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ORIGINAL ARTICLE

Transgenic Rice Overexperessing a Tomato Mitochondrial Phosphate Transporter, SlMPT3;1, Promotes Phosphate Uptake and Increases Grain Yield

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Abstract Mitochondrial phosphate transporter plays an important regulatory role in promoting the uptake and transport of phosphate in plants. In this study, the *SlMPT3;1* gene, a member of mitochondrial phosphate transporter family in tomato, was isolated and transformed into the rice *Oryza sativa* L. ssp. japonica cultivar Kitaake. The SlMPT3;1 is localized to the mitochondrial membrane and functions in compensating the phosphate uptake in yeast MB192 mutant that is defective in phosphate transport under Pi deficiency. RT-qPCR showed that the *SlMPT3;1* is expressed in all of tomato tissues, but highly accumulated in the young leaves and stems under Pi deficiency. The data demonstrated that at least two copies of the *SlMPT3;1* gene are inserted into the rice genome, and the transcripts of the *SlMPT3;1* mRNA are highly accumulated in the roots of the transgenic rice. The overexpression of the *SlMPT3;1* gene not only promotes phosphate uptake by the roots, but also increases the translocation of phosphate from the roots to the shoots in the transgenic rice. The transgenic rice accumulated more chlorophyll and soluble sugar in the shoots than the wild type under Pi deficiency. Microassay sequencing showed that the differentially expressed genes in the transgenic rice are mainly involved in the regulations of biological process and molecular function under Pi deficiency. Further RT-qPCR analyses revealed that the differentially expressed genes, which are involved in the regulations of the biological process, cell component, and molecular function, are up-regulated under Pi deficiency, and exhibit similar expression trends to the relative expression folds of these partial differentially expressed genes in the transcriptomic analyses. This study suggests that the overexpression of the *SlMPT3;1* gene promoted the uptake and transport of phosphate in rice, thus leading to an enhanced increase in tiller number and effective panicle of per plant, and increasing grain yield under Pi deficiency.

Keywords: Mitochondrial phosphate transporter, Phosphorus deficiency, Phosphate uptake, SlMPT3;1 gene, Transgenic rice

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Introduction

Rice (*Oryza sativa*) is one of the most important food crops in the world, and requires sufficient phosphorus nutrition for enhancing crop productivity (Feng et al. 2017). Therefore, it is necessary to improve rice yield by promoting the uptake of phosphate and its use efficiency in soils lacking sufficient available phosphorus (Kirk et al. 1998; Shrawat et al. 2008). The shortage of available inorganic phosphate (Pi) in soils severely limits rice yield in a modern production system with nutrient-exhaustive high-yielding, thereby, breeding Pi-efficient high-yielding rice varieties is very meaningful to maintain high yield and lower excess application of phosphate fertilizer (Mehra et al. 2015). Phosphorus (P) is an essential element for plant's growth and development, and is required for the biosynthesis such as nucleic acids, nucleoprotein and phospholipids, and simultaneously plays important regulatory roles in energy transfer, signal transduction, and metabolic pathways in plant cells (Chiou and Lin 2011). Pi deficiency in soils not only negatively triggers the physiological-

biochemical changes in plants, but also leads to a decrease in the crop productivity (Hawkesford 2014; Mehra et al. 2017). Therefore, promoting Pi use efficiency is necessary to ensure a stable and sustainable rice production under limited Pi supply.

The uptake and transport of phosphate in plants usually are modulated by phosphate transporters (*PHTs*) that are responsible for the translocation of phosphate (Mimura 1999). Plant Pi transporters have been explored functionally and are basically composed of the *PHT1*, *PHT2*, *PHT3*, *PHT4* and *Pho1* families (Okumura et al. 1998; Stefanovic et al. 2007; Wang et al. 2015; Liu et al. 2016). Many *PHT1* members in diverse plants are high-affinity transporters and function in the transport and translocation of phosphate (Muchhal et al. 1996; Muchhal and Raghothama 1999; Nagarajan et al. 2011; Qin et al. 2012; Zhang et al. 2015). The *PHT2* family consist of low-affinity phosphate transporter proteins with abundant expressions in the green tissue under Pi starvation, and also facilitates the transport of phosphate in plants (Versaw 2002; Ai et al. 2009; Guo et al. 2013). The *PHT4* family exhibits different subcellular localization profiles (Roth et al. 2004; Pavon et al. 2008; Wang et al. 2011; Miyaji et al. 2015), and not only functions in promoting the transport and recycles of phosphate, but also plays important roles in regulating the leaf size and improving starch accumulation in Arabidopsis (Eicks et al. 2002; Cubero et al. 2009; Irigoyen et al. 2011). The *Pho1* gene containing *SPX* (*SYG/PHO81/XPR1*) domain participates in Pi loading to xylem and exhibits weak up-regulation under Pi stress (Hamburger et al. 2012), and *SPX* gene is involved in the regulation of Pi homeostasis and demonstrates specific induction expression in the roots under Pi starvation (Wang et al. 2009; Wang et al. 2012). Although the plant phosphate transporters have been evaluated systematically under Pi deficiency by profiling a series of adaptive mechanisms (Wu et al. 2013), the functional exploration of the *PHTs* is mostly limited to the modulation of phosphate transport in model plant Arabidopsis.

Unlike the *PHT1*, *PHT2*, *PHT4* and *Pho1* families, the *PHT3* family is specifically located to the mitochondrial membrane, and principally involved in the exchange of phosphate between the mitochondrial matrix and cytosol, thus it is assigned as mitochondrial phosphate transporter (Poirier and Bucher 2002). Hamel et al. (2004) confirmed that two members of the *PHT3* family, the *AtPHT3;1* and the *AtPHT3;2,* function in phosphate transport by using a yeast mutant lacking endogenous mitochondrial phosphate transporter. To our knowledge, the functional reports of the mitochondrial phosphate transporter in crops are still lacking (Jia et al. 2011). A mitochondrial phosphate transporter in *Lotus japonicus, LjMPT* carrying a conserved *N*-ethylmaleimide (NEM)-reactive functional *Cys* residue*,* was just characterized by site-directed mutagenesis (Nakamori et al. 2002). Particularly, genetically manipulating the mitochondrial phosphate transporter (*PHT3*) gene for crop breeding is very limited. To investigate the functional performance of the mitochondrial phosphate transporter, we isolated a mitochondrial phosphate transporter from tomato *Solanum lycopersicum* (Chinese Lichun Cv.), the S*lMPT3;1* gene, and transferred it into the model rice *Oryza sativa* L. ssp. japonica Cultivar Kitaake. Both hydroponics and pot experiments were performed. The data showed that the transgenic rice overexpressing the *SlMPT3;1* gene significantly promoted the uptake of phosphate, and thereby increased tiller number and grain yield of per plant under Pi deficiency, indicating that the *SlMPT3;1* gene plays an important regulatory role in phosphate acquision during the metabolism process, and the differentially expressed genes of the *SlMPT3;1* gene-triggered also contribute to adaptation of the transgenic rice to Pi deficiency. Our study shows that manipulating the *PHT3* family gene in rice is a promising approach to improve the phosphate uptake under low Pi condition.

Results

The *SlMPT3;1* is a Typical Member of Mitochondrial Carrier Superfamily

For predicting the structural characteristics of the *SlMPT3;1* gene, both DNA sequencing and bioinformatics analyses were performed. Sequencing showed that the *SlMPT3;1* contains six exons and five introns, and encodes 358 amino acids with 38.44 kD of protein mass. As single copy gene, the *SlMPT3;1* gene is located on the chromosome #2 of tomato genome (NCBI website). Analyses revealed that the SlMPT3;1 contains two typical conserved domains that specifically are present in the mitochondrial-carrier superfamily. Multiple alignments shows that the SlMPT3;1 protein is composed of six transmembrane domains, and shares more than 80% homology with mitochondrial phosphate transporters from soybean, maize, potato, and alfalfa, but less than 80% (79.7%) with a rice mitochondrial phosphate transporter which seems to be an ortholog of the *SlMPT3;1* gene (Fig. 1A). Phylogenetic tree analysis demonstrated that the SlMPT3;1 has a close familiarity with the mitochondrial phosphate carrier proteins in potato and tobacco, and shares more than 80% identity in amino acids (Fig. 1B; Table S2), suggesting that the *SlMPT3;1* gene encodes a mitochondrial phosphate transporter in the *PHT3* family.

Expression Profiles of the *SlMPT3;1* Gene and Subcellular Localization

To examine the expression profiles of the *SlMPT3;1* gene in tomato in response to different levels of Pi supply, the RT-qPCR was performed. When tomato seedlings were cultured under Pi deficiency, the *SlMPT3;1* transcripts were highly

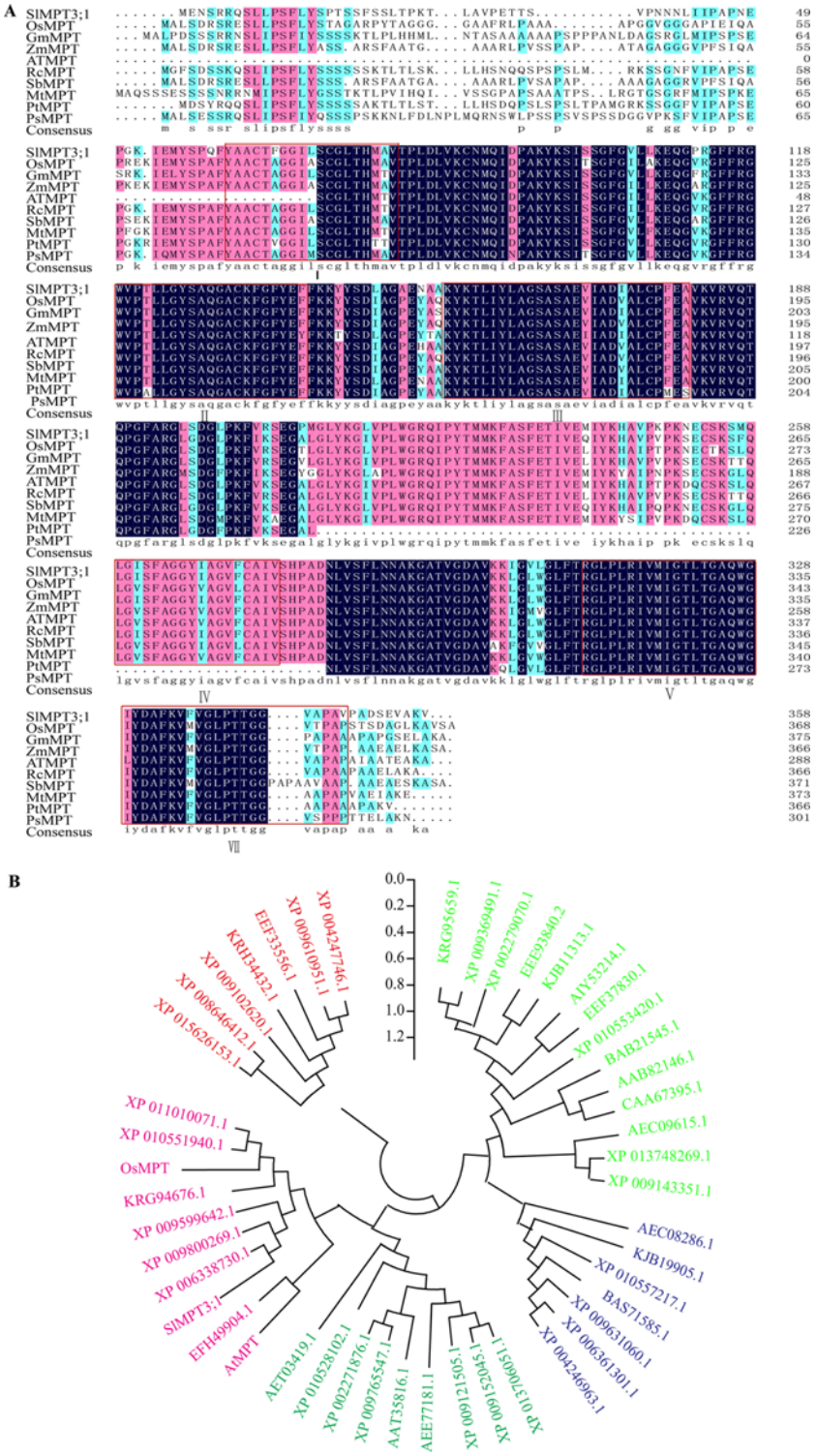

Fig. 1. Analyses of homology alignment and phylogenetic tree of the SlMPT3;1. (A) The alignment of the SlMPT3;1 with other phosphate transporters. OsMPT (*Oryza sativa Japonica Group*), Os02g0767500; GmMPT (*Glycine max*), NP_001237304.1; ZmMPT (*Zea mays*), NP_001104842.1; AtMPT (*Arabidopsis thaliana*), BAA31585.1; RcMPT (*Ricinus communis*), XP_002512859.1; SbMPT (Sorghum bicolor), XP_002454610.1; MtMPT (*Medicago truncatul*), XP_003624478.1; PtMPT (*Populus trichocarpa*), ACJ65020.1 and PsMPT (*Paeonia suffruticosa*), ABW74474.1. The membrane-spanning domains were predicted by TopPred and are boxed. (B) Phylogenetic tree of SlMPT3;1 with other plants phosphate transporters. Different colors indicate different MPT family phosphate transporters. Light green represents MPT1 family phosphate transporters, dark green represents MPT2 family phosphate transporters, pink represents MPT3 family phosphate transporters, blue represents MPT4 family phosphate transporters, red represents PHO1 family phosphate transporters.

accumulated in young leaves and stems, and was 9.9-fold and 6.5-fold higher than those in the roots, respectively (Fig. 2A). Although plants grown with sufficient phosphorus supply (NP) showed similar trends, the relative expressions of the *SlMPT3;1* gene in old leaves are increased by 2.7-fold, and by 2.6-fold in the cotyledons compared to those in the roots, respectively (Fig. 2A). These data indicate that Pi deficiency significantly induces the expressions of the *SlMPT3;1* gene in young leaves and stems of tomato seedlings. Subcellular localization showed that the signal of 16318hGFP vector are observed throughout the whole cell, while the SlMPT3;1:: 16318hGFP are clearly expressed in the mitochondria, showing that the *SlMPT3;1* gene is located to the mitochondria of tobacco (Fig. 2B).

The *SlMPT3;1* Compensates for the Uptake of Phosphate in Yeast

To explore the regulatory role of the *SlMPT3;1* in promoting phosphate uptake in yeast strain, a Pi functional complementary

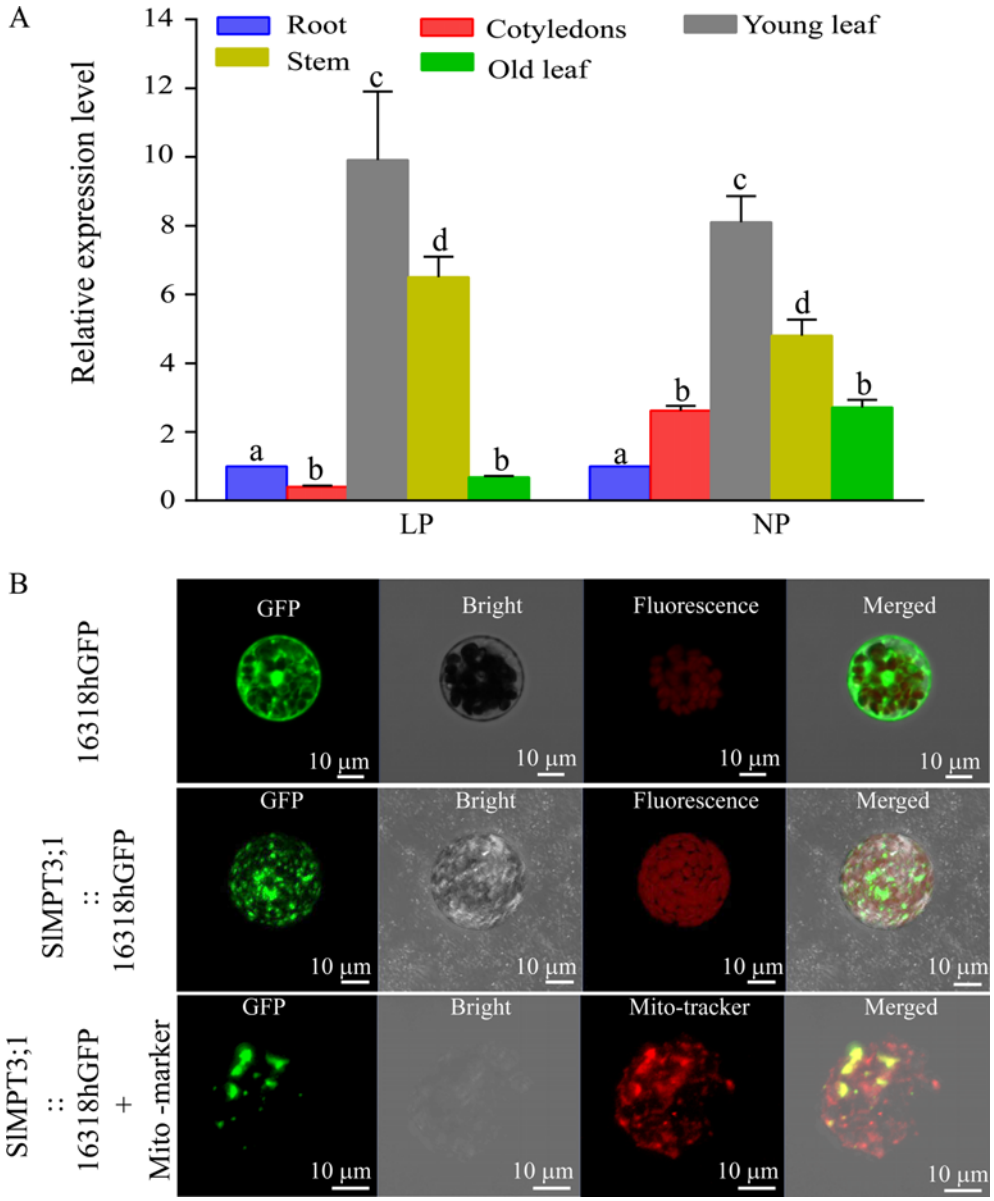

Fig. 2. The expression profiles of the *SlMPT3;1* in tomato and SlMPT3;1 fusion protein. (A) The tissue-specific expression pattern of *SlMPT3;1* in tomato. Relative expressions were normalized by internal reference gene *Actin*. LP represented 50 μM Pi, NP represented 1.25 mM Pi. Data are given as means ± SD (n=6). Letters on the histograms show significance differences at level of $P<0.05$ compared with the control. (B) Subcellular localization of the SlMPT3;1::16318hGFP in tobacco protoplasts. The green signals indicate GFP, the red signals in the second row indicate chloroplast, the red signals in the third row indicate mitochondria, and the yellow signals indicate the overlap of green and red signals.

experiment was performed. Measurement showed that the growth of the mutant MB192 strains was inhibited in YNB medium with Pi supply of 20 μM or 60 μM Pi or 100 μM Pi, while the wild-type strains demonstrated better growth. However, although the strains expressing the *SlMPT3;1*:: *Yp112AINE* exhibit growth inhibition in the liquid YNB medium containing Pi of 20 μM, or 60 μM or 100 μM compared to the wild-type strain (Fig. 3A), the detection densities at OD600 of the *SlMPT3;1*:: *Yp112AINE* strain were increased by 3.23-fold in the presence of 20 μM Pi, by 3.79-fold in the presence of 60 μM Pi, and by 2.71-fold in the presence of 100 μM Pi compared to those in the mutant MB192 strain lacking phosphate transporter, respectively (Fig. 3B). Accordingly, Pi measurements showed that the strains expressing the *SlMPT3;1*::*Yp112AINE* fusion lowered the level of residual Pi in the supernatants, which are decreased by 0.03 μg mL^{-1} in the presence of 20 μM Pi, by 0.18 μg mL^{-1} in the presence of 60 μM Pi, and by 0.20 μg mL^{-1} in the presence of 100 μM Pi compared to the mutant MB192 strain, respectively (Table 1), suggesting that the yeast strain expressing the *SlMPT3;1*:: *Yp112AINE* accumulates more Pi than the mutant MB192 strain-defective high-affinity phosphate transporter, and the *SlMPT3;1* gene, a mitochondrial phosphate transporter, promotes the uptake of phosphate in yeast cells.

The *SlMPT3;1* Gene Exhibited Stable Heredity and Expression In Rice

To determine the expressions of the *SlMPT3;1* gene in rice, both Southern blotting analyses and histochemical GUS

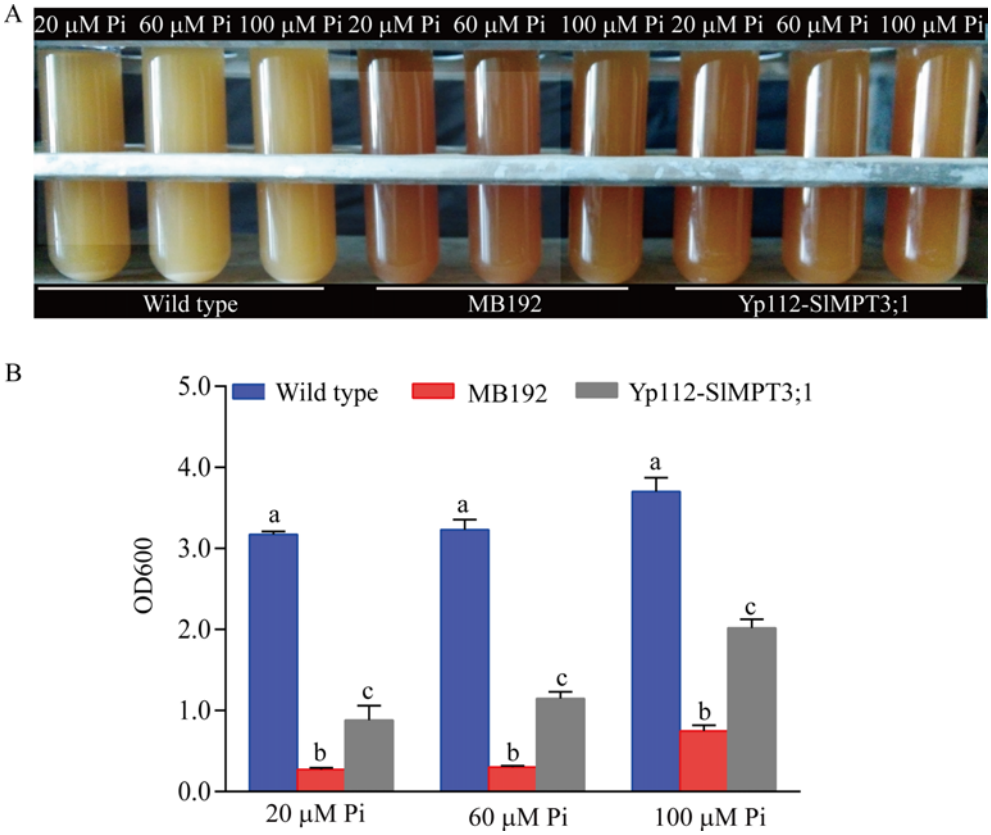

Fig. 3. Functional complementary assay of the *SlMPT3;1* in yeast. (A) Growth characteristics of the yeast strain MB192 (control), *Yp112-SlMPT3;1* (MB192 mutant carrying the *Yp112-SlMPT3;1* construct), and the wild type under different Pi supply levels. (B) OD600 in the exponential growth phase at 600 nm.

Table 1. The amounts of residual Pi in supernatants disposing of yeast cells under different Pi supply. All data (mean ± SD) in each line were statistically analyzed (n=9); Different letters for each line indicate significant differences at $P<0.05$ level

Measurements	Pi supply levels	In the supernatants disposing of yeast cells (μg mL^{-1})		
		Wild type	MB192	Yp112-SlMPT3;1
Amounts of residual Pi	20 μM	0.69 ± 0.01a	0.78 ± 0.04b	0.78 ± 0.03b
	60 μM	0.68 ± 0.03a	0.77 ± 0.04b	0.75 ± 0.04ab
	100 μM	0.66 ± 0.03a	0.72 ± 0.03b	0.71 ± 0.10ab

staining were preformed. A total of 30 transgenic lines of T0 generation were confirmed firstly by PCR detection, and 18 of these transgenic lines exhibited positive reactions (Fig. 4A). Firstly, for identifying the insertion patterns of the *SlMPT3;1* gene in rice, three transgenic lines, OE24, 0E28, and OE30, were randomly selected for Southern blotting analyses. Data revealed that no band was observed in the wild type, but the transgenic lines showed three bands in OE24, two bands in OE28, and three bands in OE30, suggesting that at least two complete copies of the *SlMPT3;1* gene were integrated into the rice genome (Fig. 4B). GUS staining assay confirmed the absence of the *SlMPT3;1* gene in the wild type (Fig. 4C-a, -c, -e) and the presences of the *SlMPT3;1* gene in the transgenic rice (Fig. 4C-b, -d, -f), showing that the *SlMPT3;1::GUS* fusion are mainly expressed in epidermis and primary phloem cells of the roots (Fig. 4C-d), and expressed throughout the leaf cells of the transgenic rice (Fig. 4C-f).

The relative expression levels of the *SlMPT3;1* gene in the roots of the transgenic lines under Pi deficiency demonstrate an increase of more than 5 folds compared with those in the roots under supply of sufficient phosphorus (NP), suggesting that the expression of the *SlMPT3;1* gene in the roots of the transgenic lines was induced by Pi deficiency (LP). However, the expressions of the *SlMPT3;1* gene in the shoots of the transgenic lines showed no significant differences compared with those under Pi deficiency (LP) or sufficient Pi (NP) (Fig. 4D).

Changes in Compatible Metabolites, Phosphate Uptake, and Grain Yield in Rice

Two independent hydroponics experiments were separately performed to understand the physiological and biomass changes in the transgenic rice before heading stage (Fig. 5A). Physiological measurements demonstrated that the transgenic rice increased accumulation of partial metabolites and phosphate compared to the wild type. The contents of chlorophyll in the transgenic lines are increased by 0.34 mg g^{-1} in OE24, 0.34 mg g^{-1} in OE28, and 0.29 mg g^{-1} in OE30 compared with the wild type under Pi deficiency (Fig. 5B), and soluble sugar in leaves of the transgenic lines are increased by 0.62 mg g^{-1} in OE24, 6.85 mg g^{-1} in OE28, and 8.72 mg g^{-1} in OE30 compared with the wild type under Pi deficiency, respectively (Fig. 5C). While both the chlorophyll

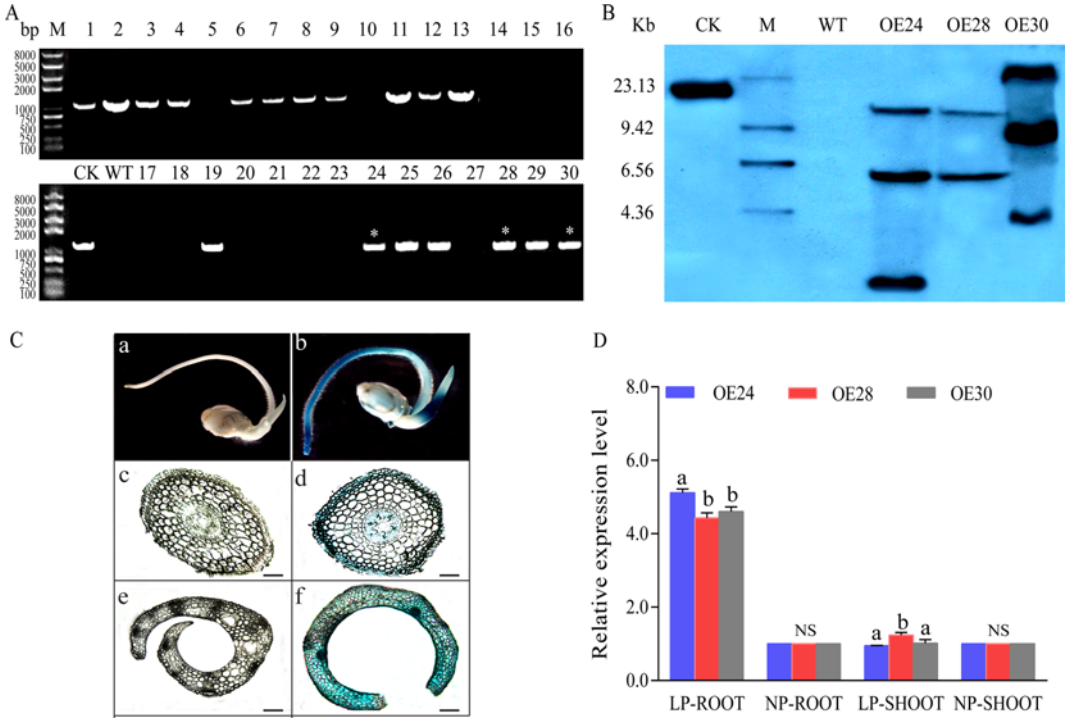

Fig. 4. PCR detection, Southern blotting, GUS staining and transcript analyses of transgenic rice. (A) PCR detection. M, DNA marker; WT, the wild type; number 1-30 represent T0 generation of the transgenic line. OE24, OE28, and OE30 represent the transgenic lines, respectively. (B) Southern blotting assay for the transgenic rice. Lane 1 represents a positive control (CK), lane 2 represents molecular markers (M), lane 3 contains a negative control (WT), lane 4-6 represent three transgenic lines. (C) Histochemical GUS staining analyses. a, The wild type rice seedling; b,The GUS-staining transgenic rice seedling; c, Paraffin section of root of wild type rice; d, Paraffin sections of roots of the transgenic rice; e, Paraffin section of stems of the wild type; f, Paraffin section of stems of the transgenic rice. Bar = 100 mm. (D) The transcript abundance of the *SlMPT3;1* in roots and shoots. Relative expressions were normalized by an *OsUbiquitin* internal reference gene. Data are indicated by means of three independent replicates, error bar indicates standard deviation (±SD, n=3 for each line). Letters on the histograms indicate significance differences at level of $P < 0.05$.

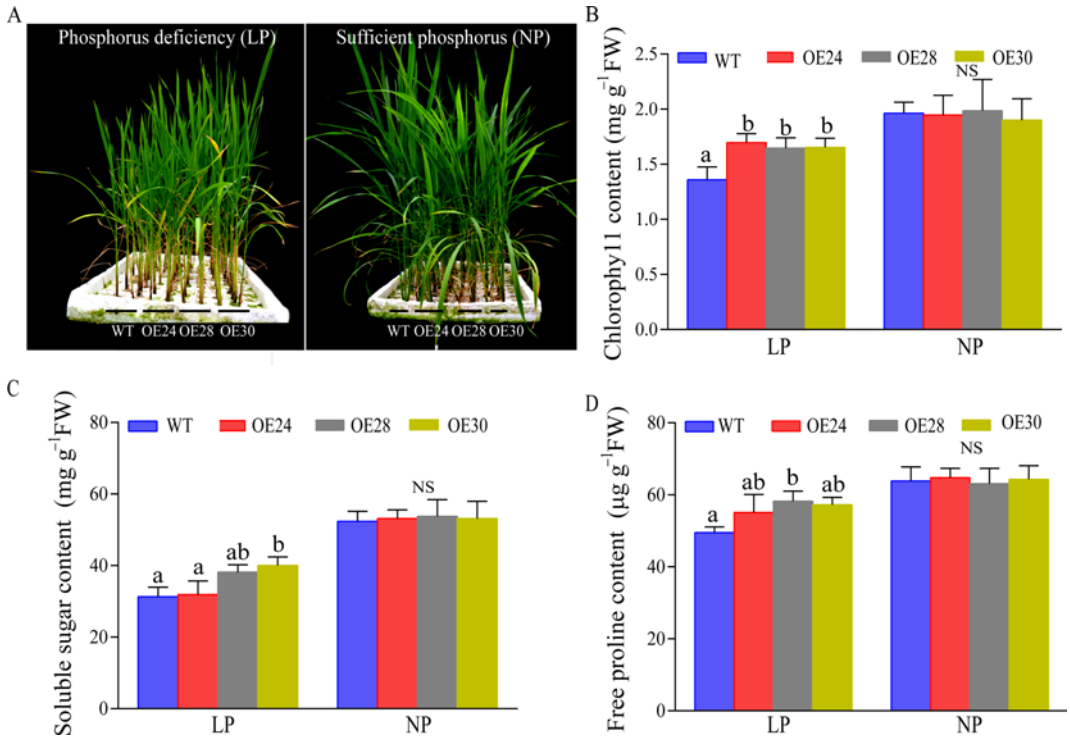

Fig. 5. Physiological responses of rice seedlings before heading to Pi supply. (A) Phenotype under culture with LP (50 μM) or NP (1.25 mM). (B) The chlorophyll contents. (C) The soluble sugar contents. (D) The free proline contents. Error bar indicates standard deviation (±SD, n=3 for each line), NS represents no significance, and different letters indicate a statistical difference at level of $P < 0.05$. FW means fresh weight.

Table 2. The Pi content in rice under Pi supply. All data (mean±SD) in each line were statistically analyzed (n=12); Different letters for each column indicate significant differences at P<0.05 level. DW, dry weight

Rice line	Pi content per plant (mg g^{-1} DW)			
	In the rice roots		In the rice shoots	
	50 μM (LP)	1.25 mM (NP)	50 μM (LP)	1.25 mM (NP)
WT	0.29 ± 0.04a	0.59 ± 0.04a	1.92 ± 0.21a	5.33 ± 0.41a
OE24	0.43 ± 0.06ab	0.58 ± 0.08a	2.42 ± 0.43a	5.50 ± 0.17a
OE28	0.55 ± 0.07b	0.57 ± 0.05a	2.21 ± 0.25a	5.42 ± 0.60a
OE30	0.45 ± 0.07ab	0.57 ± 0.06a	2.45 ± 0.21a	5.42 ± 0.26a

and soluble sugar contents in the transgenic lines did not increased compared to the wild type under sufficient phosphorus supply. However, in the case of Pi deficiency, the transgenic lines led to an increase of proline accumulation by 5.62 μg g^{-1} in leaves of OE24, 8.78 μg g^{-1} in leaves of OE28, and 7.77 μg g^{-1} in leaves of OE30 compared with the wild type, respectively (Fig. 5D). In contrast, the transgenic rice did not accumulate more proline comparing to the wild type under sufficient phosphorus (NP) (Fig. 5D).

For profiling the accumulation of phosphate, we separately measured the uptake of phosphate in the roots and shoots of the transgenic rice before heading. Data showed that the Pi contents in the roots of the transgenic lines are increased by 0.13 mg g^{-1} in the roots of per plant OE24, 0.25 mg g^{-1} in the roots of per plant OE28, and 0.16 mg g^{-1} in the roots of per plant OE30 compared with those in the roots of the wild type under Pi deficiency (LP), respectively (Table 2). But both the transgenic lines and the wild type have no obvious differences in the Pi uptake in the roots under supply of sufficient phosphorus (NP) (Table 2). The transgenic lines accumulated more Pi in the shoots, and led to an increase of 0.50 mg g^{-1} of Pi content in the shoots of per plant OE24, 0.30 mg of Pi content in the shoots of per plant OE28, and 0.53 mg g^{-1} of Pi content in the shoots of per plant OE30 compared to the shoots of per plant wild type under Pi deficiency, respectively (Table 2). Similarly, under sufficient

Pi supply, both the transgenic and the wild type have no obvious differences in the Pi content in the shoots of per plant (Table 2).

Recording data before heading shows that the fresh weights of per plant transgenic line increased by 13.9% in OE24, 15.5% in OE28, and 15.2% in OE30 relative to the wild type under Pi deficiency (LP), respectively, but biomass per plant of the transgenic lines and the wild type have no changes under supply of sufficient phosphorus (NP) (Table S3). A pot culture with a mixture of peat soils and vermicular was performed to investigate the tiller nunber and effective panicle number of per plant at heading stage, and measure the grain number and grain yield of per plant at maturity stage under Pi deficiency or sufficient phosphorus supply (Fig. 6A). Data showed that both the tiller number and efficient panicle number in the three transgenic lines are higher than those in the wild type under Pi deficiency (LP) (Fig. 6B) or sufficient phosphorus (NP) (Fig. 6C). However, in the case of Pi deficiency, the grain number of per plant in the transgenic rice are increased by 41.5 grains in OE24, 30.3 grains in OE28, and 29.5 grains in OE30 compared with those in the wild type, respectively (Table 3). Meanwhile, the

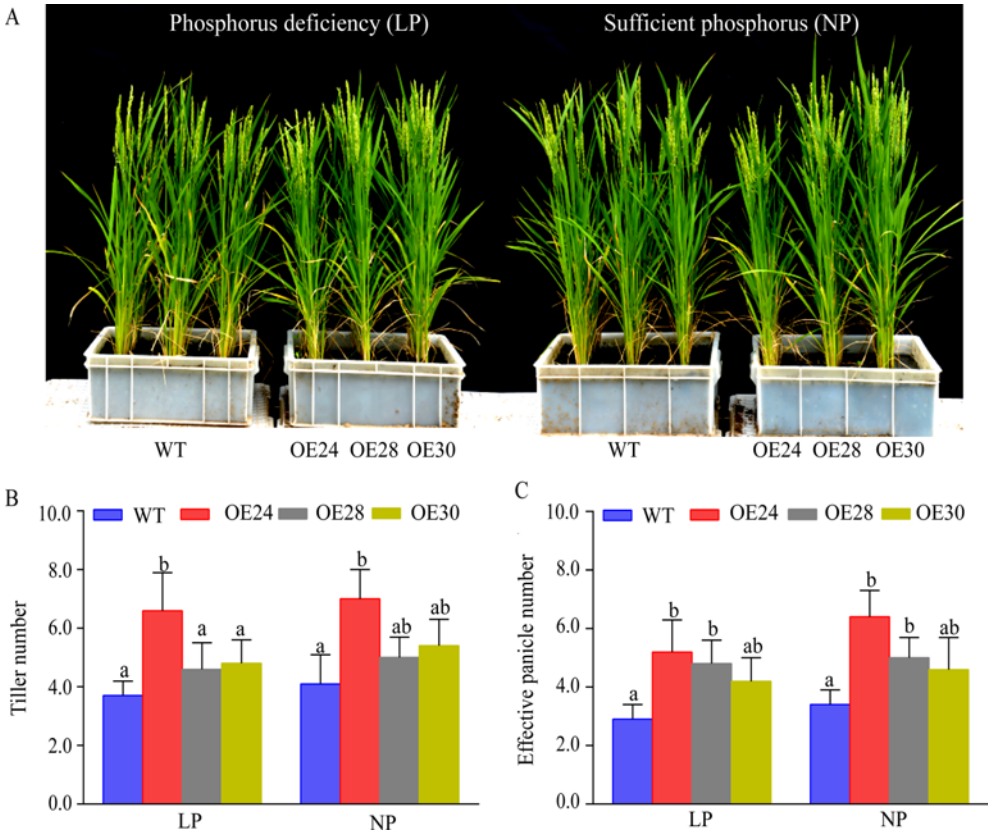

Fig. 6. Phynotypic characteristics of rice at the heading stage. (A) Phenotypic characteristics under natural culture with a disposable supply of LP (50 mM) or NP (1.25 mM). (B) The differences in tiller number of per plant; (C) The differences in effective panicle number of per plant. Error bars indicate standard deviation (±SD n=5). Different letters indicate a statistical difference at level of $P < 0.05$.

Table 3. Changes of grain traits of per plant. The data are presented by the means ± standard deviation (n=20 per line). Different letters in each column indicate significant differences at the 5% level. DW, dry weight

Rice line	Grain traits			
	Grain number		Grain yield (g DW)	
	50 μM (LP)	1.25 mM (NP)	50 μM (LP)	1.25 mM (NP)
WT	75.0 ± 3.6a	128.5 ± 3.3a	2.06 ± 0.10 a	3.52 ± 0.09a
OE24	116.5 ± 4.0b	130.5 ± 3.9a	3.19 ± 0.11b	3.58 ± 0.11a
OE28	105.3 ± 4.4c	130.6 ± 4.5a	2.89 ± 0.12c	3.58 ± 0.12a
OE30	104.5 ± 6.3c	129.3 ± 3.7a	2.86 ± 0.17c	3.54 ± 0.10a

transgenic lines show more than 30% increases in grain yield of per plant relative to the wild type under Pi deficiency (LP), but have no obvious differences in grain yield compared with the wild type under supply of sufficient phosphorus (NP) (Table 3).

The Genome-wide Analyses of the Differential Expressed Genes

Targeting at induction expressions of the *SlMPT3;1* gene in the roots under Pi deficiency, we sampled the roots of the transgenic line OE30 and the wild type, and performed transcriptomic analyses to profile the differential expressed genes. The combination of OE30/LP vs WT/LP exhibited a total of 210 up-regulated and a total of 146 of down-regulated unigenes under Pi deficiency (Fig. 7A), while the combination of OE30/NP vs WT/NP demonstrated a total of 650 of up-regulated unigenes and a total of 349 of down-regulated unigenes (Fig. 7B). The Venn diagram showed that four combinations revealed clear differences in the number of the differentially expressed genes. The combination of OE30/LP vs WT/LP (a-blue circle) showed a total of 356 of differentially expressed genes, and the combination of WT/LP vs WT/NP (b-orange circle) showed a total of 1272 of differentially expressed genes, and the combination of OE30/LP vs OE30/NP (c-purple circle) demonstrated a total of 356 of differentially expressed genes, and the combination of OE30/NP vs WT/NP (d-green circle) revealed a total of 999 of differentially expressed genes. As shown in Fig. 7C, the blue circle and green circle share a total of 170 of the differentially expressed genes, and the orange circle and purple circle share a total of 167 of the differentially expressed genes.

GO and KEGG Enrichment Analyses of the Differentially Expressed Genes

Top 20 in KEGG (Kyoto Encyclopedia of Genes and Genomes,

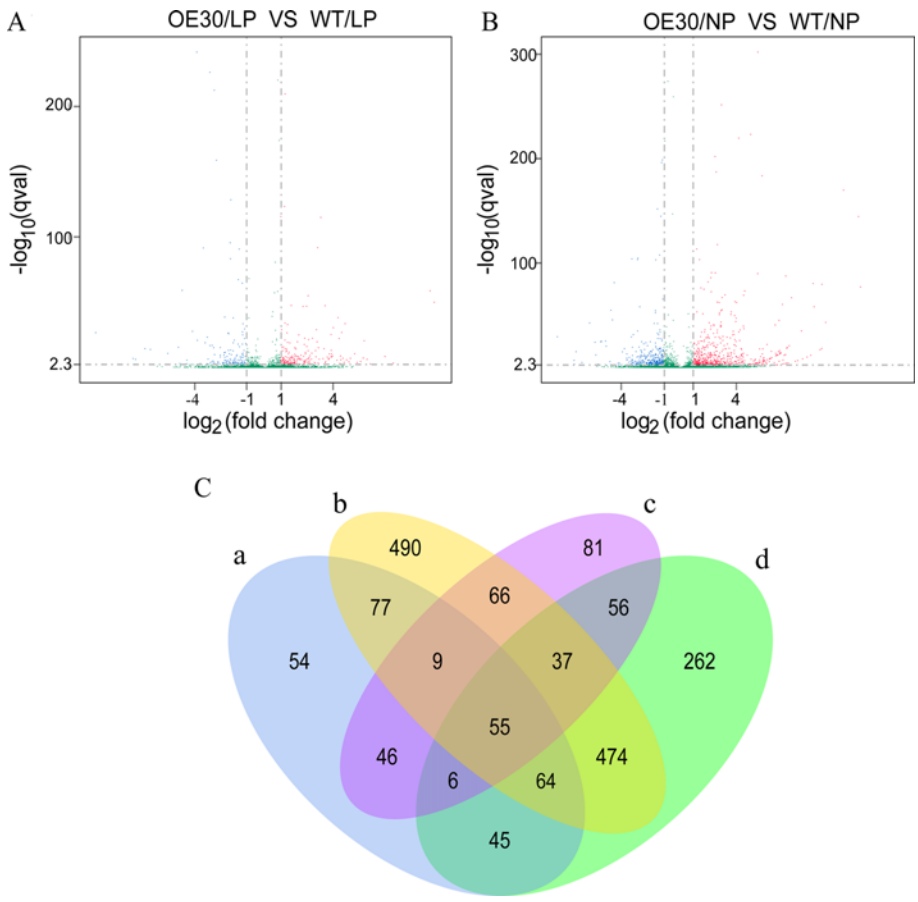

Fig. 7. The profiles of the differentially expressed genes. Red spots represent the up-regulated DEGs and blue spots indicate the down-regulated DEGs in these four volcano spots. (A) The volcano spots representing the unigenes were identified as DEGs in OE30/LP vs WT/LP combination. (B) The volcano spots representing the unigenes were identified as DEGs in the combination OE30/NP vs WT/NP. (C) The Venn diagram of DEGs. The circles represent combinations, the total number in every circle represent the number of the differentially expressed genes between two compared samples, the overlaps of circles indicates the consensus differentially expressed genes. a, OE30/LP vs WT/LP: the combination of OE30 versus WT under LP condition; b, OE30/NP vs WT/NP: the combination of OE30 versus WT under NP condition; c, WT/LP vs WT/NP: the combination of WT under LP and NP condition; d, OE30/LP vs OE30/NP: the combination of OE30 under LP and NP condition.

http://www.kegg.jp) pathways were summarized to profile the distributions of the up-regulated and down-regulated DEGs in each combination. The DEGs distributions in each GO term are shown by the Histogram of Gene Ontology (GO), and top in 30 GO categories of the up-regulated and the down-regulated transcripts are separately demonstrated, and most of the categories reveal clear differences in the number of the transcripts (Fig. 8). In the combination of OE30/LP vs WT/LP, the up-regulated genes were mainly expressed in the biological process (Bp) and cell component (Cc) (Fig. 8A), and mostly involved in the metabolism of chemical acid, water transport, and oxygen−containing in the Bp, and involved in nucleic acid binding transcript and sequence-specific DNA binding in the Cc, but no cluster of the up-regulated genes were found in the molecular function (Mf). In the combination of OE30/NP vs WT/NP, the up-regulated genes were mainly involved in the Bp that is closely related to stress defense and oxidation-reduction, and also participated in the molecular function (Mf) that is related to the activity regulations of hydrolase and oxidoreductase, and the partial DEGs were demonstrated in extracellular region in the Cc (Fig. 8B). Additionally, the up-regulated genes likely participate in three metabolic pathways, such as plant hormone signal transduction, plant-pathogen interaction, and biosynthesis of secondary metabolites under Pi deficiency, and are involved in the activity regulations of cation binding under sufficient phosphorus, suggesting that all of the

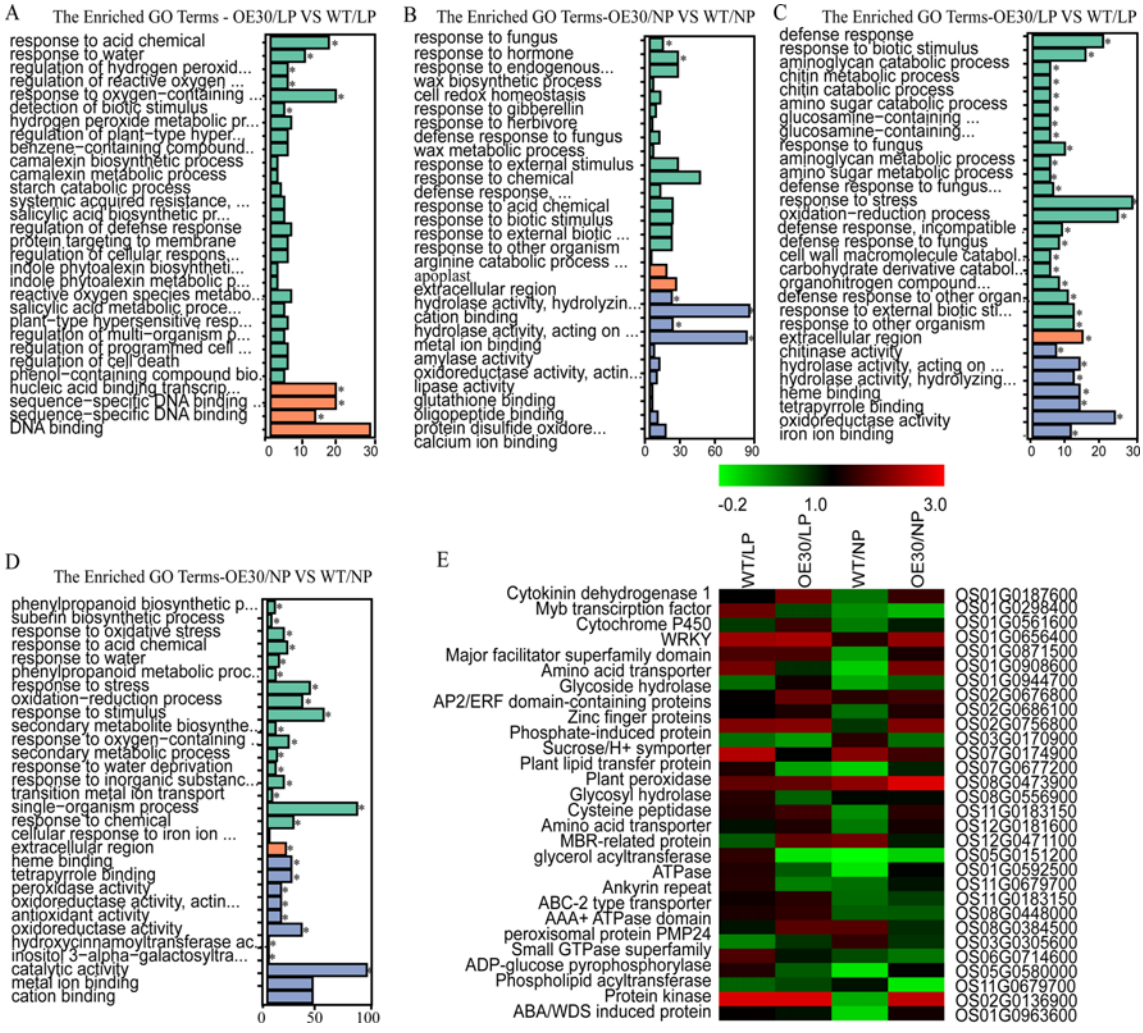

Fig. 8. GO enrichment analyses of the differentially expressed genes. Three GO categories were expressed by biological process (green), cellular component (orange) and molecular function (blue), respectively. The x-axis indicates the number of genes in one category, and the y-axis indicates different subcategories. asterisk (*) indicates significant differences at $P<0.05$ in one combination. (A) GO categories of the up-regulated unigenes in the combination of OE30 versus WT under LP condition. (B) GO categories of the up-regulated unigenes in the combination of OE30 versus WT under NP condition. (C) GO categories of the down-regulated unigenes in the combination of OE30 versus WT under LP condition. (D) GO categories of the down-regulated unigenes in the combination of OE30 versus WT under NP condition. (E) Expression profiles of the differentially expressed genes in the WT and the transgenic line OE30 under different Pi conditions, respectively. Highly expressed genes were exhibited with $p < 0.05$ under different Pi supply conditions, and the color scales represent log signal values at the top, and the color changes represent the value of log(RPKM).

up-regulated genes likely function in response to fungus and hormone in the Bp and play important roles in binding metal ion in the Mf. Similarly, the down-regulated genes are also involved in the biological process (Bp), cellular component (Cc), and molecular function (Mf) under Pi deficiency or sufficient phosphorus. In the combination of OE30/LP vs WT/LP, the down-regulated genes mainly are expressed in the Bp and Mf, and function in regulating metabolic and catalytic processes in response to stimulus and biotic stress in the Bp, and hydrolase activity in the Mf (Fig. 8C). In the combination of OE30/NP vs

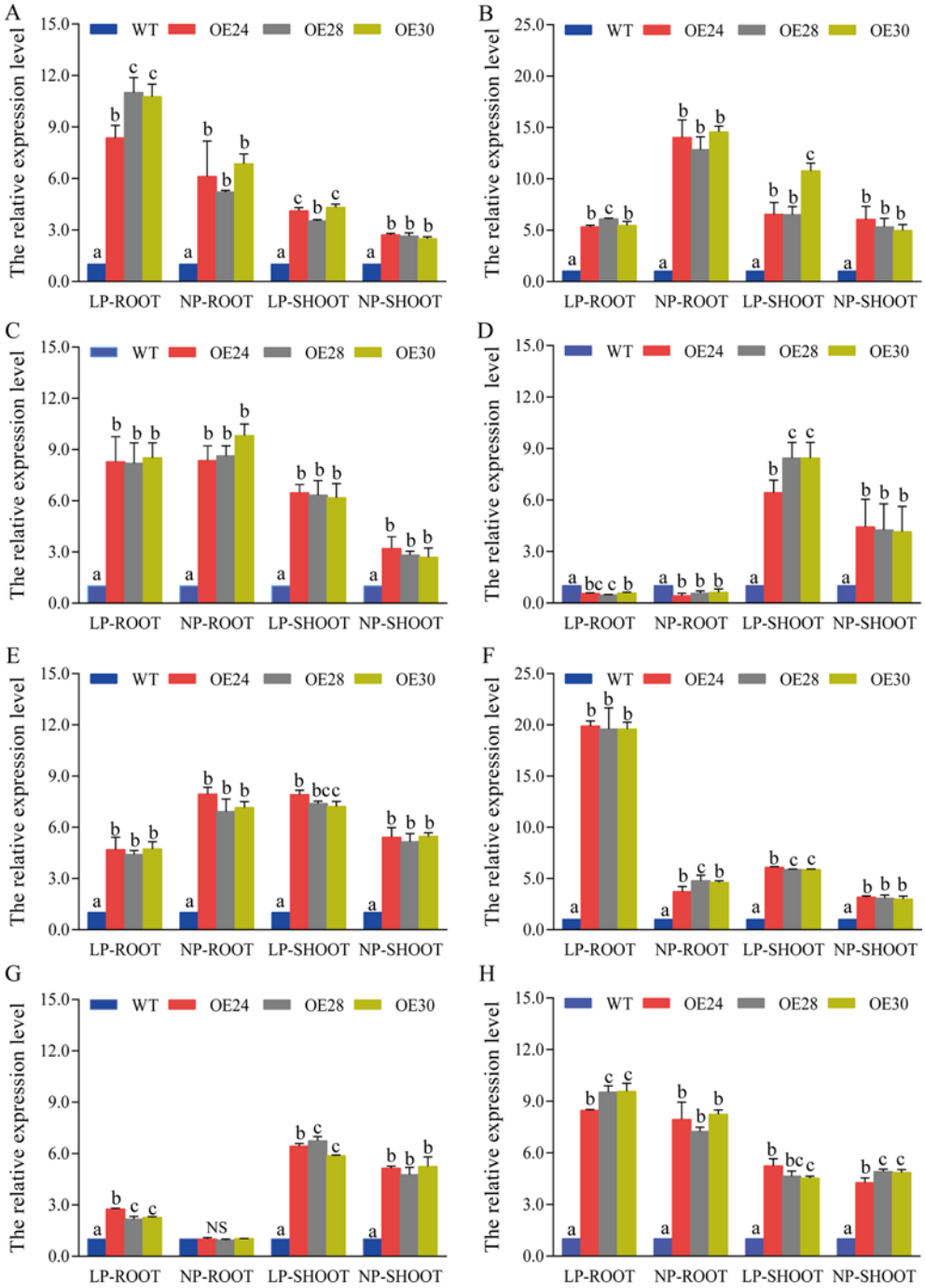

Fig. 9. Relative expression profiles of partial key DEGs in rice seedlings. Rice seedlings of two-week-old were further cultured on MS medium containing Pi with two levels for two weeks, and used for RT-qPCR analyses. (A) zinc finger-encoding gene (Os02g0686100). (B) *WRKY*-encoding gene (Os01g0656400). (C) P-type ATPase-encoding gene (Os03g0203700). (D) glycosyl hydrolase-encoding gene (Os08g0473900). (E) *LEA* gene (Os06g0110200). (F) cytokinin dehydrogenase 1-encoding gene (Os01g0187600). (G) amino acid transporter-encoding gene (Os03g0654400). (H) AAA^+ ATPase-encoding gene (Os12g0471100). Error bar indicates standard deviation (±SD, n=3 for each line); Letters indicate significance differences at level of $P<0.05$.

WT/NP, the down-regulated genes mainly were mainly involved in the physiological regulation of secondary metabolic pathways in response to stimulus, single-organism processes in the Bp, and enzymes activity and cation binding in the Mf (Fig. 8D). Thus indicates that the down-regulated genes are mainly involved in the biosynthesis of secondary metabolites, the regulations of enzymes activity, and cation binding. The differential expression profiles of these up-regulated or down-regulated genes are presented (Fig. 8E).

Expression Profiles of the Differentially Expressed Genes

To confirm the expression profiles of the partial differentially expressed genes in response to Pi deficiency, we randomly selected the partial DEGs in the OE line and the wild type by the transcriptomic analyses, and performed the RT-qPCR procedures to evaluate the transcript levels of these DEGs in the transgenic line. The relative expression level of a zinc finger gene (Os02g0686100) in the roots of the transgenic line are increased by 8.3~11.0-fold, and increased by 3.5~4.3 folds in the shoots of the transgenic line compared to those of the wild type under Pi deficiency, respectively (Fig. 9A). Sufficient phosphate supply leads to a similar increase in the transcripts of a zinc finger gene in the transgenic line, but obviously reduces 5.2~6.8-fold in mRNA accumulation of zinc finger gene in the roots and 2.4~2.7-fold in the shoots compared to the wild type relative to Pi deficiency (Fig. 9A). Similarly, the transcripts of the *OsWRKY* gene (Os01g0656400) in the roots and shoots of the transgenic line also increased several fold compared to those in the wild type under Pi deficiency or sufficient Pi (Fig. 9B), and a cation-transporting P-type ATPase (Os03g0203700) also was remarkably up-regulated in the roots and shoots of the transgenic line, especially in the roots either under Pi deficiency or sufficient Pi (Fig. 9C). However, a down-regulated gene in the roots, encodes a glycosyl hydrolase (Os08g0473900), was significantly up-regulated in the shoots of the transgenic line under Pi deficiency or sufficient phosphorus (Fig. 9D). A member of late embryogenesis abundant protein family, the *OsLEA* gene (Os06g0110200), was significantly up-regulated in the transgenic line by several folds under Pi deficiency or sufficient phosphorus (Fig. 9E). The up-regulated gene encoding a cytokinin dehydrogenase (Os01g0187600) is highly expressed in the roots of the transgenic line under Pi deficiency (Fig. 9F). An amino acid transporter, the *OsAAT* Gene (Os03g0654400), was mainly up-regulated in the roots and the shoots under Pi deficiency, and also exhibited up-regulation in the shoots under sufficient phosphate (Fig. 9G). Worth emphasizing is that the AAA^{+}ATPase (Os12g0471100) showed an enhanced increase in the transcripts in the transgenic line, especially in the roots under Pi deficiency or sufficiecy (Fig. 9H). The transcripts profiles of specific differentially expressed genes show a similarity with the relative expressions of the partial differentially expressed genes (Table S4).

Discussion

The *SlMPT3;1*, a Typical Mitochondrial Phosphate Transporter, Functions in Promoting Phosphate Uptake

The mitochondrial phosphate transporter is composed of three repeated segments covering six-transmembrane domains (Kuan and Saier 1993). The *SlMPT3;1* gene possesses two specific conserved domains representing the mitochondria-carrier superfamily, and shares high homology with other mitochondrial phosphate transporters, suggesting that the *SlMPT3;1* gene is a member of mitochondrial phosphate transporters. Similar to *PHOs* (phosphate transporters) (Sun et al. 2012) or *PHRs* (phosphate starvation responsive genes) (Lv et al. 2014) or *PHT* members in plants (Okumura et al. 1998), the *SlMPT3;1* also modulated the uptake of phosphate in rice. In our study, a yeast MB192 mutant carrying the *SlMPT3;1* gene promoted the growth of yeast strains and increased the phosphate uptake under supply of different Pi levels, and exhibited similar function to the *OsPht1;8* in MB192 mutant (Jia et al. 2011). Since the mutant MB192 yeast strain lacking *PHO84* high-affinity phosphate transporter demonstrates growth inhibition (Qin et al. 2012), thus at least proving a fact that the *SlMPT3;1* gene functions in the uptake of phosphate in yeast cells.

Theoretically, the expression levels of the *SlMPT3;1* gene in the transgenic rice are not affected by abiotic environmental factors because of utilizing a constitutive 35S promoter. However, as high-affinity phosphate transporters, multiple plasma membrane phosphate transporters exhibit differential expressions under supply with different Pi levels (Plaxton and Carswell 1999). Especially, the high-affinity phosphate transporters are usually induced by Pi starvation (Furihata et al. 1992), and accumulated more mRNA transcripts in the roots under Pi deficiency to give an enhanced capacity of roots for phosphate uptake (Duncan and Carrow 1999; Shenoy and Kalagudi 2005). This suggests the high-affinity transporters are indeed induced by Pi starvation and play crucial roles in the phosphate acquisition under Pi deficiency. Our study showed that the *SlMPT3;1* gene is a member of typical phosphate transporters, and induced in the roots by Pi deficiency. Therefore, in the case of Pi deficiency, the induction expressions of the *SlMPT3;1* gene in the transgenic rice might be closely related to the accumulation levels of organic acids in rhizosphere. Because Pi deficiency induces more exudation of organic acids like acetic, aconitic, citric, malic, fumaric, lactic, oxalic, and succinic acids from plant roots (Grierson 1992). Recent report found that more

accumulation of organic acids seems to be closely associated with enhanced intracellular APase (acid phosphatase) activity, and overexpression of *OsHAD1*, an intracellular APase, significantly raises levels of organic acids, such as oxalic acid, citric acid, malic acid, and succinic acid in the transgenic rice, and these organic acids might play important roles in catalyzing release of soluble Pi from fixed P sources (Pandey et al. 2017). Studies also confirmed that organic acids in rhizosphere could promote root growth (Dakora and Phillips 2002; Singh and Nielsen 2004; Johnson and Loeppert 2006). It is worth noting that overexpression of the *OsPAP21b* gene encoding a purple acid phosphatase not only improves root's growth and development but also increases phosphate accumulation in rice under Pi deficiency (Mehra et al. 2017).

Increased phosphate accumulation is closely associated with phosphate uptake which are modulated by phosphate transporters in plants (Mimura 1999). Study has shown that overexpressing the *OsHAD1* gene in rice significantly up-regulates the phosphate transporters, *OsPT2*, *OsPT4*, *OsPT6* and *OsPT8,* which are responsible for enhanced Pi uptake under Pi deficiency (Pandey et al. 2017). However, mitochondrial membrane-localized Pi transporters *MPT* (*PHT3*) usually function in the Pi/H^+ symport into the mitochondrial matrix, and overexpression of *AtMPT3* alters Arabidopsis growth by increasing ATP (adenosine triphosphate) and reactive oxygen species (ROS) levels, and by promoting respiration rate (Jia et al. 2015). Therefore, overexpression of the *SlPHT3;1* gene in rice likely executes Pi transport into the mitochondria, and decreases Pi efflux, thus leading an increase of phosphate accumulation in cells (Liu et al. 2011). Because the overexpression of MPT proteins in plants could result in more accumulation of ATP, which is required for the exchange of Pi between cytosol and mitochondria (Jia et al. 2015; Zhu et al. 2012; Lorenz et al. 2015). The uptake of phosphate in rice Nipponbare cultivar was confirmed to be closely associated with tiller number (Wissuwa et al. 1998). In present study, the transgenic rice not only increased the uptake of phosphate in rice, but also raised efficient tiller in rice, thereby resulting in increases of effective panicle number of per plant. Except for increasing phosphate uptake, the transgenic rice overexpressing the *SlMPT3;1* gene significantly enhanced accumulation of compatible solutes such as chlorophyll, soluble sugar and proline. Studies have shown that more accumulation of chlorophyll and soluble sugar in rice also play important role in improving carbohydrate accumulation and photosynthesis under Pi deficiency (Moscatello et al. 2017), and proline not only allows plants to increase cellular osmolarity under water stress, but also also plays regulatory roles in redox buffering and energy transfer, and particularly, the beneficial connections of proline metabolism to the oxidative pentose phosphate pathway and glutamate-glutamine metabolism are of great significance (Paul and Sandeep, 2010). It is indicated that the overexpression of the *SlMPT3;1* modulates the uptake and accumulation of phosphate and compatible solutes in rice, thereby increasing efficient tiller and grain yield of per plant under Pi deficiency.

Up-regulated Genes of the Differentially Expressed Genes Contribute to the Adaptability of the Transgenic Rice to Pi Deficiency

Transcriptomic analyses showed that Pi deficiency significantly up-regulated the expression of the differentially expressed genes which are related to DNA binding and cell component, and inhibited the expressions of the differentially expressed genes in molecular function (Fig. 8A). Usually, these up- or down-regulated differentially expressed genes mainly participate in responsive regualtions in molecular function and phenotypic changes in plants under abiotic stresses (Table S4). For example, both the *GbWRKY1* and the *OsWRKY74* are positively responded to Pi starvation by altering the sensitiveness of plant hormone (Xu et al. 2012; Dai et al. 2016). The *AP2/ERF* (Os02g0676800) and *MYB* transcription factors (Os01g0298400) were obviously up-regulated in the transgenic rice, suggesting that the up-regulations of these transcription factors might participate in the regulation of physiological responses under Pi starvation (Li et al. 2012). Particularly, the presences of three P1BS domains that specifically bind to the *MYB* transcription factors in the *SlMPT3;1* promoter (LOC101258422 gene) might involve an acclimation regulation under Pi starvation (Oropeza-Aburto et al. 2012). These studies suggested that plant transcription factors play important regulatory roles in responding to abiotic stresses through a DNA recognizing or binding pathway (Khatun et al. 2017). Two zinc finger C_2H_2 type transcription factor genes, *ZAT6* and *ZAT8,* could regulate the root development and phosphate acquisition in plants in response to Pi starvation (Devaiah et al. 2007; Ding et al. 2016). Our data showed that transgenic line OE30 accumulated more mRNA of zinc finger gene (Os02g0686100) in both the roots and the shoots compared to the wild type under Pi deficiency. The OE30 line up-regulated the expression of the cytochrome P450 gene (Os01g0561600) that functions in regulating the synthesis of physiological substances which play important roles in enhancing plant resistance to abiotic stress (Thibaud et al. 2010). Apparent up-regulation of a major facilitator superfamily member (MFS) (Os01g0871500), an amino acid transporter (Os01g0908600), and a phosphate-induced protein (Os02g0756800) in the transgenic OE30 line directly or indirectly affects the rice growth development under Pi deficiency. Additionally, the P-type ATPase can function in acidizing the rhizosphere, thereby affecting the roots architecture and increasing the uptake of phosphate in the plants (Yang et al. 2007). In our study, significant up-regulations of the ATPase (Os08g0448000 and

Os08g0384500) genes in the OE30 line might confer a modulation in rhizosphere environment and promoting phosphate uptake. It seems that the *SlMPT3;1* gene participates in the up-regulation or down-regulation of the partial differentially expressed genes that are involved in physiological metabolism in the transgenic rice in response to Pi deficiency.

Conclusion

The *SlMPT3;1* belongs to a member of mitochondrial phosphate transporters in the PHT3 family, and functions in compensating for the uptake of phosphate in the yeast mutant lacking phosphate transport system. Overexpression of the *SlMPT3;1* gene in rice profitably alters the physiological metabolites and the uptake of phosphate via a metabolism regulatory pathway, thus leading to an increase in effective panicle number and grain yield of per plant under Pi starvation. The down-regulation or the up-regulation of the differentially expressed genes might contribute to a promotion in phosphate translocation in the transgenic rice under Pi deficiency. This study suggests that the *SlMPT3;1* gene could play an important regulatory role in promoting the uptake of phosphate under Pi deficiency, and provides a novel clue for the selection of ideal candidate gene in targeting at improving the uptake and utilization of phosphate in crops under Pi deficiency.

Materials and Methods

Plant Materials and Culture

The seeds of rice *Oryza sativa* L. ssp. japonica cultivar Kitaake were sterilized by a solution of 30% hydrogen peroxide at room temperature (25°C-28°C) for 20 min, then washed with sterilized water and substantially cultured on the solid MS medium at 30°C for 14 d under favorable conditions. The generated rice seedlings were continuously cultured for one week and transferred into the Hoagland's solutions, and further cultured in greenhouse under 16 h light at 30°C with 70% humidity for 28 d, and then transferred into the fresh Hoagland's solution containing 50 μM of Pi (LP) or 1.25 mM of Pi (NP), and further cultured for 30 d by renewing nutrient solutions in an interval of 5 d. Similarly, for recording the growth parameters, the same number of two group rice seedlings of four-week-old was separately transplanted into two boxes containing Hoagland's solution of 50 μM of Pi (LP) or 1.25 mM of Pi (NP), and cultured for three months by duly renewing culture solutions. For grain yield measurements, both wild type and transgenic rice of four-week-old seedlings were transferred into the plastic boxes filled by a mixture of peat soil and vermicular (1:1, v/v), and cultured in net house under natural photo light with a disposable supply of 500 ml of Hoagland's solution containing 50 μM of Pi (LP) or 1.25 mM of Pi (NP), and continuously watered for three months. Tomato seedlings of one-week-old were planted in the pots filled by a mixture of peat soil and vermicular (1:1, v/v), and cultured under the same conditions as rice hydroponics, and used for analyses of the specific expressions of the target gene. Tobacco *N. benthamiana* were grown in the pots filled by a mixture of peat soil and vermicular (1:1, v/v) at 30°C with 70% humidity for 45 d, and used for subcellular localization. Plant tissues were sampled by at least three biological replicates, and placed in liquid nitrogen and stored at -80°C.

Nucleotides Preparation and Isolation of the *SlMPT3;1* Gene

Total RNA of the tomato and rice was extracted for cDNA synthesis using the TRIzol Extract Kit, and rice genomic DNA was extracted by CTAB method (Lota et al. 2013). RT-PCR was performed by using Eeasyscript One-step gDNA Removal and cDNA SythesisSupermix kit (TransGen Biotech, Beijing, China) with a pair of specific primers, *SlMPT3;1*-F and *SlMPT3;1*-R, by referring the expressed sequence tag (EST) database (dbEST ID: 27416938) (Table S1; TransGenBiotech), and a full length cDNA fragment was obtained and completely sequenced by both strands, and defined as the *SlMPT3;1* (Gene ID: AFQ98279.1).

DNA Sequencing Analyses and Vectors Construction

For gene manipulation, DNA sequencing was performed by Sunbiotech (Sunbiotech, Beijing, China). Multiple alignments were carried out by the software DNAMAN (version 6.0). Phylogenetic tree was established by the software MEGA5 (version 5.1). The molecular mass and structure of the protein were predicted by a specific ProtParam (http://web.expasy.org/protparam/) snd by TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html), respectively. The orthologues of the SlMPT3;1 gene were searched against the gramene database (http://ensembl.gramene.org/Tools/Blast?db=core). With a help of a specific *Nco*I restriction enzyme site, a full length cDNA of the *SlMPT3;1* gene was inserted into the binary vector pCAMBIA1304 under control of cauliflower mosaic virus 35S promoter, and the resulting constructs were transformed into *Agrobacterium tumefaciens* strain to transform rice. For localizing the target gene, the *SlMPT3;1* cDNA fragments were inserted into the restriction sites of *Pst*I and *BamH*I in a 16318hGFP vector.

Subcellular Localization

To profile subcellular localizations of the target genes, three target constructs, the empty 16318hGFP vector, the *SlMPT3;1::16318hGFP* construct, and the *SlMPT3;1::16318hGFP* construct wtih Mito-marker, were severally transformed into the *N. benthamiana* protoplast in the presence of PEG, respectively (Yoo et al. 2007). In brief, a total of 2 μg of each construct plasmid was transformed into the protoplast suspensions with approximately 2×10^5 cells, and the transformants were instantly cultured in protoplast medium (R2S plus 0.4 M mannitol) at 25°C over 16 h, and as a control, the 16318hGFP empty vector was also transformed at the same time and the generating transformant was cultured under the same condition. The GFP fluorescence signals in the transformed protoplasts were detected at the emission wavelengths ranging from 490 nm to 520 nm using a Zen LSM710 confocal laser scanning microscope.

Functional Complementation Assay of the *SlMPT3;1* in Yeast

To confirm the regulatory role in phosphate transport of the SlMPT3;1-mediated in yeast, a complementary function testing was performed by recovering yeast MB192 mutant lacking the high-affinity phosphate transport system (Jia et al. 2011). Firstly, the *SlMPT3;1::p112AINE* construct was introduced into the yeast MB192 mutant by LiAc/Single strand DNA/PEG method (Gietz and Schiestl 2007). Three independent yeast strains including the wild type, MB192 mutant, and the mutant carrying the *SlMPT3;1::p112AINE* were cultured in the liquid YNB mediums containing 20 μM or 60 μM or 100 μM of Pi with shaking at 250 rpm at 30°C for 24 h, respectively. The exponential growth profiles at 600 nm were detected by a spectrophotometer (DR/4000U; HACH, Loveland, Colorado, USA), and the supernatants of each

strain culture solution were harvested separately by removing the cells from medium by centrifugation of 10800×g, and drying the superanants at 80°C for a digestion in 4 ml of H_2SO_4. Finally, the amounts of residual Pi in the supernatants were measured by the same method as Pi measurement in rice, and thus evaluating the uptake of Pi in yeast strains.

Plant Transformation and PCR Detection

Rice seeds were sterilized and cultured on the basic solid medium of 1/2 MS containing 1 mg·L^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid) to induce embryonic callus, and the transformants of *Agrobacterium tumefaciens* strain EHA105 carrying vector *SlMPT3;1*::pCAMBIA1304 were used to infect embryonic callus on the 1/2 MS basic solid medium with 0.5 mg L^{-1} of NAA (1-Naphthaleneacetic acid) and 0.2 mg L^{-1} of 6-BA (6-Benzylaminopurine), then the generating embryonic callus were further cultured in a growth chamber at 28°C with 70% humidity for 5 weeks. The generating rice seedlings were transferred onto the MS basic medium containing 80 mg L^{-1} of hygromycin (Sigma F5506, St Louis, MO, USA), and continuously cultured under the same conditions as described above. The transgenic rice seedlings were transplanted into the soils and continuously cultured in a greenhouse at 30°C with 70% humidity under 12 h light for one month, and a total of 12 T_0 generation rice seedlings was confirmed to be the positive transgenic lines by PCR detection. All of the positive transgenic rice were transplanted into the soils and continuously grown until harvesting rice seeds under the same conditions. By continuously screening of two years, a total of 9 of T2 generation transgenic rice exhibited stable expression of the *SlMPT3;1* gene, and three representatives of T3 generation transgenic lines, OE24, OE28, and OE30, were randomly selected for subsequent experiments.

Histochemical GUS Staining

To localize the SlMPT3;1::GUS in the transgenic rice, a histochemical GUS staining was conducted (Ai et al. 2009). Briefly, the roots and shoots of one-week-old rice seedlings were soaked in the GUS staining solution containing 10 mL of phosphate buffer saline (pH 7.2), 1 mL of 0.5% (v/v) Triton X-100, 2 mL of $K_3Fe(CN)6$, 2 mL of $K_4[Fe(CN)_6]$, 2 mL of EDTA, and 104 mg of X-Gluc, and further cultured under stationary state at 37°C over 12 h, and rinsed and fixed in a mixture of FAA with 5 mL of formaldehyde, 5 mL of acetic acid, and 90 mL of 60% ethanol, and then evacuated over 24 h and sliced into the transverse sections of 10 μm thickness using a manual rotary microtome (CUT 5062; Slee, Mainz, Germany). Finally, the paraffin sections were prepared to detect the GUS signals under a stereo microscope (DFC420C; Leica, Wetzlar, Germany) (Jia et al. 2011).

Southern Blotting and RT-qPCR

For determining the copy number of the *SlMPT3;1* gene in the transgenic rice, Southern blotting was performed by a digoxigenin label Kit (Cat No. 11585614910; Roche, Mannheim, Germany). In detail, a total of 20 μg of gDNA from each rice line was separately digested by *Eco*RI enzyme at 37°C for 20 h, and separated by gel electrophoresis, and then transferred onto a nylon membrane (Kit 11209272001, Roche). The probes of the *SlMPT3;1* cDNA were labeled by digoxigenin using standard PCR procedure, and hybridized with the nylon membranes in a solution containing 50% formamide in the presence of high SDS with continuous rotation at 42°C overnight. Plants tranformed with an empty pCAMBIA1304 vector and the wild type were designated as positive control and negative control, respectively. Washing steps were continuously performed under high-stringency conditions, and the hybridized bands were detected and imaged by anti-digoxigenin-alkaline phosphatase using immunoassay method (Dai et al. 2012).

For verifying the accumulation patterns of the *SlMPT3;1* transcripts, we performed RT-qPCR to profile the relative expression levels of the *SlMPT3;1* gene in tomato and rice. Similarly, the relative expression leves of the partial DEGs were also detected by RT-qPCR. Two internal reference genes, the *SlActin* gene in tomato (Gene ID: Sl101260631) and the *OsUbiquitin* gene in rice (Gene ID: Os4333728), were used as control. Gene-specific primers used for RT-qPCR are shown in Table S1. RT-qPCR procedures were performed in a reaction solution of 25 μl by using a kit of TransStartTipTop Green qPCR SuperMix (TransGenBiotech, Beijing, China). All data were analyzed by SPSS.PASW.Statistics.version18.

Tiller Number, Efficient Panicle, Grain Yield, and Physiological Measurements

For observing grain yield of rice, a pot culture experiment was performed to record average tiller number and efficient panicle of per plant at the heading stage, and measure grain number and grain yield of per plant at the maturity stage. For physiological measurements, the fresh leaves of rice from hydroponics before heading were sampled by at least three biological replicates.

Approximately 0.5 g of fresh leaves was extracted with 10 mL of 95% ethanol, and the chlorophyll contents were determined by the absorbance readings at 645 nm and 663 nm with a spectrophotometer (HACH). Total chlorophyll was calculated by the following formula: chlorophyll contents (mg g^{-1} FW) =[C_T (mg/L) × V_T mL × V_F] / (W (g) ×1000). Here, C_T (mg/L)=$20.29A_{645}$+ $8.05A_{663}$; C_T: Total chlorophyll; V_T: Testing volume; V_F: Folds of diluted volume; W: Weights of samples (Wintermans and De Mots 1965).

Soluble sugar content in leaves was determined by referring the anthrone method (Irigoyen et al. 1992). A total of 0.5 g of fresh leaf was ground into power with liquid nitrogen in mortar and boiled in 10 mL of distilled water for 1 h, and the resulting solution was centrifuged by 10,800 × g at 4°C for 20 min. Then 0.5 mL of mixed reagent (1 g of anthrone in 50 mL of ethyl acetate) and 5 mL of H_2SO_4 (98%) were added to the supernatant, and boiled in a water bath for 1 min. When the extracts were cooled to the room temperature (25-28°C), the absorbance at 630 nm was determined using a spectrophotometer (HACH). The same procedure was performed to establish sucrose standard curve by determining the contents of sucrose in standard solutions containing 0, 20, 40, 60, 80 and 100 μg mL^{-1}, respectively. The soluble sugar contents were expressed by the sucrose standard curve using mg g^{-1} FW.

A total of 0.5 g of fresh leaf was cut into pieces and boiled in a solution of 5 mL of 3% sulfosalicylic acid for 10 min, then cooled to room temperature (25-28°C) and filtered. The filtrate was used to measure the proline contents based on the absorbance of the extract at 520 nm using the same spectrophotometer as described above (Bates et al. 1973). Similarly, a standard curve also was established by detecting each independent solution containing appreciate concentration of proline using the same procedure as sample testing.

For Pi measurements, a total of 0.25 g of dried plants was powdered in grinder, and soaked in H_2SO_4 overnight, and digested at 180°C for 1 h, and then continuously digested by adding a little H_2O_2 at 300°C for 2 h. After cooling to room temperature, the digested solution was diluted to a volume of 50 mL by distilled water, then the contents of phosphate were measured by the molybdenum blue colorimetric method using a continuous flow analytical system (Auto analytical 3 flow injection analysis, Germany).

Microarray Sequencing Analyses

To profile the differentially expressed genes and metabolic regulation of the *SlMPT3;1* gene-triggered, both the wild type and the transgenic OE30 rice seedlings of two-week-old were cultured on the MS medium containing 50 mM of Pi (LP) or 1.25 μM of Pi (NP) for 15 d in a growth chamber, then the roots of each treatment with three

biological replicates were sampled to construct cDNA libraries, and high-throughput microarray sequencing was performed. In brief, the roots samples of each line were harvested and stored at -80°C. Total RNA was prepared using the Trizol kit (Invitrogen, Carlsbad, CA, USA), and standard RNAs with high quality were transcripted into double strands cDNA, and then cDNA libraries were established by the end-repaired with T_4 polymerase and adapter addition with T_4 ligase, and finally detected by Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA). Specific-DNA sequencing was conducted by using a HiSeqTM2500 system (Beijing Auwigene Ltd. China). The reads and the differentially expressed genes were confirmed by the DEGseq software (Anders and Huber 2010). Both volcano and venn diagrams were predicted to exhibit the distribution profiles of overlapping genes. Three ontologies, biological processes, cellular component, and molecular function, were explained by the Gene Ontology (GO) enrichments that specially illuminate the biological functions of DEGs by GOseq (Young et al. 2010). Each GO term represents one function or property.

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Author's Contributions

GY performed hydroponics, tissue culture, physiological measurements, and preparation of manuscript; YL supervised and advised to the experiments; SH participated in the measurements; RH analyzed the data; XCh designed the experiment and corrected the manuscript.

Supporting Information

Table S1. Specific primers used for vectors construction and qRT-PCR.
Table S2. Phosphate transporters information for constructing phylogenetic tree of SlMPT3;1.
Table S3. The biomass of the transgenic lines and the wild type under different Pi supply.
Table S4. Basic information of the partial up-regulated or down-regulated genes.

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