR2* on the elongation zone (EZ) and maturation zone (MZ) of the primary root, seedlings (7-d-old) of the WT, Ri2, and Ri9 were grown hydroponically under +P condition for 10 d (**Fig. 4**). Roots were harvested and stained with a cationic dye propidium iodide (PI), which stains cell walls and give red fluorescence. The cell length and diameter (transverse section) in the MZ and EZ of the root were measured by using an open source Java ImageJ processing program (Collins 2007, www.rsb.info.nih.gov/i). Although the cell length in the EZ was comparable between the WT and the Ri lines, it was significantly reduced in the latter than the former in the MZ zone (**Fig. 4A, B**). Further, transverse sections of the EZ and MZ of the primary root of the WT and Ri lines (Ri2 and Ri9) were taken for documenting the cell diameter (**Fig. 4C**). A strip of the transverse section of the primary root shows the layers of the cortical cell numbered 1 to 9 from the endodermis towards epidermis (right of **Fig. 4D**). There was a significant reduction in the cell diameter of the cortical layers 1 and 2 (proximal to the endodermis) in the EZ of the Ri lines compared with their respective WT (**Fig. 4D**). A similar attenuating effect on the cell diameter was also observed in the cortical layers 1 and 2 of Ri2 and Ri2 and Ri9, respectively compared with their respective WT in the MZ (**Fig. 4D**). However, the cell diameter of the cortical layers 3-9 in both the EZ and MZ were comparable in the WT and Ri lines (Ri2 and Ri9) (**Supplementary Fig. S3**). The results suggested the role of *OsPDR2* in both the longitudinal elongation and lateral expansion of the root cells, which concomitantly influences the primary root growth.

RNAi-mediated suppression of *OsPDR2* affects the growth performance, male reproductive traits, and yield

Since in *Arabidopsis* *PDR2* also annotated as *MIA* has been implicated in playing a role in pollen development and fertilization (Jakobsen et al. 2005), we investigated whether RNAi-mediated attenuation in the expression of *OsPDR2* exerts any influence on the growth performance, male reproductive traits, and consequently the yield performance. The WT and Ri lines (Ri2, Ri9, and Ri18) were grown for 20 weeks in the pot up to the maturity (**Fig. 5**). Compared with the WT, Ri2 and Ri9 exhibited stunted growth (**Fig. 5A**) and their height was significantly reduced (**Fig. 5B**). Notably, the growth performance and the plant height of Ri18 were comparable with the WT (**Fig. 5A, B**). The differential responses of these Ri lines could be due to the variable level of RNAi-mediated suppression in the expression of *OsPDR2* in them. For

instance, the reduction in the relative expression level of *OsPDR2* in both Ri2 and Ri9 was relatively more accentuated than Ri18 (**Supplementary Fig. S2B**). Further, the effects of Ri lines on the male reproductive traits were investigated (**Fig. 5C-F**). The WT anthers showed normal dehiscence to release the fertile pollen grains, whereas more than 50% Ri2 and Ri9 and ~50% Ri18 anthers remained indehiscent after anthesis (**Fig. 5C, D**). I₂-KI staining procedure (Shivanna and Rangaswamy 1992) was employed to compute the per cent mature pollen grains in the WT and Ri lines. Pollen grains stained dark were counted as mature and viable (**Fig. 5E**). There was a significant reduction in the per cent pollen grains in the Ri lines compared with the WT (**Fig. 5F**). The results thus corroborate the earlier study by Jakobsen et al. (2005) suggesting the role of *OsPDR2* in pollen development and its influence on male fecundity. The adverse effects of the RNAi-mediated attenuated expression of *OsPDR2* in the Ri lines were also evident on the panicle development (**Fig. 6A**), per cent seed setting rate (**Fig. 6B**), yield (**Fig. 6B**), and the length and width of the seeds (**Fig. 6D-F**). The study thus highlighted a wide-spectrum role of *OsPDR2* in influencing various reproductive traits.

RNAi-mediated suppression of *OsPDR2* affects Pi homeostasis

The *spatial*-specific effects of RNAi-mediated suppression of *OsPDR2* on the concentrations of Pi (under +P and -P conditions in a hydroponic system for 21 d) and total P (10 d before harvest and post-harvest during growth in a pot soil) in Ri lines (Ri2, Ri9, and Ri18) were compared with the WT (**Fig. 7**). Although under +P condition the Pi concentration of the WT and Ri lines were comparable in the new blade, the values in the Ri lines compared with the WT in the root (Ri9 and Ri18), and leaf (old blade and sheath of Ri2, Ri9, and Ri18) were significantly higher (**Fig. 7A**). A similar trend of an elevated Pi concentration during Pi deficiency was also observed in the Ri lines than WT in the leaf (old blade [Ri2 and Ri18] and sheath [Ri2, Ri9, and Ri18]) (**Fig. 7A**). The variable effects of RNAi-mediated suppression of *OsPDR2* were also evident on the total P concentration in some of the tissues collected 10 d before harvest and post-harvest in Ri lines compared with the WT (**Fig. 7B**). For instance, the effects varied from the reduction in the total P concentration of Ri9 leaf blade and its significant increase in the leaf sheath and culm (Ri2, Ri9, and Ri18) than the corresponding WT at 10 d before harvest stage (**Fig. 7B**). At the post-harvest stage, a significant increase in the total P concentration was also detected in some of the tissues of Ri lines (leaf sheath [Ri2]) and culm [Ri2, Ri9, and Ri18]) compared with the WT (**Fig. 7B**). The results suggested a tissue-specific negative regulatory influence of *OsPDR2* on the concentrations of Pi (independent of Pi regime) and total P (at different growth stages).

Split-root experiment is an attractive technique for determining whether morphophysiological and/or molecular responses are regulated by an external availability of Pi (local sensing) or by the internal status of the whole plant (systemic or long-distance sensing) (Burleigh and Harrison 1999, Cao et al. 2016; Giri et al. 2018). Therefore, a split-root experiment was designed to determine the effects of RNAi-mediated suppression of *OsPDR2* on the total P concentration and ³²Pi distribution (**Fig. 8**). The total P concentration was assayed in the leaf (blade and sheath) of the WT and Ri lines (Ri2 and Ri9)

seedlings (10-d-old) whose roots were supplied with +P/-P media (split root system), and only with +P and -P medium for 14 d (**Fig. 8A**). Although during +P/+P treatment the total P concentration in +P and -P split roots, and the leaf blade were comparable between the WT and Ri lines, the value was significantly higher in the leaf sheath of the Ri lines compared with the WT. When the roots were treated with only +P medium, the total P concentration was comparable in the roots of the WT and Ri lines but it was significantly higher in the leaf (blade and sheath) of Ri lines than the WT. Whereas, the total P concentration was not significantly different in the root and leaf (blade and sheath) of the WT and Ri lines when their roots were exposed to -P condition. It was interesting to observe that an increase in the total P concentration in the leaf sheath of the Ri lines compared with their respective WT was ~20% lower under +P/-P treatment compared with +P/+P treatment. This could be due to the trigger of local Pi deficiency signal from -P root in a split root system. To further investigate the effects of RNAi-mediated suppression of *OsPDR2* on the distribution of Pi between the root and shoot, again the split-root system was employed where each half of the root of the WT and Ri9 seedlings (10-d-old) was supplied with $+^{32}\text{P}/-P$, $+^{32}\text{P}/+P$, and $-^{32}\text{P}/-P$ media for 7.5 h (**Fig. 8B**). Per cent ^{32}P distribution was significantly lower in $+^{32}\text{P}$ split root and higher in $-^{32}\text{P}$ split root and shoot of Ri9 compared with their corresponding WT (**Fig. 8B, left panel**). Under $+^{32}\text{P}/+P$ condition, per cent ^{32}P distribution was significantly lower in $+^{32}\text{P}$ split root, higher in shoot and no effect in +P split root of Ri9 than their corresponding WT (**Fig. 8B, middle panel**). Per cent ^{32}P distribution in the WT and Ri9 under $-^{32}\text{P}/-P$ condition showed a trend similar to that under $+^{32}\text{P}/+P$ condition (**Fig. 8B, right panel**). The results revealed the role of *OsPDR2* in the distribution of Pi between the source and sink.

Effects of RNAi-mediated suppression of *OsPDR2* on the relative expression of *OsLPR3/5*

In Arabidopsis, ER-resident proteins PDR2 and LPR1 interact genetically to regulate the developmental response of the root meristems to Pi availability (Ticconi et al. 2009). The qRT-PCR assay revealed elevated expression levels of *OsLPR3* and *OsLPR5* in the root compared with other tissues (leaf blade and sheath and basal stem at the seedling stage, and leaf blade and sheath, culm, node, and panicle axis), which increased further during Pi deficiency, and split-root experiment revealed the relative expression levels of *OsLPR3/5* were regulated systemically by whole plant Pi status (Cao et al. 2016). To determine the effect of RNAi-mediated suppression of *OsPDR2* on the relative expression of *OsLPR3/5*, the seedlings (10-d-old) of the WT and Ri lines (Ri2 and Ri9) were split into two parts with each half was supplied with +P and -P for 14 d, and then the split roots were assayed for the relative expression levels of *OsLPR3/5* by qRT-PCR (**Fig. 9**). In the WT, the relative expression levels of both *OsLPR3* and *OsLPR5* were significantly higher in -P split root compared with the +P split root. The results suggested the effect of local Pi availability on the relative expression levels of *OsLPR3* and *OsLPR5*, which was in agreement with an earlier study (Cao et al. 2016). Interestingly, in the Ri lines the RNAi-mediated suppression of *OsPDR2* triggered a decrease and increase in the relative expression levels of *OsLPR3* and *OsLPR5*, respectively in the -P split than the +P split root. The results revealed the Pi-deficiency mediated the

differential influence of *OsPDR2* on the relative expression levels of *OsLPR3* and *OsLPR5*. The study also suggested an apparent lack of functional redundancy between *OsLPR3* and *OsLPR5* during sensing and signaling cascade governing the maintenance of Pi homeostasis in rice. Transcription factor *OsPHR2* plays a pivotal role in the transcriptional regulation of Pi starvation-responsive (PSR) genes (Zhou et al. 2008, Guo et al. 2015). In an earlier study, elevated relative expression levels of *OsLPR3* and *OsLPR5* in the *osphr2* mutant indicated their negative transcriptional regulation by *OsPHR2* (Cao et al. 2016). Therefore, to determine whether *OsPHR2* exerts any regulatory influence on *OsPDR2*, the relative expression level of *OsPDR2* was assayed by qRT-PCR in the root and shoot of the seedlings (14-d-old) of the WT (ZH11) and *osphr2* grown hydroponically under +P and -P conditions for 21 d (**Supplementary Fig. S4**). The relative expression level of *OsPDR2* was comparable between the WT and *osphr2* in both +P and -P roots. However, the relative expression level of *OsPDR2* was significantly reduced in the shoot of *osphr2* compared with the WT irrespective of Pi regime. This suggested a positive regulatory influence of *OsPHR2* on *OsPDR2* in a tissue-specific manner.

Conclusions

This study presented the effects of different growth stages and Pi availability on the tissue-specific expression profile of *OsPDR2*, the silencing of this gene triggered wide-spectrum effects on phenotypes during vegetative and reproductive growth phases. We observed the reduction of biomass, inhibition of the primary root growth in *OsPDR2*-Ri materials. The RNAi-mediated suppression of *OsPDR2* also affects anthers normal dehiscence to release the fertile pollen grains and the per cent mature pollen grains, which lead to the reduce of seed setting rate. In addition, the decrease of seed length and width work together result in low yield of *OsPDR2*-Ri materials. Furthermore, the analysis of Pi concentration, total P concentration and ³²P distribution in WT and *OsPDR2*-Ri lines suggested the key roles of *OsPDR2* in the maintenance of Pi homeostasis.

Abbreviations

Cu, copper; ER, endoplasmic reticulum; EZ, elongation zone; *LPR1*, *LOW PHOSPHATE ROOT1*; MZ, maturation zone; *MIA*, *MALE GAMETOGENESIS IMPAIRED ANTERS*; Not significant, ns; P, phosphorus; *PDR2*, *PHOSPHATE DEFICIENCY RESPONSE2*; Pi, inorganic orthophosphate; PI, propidium iodide; qRT-PCR, quantitative real-time PCR; RSA, root system architecture; WT, wild-type.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All the data supporting the present findings is contained within the manuscript.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by National Program on R&D of Transgenic Plants (2016ZX08009-003-005), Chinese National Natural Science Foundation (31672226), The National Key Research and Development Program of China (2016yfd0100700), Innovative Research Team Development Plan of the Ministry of Education (IRT1256), 111 Project (number 12009) and Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX17_0592 and KYCX17_0594).

Authors' contributions

YC participated in planning and conducting the experiments, did bioinformatics analysis and helped in writing the manuscript. AJ participated in analysis of the data, and helped in writing the manuscript. HA carried out some experiments participated in writing the manuscript. XL, XW, ZH, YS, SH and XL participated in carrying out different experiments. GX participated in planning the study. SS conceived the study, participated in planning and analysis of the data, and helped in writing the manuscript. All authors read and approved the final manuscript.

Acknowledgment

We thank Prof. Yang Chengwei at South China Normal University for insightful suggestions during the course of this study.

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Figures

Figure 1

Effects of different growth stages and Pi availability on the tissue-specific expression profile of OsPDR2. Wild-type rice (Nipponbare) was used in the present study and hereafter referred as WT, unless stated otherwise. (A) WT was grown in the field and tissues were harvested at different time intervals (5-17 weeks). (B) WT seedlings (14-d-old) were grown hydroponically under +P (300 μ M Pi) and -P (10 μ M Pi) conditions for 3 d and 21d. After 21 d of the treatment, half of the -P plants were replenished with +P for 1

d. (A and B) qRT-PCR analysis of the expression of OsPDR2 in different tissues. OsActin was used as an internal control. Data presented are means $\pm$ SE (n = 3). In (B), different letters on the histograms indicate significant differences in the relative expression of OsPDR2 ($P < 0.05$, one-way ANOVA). ns = not significant.

Figure 2

Subcellular localization of OsPDR2. *N. benthamiana* was infected with *Agrobacterium* EHA105 harboring the GFP constructs 35S::eGFP::OsPDR2 and 35S::mCherry::HDEL. Confocal microscopy was used for capturing the images of the epidermal leaf cells showing the transient expression of 35S::eGFP::OsPDR2 (left) and 35S::mCherry::HDEL (middle). Green (GFP) and red (mCherry) fluorescence images were merged (right) to reveal the localization of OsPDR2 in the endoplasmic reticulum. Bar = 10 μ m.

Figure 3

RNAi-mediated suppression of *OsPDR2* affects biomass and root traits under different Pi regime. (A and B) Seedlings (10-d-old) of the WT and RNAi lines were grown hydroponically under +P and -P conditions for 21 d. (A) Photos showing the growth performance of the WT and RNAi lines under different Pi regime are representative of 6 plants each. Bar = 5 cm. (B) Data are presented for the biomass. (C and D) Seedlings (7-d-old) of the WT and RNAi lines were grown hydroponically under +P and -P conditions for

10 d. Roots were harvested and data are presented for (C) primary root length and (D) length of three longest adventitious roots. (B-D) Values are means $\pm$ SE (n = 3 and 8 for [B] and [C and D], respectively) and different letters on the histograms indicate significant differences (P < 0.05, one-way ANOVA).

Figure 4

RNAi-mediated suppression of OsPDR2 affects the elongation and maturation zones of the primary root. (A) Seedlings (7-d-old) of the WT and RNAi lines were grown hydroponically under +P condition for 10 d. Roots were harvested and stained with propidium iodide. The elongation zone (EZ) and maturation zone (MZ) of the primary root were then observed under fluorescence microscope by using RFP channel. (C) Transverse section (20x) of the EZ and MZ of the primary root of the WT and RNAi lines. Bar = 50 μ m and 100 μ m in (A) and (C), respectively. (B and D) Data are presented for (B) length and (D) diameter of the cells in EZ and MZ. Values are means $\pm$ SE (n = 6) and different letters on the histograms indicate significant differences (P < 0.05, one-way ANOVA). In (D), 1 and 2 represent the cortical cells close to the

endodermis. A strip of the transverse section of the primry root shows the cortical cells (1-9) from the endodermis towards epidermis.

Figure 5

RNAi-mediated suppression of OsPDR2 affects growth performance and male reproductive traits lead to low yield. (A and C) WT and RNAi lines were grown in the pot up to maturity (20 weeks) showing the phenotype of (A) plants and (C) flowered spikelets with mature stamens in the upper panel. Magnified

images of the stamens indicated by red triangles are shown in the lower panel. (E) Pollen grains were treated with I2–KI solution for 1 min and scored under the microscope the number of them that were stained (dark blue) and remained unstained. Data are presented for (B) plant height, and per cent (D) dehiscent anthers, and (F) mature pollen. Values (B, D, and F) are means $\pm$ SE ($n = 6$) and different letters on the histograms indicate significant differences ($P < 0.05$, one-way ANOVA). Bar = 10 cm in (A), 0.5 cm in (C, upper panel), 50 μ m in (C, lower panel), and 25 μ m in (E).

Figure 6

RNAi-mediated suppression of OsPDR2 affects the yield. (A and D) WT and RNAi lines were grown as described in the legend to Figure 5 showing the phenotype of the (A) panicles and (D) seeds. Bar = 1 cm and 0.5 cm in (A) and (D), respectively. Data are presented for (B) per cent seed setting rate, (C) yield/plant, (E) seed length, and (F) seed width. Values (B, C, E and F) are means $\pm$ SE ($n = 6$) and different letters on the histograms indicate significant differences ($P < 0.05$, one-way ANOVA).

Figure 7

Effects of RNAi-mediated suppression of OsPDR2 on the concentrations of Pi in different tissues under different Pi regime and total Pin pre-and post-harvest tissues. (A) Seedlings (10-d-old) of the WT and RNAi lines were grown hydroponically under +P and -P conditions for 21 d. Different tissues were harvested for assaying Pi concentration. (B) WT and RNAi lines were grown in the pot and different tissues were collected 10 d before and after harvesting for assaying total P concentration. Values are means $\pm$ SE ($n = 3$) and different letters on the histograms indicate significant differences ($P < 0.05$, one-way ANOVA). ns = not significant.

Figure 8

Effects of RNAi-mediated suppression of OsPDR2 on the total P concentration and ³²Pi distribution in a split-root experiment. (A and B) Roots of the WT and RNAi seedlings (10-d-old) were split into two parts and one half each was supplied with (A) +P/-P, +P/+P, and -P/-P media for 14 d and (B) +³²P/-P, +³²P/+P, and -³²P/-P for 7.5 h. Tissues were harvested for assaying (A) concentration of total P, and (B) per cent distribution of ³²Pi. Values are means ± SE (n = 3 and 4 for (A) and (B), respectively) and different letters on the histograms indicate significant differences (P < 0.05, one-way ANOVA). ns = not significant.

Figure 9

Effects of RNAi-mediated suppression of OsPDR2 on the relative expression of OsLPR3/5 in a split-root experiment. WT and RNAi lines were subjected to a split root experiment as described in the legend to Figure 8A. Roots were harvested for qRT-PCR analysis of the expression of OsLPR3/5. OsActin was used as an internal control. Data presented are means $\pm$ SE (n = 3). Different letters on the histograms indicate significant differences in the relative expression of OsLPR3/5 ($P < 0.05$, one-way ANOVA).

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