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Overexpression of *OsPHT1;4* Increases Phosphorus Utilization Efficiency and Improves the Agronomic Traits of Rice cv. Wuyunjing 7

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Abstract: Inorganic phosphate (Pi) is taken up by plant roots and translocated via phosphate transporters. Previously, we showed that phosphate transporter *OsPHT1;4* in the *PHT1* family participates in phosphate acquisition and mobilization; it facilitates the embryo development of Japonica rice Nipponbare. This study investigated the potential of manipulating the expression of *OsPHT1;4* to increase Pi acquisition efficiency and crop productivity in rice cv. Wuyunjing 7 (WYJ 7), a cultivar widely grown in Yangtze River Delta of China. The *OsPHT1;4* overexpression lines and wild-type WYJ 7 were treated under different Pi conditions in hydroponic and field experiments. Quantitative real-time RT-PCR analysis and the transgenic plants expressing GUS reporter gene indicate strong expression of *OsPHT1;4* in roots and leaf collars of cv. WYJ 7. The total P contents in shoots of the *OsPHT1;4*-overexpressing plants were significantly higher under Pi-deficient hydroponic conditions than the wild type under Pi sufficiency and deficiency. ³³Pi uptake and translocation assays confirmed the results. In the field condition, *OsPHT1;4* overexpression lines had a higher P concentration in tissues than the wild type control, and the panicle performance of the overexpression lines including the grain yield was improved as well. Taken together, our results show that *OsPHT1;4* plays an important role in the acquisition and mobilization of Pi in WYJ 7, especially under Pi deficiency. The study highlights the importance of *OsPHT1;4* in improving the agronomic traits of the widely grown rice cultivar in China.

Keywords: phosphate transporter; phosphorus nutrition; P uptake and translocation; grain yield; rice cultivar

1. Introduction

The essential macronutrient phosphorus (P) is a constituent of many biologically important molecules such as nucleic acids and phospholipids. It plays a pivotal role in plant growth and development and several metabolic pathways and energy transfer processes in plants [1]. However, inorganic phosphate (Pi), the major form of P taken up by the roots from the soil, is often a limiting nutrient in the rhizosphere [1]. The acquisition of Pi from the rhizosphere by the plant root and its translocation between cells is via Pi transporters (PTs) of the *PHT1* gene family [1,2]. The first genes encoding putative

plant PTs were isolated from *Arabidopsis* [3]. Since then, a large number of PT genes have been identified from different plant families, including cereals, legumes, and Solanaceous species [4].

In rice (*Oryza sativa* L.), 13 genes encode proteins belonging to the PHT1 family [5,6]. Several of them have been functionally characterized. Generally, these PHT1 members display different characteristics either in response to Pi concentration or in physiological functions. *OsPHT1;2* and *OsPHT1;6* are responsive to Pi starvation. *OsPHT1;2* is expressed throughout the stele in the primary and lateral roots under Pi deprivation; it is responsible for transporting Pi from roots to shoots, whereas *OsPHT1;6* is expressed throughout the young primary and lateral roots under P deficiency. It plays an important role in both Pi uptake and translocation throughout the plant [7]. *OsPHT1;1* is constitutively expressed in roots and shoots under both Pi-sufficient and -deficient conditions. It participates in Pi uptake and translocation under Pi-sufficient conditions [8]. Similarly, *OsPHT1;8* is expressed in various tissues independent of Pi supply, and overexpression of *OsPHT1;8* resulted in excessive Pi in both roots and shoots [9]. *OsPHT1;9* and *OsPHT1;10* are induced in root epidermis, root hairs, and lateral roots by Pi starvation [10]. *OsPHT1;3* mediates Pi uptake, translocation, and remobilization under extremely low Pi regimes [11], while *OsPHT1;11* and *OsPHT1;13* are specifically induced during mycorrhizal symbiosis [6,12].

We previously investigated the functions of *OsPHT1;4* in rice cv. *Nipponbare* [13]. Our findings suggest that *OsPHT1;4* facilitates the acquisition and mobilization of Pi, and it plays an important role in the development of the embryo [13]. Since the Pi uptake efficiency and the development of the embryo are important events to crop production, we hypothesized that *OsPHT1;4* may play a major role in enhancing grain yield. Rice is one of the most important staple food crops for almost half of the world's population, of which about 90% is consumed in Asia alone. To meet the food demand of the rapidly growing world population, increasing crop yield through various biotechnologies including manipulating valuable gene expression proved to be promising [14]. Rice cv. Wuyunjing 7 (WYJ 7) is a japonica variety widely cultivated in China's Yangtze River Delta. Therefore, the study on the improvement of yield per plant for the crop has practical value.

In the present study, we investigated the functions of *OsPHT1;4* in WYJ 7 on phosphate uptake efficiency and its potential in yield improvement. We found that the uptake and mobilization of Pi significantly increased in all tissues of *OsPHT1;4*-overexpressing plants tested, and a pivotal role of *OsPHT1;4* in the agronomic traits of the cultivar was identified. The results lay the foundation to create rice plants that require less P fertilizer without sacrificing production by genetic engineering.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Rice [*Oryza sativa* L. ssp. *Japonica* cv. Wuyunjing 7 (WYJ 7)] was used as the wild-type and for transformation in this study.

Light-proofed plastic boxes of 7L were used for hydroponic cultivation. Seeds were surface-sterilized with diluted (1:3, *v/v*) NaClO₄ for 30 min, followed by rinsing with deionized water for 30 min. They were germinated in the dark at 25 °C for 3 days. Seedlings (10 days old) were transferred to the hydroponic boxes containing IRRI solution (1.25 mM NH₄NO₃, 1 mM each of CaCl₂ and MgSO₄, 0.5 mM Na₂SiO₃, 0.4 mM K₂SO₄, 0.2 mM KH₂PO₄, 20 μM Fe-EDTA, 20 μM H₃BO₃, 9.0 μM MnCl₂, 0.77 μM ZnSO₄, 0.39 μM Na₂MoO₄, and 0.32 μM CuSO₄). Each plastic box contains 20 rice seedlings, and rice seedlings were fixed with a sponge on a foam board covering the plastic boxes. +P treatment was implemented by following the recipe while -P treatment was carried out by reducing the KH₂PO₄ concentration of the recipe to 10 μM. Plants are grown hydroponically in an artificial greenhouse (16-h light, 30 °C/8-h dark, 22 °C; 70% relative humidity). Plant height and root length were measured after the plants had been treated in the solution for 21 days, and the shoots and roots were collected separately and dried in an oven at 70 °C for 1 week for phosphorus content determination. For qRT-PCR experiments, rice

plants grown for 1 week under different treatments were put into liquid nitrogen before being stored at -80°C .

For field experiments, seeds were first sterilized with 30% hydrogen peroxide for 30 min and then rinsed thoroughly with deionized water. Transgenic seeds were soaked in water containing 25 mg/L hygromycin, and wild-type seeds were soaked in water for 3 days, then the seedlings were sown in the Pailou Experimental Base of Nanjing Agricultural University for field growth. One month later, seedlings of the wild-type and OsPT4-Ox lines with the same growth vigor were selected and transplanted in the paddy fields at the interval of 21 cm between rows and 12 cm between plants. To follow the normal management, the paddy field was adjusted to contain 120 kg N/ha (urea), 70 kg P/ha (potassium dihydrogen phosphate), and 260 kg K/ha (potassium chloride). The rice plants mature after about 130 days of growth. After measuring agronomic traits such as plant height and tiller number, it was dissected into different parts and moved to a 70°C oven to dry for a week, and then the samples were prepared to extract total phosphate. For qRT-PCR experiments, the fresh plant samples were put into liquid nitrogen before being stored at -80°C after collection from the field.

2.2. RT-PCR and qRT-PCR Analyses

Total RNAs from various tissues of rice cv. WYJ 7 were isolated using the Trizol reagent (Invitrogen). Reverse Transcription-PCR (RT-PCR) was carried out by using gene-specific primers for *OsPHT1;4* (primers were listed in Supplementary Table S1). The PCR products were analyzed on an agarose gel (1%, *w/v*) and images were captured with a CCD camera. For qRT-PCR, first-strand cDNAs were synthesized from the total RNAs (HiScript II Q RT SuperMix for qPCR + gDNA wiper, Vazyme, Nanjing, China) according to the manufacturer's instructions followed by qRT-PCR (AceQ qPCR SYBR Green Master Mix, Vazyme on the StepOnePlus™ Real-Time PCR System, Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions. *OsActin1* (LOC_Os03g50885) was used as an internal control for RT-PCR and qRT-PCR analyses.

2.3. Plasmids Construction and Plant Transformation

The full-length open reading frame (ORF) of *OsPHT1;4* was ligated under the control of a ubiquitin promoter in the pS1a-4 for *OsPHT1;4* overexpression (*OsPHT1;4*-Ox). The final constructs were transferred into *Agrobacterium tumefaciens* strain EHA105 by electroporation and subsequently transformed into rice WYJ 7 as described previously [15]. Three independent *OsPHT1;4*-Ox transgenic lines were used in this study. A list of primers used for the cloning is provided in Supplementary Table S2.

2.4. Southern Blot Analysis

The three independent *OsPHT1;4*-Ox transgenic lines were confirmed by Southern blot analysis. Genomic DNA (80–100 μg) was digested overnight with *Bam*HI and *Xho*I at 37°C , separated by agarose gel (1%, *w/v*) electrophoresis, and blotted onto a nylon membrane (Hybond N+, Amersham, UK). The membrane was hybridized with a probe based on the hygromycin resistance gene fragment presented in the plasmid. The membrane was washed twice with a solution containing $1\times\text{SSC}$ and SDS (0.1%, *w/v*) for 15 min at 65°C . The signal on the washed membrane was captured (ScanMaker S260, Microtek, Budapest, Hungary).

2.5. Measurement of Total P Concentrations

Total P concentrations were determined after the plant samples had been dried at 70°C for 3 days. The dried sample (50 mg) was predigested in glass tubes containing 1 mL of deionized water and 5 mL of H_2SO_4 overnight. Afterward, the tubes were heated at 180°C for 20 min and then at 280°C for 10 min. During the 30 min heating step, 30% hydrogen peroxide (50 μL) was added slowly every 10 min until the solution turned colorless [8,16]. After cooling to ambient temperature, the digested sample was diluted to 100 mL with distilled water. The total P concentration in the solution was assayed as described [16].

2.6. ^{33}P Uptake Assay

Ten-day-old wild-type and *OsPHT1;4-Ox* seedlings were transferred to +P medium (200 μM Pi) or –P medium (10 μM Pi) for a further 7 days, then transferred to medium containing 0.5 mM CaCl_2 and 2 mM MES (pH 5.5) for 10 min. Then plants were transferred to a 300 mL growth medium (as described above) containing 200 kBq of $\text{H}_3^{33}\text{PO}_4$ and incubated at 28 °C for 3 h. After labelling, roots were immersed in ice-cold desorption solution (0.5 mM CaCl_2 , 100 μM unlabeled NaH_2PO_4 , 2 mM MES, pH 5.5) for 10 min to remove the apoplastic ^{33}P . Roots were harvested and digested using HClO_4 and H_2O_2 at 70 °C for 2 h to 3 h with intermittent shaking until the solution was colorless. Following digestion, 1 mL TritonX-100 and 3 mL scintillation cocktail (Ultima Gold; Perkin-Elmer, Waltham, MA, USA) was added to the digested material and incubated for 24 h. ^{33}P radioactivity was determined by liquid scintillation counting (Tri-Carb 2100, Packard, Detroit, MI, USA). The sum of the ^{33}P cpm in the root and shoot portion of each plant was divided by the mass of the root to obtain the specific uptake of ^{33}P over the incubation period.

2.7. Statistical Analysis

Data were collected from two or three independent biological experiments and analyzed for significant differences (SPSS Statistics 20, IBM, Armonk, NY, USA). Duncan's multiple range test at $p < 0.05$ was carried out for all experiments to determine the significance of differences between the control and treatment plants.

3. Results

3.1. *OsPHT1;4* Is Responsive to Pi Starvation and Is Abundantly Expressed in the Collar and Leaf Sheath of Rice cv WYJ 7

To determine the transcriptional expression of *OsPHT1;4* in response to Pi starvation and in different tissues of rice cv. WYJ 7, quantitative real-time PCR (qRT-PCR) analysis was employed (Figure 1). The transcript level of *OsPHT1;4* was higher in the root compared with the leaf blade under Pi-sufficient (+P) and -deficient (–P) conditions. Moreover, the expression of *OsPHT1;4* was significantly upregulated in response to Pi starvation in both root and leaf blade (Figure 1A). Previous studies have shown that the expression of *OsPHT1;4* was high in spikes [17], but we found different results in WYJ7. In the field growth conditions, the expression of this gene was highest in leaves and lowest in spike at the heading stage (Figure 1B), and *OsPHT1;4* was expressed significantly higher in leaf collar and leaf sheath compared to leaf blade at the mature stage (Figure 1C).

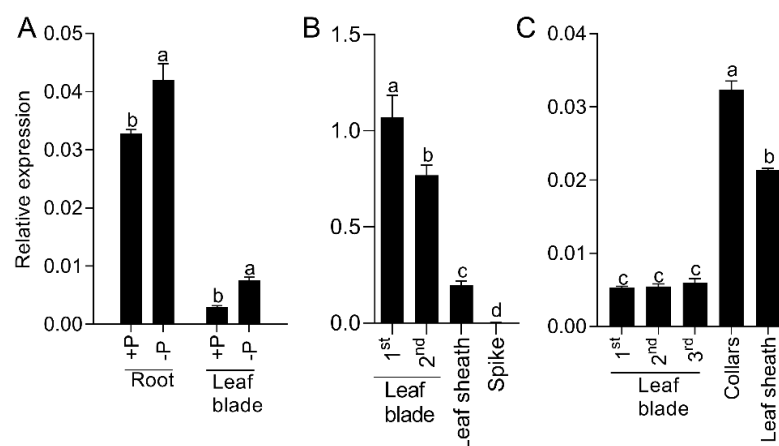


Figure 1. Relative transcript levels of *OsPHT1;4* in rice cv. WYJ 7. The seedlings (3 days old) were grown hydroponically (A) and in pot soil (B,C) for 13 and 20 weeks. Different tissues were harvested and qRT-PCR analysis was carried out to determine the relative transcript levels of *OsPHT1;4*. *OsActin1* was used as an internal control. Values are means \pm SE ($n = 3$). Different letters above the bars indicate significant differences ($p < 0.05$, one-way ANOVA).

3.2. Overexpression of *OsPHT1;4* Promotes the Rice Growth under Both Pi-Sufficient and Pi-Deficient Conditions

We created *OsPHT1;4*-overexpressing transgenic lines based on WYJ 7, hereafter referred to as *OsPHT1;4*-Ox lines, to test the gene's effect on plant performance. According to the results of RT-PCR, qRT-PCR, and Southern blot analysis (Supplementary Figure S1), we selected three independent homozygous *OsPHT1;4*-Ox lines, named Ox1, Ox2, and Ox3, for further study.

Wild-type and *OsPHT1;4*-Ox plants were grown in hydroponic culture and treated under Pi-sufficient (200 μ M) and Pi-deficient (10 μ M) conditions for 3 weeks. The primary root length of the *OsPHT1;4*-Ox lines was about 11.75% longer than that of the wild-type plants under the Pi-deficient condition, but no significant difference was observed under the Pi-sufficient condition (Figure 2A,B). Overexpression of *OsPHT1;4* resulted in a 7–12.6% increase in the shoot and root biomass independent of Pi supply conditions (Figure 2B–D). As a result, overexpression of *OsPHT1;4* in WYJ 7 promoted the growth of the Ox lines under both Pi-sufficient and Pi-deficient conditions by hydroponic cultivation.

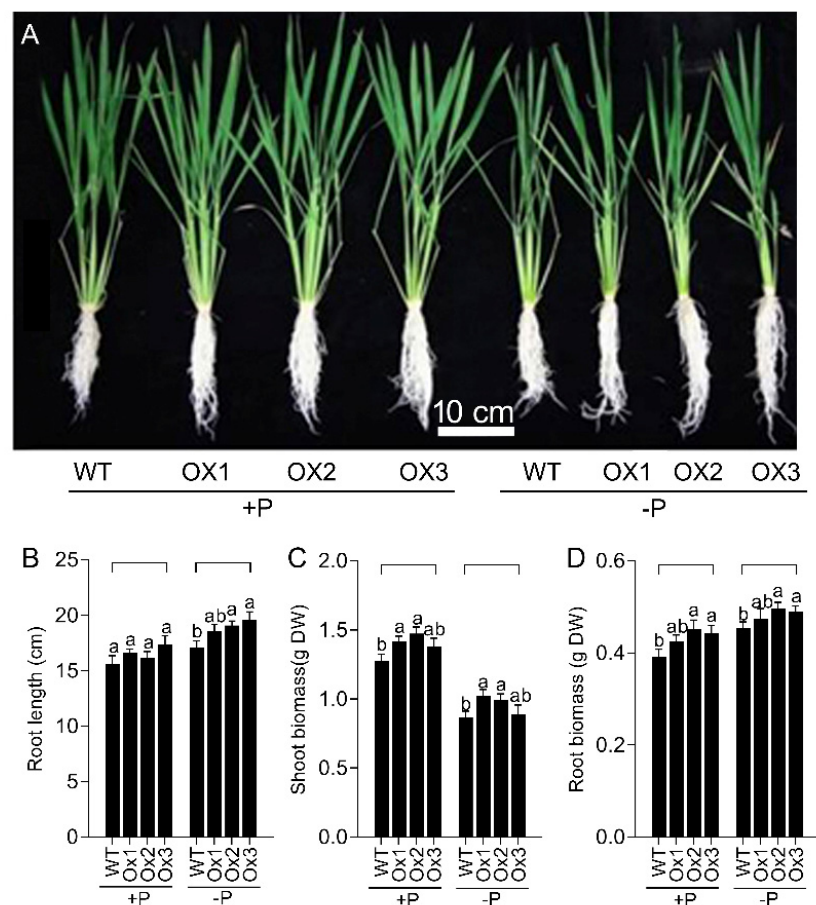


Figure 2. Overexpression of *OsPHT1;4* promotes rice vegetative growth. WT and *OsPHT1;4*-Ox lines (Ox1–Ox3) were grown hydroponically in (A) +P and –P media for 3 weeks. (A) Morphological comparison of WT and Ox1–Ox3 plants grown under +Pi and –Pi regimes. Comparisons of primary root length (B), shoot biomass (C), and root biomass (D) of the Ox plants and WT control. DW: Dry weight. Values are means \pm SE ($n = 5$). Different letters above the bars indicate significant differences ($p < 0.05$, one-way ANOVA).

3.3. Overexpression of *OsPHT1;4* Increases Pi Uptake and Translocation Efficiency under the Hydroponic Condition

To determine the effect of overexpressing *OsPHT1;4* on the maintenance of Pi status, we measured the total P concentration and content in shoots and roots of *OsPHT1;4*-Ox plants under both Pi-sufficient and Pi-deficient conditions. The total P contents in roots of *OsPHT1;4*-Ox plants were comparable to that of WT under both +P and –P. However, the *OsPHT1;4*-Ox plants had 3.37% and 42% higher total P content in shoots than the control under +P and –P, respectively (Figure 3A,B). The P uptake efficiency was calculated according to the modified method of the reference [18]. As shown in Figure 3C, the P uptake efficiency of the *OsPHT1;4*-overexpressing plants was 23% higher than that of the wild-type plants under the Pi-deficient conditions, although the concentrations were comparable between them under Pi-sufficiency (Figure 3C). We also calculated the root/shoot ratio of total P under different conditions, and the results showed that the overexpression tissues under the –P condition were significantly lower than that of the wild-type (Figure 3D). Based on these results, we conclude that overexpression of *OsPHT1;4* in WYJ 7 promoted the transport of P from roots to shoots and enhanced P uptake efficiency under Pi-deficient conditions.

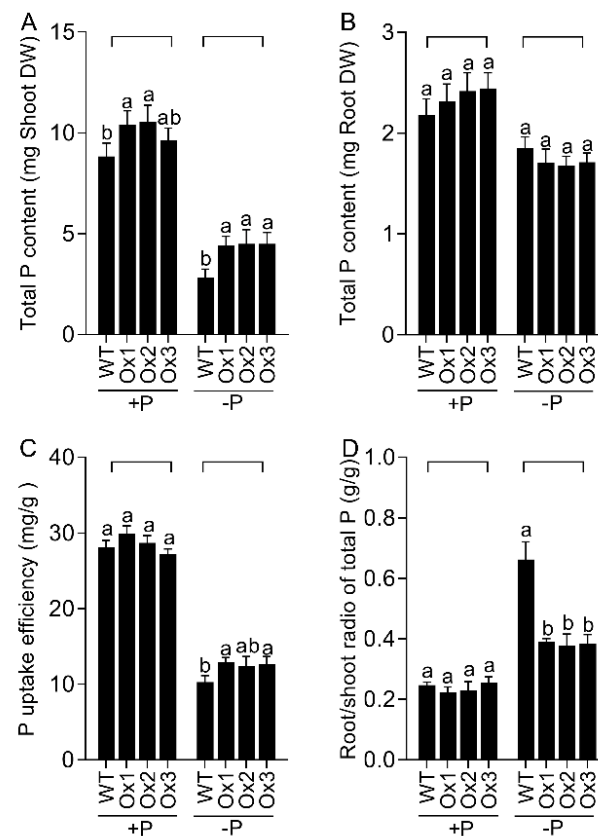


Figure 3. Overexpression of *OsPHT1;4* elevated total P concentration in Pi-deprived shoot. WT and Ox1–Ox3 seedlings (10-d-old) were grown hydroponically in +P and –P media for 3 weeks. Tissues were harvested and assayed for their total P concentrations. Comparisons of total P content of shoot (A), total P content of root (B), P uptake efficiency (C) and (D) root/shoot ratio of total P of WT and Ox1–Ox3 plants grown under +Pi and –Pi regimes. Values are means \pm SE ($n = 5$). Different letters above the bars indicate significant differences ($p < 0.05$, one-way ANOVA).

To further determine the contribution of overexpression of *OsPHT1;4* in WYJ 7, Pi uptake assays were performed using isotope ^{33}P . The results showed that overexpression of the gene led to a significant increase of Pi uptake in roots under both Pi-sufficient and -deficient conditions. The ^{33}P uptake by roots of *OsPHT1;4*-Ox plants was 25–50% higher

than that of the wild-type plants under Pi-sufficient conditions (Figure 4). Similarly, under Pi-deficient condition, ^{33}P uptake by roots of *OsPHT1;4*-Ox transgenic plants was about 15–35% higher compared with the wild-type plants (Figure 4). ^{33}P isotope experiment results further verified the roles of *OsPHT1;4* in the uptake of Pi in roots.

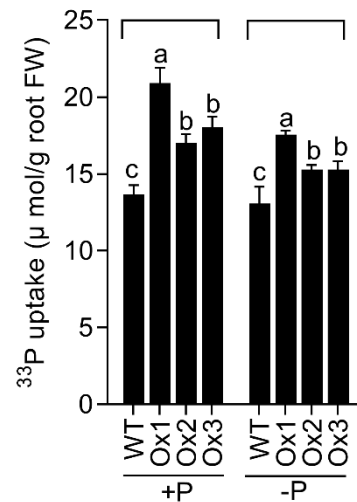


Figure 4. Overexpression of *OsPHT1;4* increases ^{33}P uptake rate. WT and Ox1–Ox3 seedlings (10 days old) were grown hydroponically in +P and –P media for 1 week and then transferred to the respective media containing ^{33}P to incubate for 3 h. Roots were harvested to determine the ^{33}P uptake. Values are means \pm SE ($n = 5$). Different letters above the bars indicate significant differences ($p < 0.05$, one-way ANOVA). FW: Fresh weight.

The expression of *OsPHT1* family genes is a diversely regulated process, we thus examined the expression of other well-characterized *OsPHT1* family genes in the *OsPHT1;4*-overexpressing lines. The results showed that most genes did not change their expressions significantly in the lines, and only the expression of *OsPHT1;10* was significantly inhibited (Figure 5). This result shows that the overexpression of *OsPHT1;4* inhibits the expression of some other *OsPHT1* family genes under phosphorus-sufficient conditions, which is consistent with the previous investigation reported by Ye et al. [19].

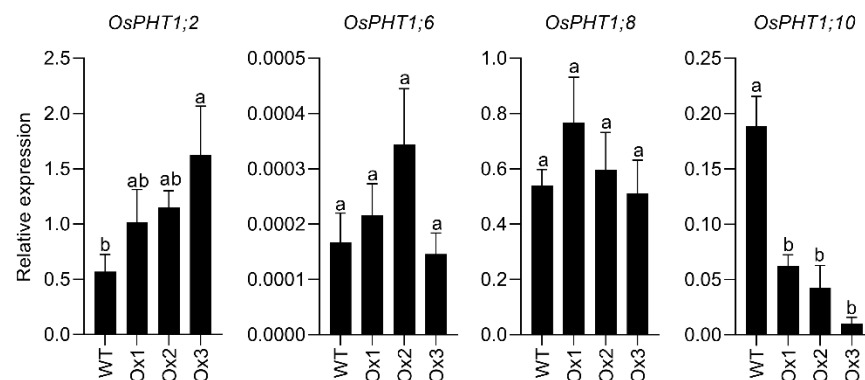


Figure 5. Expression of other *OsPHT1* family genes (*OsPHT1;2*, *OsPHT1;6*, *OsPHT1;8*, and *OsPHT1;10*) in the *OsPHT1;4*-Ox lines. WT and Ox1–Ox3 seedlings (10-d-old) were grown hydroponically in the +P media for 1 week. Values are means \pm SE ($n = 3$). Different letters above the bars indicate significant differences ($p < 0.05$, one-way ANOVA).

3.4. Overexpression of *OsPHT1;4* Enhances Translocation of P under Field Condition

To assess the impact of *OsPHT1;4* overexpression on Pi uptake and translocation during the entire rice growth cycle, we performed experiments on wild-type and *OsPHT1;4*-Ox plants grown in the paddy soil containing 25.25 mg kg⁻¹ Pi. The total P concentrations in various tissues dissected from mature plants under the field were measured. They were significantly higher in all tissues of *OsPHT1;4*-Ox plants as compared to WT plants (Figure 6). The culm and leaf sheath from mature *OsPHT1;4*-Ox plants had 18% and 15% higher P concentrations, respectively, than those from the wild-type plants (Figure 6). Therefore, overexpression of *OsPHT1;4* in WYJ 7 significantly enhanced the accumulation of P in vegetative organs of the transgenic plants. Compared with that of the wild-type plants, the total P concentrations were higher in the panicle axis by 53%, in the unfilled rice hull by 58%, in the rice hull by 21%, and in brown rice by 14% (Figure 6), respectively. These results indicate that overexpression of *OsPHT1;4* was also effective in the accumulation of P in rice reproductive organs.

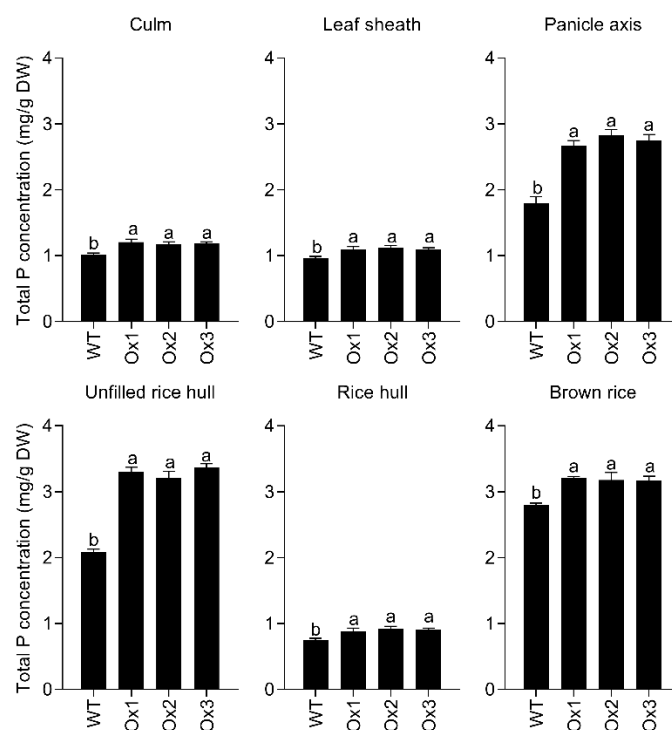


Figure 6. Overexpression of *OsPHT1;4* elevates total P concentration. WT and Ox1–Ox3 seedlings (10 days old) were grown to maturity in the field for 20 weeks. Various tissues indicated on the panel were harvested and the total P concentration was determined. Values are means \pm SE ($n = 5$). Different letters above the bars indicate significant differences ($p < 0.05$, one-way ANOVA).

3.5. Enhanced Pi Utilization in *OsPHT1;4*-Ox Lines Promotes Grain Yield and Agronomic Traits of Rice cv. WYJ7 under Field Growth Conditions

The three *OsPHT1;4*-Ox lines were used for a field experiment in Nanjing, China, over three consecutive years. It was found that the grain density of each panicle of the *OsPHT1;4*-Ox plants was slightly bigger than those of the wild-type plants (Figure 7A). The grain yield per plant and 1000-grain weight of the *OsPHT1;4*-Ox plants were 5% and 7% greater than those of the wild-type, respectively (Figure 7B,C). However, the seed setting rate of the *OsPHT1;4*-Ox plants was not significantly different from that of the wild-type plants (Figure 7B). These results may be caused by the accumulation and translocation of P in the vegetative organs and its subsequent translocation to the reproductive organs. Since the grain yield per plant and 1000-grain weight of the *OsPHT1;4*-Ox plants increased without increasing the seed setting rate of the plants, the length and width of the wild-type

and *OsPHT1;4*-Ox plant seeds were measured. As shown in Figure 7, the seeds from the *OsPHT1;4*-Ox plants were bigger, by 6% longer and 3% wider, than those from wild-type plants (Figure 7E–H). Taken together, the overexpression of *OsPHT1;4* promotes agronomic traits of rice under field growth conditions.

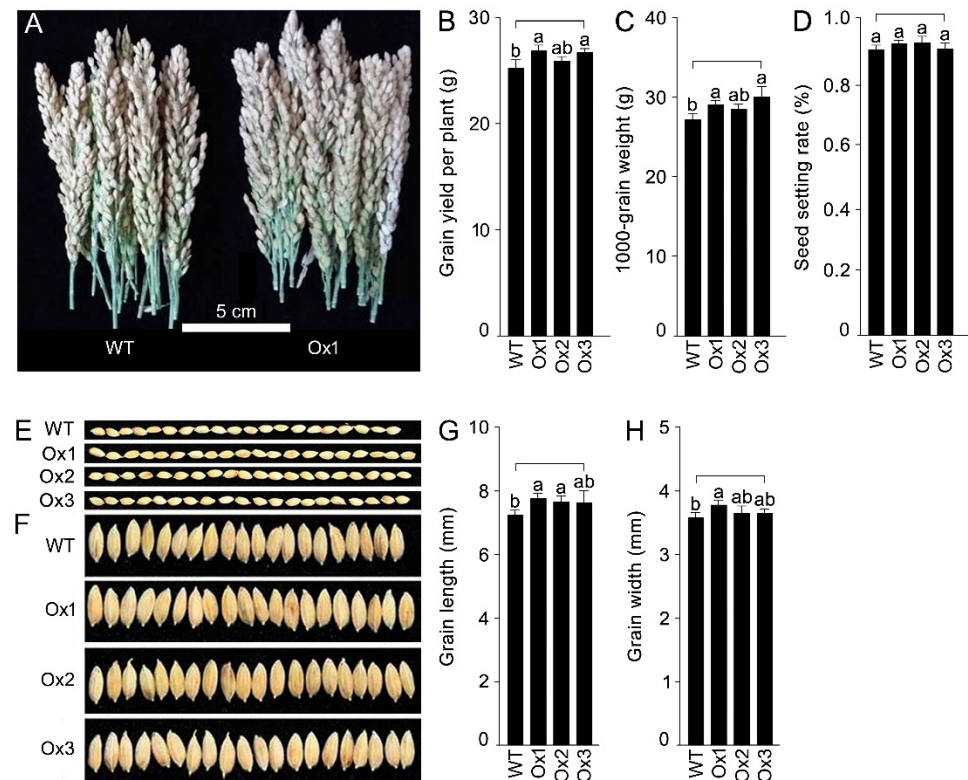


Figure 7. Overexpression of *OsPHT1;4* accentuates yield performance of WYJ 7. WT and Ox1–Ox3 plants were grown in the field as described in the legend of Figure 6. Photographs of material from the WT and Ox1 plants show the phenotypes of panicle (A), grain length (E), and grain width (F). Data are presented for grain yield per plant (B), percent seed setting rate (C), 1000-grain weight (D), grain length (G), and grain width (H). Values are means \pm SE ($n = 5$). Different letters above the bars indicate significant differences ($p < 0.05$, one-way ANOVA).

4. Discussion

4.1. Overexpression of *OsPHT1;4* Promotes the Growth and Grain Yield per Plant

The overexpression of *OsPHT1;4* in WYJ 7 promoted growth in the seedling stage. Both the shoot and root biomass of *OsPHT1;4*-Ox lines were greater than those of the wild type under both Pi-sufficient and Pi-deficient conditions. Moreover, under Pi-deficiency, the primary root length of the *OsPHT1;4*-Ox lines was longer than that of the wild-type plants. In previous work, *OsPht1;1*-Ox lines developed much longer and more dense root hairs, which led to an increase in Pi accumulation under P-replete conditions [8]. The overexpression of low-affinity phosphate transporter *OsPht1;2* in rice enhanced the absorption and translocation of P from roots to shoots, but with yellow leaves and short plants [20]. Although *OsPht1;8*-Ox lines improved the accumulation of P in plants, it hindered the growth of rice as the plant became shorter and displayed Pi toxicity symptoms [9]. The biomass of *OsPht1;3* overexpression lines was significantly decreased under both Pi-sufficient and -deficient conditions, and Pi toxicity symptoms could be observed under Pi-sufficiency [11]. We previously investigated the functions *OsPht1;6* in WYJ 7, the same rice cv. as we used in the present study, and found that the overexpression of *OsPht1;6* improved the absorption and utilization of P in plants. It also promoted the growth and development of the

overexpression of plants [17]. In comparison with *OsPht1;6*, *OsPHT1;4* in WYJ 7 acts more effectively on the agronomic traits, such as grain size and yield per plant (Figure 7).

Improving rice grain yield is an important goal of agricultural production, as it is critical to ensure food security for nearly half of the world's population that are dependent on rice as a staple food. Our previous work demonstrated that the overexpression of *OsPht1;6* improved agronomic traits in rice WYJ 7, the main rice cultivar grown in China's Yangtze River Delta [17], by increasing the effective tiller number, grain weight per panicle, and grain yield per plant compared with the wild-type. In this study, our data showed that the overexpression of *OsPHT1;4* increases the 1000-grain weight and grain yield per plant compared with the wild-type of rice WYJ 7 (Figure 7B,C). These results indicate that both the overexpression of *OsPHT1;4* and *OsPht1;6* in rice WYJ 7 promote the growth of the plants and the grain yield, but the mechanisms of the gene's effects are different.

4.2. *OsPHT1;4* Is Responsive to Pi Deficiency in WYJ 7 and Promotes P Uptake and Transport under Pi Deficiency

The transcript level of *OsPHT1;4* was higher in the roots than in the leaf blades of WYJ 7 under various Pi regimes (Figure 1A), and the transcript levels of *OsPHT1;4* were significantly upregulated by Pi starvation in both the roots and leaf blades (Figure 1A). We previously reported that the transcript level of *OsPHT1;4* was also higher in the root than in the leaf blade in rice cv. *Nipponbare* under Pi-sufficient (+P) and -deficient (-P) conditions [13]. However, the expression of *OsPHT1;4* was comparable in the roots under +P and -P conditions although the transcript level of *OsPHT1;4* was moderately induced by -P conditions in leaf blades [13]. These results show that their expression patterns of *OsPHT1;4* in rice cv. WYJ 7 and cv. *Nipponbare* are different.

The P concentrations of *OsPHT1;4*-overexpressing plants in rice cv. *Nipponbare* and *OsPht1;6*-overexpressing plants in rice cv. WYJ 7 are much higher than wild-type in both shoots and roots under Pi-sufficient (+P) and Pi-deficient (-P) conditions [13,17]. In the present work, the total P contents of *OsPHT1;4*-Ox plants in the shoots were significantly higher than that of the wild-type rice under the Pi-deficient conditions, while the total P contents in the roots were comparable to that of the wild type (Figure 3A,B,D). These data indicate that the overexpression of *OsPHT1;4* in WYJ 7 promotes the transport of P from roots to shoots, especially under Pi-deficient conditions. In addition, the P uptake efficiency of *OsPHT1;4*-overexpressing plants in WYJ 7 was significantly improved compared with the wide-type under Pi-deficient conditions (Figure 3C). The ratio of total P concentration in young leaves to that in old leaves of *OsPht1;6*-Ox plants was significantly higher than that of the wild-type plants under the two different Pi levels (40 and 80 mg fertilizer Pi kg⁻¹ soil) tested in WYJ 7 [13]. These results indicate that both members in the rice PHT1 family can facilitate the uptake, translocation, and remobilization of Pi in rice [7,13,17]. Hopefully, the overexpression of *OsPHT1;4* in WYJ 7 is capable of maintaining normal growth in low-P soil or in the soil with less Pi fertilizer applied, which is one of the foci of agricultural production and environmental protection.

4.3. Overexpression of *OsPHT1;4* Promotes Grain Yield Partly through Increasing Grain Size

Grain size is one of the key factors determining grain yield in crops [21,22]. However, the underlying molecular mechanism is largely unclear. OsMAPK6, a mitogen-activated protein kinase, influences rice grain size by influencing cell proliferation and reducing endogenous brassinosteroid levels of rice [23]. Similarly, mitogen-activated protein kinase 4 is a factor capable of affecting the grain size of rice [24]. In this study, we showed that *OsPHT1;4*-Ox plants increase the 1000-grain weight and grain yield per plant compared with wild-type WYJ 7 (Figure 7B,C). Furthermore, the lengths and widths of *OsPHT1;4*-Ox plants seeds increased significantly compared with the wild-type plants (Figure 7E-H). In our previous work in rice cv. *Nipponbare*, there were significant increases in 1000-grain weight and grain yield per plant in *OsPHT1;4*-Ox1 and -Ox2 compared with the WT [13]. A detailed investigation exhibited the genetic evidence for the role of *OsPHT1;4* during

embryogenesis, and the embryo surface areas of Ox1 and Ox2 were 9–14% larger than that of the WT control [13]. These results provide a possible explanation for the increases of length and width of the grains in the *OsPHT1;4*-Ox plants (Figure 7), and *OsPHT1;4* may exert influence on the embryogenesis and seed development in rice cv. WYJ 7. Alternatively, the increase in grain size is the consequence of the enhanced uptake, translocation, and accumulation of P in above-ground parts of plants via the overexpression of *OsPHT1;4* in WYJ 7. In addition, *OsPht1;8*, another member of the rice PHIT1 family, demonstrated its effect on the allocation of Pi between the embryo and the endosperm [25]. This raised the possibility that a connection may exist between *OsPHT1;4* and *OsPht1;8* during embryogenesis and seed development, which is an interesting and important topic that deserves more detailed investigations.

The ultimate goal of agricultural research is to nurture more people with limited arable land on earth. How to improve yield per unit is critical to achieving the goal. Traditionally, different crop cultivars provide many valuable solutions for yield improvement. In rice, many cultivars including WYJ 7 show much better performance in agronomic traits than Nipponbare. This suggests that these practically used rice cultivars have their unique advantages in agriculture. In this study, our investigation shows that overexpression of *OsPHT1;4* in WYJ 7 increases phosphate utilization efficiency and improves the agronomic traits of the cultivar. It implies that the future improvement of rice yield by manipulating critical gene expression in optimized cultivar is still a feasible method in practical application.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12061332/s1>, Figure S1: Identification of *OsPHT1;4* Overexpressing Lines in WYJ 7. Table S1: List of primers used for semi-quantitative RT-PCR and quantitative real-time PCR analysis. Table S2. Primers used for vector construction.

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