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ORIGINAL ARTICLE

A systemic view of phosphate starvation-responsive genes in rice roots to enhance phosphate use efficiency in rice

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Abstract

Phosphate (Pi) is one of the major nutrients for crop growth and yield. Although several studies have revealed a global view of Pi starvation responses in rice, the detailed features were not well-addressed. To identify differentially expressed genes associated with phosphate starvation on a genome scale, we analyzed transcriptome data of roots from 2-week old seedlings that were grown on Pi-sufficient or -deficient media for 7 and 21 days. Using publicly available RNA-sequencing data, we subsequently identified 820 up-regulated genes in roots under Pi starvation. Gene ontology enrichment analysis of these genes indicated that secondary metabolic processes are most significantly enriched under Pi starvation, and Pi transport and defense to biotic stress also play significant roles in response against Pi deficiency. Functional classification analysis using MapMan emphasizes the significance of transcription factors, such as MYB, WRKY, and bHLH, various transporters, and genes in secondary metabolic processes. Use of promoter trap lines or transgenic plants expressing the GUS reporter gene under the control of Pi starvation-inducible gene promoters confirmed the meta-expression patterns of two genes stimulated by Pi starvation, suggesting novel promoters for enhancing Pi use efficiency. In addition to the identification of two novel promoters for Pi starvation response, *cis*-regulatory elements for the regulation of Pi starvation are suggested. Overall, our study provides a global view of Pi starvation response based on transcriptome data and novel tools for improving PUE and Pi uptake in rice, a model crop plant.

Keywords Rice · Phosphate starvation · Transcriptome · Gene ontology enrichment · MapMan analysis

Introduction

Phosphate (Pi) is one of the major nutrients of growth, development, and reproduction in crop plants and functions in energy metabolism and signal transduction cascades and regulates enzymatic activities (Schachtman et al. 1998). Root growth plasticity determines the survival of plants in continuously changing environmental conditions (Koevoets et al. 2016). Most rice ecosystems frequently suffer from Pi deficiency. Pi fertilizer is a limited and unrenewable resource that is supplied by only a few countries. In addition, Pi as a fertilizer is rapidly immobilized owing to fixation and microbial activity. Only approximately 20–30% of the Pi applied is utilized by plants, and the rest is lost (Cordell et al. 2011; Schroder et al. 2011). An increase in the efficiency of Pi use is a very important target for current and future crop breeding.

In rice, a model crop plant, the functions of 18 genes relating to Pi use efficiency (PUE) have been reported. The genes include five Pi transporters, two auxin response factors, three SYG1/Pho81/XPR1 (SPX) domain-containing proteins, two MYB-like transcription factors (TFs), an MYB TF, and a phosphatase (Yamamoto et al. 2012). Interestingly, most of them are significantly stimulated in conditions of Pi starvation. Transcriptome data is an effective way to address limitations in current understanding,

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quickly expand current knowledge, and provide an improved strategy to enhance PUE in crops.

Pi starvation transcriptomes in rice cultivars were revealed through microarray or RNA-seq technologies. Transcriptomes in rice under low Pi stress were analyzed using a BGI 60 K rice array (Li et al. 2009). RNA-seq-based mRNA transcriptome analysis was performed using rice seedling roots and shoots under Pi starvation and excess Pi conditions (Oono et al. 2011). Later, mRNA sequencing of four rice cultivars with different growth and physiological responses to Pi starvation were produced, and the potential for the development of novel strategies for improving tolerance to Pi starvation in rice was suggested (Oono et al. 2013). More intensive transcriptomes under Pi starvation using RNA-seq technologies have been produced. The expression profiles for 126 samples were analyzed and revealed a very complex diversity of transcriptomes including short-, middle-, and long-term Pi starvation responses (Secco et al. 2013). Although several studies have reported global transcriptome responses to Pi starvation in rice, the functional analysis of candidate genes in roots that show primary responses to Pi starvation has been less intensively studied. The functions of 47 genes obtained through genetic and molecular studies have been reported and are well-summarized in the Overview of functionally characterized Genes in Rice Online (OGRO) database (http://qtaro.abr.affrc.go.jp/ogro) (Yamamoto et al. 2012). The functions of the remaining genes could be studied with gene indexed mutants produced by the use of T-DNA or transposons, such as Tos17 or Ac/Ds, or ds/espm insertion and then used for further functional genomic studies. At present, 65% of Rice Genome Annotation Project (RGAP) non-transposable element (TE) genes have gene indexed mutants (Chandran et al. 2016). Categorization of candidate genes from previous studies can be improved through an advanced integrating omics analysis.

In this study, we focus on 820 genes with a significant up-regulation in rice roots under long-term Pi starvation and the recovery process obtained through the reanalysis of previous global transcriptome data produced by Secco et al. (2013). MapMan analysis was used to categorize our candidate genes in terms of metabolic and regulatory pathways and transporting activities, which may assist researchers in prioritizing the candidate genes for further studies. In addition, we validated the expression patterns of two candidate genes showing up-regulation under Pi starvation through a GUS reporter system/promoter-GUS transgenic line and RT-PCR analyses, providing a new tool to optimize the function of candidate genes for enhanced PUE. Analysis of functionally characterized candidate genes identified in this study support the significance of selected candidate genes for further study to enhance PUE. Detailed data analyses and discussion are presented.

Materials and methods

Plant growth

The experiment was conducted using rice cultivar Dongjin seedlings that were first germinated for 2 weeks in Yoshida solution and then grown for 7 or 21 days in Pi-sufficient (0.320 mM Pi) or -deficient Yoshida solution (Yoshida et al. 1976). The pH of the culture solution was adjusted to 5.5 using 1 M NaOH. The Yoshida solution was replaced with new solution every 3 days. In all the hydroponic experiments, seedlings were directly grown in each culture solution (8 L) with an 8-h light (28°C)/16-h dark (22 °C) photoperiod. To verify that the samples responded to the Pi starvation treatment, we assessed the expression patterns of two Pi starvation marker genes, *SULFOQUINOVOSYLDIACYLGLYCEROL 2 (*Os*SQD2, LOC_Os01g04920*) and *PHOSPHATE TRANSPORTER 6 (*Os*PT6, LOC_Os08g45000*), through RT-PCR and qRT-PCR analyses.

Global identification of rice genes stimulated under phosphate starvation using RNA-sequencing data

We used RNA-seq data obtained under Pi starvation conditions (Secco et al. 2013). Fragments per kilobase of transcript per million mapped reads (FPKM) values from whole samples used in this study were downloaded from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sra) under accession number SRA097415. We then generated fold changes of roots under Pi starvation for 1 h, 6 h, 24 h, 3 days, 7 days, 21 days, + 1 h, 21 days + 6 h, and 21 days + 24 h with roots under normal conditions. In addition, fold changes were generated to compare roots under recovery conditions (+ 1, + 6, + 24 h) after Pi starvation for 21 days over roots under Pi starvation for 21 days + 1 h, 21 days + 6 h, and 21 days + 24 h. With this fold change data, we carried out k-means clustering analysis and produced 10 clusters with differential expression patterns. As a result, we identified 820 candidate genes showing more than a significant $\log_2$ 2.5-fold change up-regulation at 21 days under Pi starvation from three clusters (Fig. 2).

Analysis of phosphate starvation-inducible genes with known function

To determine the functional features of our candidate genes associated with Pi starvation, we searched the OGRO database, which contains summaries of rice genes with known functions obtained by genetic and molecular studies

Table 1 Summary of functionally characterized phosphate starvation-inducible candidate genes through a literature search

MSU-Locus	Gene symbol	Category[a]	Objective character	Isolation[b]	Reference (DOI)[c]
LOC_Os03g05640	OsPT2	Phosphate	Grain weight through selenite uptake	KD OX	https://doi.org/10.1111/nph.12596
LOC_Os08g45000	OsPT6	Phosphate	Phosphate uptake and translocation	KD	https://doi.org/10.1111/j.1365-313X.2008.03726.x
LOC_Os10g30790	OsPT8	Phosphate	Phosphate uptake	KD OX	https://doi.org/10.1104/pp.111.175240
LOC_Os06g21920	OsPT9	Phosphate	Pi uptake, Pi translocation	KD OX	https://doi.org/10.1111/pce.12224
LOC_Os06g21950	OsPT10	Phosphate	Pi uptake, Pi translocation	KD OX	https://doi.org/10.1111/pce.12224
LOC_Os06g40120	OsSPX1	Phosphate	Cold and oxidative stresses tolerance	KD	https://doi.org/10.1371/journal.pone.0081849
LOC_Os10g25310	OsSPX3	Phosphate	Pi starvation response	OX	https://doi.org/10.1111/j.1744-7909.2009.00834.x
LOC_Os06g40120	SPK1(SYG1)	Phosphate	Pi-dependent inhibitor of phosphate starvation response regulator 2 (PHR2)	KD OX	https://doi.org/10.1073/pnas.1404680111
LOC_Os02g10780	SPK2(SYG2)	Phosphate	Pi-dependent inhibitor of phosphate starvation response regulator 2 (PHR)	KD OX	https://doi.org/10.1073/pnas.1404680111
LOC_Os01g56880	OsPAP10a	Phosphate	Phosphate uptake and translocation	OX	https://doi.org/10.1111/j.1744-7909.2012.01143.x
LOC_Os01g54270	d10	Root	Strigolactone biosynthesis. Crown root length	MT	https://doi.org/10.1007/s00344-011-9228-6
LOC_Os04g46990	cZOGT2	Root	Dwarfism. Leaf senescence. Crown root	OX	https://doi.org/10.1104/pp.112.196733
LOC_Os05g13900	RePRP1.1	Root	Root growth. Root cell elongation. ABA sensitivity	KD OX	https://doi.org/10.1104/pp.113.217547
LOC_Os03g17350	rcn1	Root	Root length and flexible under hypoxic conditions	MT	https://doi.org/10.1111/tpj.12614
LOC_Os05g13940	RePRP1.2	Root	Root growth. Root cell elongation. ABA sensitivity	KD OX	https://doi.org/10.1104/pp.113.217547
LOC_Os06g21920	OsPT9	Root	Pi uptake, Pi translocation	KD OX	https://doi.org/10.1111/pce.12224
LOC_Os06g21950	OsPT10	Root	Pi uptake, Pi translocation	KD OX	https://doi.org/10.1111/pce.12224
LOC_Os05g41090	OsCCaMK	Root	CH_4 oxidation, N_2 fixation activation	MT	https://doi.org/10.1128/AEM.03646-13
LOC_Os03g05640	OsPT2	Root	Selenite uptake	KD OX	https://doi.org/10.1111/nph.12596
LOC_Os01g54270	d10	Dwarf	Tillering dwarf. Tiller bud outgrowth	MT	https://doi.org/10.1111/j.1365-313X.2007.03210.x
LOC_Os02g17780	oscps1	Dwarf	Dwarfism. Gibberellin biosynthesis	MT	https://doi.org/10.1104/pp.103.033696
LOC_Os04g46990	cZOGT2	Dwarf	Dwarfism. Leaf senescence. Crown root	OX	https://doi.org/10.1104/pp.112.196733
LOC_Os04g52230	osks1	Dwarf	Dwarfism. Gibberellin biosynthesis	MT	https://doi.org/10.1104/pp.103.033696
LOC_Os05g15630	OsBLE3	Dwarf	Internode elongation. Cell elongation. Leaf angle. Brassinosteroid sensitivity	KD	https://doi.org/10.1016/j.phytochem.2006.05.026
LOC_Os02g08100	Os4CL3	Dwarf	Lignin content. Dwarfism. Culm length. Anther development	KD	https://doi.org/10.1104/pp.111.178301

Table 1 (continued)

MSU-Locus	Gene symbol	Category[a]	Objective character	Isolation[b]	Reference (DOI)[c]
LOC_Os07g08840	OsTRXh1	Dwarf	Dwarfism with fewer tillers. ABA sensitivity during seed germination and seedling stage. Hydrogen peroxide production under salt stress	KD OX	https://doi.org/10.1104/pp.111.182808
LOC_Os10g26340	pla1	Dwarf	Plastochron and phyllotaxy. Dwarfism	MT	https://doi.org/10.1073/pnas.2636936100
LOC_Os04g54900	ili1	Dwarf	Leaf angle. Cell length. Brassinosteroid sensitivity	MT	https://doi.org/10.1105/tpc.109.070441
LOC_Os04g23550	RERJ1	Dwarf	Dwarfism. JA sensitivity during seedling stage	KD OX	https://doi.org/10.1016/j.bbrc.2004.10.126
LOC_Os09g36680	OsRNS4	Dwarf	Salinity tolerance. Seedling growth	OX	https://doi.org/10.1016/j.plantsci.2013.10.003
LOC_Os07g08840	OsTRXh1	Dwarf	Dwarfism with fewer tillers. ABA sensitivity during seed germination and seedling stage. Hydrogen peroxide production under salt stress	KD OX	https://doi.org/10.1104/pp.111.182808
LOC_Os04g23550	RERJ1	Dwarf	Dwarfism. JA sensitivity during seedling stage	KD OX	https://doi.org/10.1016/j.bbrc.2004.10.126
LOC_Os01g54270	d10	Culm leaf	Tillering dwarf. Tiller bud outgrowth	MT	https://doi.org/10.1111/j.1365-313X.2007.03210.x
LOC_Os03g29480	NSP1	Culm leaf	Striga resistance. Regulation of strigolactone biosynthesis	KD	https://doi.org/10.1105/tpc.111.089771
LOC_Os04g39780	DWA1	Culm leaf	Leaf wax crystallization. Formation of cuticular layer. Drought tolerance	MT	https://doi.org/10.1073/pnas.1316412110
LOC_Os03g15680	NSP2	Culm leaf	Striga resistance. Regulation of strigolactone biosynthesis	KD	https://doi.org/10.1105/tpc.111.089771
LOC_Os05g15630	OsBLE3	Culm leaf	Internode elongation. Cell elongation. Leaf angle. Brassinosteroid sensitivity	KD	https://doi.org/10.1016/j.phytochem.2006.05.026
LOC_Os03g17350	rcn1	Culm leaf	Tillering bud outgrowth	MT	https://doi.org/10.1111/j.1469-8137.2008.02724.x
LOC_Os10g26340	pla1	Culm leaf	Plastochron and phyllotaxy. Dwarfism	MT	https://doi.org/10.1073/pnas.2636936100
LOC_Os03g30250	cwa1/bc1	Culm leaf	Brittle culm. Secondary cell wall synthesis	MT	https://doi.org/10.1007/s00425-010-1171-4
LOC_Os06g21920	OsPT9	Culm leaf	Pi uptake, Pi translocation	KD OX	https://doi.org/10.1111/pce.12224
LOC_Os06g21950	OsPT10	Culm leaf	Pi uptake, Pi translocation	KD OX	https://doi.org/10.1111/pce.12224
LOC_Os01g54270	d10/CCD	Secondary metabolite	Tillering dwarf. Tiller bud outgrowth	MT	https://doi.org/10.1111/j.1365-313X.2007.03210.x
LOC_Os03g29480	NSP1	Secondary metabolite	Striga resistance. Regulation of strigolactone biosynthesis	KD	https://doi.org/10.1105/tpc.111.089771
LOC_Os03g15680	NSP2	Secondary metabolite	Striga resistance. Regulation of strigolactone biosynthesis	KD	https://doi.org/10.1105/tpc.111.089771
LOC_Os04g09920	CYP99A3	Secondary metabolite	Production phytoalexins and allelochemicals	KD	https://doi.org/10.1111/j.1365-313X.2010.04408.x
LOC_Os04g10160	CYP99A2	Secondary metabolite	Production phytoalexins and allelochemicals	KD	https://doi.org/10.1111/j.1365-313X.2010.04408.x
LOC_Os06g40120	OsSPX1	Cold tolerance	Cold and oxidative stresses tolerance	KD	https://doi.org/10.1371/journal.pone.0081849
LOC_Os03g01320	qLTG3?1	Cold tolerance	Cold tolerance at germination stage	NV	https://doi.org/10.1073/pnas.0805303105

Table 1 (continued)

MSU-Locus	Gene symbol	Category[a]	Objective character	Isolation[b]	Reference (DOI)[c]
LOC_Os09g35030	OsDREB1A	Cold tolerance	Cold, drought and salinity tolerance	OX	https://doi.org/10.1093/pcp/pci230
LOC_Os09g25060	OsWRKY76	Cold tolerance	Resistance to Magnaporthe oryzae. Cold tolerance	OX	https://doi.org/10.1093/jxb/ert298
LOC_Os10g25310	OsSPX3	Cold tolerance	Cold and oxidative stresses tolerance	OX	https://doi.org/10.1371/journal.pone.0081849
LOC_Os09g36680	OsRNS4	Salinity tolerance	Salinity tolerance. Seedling growth	OX	https://doi.org/10.1016/j.plantsci.2013.10.003
LOC_Os03g17350	rcn1	Salinity tolerance	Salinity tolerance in part by reducing the Na/K ratio	MT	https://doi.org/10.1016/j.plantsci.2014.04.011
LOC_Os07g08840	OsTRXh1	Salinity tolerance	Dwarfism with fewer tillers. ABA sensitivity during seed germination and seedling stage. Hydrogen peroxide production under salt stress	KD OX	https://doi.org/10.1104/pp.111.182808
LOC_Os09g35030	OsDREB1A	Salinity tolerance	Cold, drought and salinity tolerance	OX	https://doi.org/10.1093/pcp/pci230
LOC_Os11g37970	OsPR4	Drought tolerance	Drought tolerance	OX	https://doi.org/10.1016/j.jplph.2011.07.013
LOC_Os04g39780	DWA1	Drought tolerance	Leaf wax crystallization. Formation of cuticular layer. Drought tolerance	MT	https://doi.org/10.1073/pnas.1316412110
LOC_Os09g35030	OsDREB1A	Drought tolerance	Cold, drought and salinity tolerance	OX	https://doi.org/10.1093/pcp/pci230
LOC_Os09g34250	OsSGT1	Blast resistance	Probenazole dependent blast resistance	KD	https://doi.org/10.1111/j.1365-313X.2008.03697.x
LOC_Os03g12500	OsAOS2	Blast resistance	Resistance to Magnaporthe grisea	OX	https://doi.org/10.1094/MPMI-19-1127
LOC_Os09g25060	OsWRKY76	Blast resistance	Resistance to Magnaporthe oryzae. Cold tolerance	OX	https://doi.org/10.1093/jxb/ert298
LOC_Os09g25070	OsWRKY62	Bacterial blight resistance	Resistance to Xanthomonas oryzae pv. oryzae	OX	https://doi.org/10.1093/mp/ssn024
LOC_Os09g25060	OsWRKY76	Bacterial blight resistance	Resistance to Xanthomonas oryzae pv. oryzae	OX	https://doi.org/10.1007/s12284-010-9039-6
LOC_Os09g26780	OsJAZ8	Bacterial blight resistance	JA induced resistance to Xanthomonas oryzae pv. oryzae	OX	https://doi.org/10.1093/pcp/pcs145
LOC_Os09g37540	OsLDC-like 1	Others	Resistance to reactive oxygen species	MT	https://doi.org/10.1007/s10059-012-0067-5
LOC_Os05g41090	OsDMI3	Others	Oxidative stress tolerance. ABA sensitivity	MT	https://doi.org/10.1093/mp/sss068
LOC_Os02g08100	Os4CL3	Others	Lignin content. Dwarfism. Culm length. Anther development	KD	https://doi.org/10.1104/pp.111.178301
LOC_Os03g30250	cwa1/bc1	Others	Brittle culm. Secondary cell wall synthesis	MT	https://doi.org/10.1007/s00425-010-1171-4
LOC_Os03g01320	qLTG3?1	Others	Cold tolerance at germination stage	NV	https://doi.org/10.1073/pnas.0805303105
LOC_Os07g08840	OsTRXh1	Others	Dwarfism with fewer tillers. ABA sensitivity during seed germination and seedling stage. Hydrogen peroxide production under salt stress	KD OX	https://doi.org/10.1104/pp.111.182808
LOC_Os06g06560	OsSSI	Others	Seed amylopectin content	MT	https://doi.org/10.1104/pp.105.071845
LOC_Os09g23300	OsVIT2	Others	Fe and Zn translocation. Fe and Zn content in seed	MT	https://doi.org/10.1111/j.1365-313X.2012.05088.x

Table 1 (continued)

MSU-Locus	Gene symbol	Category[a]	Objective character	Isolation[b]	Reference (DOI)[c]
LOC_Os03g52860	OsLOX2 (L-2)	Others	Promoting early seed germination, seed longevity	KD OX	https://doi.org/10.1007/s11248-014-9803-2
LOC_Os02g08100	Os4CL3	Others	Lignin content. Dwarfism. Culm length. Anther development	KD	https://doi.org/10.1104/pp.111.178301
LOC_Os08g04540	TDC	Others	Leaf senescence. Serotonin biosynthesis	KD OX	https://doi.org/10.1104/pp.109.138552
LOC_Os06g06560	SS1	Others	Starch biosynthesis in endosperm, chain elongation	Others	https://doi.org/10.1186/1471-2229-14-80
LOC_Os05g41760	MSF1	Others	Spikelet determinacy. Floral organ development	MT	https://doi.org/10.1104/pp.113.216044
LOC_Os04g01140	CYP93G1	Others	Biosynthesis of different tricin O-conjugated derivatives	KD	https://doi.org/10.1104/pp.114.239723
LOC_Os01g58290	Hwi2	Others	Activate Hwi1-perceived downstream defense responses	Others	https://doi.org/10.1038/ncomms4357
LOC_Os05g50380	LSU3	Others	Culm starch content	MT	https://doi.org/10.1071/FP12186

[a]Agronomic trait associated with functionally characterized genes out of candidate genes in this study

[b]Methods used for the functional characterization: *MT* mutants by T-DNA/Tos17/Ds insertion, *KD* knockdown mutants by RNAi or anti-sense approaches, *OX* overexpressed mutants by transgenic approaches, *NV* natural variation, and others, those by other methods besides four major methods

[c]Digital object identifier (DOI)

(Yamamoto et al. 2012). Of our 820 candidate genes, the functions of 47 have been reported and are summarized in Table 1.

MapMan analysis

Rice MapMan classification covers 36 BINs, and these BINs can be extended in a hierarchical manner into subBINs (Usadel et al. 2005; Urbanczyk-Wochniak et al. 2006). Using diverse MapMan tools, significant gene lists selected from high-throughput data analysis can be integrated into diverse overviews. To do this, we generated a dataset carrying locus IDs from the Rice Genome Annotation Project and the average $\log_2$ fold-change data of Pi starvation/control. For the functional classification of 820 genes up-regulated in Pi starvation conditions, we used Metabolism_overview (Fig. 3a), Transporter_overview (Fig. 3b), Transcription_overview (Fig. 3c), and Regulation_overview (Fig. 3d). The detailed information is presented in Table S1.

Gene ontology enrichment analysis

The gene ontology (GO) enrichment tool installed in the Rice Oligonucleotide Array Database (ROAD, http://www.ricearray.org/analysis/go_enrichment.shtml) (Cao et al. 2012) was used to determine the biological roles of high-throughput candidate genes (Jung et al. 2008). We applied GO enrichment tools for 820 genes up-regulated in rice root after 21 days of Pi starvation. Based on a p value < 0.05 and fold enrichment value > 2, we selected 36 significant GO terms (Fig. 4 and Table S2).

Construction of promoter-GUS vectors and GUS assay

The promoter region (− 1 to − 2411 bp from the initiation ATG codon) of LOC_Os02g44654/Cytochrome p450 was amplified with the 5′-GGTACCTAGTAGCCGTATCAC ATTAC-3′ (Kpn I)/5′-TCTAGAGG GTCTGGTCTACCTCA AGGCG-3′ (Xba I) primer set through genomic DNA PCR analysis. The resulting promoter DNA fragment was placed upstream of the *beta-glucuronidase* (*GUS*) reporter gene located in binary vector pGA3519 (Kim et al. 2009). Transgenic plants harboring the above construct were obtained in the *japonica* cultivar Dongjin background through the *Agrobacterium tumefaciens* mediating transformation method (Lee et al. 1999). To analyze GUS expression patterns using the GUS reporter system, we grew the promoter-GUS transgenic line for 21 days on Pi-sufficient or -deficient media after growing germinated plants for 2 weeks using Yoshida solution (Yoshida et al. 1976). Whole seedlings were soaked for 1 h in GUS staining solution. GUS staining assay was performed as previously described (Jefferson et al. 1987) on 21-day-old seedlings of one transgenic line for LOC_Os02g44654 encoding cytochrome p450 (Fig. 5a–c) carrying promoter-GUS vector and promoter trap lines for LOC_Os01g52560 (1D-014-17) (Fig. 5d–f). Photographs of GUS assayed whole plants were produced using an EOS

560 digital camera (Cannon, Tokyo, Japan), and those of roots were obtained using an Olympus microscope BX61 (Olympus, Tokyo, Japan).

Cis-acting element analysis

To identify conserved *cis*-acting elements (CREs) in the promoters of LOC_Os01g52560, LOC_Os02g44654, and LOC_Os02g44654 genes showing up-regulation under Pi starvation, we extracted 2-kb sequences upstream of ATG of these genes from PLANTPAN (http://plantpan2.itps.ncku.edu.tw/) (Chow et al. 2016), and CREs in the promoters were analyzed using OSIRIS (Morris et al. 2008) and PLACE (Higo et al. 1998). CREs in the promoters were aligned with the Motif Alignment and Search Tool (MAST) (Bailey et al. 2015). The results are displayed in Fig. 6, and known target motifs with a p value ≤ 0.05 were selected for further analysis.

Results

Morphological features of rice seedlings grown under low phosphate conditions

We identified the morphological features of rice cultivar Dongjin seedlings grown for 7 days (Fig. 1a) or 21 days (Fig. 1b) on Pi-sufficient or -deficient media. It was shown that Pi plays particularly important roles in early root development of rice (Fig. 1a). The numbers of leaves and tillers are reduced due to low Pi supplement. The length of shoots is also shorter than that of rice grown under normal conditions. Young leaves appear to be healthy, but older leaves turn brown (Fig. 1a). For evaluating the quality of these samples, we assessed the expression patterns of two Pi starvation-inducible marker genes (Fig. 1c, d). As a result, we found that two marker genes *OsSQD2 (LOC_Os01g04920)* (Yu et al. 2002) and *OsPT6* (*LOC_Os08g45000*) (Ai et al. 2009) are significantly up-regulated under Pi starvation of 7 and 21 days. This result indicates that root samples grown under Pi starvation for 7 and 21 days are relevant for analyzing the physiological response.

Identification of phosphate starvation-responsive genes using RNA-seq data

Transcriptome analysis was conducted with publicly available RNA-sequencing data using the roots of 2-week old seedlings exposed to Pi starvation for 7 and 21 days in Pi-sufficient or -deficient conditions or when Pi is re-supplemented (additional 0.320 mM Pi at 1, 6, or 24 h after 21 days). The RNA-seq data were used under the criteria of more than 5 FPKM values in Pi starvation, less than 0.5 p value, and more than 2.5 ($\log_2$ scale) fold up-regulation in response compared to the control (rice roots under sufficient Pi conditions). Subsequently, we identified 820 up-regulated genes in rice root under conditions of Pi starvation (Fig. 2a).

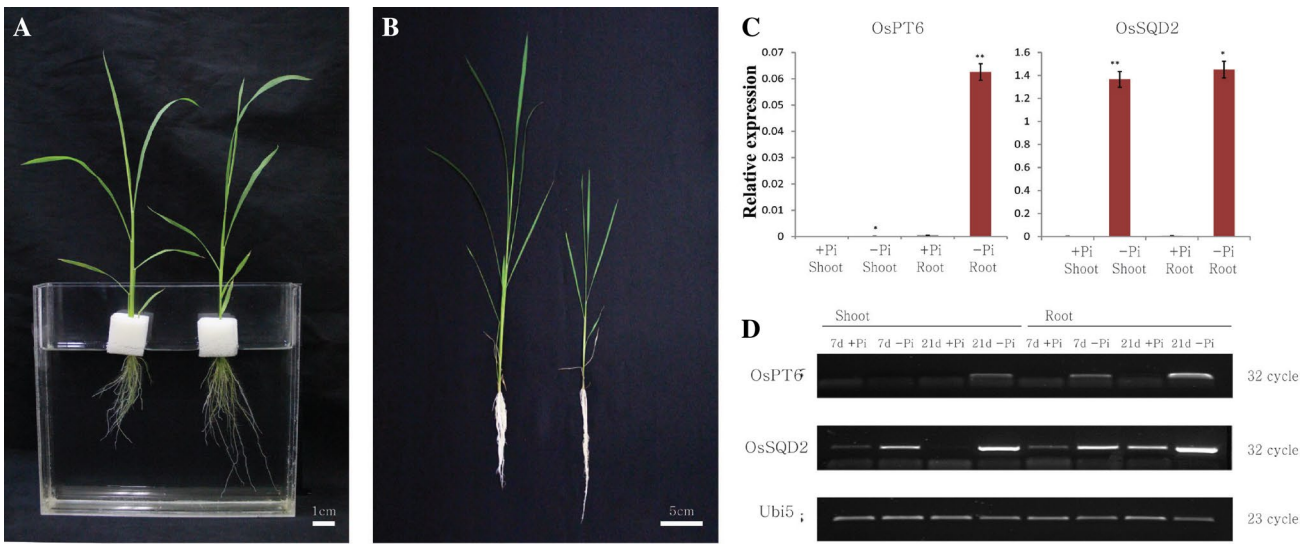

Fig. 1 Morphological appearance under phosphate starvation and schematic diagram of samples used in transcriptome analysis. Morphological appearance of Rice cultivar Dongjin seedling grown for 7 days (**a**) or 21 days (**b**) on Pi-sufficient or -deficient media after germination for 2 weeks using Yoshida solution. Preferential expression patterns based on qRT-PCR (**c**) and RT-PCR analysis (**d**) for two marker genes: Os*SQD2* (*LOC_Os01g04920*) and Os*PT6 (LOC_Os08g45000*). *OsUBI5* (*LOC_Os01g22490*) was used as internal control for both analyses. Bar = 1 cm. **P* value < 0.05; ***P* value < 0.01; ****P* value < 0.001

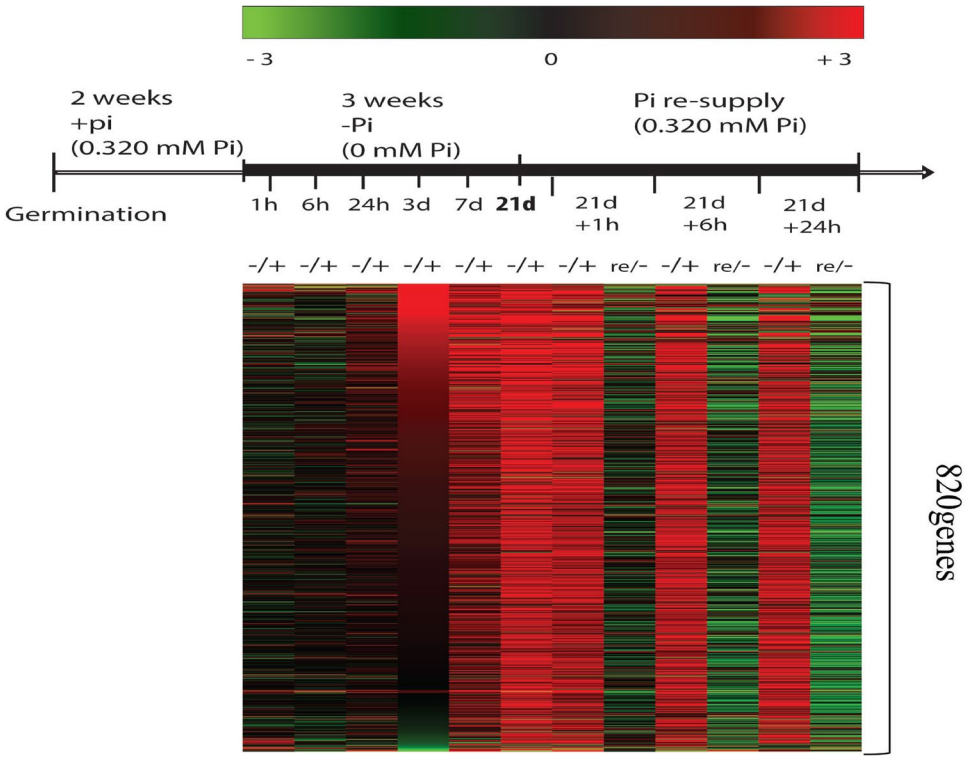

Fig. 2 Heatmap of differentially expressed genes under phosphate starvation in rice root using RNA-seq

Functional evaluation of phosphate starvation-inducible genes using literature searches

To evaluate the functional significance of our candidate genes, we searched the literature to determine whether their functions have been reported previously. Out of 820 up-regulated genes under Pi deficiency in rice roots, we identified 47 with known functions through analysis using the OGRO database, which contains functionally characterized rice genes obtained through genetic and molecular studies (Yamamoto et al. 2012). Of the 47 genes with known functions, 10 are involved in the efficiency of Pi use and include Os*PT2* (Zhang et al. 2014), Os*PT6* (Ai et al. 2009), Os*PT8* (Jia et al. 2011), Os*PT9*, Os*PT10* (Lapis-Gaza et al. 2014), Os*SPX1*, Os*SPX3* (Wang et al. 2009), Os*SPX2* (Wang et al. 2014), and *Purple acid phosphatase 10 a* (*OsPap10a*) (Wang and Liu 2012). The functions of nine genes are reported to be associated with root growth and development, and they include rice *OsDwarf10 (OsD10)* (Arite et al. 2007), rice *cis-Zeatin-O-glucosyltransferase (OscZOGT2)* (Kudo et al. 2012), rice *REPETITIVE PROLINE-RICH PROTEIN 1.1* (Os*RePRP1.1*), *OsRePRP1.2* (Tseng et al. 2013), rice *ATP-binding cassette transporter5* (Os*ABCG5*) (Shiono et al. 2014), rice Ca^{2+}*/calmodulin-dependent protein kinase* (*OsCCaMK*) (Bao et al. 2014), *OsPT2* (Zhang et al. 2014), *OsPT9*, and *OsPT10* (Lapis-Gaza et al. 2014). *OsD10* is a rice ortholog of MAX4/RMS1/DAD1 that encodes carotenoid cleavage dioxygenase 8 (CCD8) and is believed to be involved in the synthesis of an unidentified inhibitor of shoot branching by strigolactones (SL) (Arite et al. 2007). Recently, *osd10* mutants were found to have a loss of sensitivity in root responses under Pi deficiency (Sun et al. 2014; Xi et al. 2015). Mutations in rice regulators of SL, such as *NODULATION SIGNALING PATHWAY 1* (*OsNSP1*) and *OsNSP2*, induced an accumulation of SL during Pi starvation (Yokota et al. 2010; Liu et al. 2011). Moreover, SLs are mediators of root growth under low Pi conditions (Koltai and Kapulnik 2011) and play an important role in the Pi uptake of plants. The functions of 18 of the 47 genes, including *OsD10, OsCPS1*, and *OsKS1*, are related to length growth/elongation of the plant (Table 1). These results indicate that Pi starvation-inducible genes might also have roles in regulating morphological or physiological traits. Additionally, five genes, including *OsJAZ8* and *OsWRKY76*, are related to pathogen resistance (Table 1). Taken together, these findings appear to have an important role in root development, length growth, and biotic stress tolerance in association with the efficiency of Pi use, and there is the possibility of crosstalk between Pi uptake and these agronomic traits.

MapMan analysis of phosphate deficiency-related genes in rice roots

For the functional classification of 820 genes up-regulated under Pi starvation, we used the MapMan toolkit. Among them, 141 elements were assigned to the 'miscellaneous

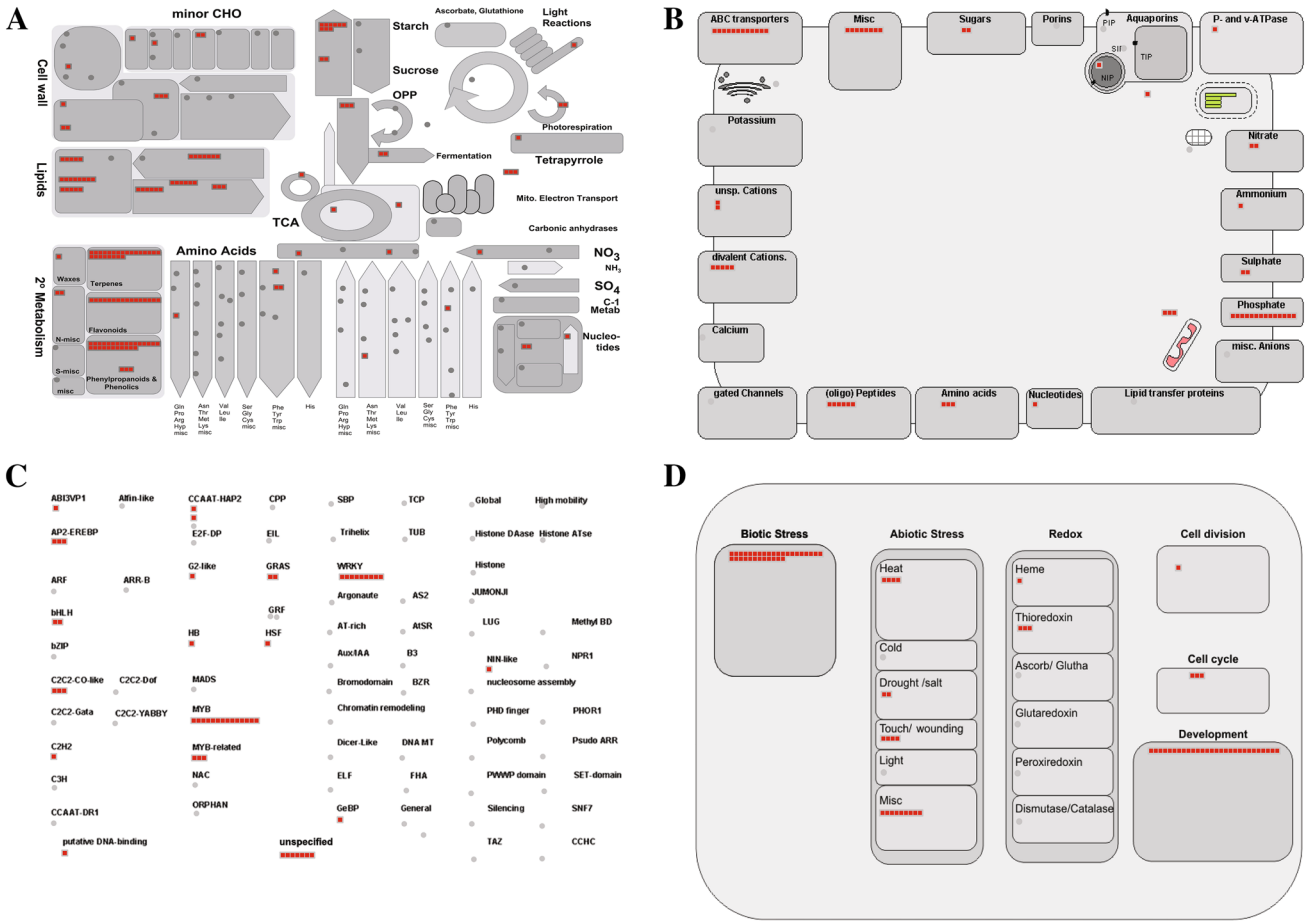

Fig. 3 MapMan analysis of genes associated with phosphate starvation in rice root. Secondary metabolism overview (**a**), Transporter (**b**), Transcription (**c**), and Regulation (**d**) were mapped with selected phosphate starvation-inducible genes in rice root. Red boxes indicate groups of genes up-regulated by phosphate starvation. Details are presented in Table S1

function' category, 81 to 'protein', 72 to 'secondary metabolism', 67 to 'transporter', 64 to 'stress', 58 to 'RNA-regulation', 41 to 'lipid metabolism', 40 to 'signaling', 32 to 'hormone', and the rest to other functional groups (Table S1). We selected four MapMan overviews for further analysis: metabolism_overview (Fig. 3a), transporter_overview (Fig. 3b), transcription_overview (Fig. 3c), and regulation_overview (Fig. 3d). With regard to metabolism overview, we found that the secondary metabolism category (72 elements) has the closest links with up-regulation under Pi starvation in rice root. From the transport overview, we found that genes involved in phosphate transporters and ATP-binding cassette (ABC) transporters are more abundant than others. From the cellular response overview, we found that the Pi starvation response is likely to be associated with the biotic stress response. In the 'RNA-regulation' category, we found that MYB, WRKY, and bHLH TFs are more important than others in response to Pi starvation. The remaining 273 genes did not have assigned MapMan terms. As in the GO enrichment analysis above, the functional category 'transport', including 'ion transport', 'cation transport', and 'metal ion transport', was more frequent under Pi starvation conditions.

Gene ontology enrichment analysis reveals the significance of 36 biological processes

To determine the biological functions of Pi deficiency-inducible genes in rice root, we performed GO term enrichment analysis of 820 genes up-regulated by Pi deficiency in root. GO term enrichment analysis suggests that there were significant GO terms in the biological process categories associated with Pi starvation (Table S2). Through the use of GO enrichment analysis data with a criteria of ≥ twofold enrichment value and ≤0.05 hyper-geometric *p* value, we showed that secondary metabolic processes such as 'Diterpene phytoalexin precursor biosynthetic process pathway' (26.4-fold enrichment value), 'L-phenylalanine catabolic process' (24.0), and 'Phenylpropanoid metabolic process' (22.6); biological processes relating to defense response such as 'defense response to fungus' (17.59), 'response to

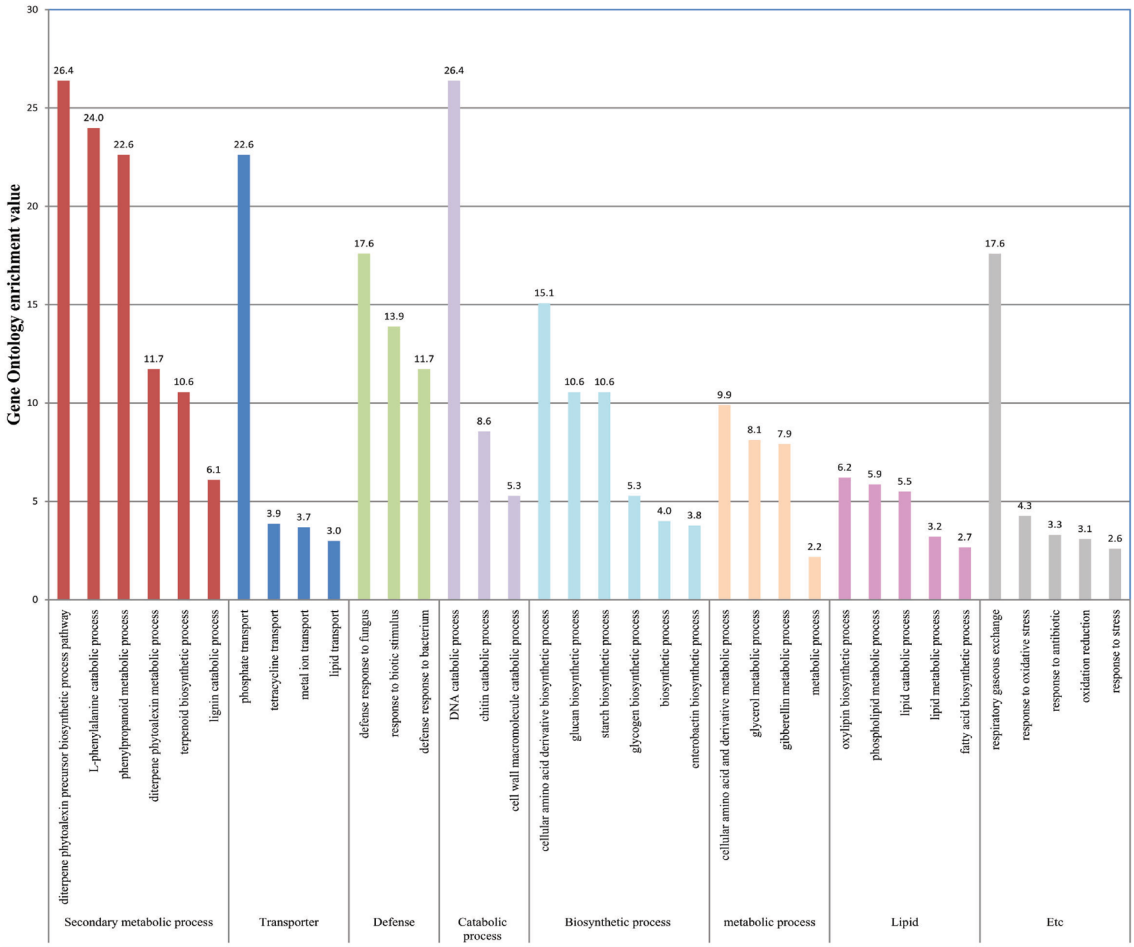

Fig. 4 Gene ontology analysis of genes associated with phosphate starvation in rice root. In all, 8 GO major categories were over-represented by > twofold enrichment value, with p values < 0.05. Details of GO assignment are presented in Table S2

biotic stimulus' (13.3), and 'defense response to bacterium' (11.7); and biological processes related to various transports such as 'phosphate transport' (22.6), 'tetracycline transport' (3.9), 'metal ion transport' (3.7), and 'lipid transport' (3.0) are highly enriched in Pi starvation-induced genes (Fig. 4).

Validation of phosphate deficiency-inducible genes in rice roots using the GUS reporter system

The GUS reporter gene system has been used to identify in vivo activity of promoters for the regulation of gene expression (Jefferson 1989). From RNA-seq data of samples under Pi starvation for 21 days compared to Pi-sufficient conditions (Control), we identified 40 genes showing the highest significant up-regulation under Pi starvation. Then, we screened a GUS reporter system-based promoter trap lines of 32 genes and carried out co-segregation analysis of the T-DNA insertion and GUS expression in T2 seedlings of these lines. As a result, the promoter trap lines showed the co-segregation of Pi starvation-induced GUS expression patterns and T-DNA insertion: Line 1D-014-17, a promoter trap line for *LOC_Os01g52560*, displayed GUS expression at the elongated region in seedling roots under Pi starvation for 3 weeks (Fig. 5 and Figure S1). In addition, we produced a promoter-GUS transgenic line for *LOC_Os02g44654* encoding CytochromeP450 and found that this line showed a similar expression pattern with the line 1D-014-17 under Pi starvation. Interestingly, application of the promoter trap system or promoter-GUS system combined with qualified genome-wide transcriptome data is a very effective way to quickly identify the activity of a novel promoter.

Cis-acting element analysis of three phosphate starvation-inducible promoters confirmed by the GUS reporter system

To find CREs for triggering Pi deficiency response in rice roots, we used promoter regions of *LOC_Os02g44654* and *LOC_Os01g52560* that showed Pi starvation-inducible expression patterns confirmed using GUS reporter systems.

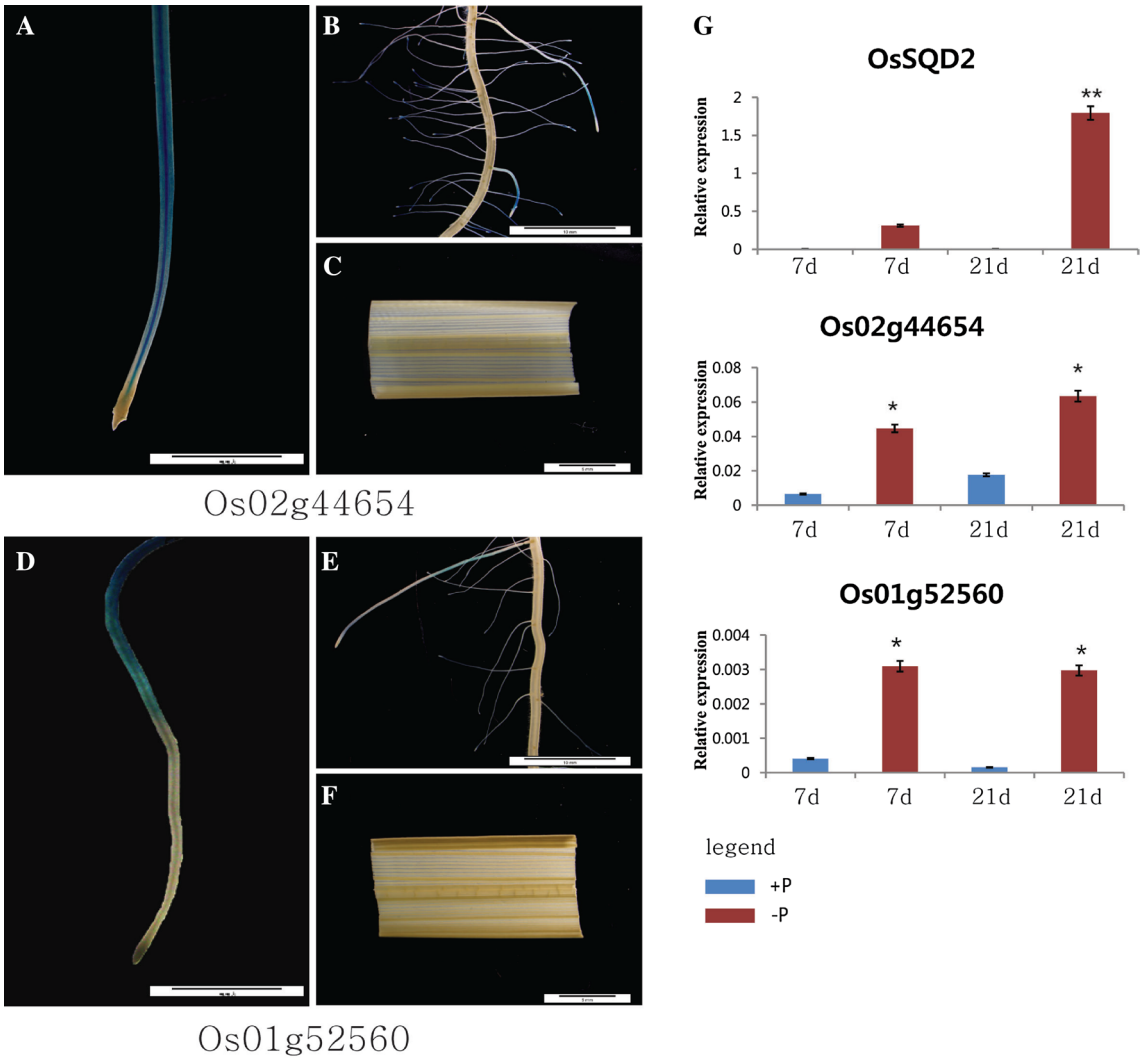

Fig. 5 Validation of expression patterns of two phosphate starvation-inducible genes using a promoter trap system and a promoter-GUS transgenic line. Expression patterns of promoter-GUS transgenic line for *LOC_Os02g44654* (**a**–**c**) and a promoter trap line (1D-014-17, D-F) for *LOC_Os01g52560* were analyzed under Pi starvation for 3 weeks a. Roots (**a, b, d, e**) and leaf (**c, f**) were then incubated for 3 h in GUS staining solution. Additional data of the validation using GUS reporter system for expression patterns of two genes under Pi-sufficient or deficient condition are presented in Figure S1. Validation of expression patterns using qRT-PCR analysis under phosphate starvation for *LOC_Os02g44654* and *LOC_Os01g52560* was prepared, and *OsSQD2* (*LOC_Os01g04920*) was used as a positive control for inducible expression under phosphate starvation (**g**). *OsUBI5* (*Os01g22490*) was used as an internal control for qRT-PCR analysis. **p* value < 0.05; ***p* value < 0.01; ****p* value < 0.001

The promoters were analyzed using the PLANTPAN 2.0 database (http://plantpan2.itps.ncku.edu.tw) (Chow et al. 2016), PLACE (Higo et al. 1998), and MEME tools (Bailey et al. 2015). As a result, we found 65 common CREs between them; from these, we obtained five consensus CREs in the promoters of the genes as candidate CREs responsible for Pi starvation-inducible expression: P1BS/GNATATNC, WBBOXPCWRKY1/TTTGACY, MYB2CONSENSUSAT/YAACKG, Helix-loop-helix DNA-binding domain/NSCACGTGSN, and IRO2OS/CACGTGG. Of these CREs, P1BS was the most frequently identified in all promoter regions (Fig. 6). P1BS is the binding site of an MYB domain transcription factor, PHR1. P1BS is present in the promoters of many crucial Pi-responsive genes (Rubio et al. 2001; Schunmann et al. 2004; Bustos et al. 2010; Nilsson et al. 2010; Oropeza-Aburto et al. 2012). WBBOXPCWRKY1 is one of the WRKY box (W-box) elements commonly found in the promoters of genes relevant for Pi-retranslocation and scavenging including PT1, PT3, PT4, PT5, PT7, INDUCED BY PHOSPHATE STARVATION 1 (IPS1), ACID PHOSPHATASE GENES2 (PS2), Purple Acid Phosphatase (PAP11), and PHOSPHATE STARVATION RESPONSE 1 (PHR1) (Devaiah et al. 2007). MYB2CONSENSUSAT is a MYB recognition site found in the promoters of the

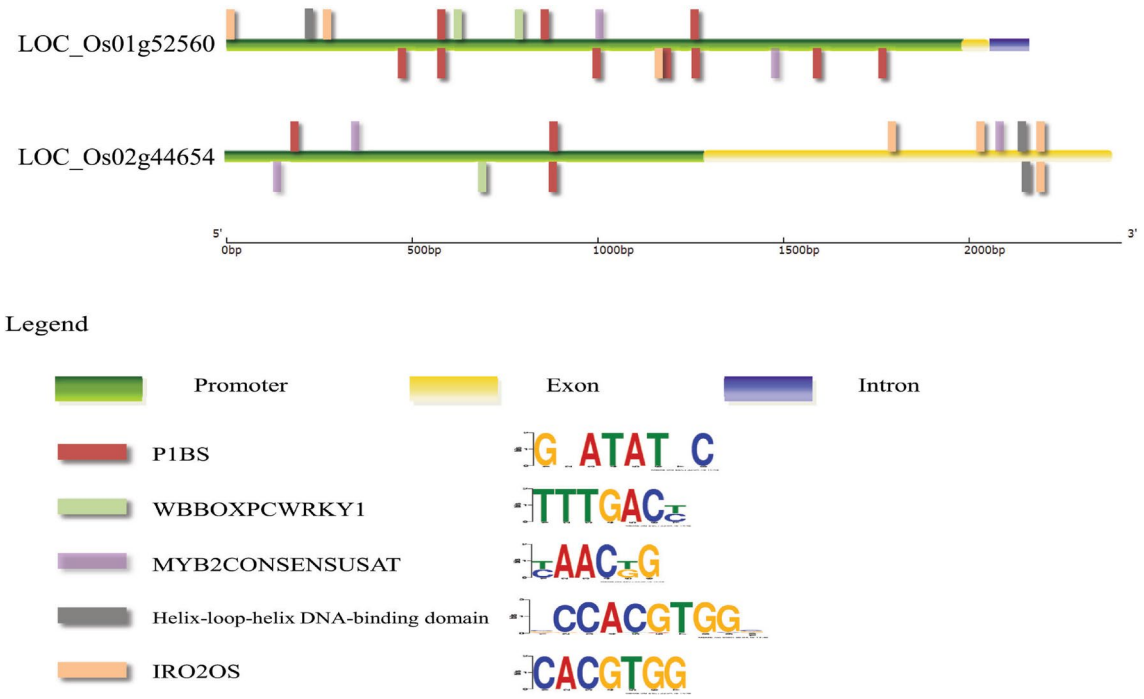

Fig. 6 Identification of conserved *cis*-acting elements of phosphate starvation-inducible genes. Conserved CRE was indicated by node color red nodes indicate P1BS motif, WBBOXPCWRKY1 motif; light green nodes, MYB2CONSENSUSAT motif; purple nodes, Helix-loop-helix DNA-binding domain motif; gray nodes, IRO2OS motif; and orange nodes. Detailed information is presented in Table S3

dehydration-responsive gene in *Arabidopsis*. The bHLH transcription factor-binding domain (NSCACGTGSN) is the most similar to E-box (CANNTG) and is found in all promoters of *OsPT* genes except *OsPT1, OsPT6*, and *OsPT8* (Hatorangan et al. 2009) and phosphate starvation-induced bHLH transcription factor (Os*PTF1*) (Yi et al. 2005). In addition, IRO2OS is also an OsIRO2-binding core sequence that is an essential regulatory element of the genes involved in Fe uptake under Fe-deficient conditions (Ogo et al. 2006). Recent studies in response to Pi starvation revealed its relationship with the accumulation of iron (Hirsch et al. 2006; Misson et al. 2005). Based on these findings, P1BS and WBBOXPCWRKY1 might be related to Pi starvation-inducible expression, and bHLH transcription factor-binding domain and MYB2CONSENSUSAT might be associated with crosstalk between various TFs and Pi starvation-inducible expression. The other CREs not mentioned here could have novel roles in driving Pi starvation-inducible expression. Future experiments will be necessary to confirm our predictions (Table S3).

Analyses of predicted protein–protein interactions associated with phosphate starvation

Regulatory genes are primary targets when investigating diverse stress responses and developmental processes. Among the 820 genes that were up-regulated under Pi starvation, we identified 22 TFs, 43 protein degradation and modification pathways, 16 transporters, and 47 functionally characterized genes (Fig. 7, Table S4, and Table S5). Understanding the regulatory relationships among them might improve our ability to develop novel strategies for enhancing PUE. To do this, we utilized the Rice Interactions Viewer to generate a hypothetical protein–protein interaction network associated with the up-regulated genes mentioned above. The network was further refined by highlighting genes in two categories: 60 functionally characterized genes and 52 up-regulated genes under low Pi in rice root. Of the latter, 31 genes have been functionally characterized. We also highlighted elements with multiple interactions among these regulatory genes or functionally characterized genes. This network data will be a useful resource in designing the hypothetical molecular model for detailed regulatory pathways in response to low Pi stress. Details on locus IDs, gene names, and putative functions of elements used in this refined network are provided in Table S5.

Discussion

Pi is a major nutrient with unequivocal importance for plant development such as root development, shoot length, and the number of tillers. Our data has shown that root development

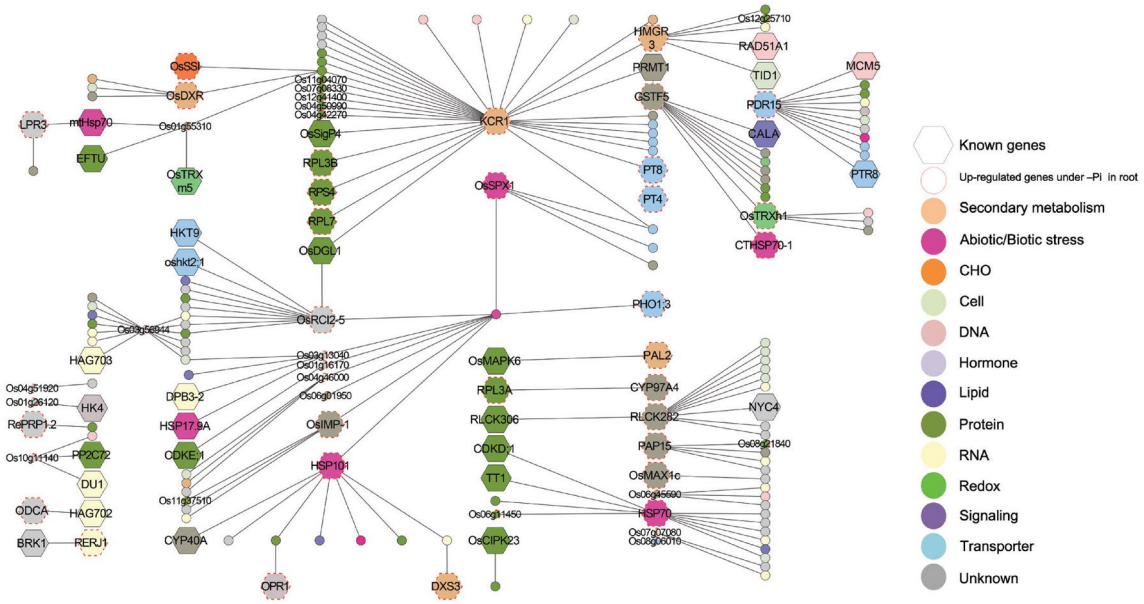

Fig. 7 Construction of a regulatory network associated with genes up-regulated in root under phosphate starvation. Predicted protein–protein interaction analysis using the Rice interaction viewer and Cytoscape 3.2.1 is prepared. We queried predicted protein to protein interaction network associated with functionally characterized 47 genes (large hexagons) from the OGRO database and 59 up-regulated regulatory genes under low Pi in rice root (red dotted circles or red dotted hexagons). In total, 197 interactions with 209 proteins including 21 up-regulated uncharacterized genes under low Pi in rice root and 60 functionally characterized genes were visualized. Functional classifications using MapMan annotation were indicated by 13 node colors Detailed information of the network is presented in Table S4 and S5

is enhanced for the first 7 days under low Pi conditions, but after 21 days, low Pi causes growth retardation and brown roots. The global understanding on the molecular mechanism of rice roots in response to low Pi stress will be very important in studies to enhance PUE. MapMan and GO enrichment analyses suggest that the Pi starvation response primary affect root development, secondary metabolism process, biotic stress response, and various transporter activities.

Of all the enriched GO terms, 'diterpene metabolic process' was the most significantly enriched by Pi starvation in rice roots. Rice produces diterpene phytoalexins, such as momilactones, oryzalexins, and phytocassanes. It was also reported that the production and secretion of momilactone A and B, which act as potent phytoalexins and allelochemicals, are increased in rice roots under various stress conditions (Kato-Noguchi 2011; Zhao et al. 2005; Kong et al. 2006). Recently, it was reported that rice exhibited high allelopathic activity when incubated under Pi- or nitrogen-limited conditions (Shen and Lin 2007; Song et al. 2008), indicating that the allelopathic activity of rice may be increased by nutrient starvation. Kim et al. (2005) also reported that nutrient competitive conditions with barnyard grass increased allelopathic activity in aqueous extracts of rice plants. These results suggest that phytoalexin diterpene probably led to allelopathic activity in rice under nutrient competitive conditions. Strigolactone (SL) resulting from terpenoid biosynthetic processes accumulates in *Arabidopsis* and rice roots under Pi starvation and is known to suppress shoot branching by inhibiting the outgrowth of axillary buds (Gomez-Roldan et al. 2008) and to promote the elongation of seminal/primary roots and adventitious roots (ARs) (Kapulnik et al. 2011Arite et al. 2012). The elevated endogenous SL concentration by Pi deficiency contributes to inhibition of tiller bud outgrowth (Umehara et al. 2010) and enhances root development in rice (Sun et al. 2014; Kumar et al. 2015). Elevated endogenous SL-accumulating phenotypes are consistent with the major phenotypes seen in Pi deficiency. In addition, a protein, PLEIOTROPIC DRUG RESISTANCE 1 (PDR1), belonging to the ABC transporters is known to be involved in the long-distance transport of SLs from root to shoot and also in root tissues (Kretzschmar et al. 2012).

Furthermore, changes of the secondary metabolites in response to low Pi stress resulted in accumulation of anthocyanin and total phenolic compounds in leaves under long-term Pi deficiency (Misson et al. 2005). Similar results were observed in *Zea mays*, with genes related to the phenylpropanoid pathway identified as up-regulated or down-regulated under Pi starvation (Juszczuk et al. 2004; Calderon-Vazquez et al. 2008). Our data suggest that Pi deficiency stress may affect the accumulation of anthocyanin as well as lignin

development in rice roots. In addition, secondary metabolites in rice may play an important role in enhancing survival rate under nutrient-deficient conditions.

From both MapMan and GO enrichment analyses, phosphate transporters were significantly identified in genes up-regulated under Pi starvation conditions, supporting the quality of samples used for transcriptome analysis. Interestingly, biotic stress or defense-related genes are also closely associated with Pi deficiency (Fig. 3e). We also identified other significant GO terms such as 'DNA catabolic process' and 'respiratory gaseous exchange' (Fig. 4). Therefore, the biological processes identified as being closely associated with Pi-limited conditions in rice root might be novel resources for improving our understanding of the molecular mechanism and PUE through further applications.

Under Pi starvation, the expression of the rice Phosphate transporter 1 (PHT1) gene group belonging to high-affinity transporters is strongly induced to increase the ability of the roots in acquiring Pi from soils and remobilizing Pi within plants (Mudge et al. 2003; Raghothama and Karthikeyan 2005). Most transporters in the PHT1 group are known to be strongly expressed in roots of *Arabidopsis, Zea mays*, and rice (Muchhal et al. 1996; Koyama et al. 2005; Nagy et al. 2006). For regulation of genes in the PHT1 group, TFs might have important roles. From the transcription overview installed in the MapMan toolkit, we found that MYB, WRKY, and bHLH TFs are more important than others. Previous studies have shown that MYB, WRKY, and bHLH TFs were involved in a regulation system under Pi starvation. *PHR1*, a MYB TF (Rubio et al. 2001); *WRKY75*, a WRKY family TF (Devaiah et al. 2007) in *Arabidopsis;* another WRKY family TF named *OsWRKY74;* and a bHLH transcription factor named *OsPTF1* have been reported to play a role in providing tolerance against Pi starvation stress by improving root system architecture in rice (Dai et al. 2016; Yi et al. 2005). Other TFs than those mentioned might have important roles in regulating Pi starvation responses in rice.

Our study provides a global view of Pi starvation response based on transcriptome data and novel promoters for improving PUE and Pi uptake. This strategy is very promising for future applications because recent studies using both ubiquitous and root-preferred promoters for drought stress tolerance clarified that root-preferred promoters are more effective at enhancing *OsNAC6*, which is involved in the drought tolerance regulatory pathway (Lee et al. 2017). Histochemical GUS assays were carried out on 21-day-old seedlings under Pi starvation and Pi-sufficient conditions using Yoshida solution. Interestingly, a GUS staining pattern was observed only in initiated or elongated areas of crown roots and lateral roots under Pi starvation and was not identified in any tissue under sufficient Pi conditions (Fig. 4d). These expression patterns are consistent with physiological changes in the rice root under Pi deficiency. In addition, the promoter of OsPTF1, a known TF involved in tolerance to Pi starvation in rice, was expressed in all cells of newly developed lateral roots and in the elongation zone of primary roots in the Pi starvation condition (Yi et al. 2005). We anticipate that the use of a Pi starvation-inducible promoter will more effectively improve root quality and Pi uptake in a Pi-limited field than that of ubiquitous promoters.

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