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Full Length Research Paper

Phosphate uptake and growth characteristics of transgenic rice with phosphate transporter 1 (*OsPT1*) gene overexpression under high phosphate soils

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Farmers have used phosphate fertilizer to provide sufficient yields. However, overuse of phosphorus accumulate in soil and causes soil and water pollution. We evaluated the phosphate acquisition and growth characteristics of *OsPT1* transgenic rice (OsPT1-OX, over-expressing the high affinity phosphate transporter 1) in high phosphate soils with different level of nitrogen fertilizer treatment to investigate its removal ability of excessive phosphate from soil. OsPT1-OX had shorter culm length but more tillers than those of wild-type plants in each soil conditions. Phosphate content per dry weight of OsPT1-OX was 1.8 times higher than that of wild-type under control fertilizer treated conditions. Although the dry weight of OsPT1-OX was not different from that of wild-type plants, whole plant phosphate content was 1.7 times higher than that of wild-type plants under control fertilizer conditions. Tiller number and phosphate content per dry weight of wild-type plants increased following high levels of phosphate application, but did not change following additional nitrogen application. Tiller number and phosphate content per dry weight of OsPT1-OX did not also change under the high phosphate condition, but increased following nitrogen application under similar conditions. Whole plant phosphate content was also highest under high nitrogen and high phosphate application conditions. These results suggest that OsPT1-OX may reduce phosphate content in soils containing excess phosphate and may be further effective under high nitrogen condition.

Key words: Phosphate content, fertilizer treatment, phosphate transporter, rice, soil.

INTRODUCTION

Phosphate is one of the most important elements for plant growth and development, as it is incorporated with sugar phosphates, nuclear acids, nucleotides and co-enzymes. Plants absorb phosphate in the form of phosphate ions, represented by $(H_2PO_4)^{2-}$ or $(HPO_4)^2$. Phosphate easily combines with other minerals such as calcium and magnesium, resulting in an available phosphate of about 0.3% in the most cultivated soils of the world. To achieve higher yields under phosphate deficient condition, farmers have used surplus amounts of chemical fertilizers and animal manure especially in developed countries. Although fertilizer applications increase crop yield, they have caused several problems.

The overuse of phosphate fertilizers increase algal productivity in fresh water, potentially leading to ecological problems associated with eutrophication (Gibson, 1997; Haijun and Hongzhu, 2009). Countries including the UK, USA, Canada, France, Germany and China are now paying more attention to the problems posed by lake

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eutrophication due to the continuous and increasing losses of soil phosphorus by surface runoff (Zhou and Zhu, 2003; Edwards and Withers ., 1998; Zhang et al., 2008). Phosphates are mostly obtained from mined rock phosphate and existing reserves may be exhausted in the next 50~100 years (Steen, 1998; Smil, 2000; Smit et al., 2009; Vaccari, 2009; Cordell et al., 2011) Therefore, developing crops with improved phosphate uptake is critical to prevent environmental pollution and sustain agricultural production systems.

Uptake of phosphate at the roots is regulated by membrane-spanning phosphate transporter (PT) proteins (Raghothama and Karthikeyan, 2005; Mimura, 2001). In plant, *PT*s are classified into two forms based on phosphate absorption kinetics and affinity to target phosphate (Furihata et al., 1992). High-affinity *PTs* are induced under phosphate deficient conditions particularly in the roots, whereas low-affinity *PTs* are expressed constitutively in the aerial parts of plants (Daram et al., 1998; Rae et al., 2003). Many high-affinity *PT* genes have been isolated from *Arabidopsis*, potato, maize and tomato (del Pozo et al., 1999; Leggewie et al., 1997; Liu et al., 1998; Miller et al., 2001; Muchhal et al., 1996), suggesting potential targets for improving phosphate uptake (Mitsukawa et al., 1997; Vance et al., 2003). In tomato, transcript levels of high-affinity *PT*s increase under a phosphate deficient condition and transporter protein and phosphate acquisition also increase concurrently (Muchhal and Raghothama, 1999). Similarly, over-expression of a barley high-affinity *PT* gene in rice increased phosphate uptake rate over 2.5-fold (Rae et al., 2003) and in transgenic tobacco, over-expressing the Arabidopsis *PT* gene shows increased phosphate uptake and cell growth (Mitsukawa et al., 1997).

In a previous study, we identified nine kinds of high affinity *PTs* in rice and confirmed their expression pattern under a variety of phosphate conditions (Seo et al., 2008). Among the nine *OsPTs* genes, the Os*PT1* gene was highly expressed under phosphate deficient conditions. To test the possibility that overexpression the *OsPT1* gene would increase phosphate uptake, we generated transgenic rice (OsPT1-OX) that over-expresses the *OsPT1* gene under the control of the CaMV 35S promoter. OsPT1-OX accumulates almost twice as much phosphate in the shoots compared to that in wild-type plants grown under phosphate deficient soil conditions (Seo et al., 2008). Although several studies have been conducted to generate phosphate uptake enhanced transgenic rice, they focused on phosphate deficient condition. Furthermore, the phosphate uptake ability of OsPT1-OX under sufficient phosphate conditions has not been clearly determined. However, excess phosphate in soil has emerged as an important issue of water and soil pollution as well as the phosphate deficient problem.

In this study, we investigated phosphate uptake patterns and growth characteristic of OsPT1-OX under a high phosphate soil condition. We also investigated the possibility of enhancing phosphate uptake by applying additional nitrogen fertilizer.

MATERIALS AND METHODS

Transgenic rice (OsPT1-OX) overexpressing the high affinity *PT1* gene was generated by *Agrobacterium*-mediated transformation in the Japonica rice cultivar, Dongjinbyeo. The *OsPT1* gene was amplified with a specific primer set that included the XbaI site (forward: tgtctagacatggcgggagggcagct, reverse: gctctagaattacttc-gggtaggccgcc) to generate the transformation vector. After digestion of the polymerase chain reaction (PCR) product with XbaI, the *OsPT1* gene was fused into the pBTEX binary vector. The expression construct was introduced into *Agrobacterium tumefaciens* (EHA105) by tri-parental mating (Seo et al., 2008). Transgenic lines were selected based on the *OsPT1* gene expression level. Homozygous plants of the T_9 generation were used for further studies.

Nutrient-treatment for hydroponic culture and the reverse transcription-polymerase chain reaction (RT-PCR)

Seeds of OsPT1-OX and wild-type plants were sterilized in 30% sodium hypochlorite for 30 min and germinated on MS medium to determine *OsPT1* expression. After three days of germination, the plants were transferred and cultured in nutrient solution for two weeks in the greenhouse under a natural photoperiod. The nutrient composition and concentration for the standard solution [S] was as follows: N (1.43 mM), P (0.32 mM), K (0.51 mM), Ca (0.75 mM), Mg (1.64 mM), Fe (0.51 µM), B (18.92 µM), Mn (9.50 µM), Mo (0.10 µM), Zn (0.15 µM) (Yoshida et al., 1976). For the [high-P] solution, 1.6 mM of phosphate was added and 2.86 mM nitrogen was added in high-P solution to prepare a [high-N·P] solution. The pH of the nutrient solution was adjusted to 5.5 using NaOH and the solution was changed every three days.

Total RNA of fresh shoots and roots was isolated 14 days after germination using the TRI reagent (Invitrogen, USA). Total RNA was reverse-transcribed to cDNA using a Prime Script™ One Shot RT-PCR kit (Takara, Japan). The following PCR conditions were employed: first strand cDNA synthesis for 30 min at 50℃ *OsPT1* and actin amplication, second strand cDNA synthesis for 2 min at 94℃, denaturation for 30 s at 94℃ , annealing for 30 s at 58℃, and extension for 1 min at 72 ℃ . After 30 amplification cycles, the PCR product was examined by agarose gel electrophoresis with ethidium bromide staining. PCR primers for synthesized for *OsPT1* (forward: 5′-AGGAGCAGGA-GAAGGCTGACG-3′, reverse: 5′-CACATCGTCATCGTCCTCGTTC-3′) and actin (forward: 5′-ATTCACCACAACGGCCGAGC-3′, reverse: 5′-GGAGGGGCGACCACCTTGAT-3′).

Field experiment

A field experiment was conducted in the GMO field of the National Institute of Crop Science (Miryang, Republic of Korea) from June to October in 2009. Three different fertilization conditions were created in a paddy field (Table 1). Fertilizer application rates in control condition were 9 kg of nitrogen (N), 4.5 kg of phosphorus (P_20_5), 5.7 kg of potassium (K_20) per 10 acre. A five-time greater amount of phosphate fertilizer (22.5 kg/10 a) than control condition was added in excess phosphate condition [high-P]. To investigate effect of nitrogen on OsPT1-OX growth and phosphate uptake, two times higher amount of nitrogen fertilizer (18 kg/10 a) was applied to the excess phosphate condition [High- N·P]. After a 30-day cultivation in the greenhouse, OsPT1-OX and wild-type plants were transplanted to a paddy field. The plant culm length and tiller numbers in each plot were determined by 10 replications at maximum tillering, booting and at the ripening stage.

Table 1. Design of field experiment to test the different fertilizer conditions

Fertilizer Treatment[a]	Fertilizer dose (kg/10 acre)		
	N (Nitrogen) 50 - 20 - 30[b]	P (Phosphorus,$P_2 0_5$) 100 - 0 - 0	K (Potassium,$K_2 0$) 70 - 0 - 30
Control condition	9	4.5	5.7
High-P	9	22.5	5.7
High- N·P	18	22.5	5.7

[a]Fertilizer treatment: Control conditions; moderate combination of NPK fertilizer application, high-P; five-folds phosphate fertilizer compare to control condition, High-NP; two-folds nitrogen and five-folds phosphate fertilizer to compare to control condition. [b]A split method was used for the nitrogen and potassium application at different growth stage of rice; pre-planting (%) – beginning of tillering (%) – and the panicle differentiation stage(%).

Plant nutrient analysis

Plants were cut at ground level, washed in tap water and dried at 60℃ for three days. Dried plants were divided into leaves, stems and grains, and dry weight was measured. The phosphate content of each sample was measured using the Vanadate method (NIAST, 2000) and total nitrogen and protein contents of the plants were measured using the Kjeldahl method (Varley, 1996) using an Auto Kjeldahl Foss 2300 (Foss, Denmark).

Statistical analysis

SAS version 9.2 (SPSS Inc) was used for data analysis. Duncan's multiple range test (DMRT) was carried out to identify significant differences ($P < 0.05$) between individual treatments.

RESULTS

OsPT1 expression of OsPT1-OX

For analysis of *OsPT1* gene expression in OsPT1-OX and wild-type plants according to different nitrogen and phosphate levels, plants were cultured in nutrient solution with different phosphate and nitrogen levels: standard conditions (S), high-P, and high-N·P conditions. *OsPT1* was expressed in the wild-type regardless of nitrogen and phosphate levels, and it was more highly expressed in OsPT1-OX following every treatment with the help of the CAMV 35S promoter (Supplementary Figure 1).

Crop growth responses to different phosphate and nitrogen levels

The plant height of OsPT1-OX was not significantly different from that of wild-type plant until maximum tillering stage in each treatment. However, culm length of OsPT1-OX was shorter than that of the wild-type plants after booting stage in each treatment (data not shown). Plant culm lengths at ripening stage were between 88.08~93.61 cm in OsPT1-OX, and 100.75~105.95 cm in wild-type plant (Table 2). OsPT1-OX culm length did not change under high-P conditions compared to that under control conditions, whereas that of wild type increased significantly. Culm length of both varieties further increased under high-N·P conditions. The number of tillers per plant of OsPT1-OX and wild-type plant was 13.2~17.79 and 11.7~14.4, respectively, under the different fertilizer conditions. OsPT1-OX had 1.2 times more tillers than that of wild-type plants regardless of the fertilizer conditions in all stage (data not shown). The change in the tiller number was in accordance with the change in culm length under the different fertilizer conditions (Table 2). The tiller number of wild-type plants increased significantly in high-P conditions, but did increase further under high-N·P conditions. The tiller number of OsPT1-OX increased significantly under high-N·P conditions, but not under high-P conditions.

Meanwhile, the dry weight of the stem in OsPT1-OX did not increase under high-P conditions compared to that under control conditions (Table 2). However, the dry weight of the stem in wild-type plants increased significantly under high-P conditions. The dry weight of both cultivars significantly increased under high-N·P conditions.

Change in phosphate accumulation according to different phosphate and nitrogen levels

The average phosphate content of stem was 0.64% per dry weight in OsPT1-OX, and 0.32% in wild-type plants (Table 3). Phosphate content of OsPT1-OX in leaves, stems and grains was 1.56, 1.96 and 1.46 times higher than that in wild-type, respectively. Under high-P conditions, phosphate content of stems in wild-type plant increased compared to that in plant under control conditions, but did not increase in OsPT1-OX. While phosphate content of stems in OsPT1-OX plants significantly increased under the high- N·P condition, it did not increase in wild-type plants. The plant phosphate content (mg) was calculated by multiplying dry weight and phosphate contents (%) per dry weight (Figure 1). The phosphate contents of leaves, stems and grains of OsPT1-OX plants were higher than those in wild-type plants for all treatments. Phosphate contents in the

Table 2. Change in growth characteristics and dry weight (g) by different fertilizer treatment at the ripening stage.

Cultivar	Fertilizer treatment	Growth characteristics		Dry weight (g)			
		Culm length (cm)	No. Tillers per plant	Leaf	Stem	Grain	Total
OsPT1-OX	Control Condition	88.08 ± 2.2^{b*}	13.20 ± 2.2^{b}	6.58 ± 0.58^{b}	20.21 ± 3.23^{b}	23.05 ± 3.70^{b}	50.26 ± 7.94^{b}
	High-P	89.19 ± 2.6^{b}	14.90 ± 2.3^{b}	8.52 ± 1.43^{ab}	21.69 ± 3.48^{b}	26.31 ± 4.76^{ab}	57.15 ± 9.71^{ab}
	High- N·P	93.61 ± 3.0^{a}	17.79 ± 2.3^{a}	10.39 ± 1.1^{a}	25.43 ± 2.98^{a}	30.76 ± 3.60^{a}	64.74 ± 5.82^{a}
WT	Control Condition	100.75 ± 2.7^{c}	11.70 ± 1.7^{b}	7.31 ± 0.96^{c}	18.35 ± 1.29^{c}	25.31 ± 3.47^{b}	50.97 ± 5.58^{b}
	High-P	102.86 ± 2.7^{b}	13.10 ± 2.0^{a}	9.04 ± 1.24^{b}	21.69 ± 3.47^{b}	26.84 ± 2.18^{b}	58.22 ± 6.97^{b}
	High- N·P	105.95 ± 2.9^{a}	14.40 ± 1.9^{a}	12.41 ± 1.48^{a}	27.86 ± 2.69^{a}	33.57 ± 4.14^{a}	74.46 ± 7.98^{a}

All values are mean ± SD. *Different letters in a column indicate a significant different at the 5% level by Duncan's multiple range test.

Table 3. Change in available phosphate and nitrogen content (%) per dry weight by different fertilizer treatment at the ripening stage.

Cultivar	Fertilizer treatment	Phosphate content (%)			Nitrogen content (%)		
		Leaf	Stem	Grain	Leaf	Stem	Grain
OsPT1-OX	Control Condition	0.48 ± 0.052^{a}	0.58 ± 0.063^{b}	0.73 ± 0.139^{a}	1.40 ± 0.04^{b}	0.62 ± 0.05^{b}	1.11 ± 0.03^{b}
	High-P	0.51 ± 0.102^{a}	0.61 ± 0.029^{b}	0.73 ± 0.064^{a}	1.40 ± 0.08^{b}	0.62 ± 0.05^{b}	1.09 ± 0.04^{b}
	High- N·P	0.51 ± 0.055^{a}	0.76 ± 0.077^{a}	0.71 ± 0.041^{a}	1.70 ± 0.09^{a}	0.76 ± 0.03^{a}	1.35 ± 0.36^{a}
	Average	0.50 ± 0.083	0.64 ± 0.104	0.72 ± 0.863	1.50 ± 0.05	0.67 ± 0.04	1.18 ± 0.15
WT	Control Condition	0.28 ± 0.029^{b}	0.25 ± 0.052^{b}	0.50 ± 0.088^{a}	1.11 ± 0.03^{b}	0.47 ± 0.02^{b}	0.99 ± 0.02^{c}
	High-P	0.37 ± 0.058^{a}	0.38 ± 0.056^{a}	0.46 ± 0.079^{a}	1.19 ± 0.08^{b}	0.50 ± 0.04^{b}	1.04 ± 0.03^{b}
	High- N·P	0.31 ± 0.027^{ab}	0.36 ± 0.043^{a}	0.53 ± 0.084^{a}	1.43 ± 0.14^{a}	0.60 ± 0.03^{a}	1.15 ± 0.03^{a}
	Average	0.32 ± 0.0569	0.32 ± 0.0694	0.50 ± 0.0925	1.24 ± 0.08	0.52 ± 0.03	1.06 ± 0.02

All values are mean ± SD. Different letters in a column indicate a significant different at the 5% level by Duncan's multiple range test.

leaves and grains of OsPT1-OX gradually increased according to high P and N fertilizer. However, stem phosphate content increased significantly under high-N·P conditions compared to that under control fertilizer conditions. The total phosphate content in both cultivars of plants was higher under high-N·P conditions compared to that of other treatments.

The nitrogen content of stems was 0.47~0.60% in wild-type, and 0.62~0.76% in OsPT1-OX plants (Table 3). Nitrogen content of OsPT1-OX plants was slightly higher than in wild-type plants. The nitrogen content in both cultivars did not increase under high-P conditions, but increased significantly under the high-N·P condition.

DISCUSSION

Many phosphate transporters have been characterized to overcome phosphate-deficient

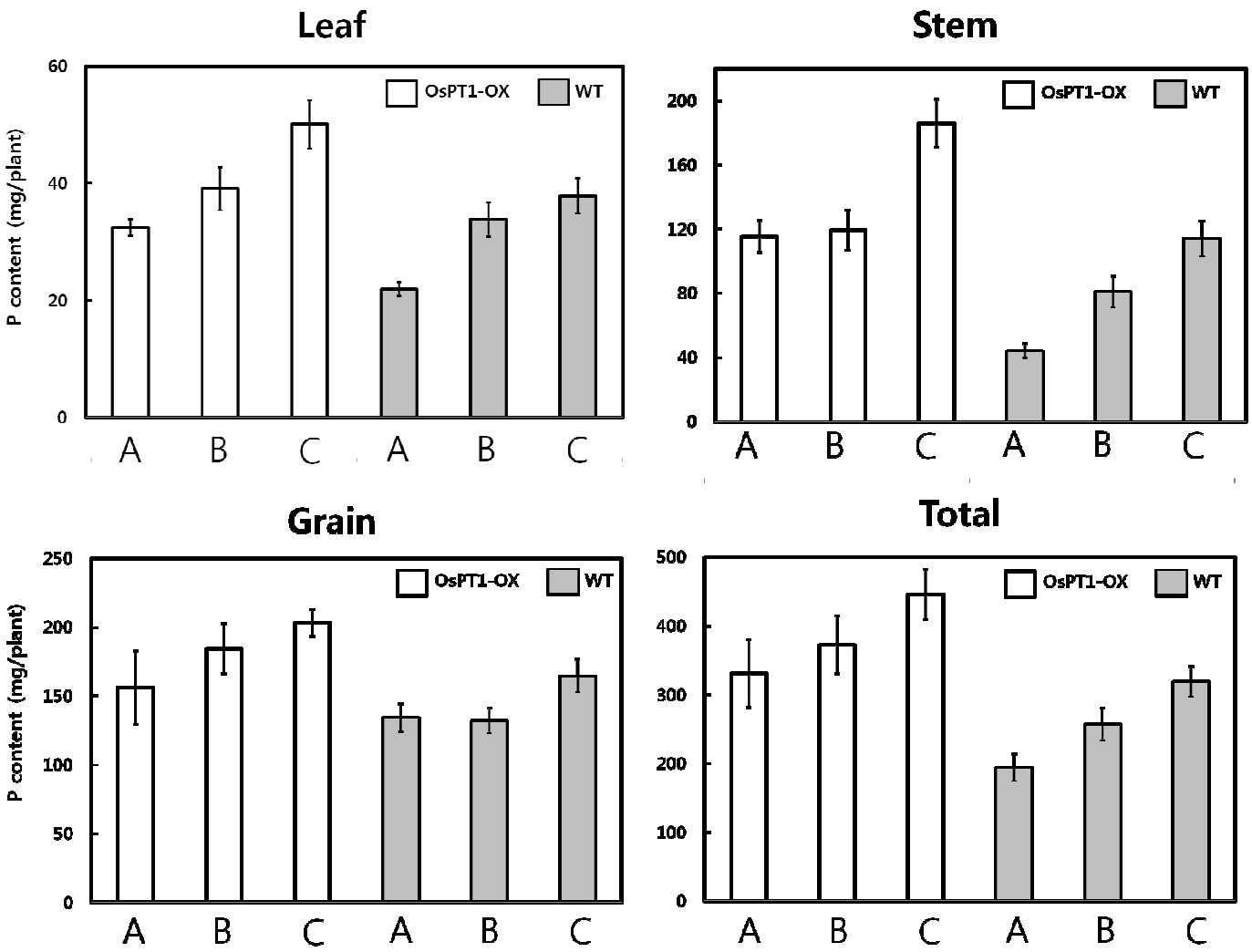

Figure 1. Plant phosphate content (mg) at the ripening stage. The plant phosphate content was calculated by multiplying phosphate content (%) with dry weight. Total P content was summed with phosphate contents of leaves, stems and grains. A: Control condition, B; High-P, C; High- N·P

soil conditions. Phosphate transporters (*PT*) are divided into the high-affinity *PTs* and low-affinity *PTs*. The low-affinity *PTs* are mainly active in vascular loading, unloading and remobilization of acquired phosphate, whereas the high-affinity *PT*s are active in acquisition of phosphate from soils (Smit et al., 2001). Since various high-affinity *PTs* were identified (Paszkowski et al., 2002; Seo et al., 2008), many researchers have investigated the expression pattern and phosphate acquisition by high-affinity *PTs* genes. Among the high-affinity *PTs*, we selected the *OsPT1* gene to generate phosphate acquisition enhanced transgenic rice (OsPT1-OX), which is highly expressed in rice regardless of the phosphate condition. In a previous study, we confirmed enhanced acquisition of phosphate by overexpressing the *OsPT1* gene. OsPT1-OX had 1.7~2 times more phosphate than that of wild-type plants under phosphate deficient soil condition (Seo et al., 2008; Song et al., 2011).

The morphological characteristics of rice related to phosphate uptake efficiency have been researched to develop phosphate uptake enhanced rice. Hung (1985) reported that tillering ability is the best marker of phosphate deficient tolerant rice cultivars. Additionally, Wissuwa et al. (1998) reported that phosphate uptake and phosphate-use efficiency could be monitored indirectly by dry weight and tiller number. In our study, OsPT1-OX had more tillers and 1.18 ~ 1.7 times higher phosphate content than that of wild-type plants under control phosphate conditions (Tables 2 and 3).

Since the use of chemical fertilizers, excess phosphate has been regarded as a serious problem that causes soil and water pollution. Hence, we investigated the possibility of removing excess phosphate from excessive phosphate treated soil using OsPT1-OX plants. Phosphate content (%) of OsPT1-OX was 1.37~1.6 times higher than that of wild-type plants under excess

phosphate condition. Furthermore, OsPT1-OX plant phosphate content was 1.15~1.47 times higher than that of wild-type plants despite a lower biomass (Figure 1). From these results, we confirmed that OsPT1-OX can take up more phosphate than wild-type plant under phosphate excessive and control phosphate conditions.

Although the tillering ability and phosphate uptake of OsPT1-OX was more effective than those of wild-type plant, OsPT1-OX biomass was not different from that of wild-type plants due to short culm length. Moiser et al. (2004) reported that nutrient recoveries are higher in plots treated with both nitrogen and phosphate combined than with nitrogen or phosphate alone in addition to enhanced crop yield. Similarly, wheat dry matter was highest under nitrogen and phosphate combined treatment among nitrogen, phosphate, nitrogen and phosphate combined treatment (Dordas, 2009). Additionally, Juan et al., 2009) reported that rapeseed yield was higher in NPK and NPB treatments than that in PKB and NKB treatments. Similarly, the short culm length of OsPT1-OX plants was considered for nutrient imbalance from the high phosphate contents. Additional nitrogen content is needed to adjust nitrogen and phosphate balance in OsPT1-OX plants. In this study, OsPT1-OX tiller number under high-P conditions did not increase compared to that under the control condition, whereas tiller number of wild-type plants increased significantly (Table 2). Adding nitrogen to the high phosphate condition (high-N·P) significantly increased tiller number in OsPT1-OX plants, followed by an increase in plant dry weight. We expected that OsPT1-OX took up enough phosphate to increase tiller number under control phosphate conditions, but tiller number did not increase under high phosphate conditions without additional nitrogen due to imbalance of nitrogen and phosphate in the plants. From these results, we suggest that additional nitrogen treatment improved the growth of OsPT1-OX.

Phosphate uptake and homeostasis are affected by the presence of several other elements and physical soil factors. The balance between nitrogen and phosphate is important for phosphate uptake and homeostasis (Jain et al., 2007; Ziadi et al., 2008). Dordas (2009) reported that wheat nitrogen content is higher under phosphate fertilizer treatment condition than a no fertilizer treatment condition. Additionally, wheat phosphate content is higher in nitrogen and phosphate combined treatment than nitrogen or phosphate treatment. Our results showed that nitrogen content of OsPT1-OX was significantly higher than that of wild-type plants under control condition (Table 3). The percentage of phosphate content in OsPT1-OX plants did not increase under the high-P conditions but increased significantly under high-N·P conditions, particularly in the stem. In addition, nitrogen content did not change in either cultivar under high phosphate conditions compared to that under the control condition, but increased significantly under high-P·N conditions (Table 3). The biomass of OsPT1-OX was the highest under high-P·N conditions, and phosphate acquisition was also the highest under the same conditions (Table 2, Figure 1).

Based on our results, OsPT1-OX was considered to be effective in the removing of phosphate from soils. Additional nitrogen is needed to improve growth and phosphate acquisition of OsPT1-OX. Meanwhile, further research on the control of nitrogen uptake genes as well as phosphate transporters is warranted to develop more effective phosphate acquisition transgenic plants.

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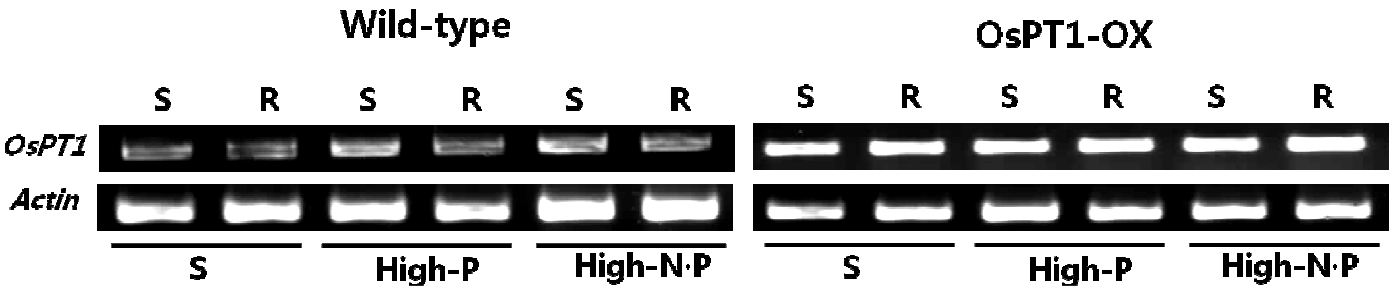

Supplementary Figure 1. *OsPT1* expression in OsPT1-OX and wild-type plants in different nutrient solutions. S, Shoot; R, root; S, standard solution.