Phosphate Utilization Efficiency Correlates with Expression of Low-Affinity Phosphate Transporters and Noncoding RNA, IPS1, in Barley

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Genetic variation in phosphorus (P) efficiency exists among wheat (Triticum aestivum) and barley (Hordeum vulgare) genotypes, but the underlying mechanisms for the variation remain elusive. High- and low-affinity phosphate (Pi) PHT1 transporters play an indispensable role in P acquisition and remobilization. However, little is known about genetic variation in PHT1 gene expression and association with P acquisition efficiency (PAE) and P utilization efficiency (PUE). Here, we present quantitative analyses of transcript levels of high- and low-affinity PHT1 Pi transporters in four barley genotypes differing in PAE. The results showed that there was no clear pattern in the expression of four paralogs of the high-affinity Pi transporter HvHPT1;1 among the four barley genotypes, but the expression of a low-affinity Pi transporter, HvHPT1;6, and its close homolog HvHPT1;3 was correlated with the genotypes differing in PUE. Interestingly, the expression of HvHPT1;6 and HvHPT1;3 was correlated with the expression of HvIPS1 (for P starvation inducible; noncoding RNA) but not with HvIPS2, suggesting that HvIPS1 plays a distinct role in the regulation of the low-affinity Pi transporters. In addition, high PUE was found to be associated with high root-shoot ratios in low-P conditions, indicating that high carbohydrate partitioning into roots occurs simultaneously with high PUE. However, high PUE accompanying high carbon partitioning into roots could result in low PAE. Therefore, the optimization of PUE through the modification of low-affinity Pi transporter expression may assist further improvement of PAE for low-input agriculture systems.

Phosphorus (P) is an essential macronutrient for plant growth and development. The availability of P is often low in soil, and a large amount of inorganic phosphate (Pi) fertilizers is applied to achieve high crop yields. Wheat (Triticum aestivum) and barley (Hordeum vulgare) production uses approximately 46% of the P fertilizers applied to cereals (FAO Fertilizer and Plant Nutrition Bulletin 17; http://www.fao.org). Extensive fertilization with P leads to rapid depletion of nonrenewable P resources and contributes to environmental pollution (Vance et al., 2003). P deficiency in cereal crops is widespread and causes significant yield reductions (Elliott et al., 1997; Gahoonia and Nielsen, 2004). The improvement of P efficiency in winter cereals through breeding is crucial for sustainable agriculture and world food security (Godfray et al., 2010; Parry and Hawkesford, 2010).

There are many definitions of P efficiency in the literature (Gourley et al., 1993; Hammond et al., 2009; Wang et al., 2010), but it can generally be divided into P acquisition efficiency (PAE) and P utilization efficiency (PUE). PAE refers to the ability of the plant to mobilize P from poorly soluble sources and/or to take up the soluble P available in the soil solution (Narang et al., 2000), whereas PUE is the amount of biomass produced per unit of acquired P (Ozturk et al., 2005; Wang et al., 2010). PAE is considered to be a major component of overall P efficiency (Ozturk et al., 2005; Imsmail et al., 2007; Ramaekers et al., 2010), but PUE can influence PAE. P supply to plants is often fluctuating; therefore, P remobilization within the plant is critical for plant survival (Drew and Saker, 1984; Marschner and Cakmak, 1986; Jeschke et al., 1997; Vance et al., 2003; Huang et al., 2008). Remobilization and translocation of P from shoots to roots can lead to a larger root system, and hence greater exploitation of limited soil P resources for more P. However, the effect of PUE and simultaneous carbohydrate partitioning on PAE (Hermans et al., 2006; Liao et al., 2008) is not clear (Manske et al., 2001; Wang et al., 2010).

PHT1 Pi transporters play a critical role in Pi acquisition from soil solution and Pi remobilization within the plant. Nine members of the PHT1 gene family have been found in the Arabidopsis (Arabidopsis thaliana)
MicroRNAs represent a class of noncoding small RNAs that generally function as posttranscriptional negative regulators through base pairing to nearly complementary sequences in target mRNAs (Jones-Rhoades et al., 2006). miR399 is the first microRNA found to be involved in Pi homeostasis (Sunkar and Zhu, 2004; Fujii et al., 2005; Bari et al., 2006; Chiou et al., 2006). miR399-guided degradation of mRNA of the target gene PHO2, encoding for a ubiquitin E2 conjugase, regulates Pi homeostasis in the plant (Aung et al., 2006; Bari et al., 2006; Mallory and Bouché, 2008). IPS genes are also involved in the regulation of the miR399-PHO2 pathway (Doerner, 2008). Incomplete sequence complementarity between AtIPS1 and AtmiR399 forms an RNA duplex with a mismatch loop, which inhibits the degradation of AtPHO2 mRNA (Franco-Zorrilla et al., 2007). AtPHO2 regulates the expression of AtPHT1.8 and AtPHT1.9, and controls the remobilization and translocation of Pi via an unknown process (Aung et al., 2006; Bari et al., 2006; Chiou et al., 2006; Doerner, 2008).

Considering genetic variation in PAE and PUE has been reported in a wide range of barley and wheat genotypes (Gahoonia and Nielsen, 1996; Manske et al., 2001; Ozturk et al., 2005; Liao et al., 2008; George et al., 2011), but there have been few studies on the genetic variation in expression levels of Pi transporters and the relationships between the expression of Pi transporters and PAE or PUE (Ramaekers et al., 2010). To address this issue, we used barley, a diploid relative of hexaploid wheat, as a model system for winter cereals to study the functions of PHT1 genes involved in the acquisition and remobilization of Pi (Preuss et al., 2010, 2011). In this report, we show that differences in expression levels of four paralogs of the high-affinity Pi transporter gene HvPHT1;1, are not apparent among four barley genotypes differing in PAE, but there is a large difference in the expression of the low-affinity Pi transporter HvPHT1;6 and a close homolog, HvPHT1;3. In addition, the expression of HvPHT1;6 and HvPHT1;3 correlates with the expression of HvIPS1 and PUE. The implications of these findings in the improvement of PAE for barley and wheat are discussed.

RESULTS

Plant Growth, P Concentration, PAE, and PUE among Four Distinct Barley Genotypes

Growing plants in a soil is essential for the evaluation of PAE because it allows the efficient mechanisms operating at the root-soil interface to be functional. These mechanisms include greater exploitation of soil volume by roots, including root hairs, root exudates, and microbial activities. These morphological and biochemical changes can increase P availability by mobilizing sparingly soluble mineral P and organic P sources (Gahoonia and Nielsen, 1997;...
We grew barley plants in fine sand mixed with calcium carbonate to simulate calcareous sandy soil. Four distinct barley genotypes (Clipper, Sahara, Pallas, and a hairless mutant, brb, derived from Pallas) were selected. These four barley genotypes were grown in the simulated calcareous sandy soil with three rates of P. At high P supply (75 mg P kg\(^{-1}\); referred to as P75), shoot dry weights of Pallas, brb, and Sahara were significantly higher than for Clipper, but there were no differences between Pallas, brb, and Sahara (Fig. 1A). At moderately low P supply (22.5 mg P kg\(^{-1}\); referred to as P22.5), shoot dry weights of all four genotypes were significantly reduced compared with those at P75 (Fig. 1A). Shoot dry weight of Pallas was significantly higher than that of brb, Clipper, or Sahara. When P supply was further reduced (7.5 mg P kg\(^{-1}\); referred to as P7.5), a further reduction in shoot dry weight was observed for all four genotypes, but the shoot dry weight of Pallas was significantly higher than that of brb and Sahara and the shoot dry weight of Clipper was significantly higher than that of Sahara (Fig. 1A).

Root growth on a fresh weight basis was reduced by the low rates of P supply, but the reduction was much less than for shoot growth (Fig. 1A and B). There was no significant interaction between P rates and genotypes. The root fresh weight of Pallas on average was significantly higher than that of the three other genotypes, but no differences were found among brb, Clipper, and Sahara. These results indicate that the low rates of P supply have a smaller impact on root growth than shoot growth. A genotypic difference in root growth was found between Pallas and brb but not between Clipper and Sahara.

An increase in root-shoot ratio is often observed in plants grown at low P supply and is attributed to increased carbohydrate partitioning to the root (Zhu et al., 2002; Hermans et al., 2006; Liao et al., 2008). Root-shoot ratios on a fresh weight basis increased as P supply decreased in all genotypes (Fig. 1C). At low P supply (P7.5), root-shoot ratios of brb and Sahara were significantly higher than those of Pallas and Clipper, while the difference between Pallas and Clipper was not significant. At moderately low P supply (P22.5), a similar trend to that at low P supply (P7.5) was observed (Fig. 1C). At high P supply (P75), root-shoot ratios in brb and Clipper were significantly higher than that in Sahara and the root-shoot ratio of Pallas was between these two groups (Fig. 1C).

P concentrations of shoots in all four genotypes at P75 were above the critical level of 4,000 μg P g\(^{-1}\) dry weight (Fig. 2A), which is required for normal plant growth (Reuter and Robison, 1997; George et al., 2011). Genotypic differences in P concentrations of shoots were observed at P75. Clipper had a significantly higher P concentration than the other genotypes, and the P concentration in Pallas was higher than that in brb or Sahara. When P supply was reduced to P22.5, P concentrations of shoots were reduced to a level below the critical level (Fig. 2A). The shoot P concentration of Pallas was significantly lower than that of brb, Clipper, or Sahara. When P supply was further reduced (P7.5), a further reduction in P concentration was observed for all four genotypes, but the P concentration of Pallas was significantly higher than that of brb and Sahara and the P concentration of Clipper was significantly higher than that of Sahara (Fig. 2A).

Figure 1. Shoot dry weight and root fresh weight of four barley genotypes. A, Shoot dry weight. B, Root fresh weight. C, Root-shoot ratio. Barley seedlings were grown in a calcareous sandy soil with three rates of P (7.5, 22.5, and 75 mg P kg\(^{-1}\) sand, referred to as P7.5, P22.5, and P75, respectively). Plants were harvested at day 16 after seed imbibition. Means and SE values of four replicates are presented. There are significant differences in shoot dry weight and root-shoot ratio for interactions of P rate × genotype (P < 0.001). Error bars indicate LSD (LSD0.05) for shoot dry weight and root-shoot ratio. There are no significant differences in root fresh weight for interactions of P rate × genotype (P = 0.25).
Clipper remained higher than in the other genotypes, and Pallas had a significantly higher P concentration than either \textit{brb} or Sahara. When P supply further decreased to P7.5, P concentrations of shoots were further reduced in all four genotypes (Fig. 2A). The shoot P concentration of Pallas remained higher than that of \textit{brb} or Sahara. The shoot P concentration of Clipper was also higher than that of Sahara but similar to that of Pallas.

Shoot P content, a measure of PAE (Ozturk et al., 2005), was significantly reduced at the two low rates of P supply (Fig. 2B), but there was no significant interaction between P rates and genotypes. There was a significant difference among the four genotypes. The shoot P content was significantly higher in Pallas and Clipper than in \textit{brb} or Sahara, but no difference between Pallas and Clipper was seen. These results indicate that Pallas and Clipper are efficient in P acquisition and that \textit{brb} and Sahara are inefficient in P acquisition.

When biomass production per unit of P in shoots of four genotypes was calculated for PUE, there were no significant differences at the high P supply (P75) among the four genotypes (Fig. 2C). The low rates of P supply at P22.5 and P7.5 increased PUE in all four genotypes, but differences in PUE were found among the four genotypes. The genotypes low in PAE (\textit{brb} and Sahara) had significantly higher PUE than those with high PAE (Pallas and Clipper) at both P22.5 and P7.5 (Fig. 2C). These results show that all genotypes increase PUE when P supply is limited for optimal growth, and the genotypes with greater PUE at low P conditions have a higher root-shoot ratio (Fig. 1C). This suggests that high remobilization of P occurs simultaneously with enhanced carbohydrate partitioning into roots.

Identification of Additional \textit{HvPHT1;1} Paralogs in the Barley Genome

Two paralogs of \textit{HvPHT1;1} genes have been described by Smith et al. (1999). We isolated two additional \textit{HvPHT1;1} paralogs from a Haruna Nijo bacterial artificial chromosome (BAC) library (Saisho et al., 2007) using a 388-bp probe derived from the coding sequence of \textit{HvPHT1;1}. The first gene encoding a protein was identical to \textit{HvPHT1;1} (Fig. 3A) but differed in the 3′ untranslated region (UTR). We designated this new \textit{HvPHT1} gene as \textit{HvPHT1;9} (GenBank accession no. AM904733). The transcript of \textit{HvPHT1;9} had a 56-nucleotide deletion in the 3′ UTR compared with that of \textit{HvPHT1;1} (Fig. 3B). The second new \textit{HvPHT1;1} paralog is similar to \textit{HvPHT1;2} and is designated as \textit{HvPHT1;10} (GenBank accession no. FN392213). The protein sequence of \textit{HvPHT1;10} differs only by four amino acid residues from \textit{HvPHT1;2} (Fig. 3A).

Database searches identified an ortholog of \textit{HvPHT1;9} in wheat (GenBank accession no. BJ277773), which also has a 30-nucleotide deletion in the 3′ UTR compared with that of \textit{TaPHT1;2} (Fig. 3B). The second new \textit{HvPHT1;1} paralog is similar to \textit{HvPHT1;2} and is designated as \textit{HvPHT1;10} (GenBank accession no. CD871730). No \textit{Brachypodium} sequences similar to either \textit{HvPHT1;1} or \textit{HvPHT1;9} were identified (Supplemental Fig. S1).

Database searches using the coding sequences of \textit{HvPHT1;2} and \textit{HvPHT1;10} identified four orthologs in wheat and one ortholog in \textit{Brachypodium}. All five orthologs of \textit{HvPHT1;2}/\textit{HvPHT1;10} are 525 amino acid residues in length. The four wheat orthologs differ from each other only by two amino acid residues (data not shown). One of the four wheat orthologs (GenBank accession no. AJ344240) was used to represent \textit{TaPHT1;2} in the phylogenetic analysis of PHT1 proteins.
Expression of Two HvIPS Genes among Four Barley Genotypes

IPS genes are highly responsive to P deficiency, and their transcript levels are a good biomarker for P deficiency responses (Liu et al., 1997; Martin et al., 2000; Wasaki et al., 2003). IPS transcripts also interact with miR399s, regulating P translocation (Franco-Zorrilla et al., 2007). Therefore, the transcript levels of two barley IPS genes (HvIPS1 and HvIPS2) in roots were determined for four barley genotypes. At the sufficient P supply (P75), a moderate level of HvIPS1 and HvIPS2 transcripts (4 to 7 × 10^6 normalized copies μg⁻¹ RNA) was detected in each of the four genotypes (Fig. 4). Lower P supply enhanced transcript levels of HvIPS2 in all four genotypes to 64 to 74 × 10^6 normalized copies μg⁻¹ RNA at P22.5 and 85 to 109 × 10^6 normalized copies μg⁻¹ RNA at P7.5 (Fig. 4). There was no significant interaction in the transcript levels of HvIPS2 between P rates and genotypes, but there was a significant difference at all P rates among the genotypes. The transcript level of HvIPS2 in brb was significantly higher at all P rates than that in Pallas, and the transcript level of HvIPS2 in Sahara was marginally higher at all P rates than that in Clipper.

In contrast, the transcript levels of HvIPS1 at the high P supply (P75) were hardly detectable in any barley genotype (Fig. 4). When P supply was reduced to the moderately low level (P22.5), the transcript levels of HvIPS1 in brb and Sahara were sharply increased to approximately 30 × 10^6 normalized copies μg⁻¹ RNA and 80 × 10^6 normalized copies μg⁻¹ RNA, respectively. However, the transcript levels of HvIPS1 in Pallas and Clipper at P22.5 increased only to less than 3 × 10^6 normalized copies μg⁻¹ RNA (Fig. 4). A further reduction in P supply to P7.5 increased the transcript level of HvIPS1 only slightly in brb, Pallas, and Clipper, but the transcript level of HvIPS1 in Sahara fell sharply relative to that at P22.5 (Fig. 4). These results show that HvIPS1 and HvIPS2 in all four genotypes are highly inducible under the low rates of P supply, indicating that the plants grown at P22.5 are moderately P deficient and those at P7.5 are severely P deficient. There is a basal level of HvIPS2 expression in the roots of the P-adequate plants but hardly any HvIPS1 transcripts in the roots of the P-adequate plants (Fig. 4). A large variation in the expression of HvIPS1 is found between the P-acquisition-efficient genotypes, Pallas and Clipper, and the P-acquisition-inefficient genotypes, brb and Sahara, especially at the moderately low P supply (P22.5), but this is not seen for HvIPS2 expression.

Expression of Four HvPHT1 Paralogs in Four Barley Genotypes

At P75, a moderate level of HvPHT1:1 transcripts (3 to 12 × 10^6 normalized copies μg⁻¹ RNA) was detected in the roots of all four genotypes (Fig. 5). The transcript level of HvPHT1:1 in brb was the highest among the four genotypes, and the transcript level of HvPHT1:1 in Sahara was higher than that in Clipper or Pallas (Fig. 5). The decrease in P supply to P22.5 increased HvPHT1:1 transcripts in all four genotypes, especially for Sahara (Fig. 5). Sahara had a much higher level of HvPHT1:1 transcripts than the three other genotypes.

In contrast, the transcript levels of HvPHT1:1 at the high P supply (P75) were hardly detectable in any barley genotype (Fig. 4). When P supply was reduced to the moderately low level (P22.5), the transcript levels of HvPHT1:1 in brb and Sahara were sharply increased to approximately 30 × 10^6 normalized copies μg⁻¹ RNA and 80 × 10^6 normalized copies μg⁻¹ RNA, respectively. However, the transcript levels of HvPHT1:1 in Pallas and Clipper at P22.5 increased only to less than 3 × 10^6 normalized copies μg⁻¹ RNA (Fig. 4). A further reduction in P supply to P7.5 increased the transcript level of HvPHT1:1 only slightly in brb, Pallas, and Clipper, but the transcript level of HvPHT1:1 in Sahara fell sharply relative to that at P22.5 (Fig. 4). These results show that HvPHT1:1 and HvIPS1 in Sahara were sharply increased to 3 × 10^6 normalized copies μg⁻¹ RNA at P7.5 (Fig. 4). There was no significant difference at all P rates among the genotypes. The transcript level of HvIPS1 in all four genotypes are highly inducible under the low rates of P supply, indicating that the plants grown at P22.5 are moderately P deficient and those at P7.5 are severely P deficient. There is a basal level of HvIPS2 expression in the roots of the P-adequate plants but hardly any HvIPS1 transcripts in the roots of the P-adequate plants (Fig. 4). A large variation in the expression of HvIPS1 is found between the P-acquisition-efficient genotypes, Pallas and Clipper, and the P-acquisition-inefficient genotypes, brb and Sahara, especially at the moderately low P supply (P22.5), but this is not seen for HvIPS2 expression.

Expression of Four HvIPS Genes among Four Barley Genotypes

IPS genes are highly responsive to P deficiency, and their transcript levels are a good biomarker for P deficiency responses (Liu et al., 1997; Martin et al., 2000; Wasaki et al., 2003). IPS transcripts also interact with miR399s, regulating P translocation (Franco-Zorrilla et al., 2007). Therefore, the transcript levels of two barley IPS genes (HvIPS1 and HvIPS2) in roots were determined for four barley genotypes. At the sufficient P supply (P75), a moderate level of HvIPS1 and HvIPS2 transcripts (4 to 7 × 10^6 normalized copies μg⁻¹ RNA) was detected in each of the four genotypes (Fig. 4). Lower P supply enhanced transcript levels of HvIPS2 in all four genotypes to 64 to 74 × 10^6 normalized copies μg⁻¹ RNA at P22.5 and 85 to 109 × 10^6 normalized copies μg⁻¹ RNA at P7.5 (Fig. 4). There was no significant interaction in the transcript levels of HvIPS2 between P rates and genotypes, but there was a significant difference at all P rates among the genotypes. The transcript level of HvIPS2 in brb was significantly higher at all P rates than that in Pallas, and the transcript level of HvIPS2 in Sahara was marginally higher at all P rates than that in Clipper.

In contrast, the transcript levels of HvIPS1 at the high P supply (P75) were hardly detectable in any barley genotype (Fig. 4). When P supply was reduced to the moderately low level (P22.5), the transcript levels of HvIPS1 in brb and Sahara were sharply increased to approximately 30 × 10^6 normalized copies μg⁻¹ RNA and 80 × 10^6 normalized copies μg⁻¹ RNA, respectively. However, the transcript levels of HvIPS1 in Pallas and Clipper at P22.5 increased only to less than 3 × 10^6 normalized copies μg⁻¹ RNA (Fig. 4). A further reduction in P supply to P7.5 increased the transcript level of HvIPS1 only slightly in brb, Pallas, and Clipper, but the transcript level of HvIPS1 in Sahara fell sharply relative to that at P22.5 (Fig. 4). These results show that HvIPS1 and HvIPS2 in all four genotypes are highly inducible under the low rates of P supply, indicating that the plants grown at P22.5 are moderately P deficient and those at P7.5 are severely P deficient. There is a basal level of HvIPS2 expression in the roots of the P-adequate plants but hardly any HvIPS1 transcripts in the roots of the P-adequate plants (Fig. 4). A large variation in the expression of HvIPS1 is found between the P-acquisition-efficient genotypes, Pallas and Clipper, and the P-acquisition-inefficient genotypes, brb and Sahara, especially at the moderately low P supply (P22.5), but this is not seen for HvIPS2 expression.
Much lower levels of HvPHT1;9 transcripts (0.4 to 2.3 × 10^6 normalized copies µg^-1 RNA) were detected in the roots of Pallas, brb, and Clipper at the sufficient P supply (P75) but not in the roots of Sahara at any P rate (Fig. 5). The low rates of P supply (P22.5) increased the transcript levels of HvPHT1;9 in Pallas, brb, and Clipper, but the transcript levels of HvPHT1;9 were approximately 3 times lower than those of HvPHT1;1 (Fig. 5). A genotypic difference in the transcript level of HvPHT1;9 was found at P75 but not at the two low P rates. brb had a significantly higher level of HvPHT1;9 than Pallas or Clipper at P75. PCR amplification of genomic DNA using HvPHT1;9-specific primer pairs generated PCR products of HvPHT1;9 from Pallas, Clipper, and Haruna Nijo (note that the Haruna Nijo BAC library was used to obtain HvPHT1;9 sequence), but no PCR product of similar size was present in Sahara. Instead, a larger PCR product was observed in Sahara (Supplemental Fig. S2). The sequence analysis of this larger PCR product of HvPHT1;9 from Sahara revealed that it was identical to that of HvPHT1;1. Therefore, the higher transcript levels of HvPHT1;1 detected in Sahara relative to the other genotypes (Fig. 5) could result from combined gene products of both HvPHT1;1 and HvPHT1;9.

The transcript levels of HvPHT1;2 (2 to 9 × 10^6 normalized copies µg^-1 RNA) at P75 were detected in the roots of all four genotypes (Fig. 5). The moderately low P supply (P22.5) increased the transcript levels of HvPHT1;2 in all four genotypes to more than 4 times those at P75. The low P supply (P7.5) further increased the transcript levels of HvPHT1;2 in Pallas, brb, and Clipper, but the transcript levels of HvPHT1;2 were slightly reduced in Sahara (Fig. 5). There was a significant difference in the transcript level of HvPHT1;2 among the four genotypes. brb and Clipper had much higher transcript levels of HvPHT1;2 than Pallas and Sahara across all three rates of P supply (Fig. 5).

The expression of HvPHT1;10 was similar to that of HvPHT1;2 in Pallas and brb but was different in Clipper and Sahara (Fig. 5). The transcript levels of HvPHT1;10 were similar to that of HvPHT1;2 in Pallas and brb but was different in Clipper and Sahara (Fig. 5). The transcript levels of

**Figure 4.** Transcript levels of two HvIPS genes in roots of four barley genotypes. Barley seedlings were grown in a calcareous sandy soil with three rates of P (7.5, 22.5, and 75 mg P kg^-1 sand, referred to as P7.5, P22.5, and P75, respectively). Plants were harvested at day 16 after seed imbibition. Quantitative real-time RT-PCR was used to determine transcript levels in roots. The means (n = 4) of normalized copies µg^-1 RNA and s are presented. Log-transformed data were used in the statistical analysis for comparisons of means. There is a significant difference in the expression of HvIPS1 for interactions of P rate × genotype (P < 0.001) but not in the expression of HvIPS2 (P = 0.16).

**Figure 5.** Transcript levels of four paralogs of HvPHT1;1 in roots of four barley genotypes. Barley seedlings were grown in a calcareous sandy soil with three rates of P (7.5, 22.5, and 75 mg P kg^-1 sand, referred to as P7.5, P22.5, and P75, respectively). Plants were harvested at day 16 after seed imbibition. Quantitative real-time RT-PCR was used to determine the levels of transcripts in roots. The means (n = 4) of normalized copies µg^-1 RNA and s are presented. Log-transformed data were used in the statistical analysis for comparisons of means. There are significant differences in the expression of HvPHT1;1, HvPHT1;9, and HvPHT1;2 for interactions of P rate × genotype (P < 0.001 for all three genes) but not for HvPHT1;10 (P = 0.49).
HoPHT1;10 in Clipper and Sahara were at least twice as much as those of HoPHT1;2 at all three rates of P. There was no difference in the transcript level of HoPHT1;10 between Clipper and Sahara across three rates of P supply (Fig. 5).

The transcript levels of the four HoPHT1;1 paralogs reveal that all four genes are highly responsive to P deficiency. Although there were genotypic differences in the expression of the four HoPHT1;1 paralogs, the expression pattern was not apparent between two P-acquisition-inefficient genotypes and two P-acquisition-efficient genotypes.

Expression of HoPHT1;6 and HoPHT1;3 in Four Barley Genotypes

At sufficient P supply (P75), the transcript levels of HoPHT1;6 in Pallas, brb, and Clipper were less than 0.5 × 10^6 normalized copies μg⁻¹ RNA, but a higher transcript level (9.3 × 10^6 normalized copies μg⁻¹ RNA) was detected in Sahara (Fig. 6). When moderately low P (P22.5) was supplied, the transcript levels of HoPHT1;6 increased to 9 × 10^6 normalized copies μg⁻¹ RNA in brb and 20 × 10^6 normalized copies μg⁻¹ RNA in Sahara (Fig. 6). However, the transcript levels of HoPHT1;6 increased only to 0.7 to 1.3 × 10^6 normalized copies μg⁻¹ RNA in Pallas and Clipper (Fig. 6). With low P supply (P7.5), the transcript level of HoPHT1;6 was further increased in brb to approximately 20 × 10^6 normalized copies μg⁻¹ RNA but was greatly reduced in Sahara relative to that at P22.5 (Fig. 6). The transcript levels of HoPHT1;6 in Pallas and Clipper were also increased at P7.5 but remained lower than 3.0 × 10^6 normalized copies μg⁻¹ RNA.

HoPHT1;3 protein has the same number of amino acid residues as HoPHT1;6. HoPHT1;3 protein is more similar to HoPHT1;6 (77%) than the four HoPHT1;1 paralogs (70% identity). The transcript levels of HoPHT1;3 almost mimicked those of HoPHT1;6 (Fig. 6). A large difference in the expression of HoPHT1;6 and HoPHT1;3 was evident between the P-acquisition-efficient and P-acquisition-inefficient genotypes (Fig. 6). Interestingly, the difference in the expression of HoPHT1;6 and HoPHT1;3 is significantly correlated with the expression of HvIPS1 under the low P rates (P7.5 and P22.5) across the four genotypes (r = 0.91 and r = 0.95 at degrees of freedom = 30, respectively; P < 0.001) but not with that of HoIPS2. The P-acquisition-inefficient genotypes, brb and Sahara, had much higher expression levels of HoPHT1;3, HoPHT1;6, and HoIPS1 in roots under the low-P conditions than the P-acquisition-efficient genotypes, Pallas and Clipper (Figs. 4 and 6).

Stability of RNA Duplexes Formed between Conserved 24-Nucleotide Motifs of HvIPS1 or HoIPS2 and HvmiR399s

The conserved 24-nucleotide motif of HvIPS1 differs from that of HoIPS2 by six nucleotides in the 11th to 16th nucleotide positions, which correspond to the 10th to 14th nucleotide positions of HvmiR399s from the 5’ end (Fig. 7, A and B). These HvmiR399s were recently identified by deep sequencing of short reads of P-deficient barley plants of Pallas (Schreiber et al., 2011). By using RNAhybrid software (Rehmsmeier et al., 2004), minimum free energy (MFE) was calculated for the incomplete double-stranded RNA molecules formed between the conserved 24-nucleotide motif of HvIPS1 or HoIPS2 and HvmiR399s (Fig. 7, A and B). The HvIPS1 motif with HvmiR399s could form an RNA duplex with a mismatch loop of two nucleotides only at the 11th to 12th nucleotide positions of HvmiR399s, and the RNA duplexes possess MFE more negative than −30.9 kcal mol⁻¹ (Fig. 7, A and C). In contrast, the 24-nucleotide motif of HoIPS2 with
HvmiR399s could form an RNA duplex with a mismatch loop of five nucleotides at the 10th to 11th nucleotide positions of HvmiR399s and a second loop at the 12th to 15th nucleotide positions. The RNA duplexes possess MFE less negative than $-27.1$ kcal mol$^{-1}$ except for HvmiR399d (Fig. 7, B and D). A higher MFE value was predicted in the RNA duplex formed between HvIPS1 or HvIPS2 and HvmiR399d (Fig. 7, A and B), suggesting that HvIPS1 and HvIPS2 are more effective in sequestering HvmiR399d than the other HvmiR399s. Nevertheless, the RNA duplexes formed between HvIPS1 and HvmiR399s are more stable than those between HvIPS2 and HvmiR399s.

We also determined the transcript levels of a barley PHO2 ortholog, HvPHO2. Two primer pairs were used for quantitative reverse transcription (RT)-PCR amplification of both 3′ and 5′ ends of the HvPHO2 mRNA sequence, similar to the measurement of AtPHO2 described by Bari et al. (2006), given that HvPHO2 has a long mRNA (approximately 4,000 nucleotides) and multiple miR399 target sites in the 5′ UTR (data not shown). When a pair of 5′ end primers was used (HvPHO2-5′), the transcript levels of HvPHO2 in roots were low in all four genotypes at the high rate of P supply (P75), but the transcript level of HvPHO2 in Clipper was significantly higher than in the three other genotypes (Fig. 8). When P supply was reduced to P22.5, the transcript levels of HvPHO2 were significantly increased in brb and Sahara but no change was found in Pallas and Clipper (Fig. 8). The further decrease in P supply to P7.5 enhanced the transcript levels of HvPHO2 in brb, Clipper, and Sahara to a similar level without much change in Pallas. In comparison, the transcript levels of HvPHO2 measured using a pair of primers at the 3′ end (HvPHO2-3′) were similar to those of HvPHO2-5′ in response to P rates and genotypic differences, except for a higher transcript level (Fig. 8). These results indicate that the transcript levels of HvPHO2 in brb, Clipper, and Sahara are increased in the low P supply, and Pallas appears to be less responsive to P supply.

**DISCUSSION**

**Genetic Variation in P-Acquisition Efficiency in Four Distinct Barley Genotypes**

Three of the four genotypes used in this study have a diverse genetic background. Clipper is an Australian barley cultivar, Sahara is a landrace originating from Algeria (Karakousis et al., 2003), Pallas is a northern European barley cultivar (Gahoonia et al., 2001), and the fourth genotype, brb, is a root-hairless mutant derived from Pallas (Gahoonia et al., 2001). Shoot P content can be used as an indication of P acquisition efficiency (Manske et al., 2001; Zhu et al., 2002; Ozturk et al., 2005). The higher P content of shoots in Pallas and Clipper than in brb and Sahara indicates that Pallas and Clipper are P-acquisition-efficient genotypes whereas brb and Sahara are P-acquisition-inefficient genotypes. Our results are consistent with a previous study of Clipper and Sahara where they were grown in a calcareous soil (Zhu et al., 2002). A similar result was also observed for soil-grown Pallas and brb (Gahoonia and Nielsen, 2003). Reduced root surface area in brb is likely to be responsible for its low PAE (Gahoonia and Nielsen, 2003). However, it is not known what is responsible for the low PAE in Sahara. It is noteworthy that PUE in the P-acquisition-inefficient Sahara and brb is greater than that in the P-acquisition-efficient...
transcripts in roots. The means (Quantitative real-time RT-PCR was used to determine the levels of respectively). Plants were harvested at day 16 after seed imbibition.

May Not Be the Limiting Factor

Pi Transporters in P-Acquisition-Inefficient Genotypes

Expression Levels of the Genes Related to High-Affinity Pi Transporters in P-Acquisition-Inefficient Genotypes May Not Be the Limiting Factor

All four paralogs of the high-affinity Pi transporter, HvPHT1;1, were expressed in Pallas, brb, and Clipper, whereas expression of only three of the four HvPHT1;1 paralogs could be detected in Sahara (Fig. 5) due to the identical sequences in the 3’ UTR between HvPHT1;9 and HvPHT1;1 (Figs. 3B and 5; Supplemental Fig. S2). Our recent work revealed that HvPHT1;1 is a high-affinity Pi transporter with a $K_m$ value of 1.9 $\mu$m when expressed in Xenopus oocytes (Preuss et al., 2011). Higher transcript abundance of HvPHT1;1 is found in the root hair zone than in the root tip (Preuss et al., 2011). The expression pattern of HvPHT1;1 in root tissues overlaps with HvPHT1;2 (Schünemann et al., 2004; Glassop et al., 2005), suggesting that both HvPHT1;1 and HvPHT1;2 are involved in P acquisition. The two newly identified paralogs of HvPHT1;1 (HvPHT1;9 and HvPHT1;10) are either identical or very similar in protein sequences to HvPHT1;1 and HvPHT1;2 (Fig. 3A) and highly responsive to P deficiency (Fig. 5), suggesting that they have a role in P acquisition similar to HvPHT1;1 and HvPHT1;2. Orthologs (BdPHT1;2, TaPHT1;2, TaPHT1;1, and TaPHT1;9) group with the four barley HvPHT1;1 paralogs in the phylogenetic tree (Supplemental Fig. S1), suggesting that the PHT1 genes involved in P acquisition are conserved in Brachypodium and the Triticeae lineage.

There is no apparent pattern in the expression levels of the four HvPHT1;1 paralogs between two P-acquisition-inefficient genotypes (Sahara and brb) and the two P-acquisition-efficient genotypes (Clipper and Pallas) under low-P conditions (Fig. 5). A similar result from two contrasting rice genotypes differing in PAE is also reported using a microarray analysis of seven OsPHT1 genes (Ismail et al., 2007). These data suggest that transcript levels of the high-affinity Pi transporters in P-acquisition-inefficient genotypes may not be a limiting factor if it is assumed that the high-affinity PHT1 is regulated predominantly at the transcript level.

Interestingly, the absence of root hairs did not reduce the expression levels of any of the four HvPHT1;1 paralogs. Increased expression levels of HvPHT1;2 and HvPHT1;10 in brb relative to Pallas under the low P supply (Fig. 5) could be due to enhanced systemic Pi signaling initiated by plant low-P status (Figs. 2A and 4). However, the increased expression levels of the four HvPHT1;1 paralogs were also observed in brb relative to Pallas under the high P supply (Fig. 5), when plants have adequate P (Fig. 2A), and there was no increased expression of HvIPS1 (Fig. 4). This suggests that the interception of external Pi signal (Yang and Finnegan, 2010) may be impaired due to the absence of root hairs in brb.

Expression Levels of the Genes Related to Low-Affinity Pi Transporters Are Correlated with PUE

HvPHT1;6 is a low-affinity Pi transporter (Preuss et al., 2010). It is expressed in both shoots and roots (Huang et al., 2008) and especially in phloem tissues of the leaf (Rae et al., 2003), suggesting that HvPHT1;6 plays a role in Pi remobilization in the whole plant. The protein sequence of HvPHT1;3 is more similar to HvPHT1;6 than to the HvPHT1;1 paralogs. HvPHT1;3 is grouped to the clade containing OsPHT1;8, BdPHT1;3, and TaPHT1;3 with a high bootstrap value (Supplemental Fig. S1). OsPHT1;8 and the low-affinity Pi transporter OsPHT1;2 have been shown to be involved

Figure 8. Transcript levels of HvPHO2 in roots of four barley genotypes. Barley seedlings were grown in a calcareous sandy soil with three rates of P (7.5, 22.5, and 75 mg P kg$^{-1}$ sand, referred to as P7.5, P22.5, and P75, respectively). Plants were harvested at day 16 after seed imbibition. Quantitative real-time RT-PCR was used to determine the levels of transcripts in roots. The means ($n = 4$) of normalized copies per $\mu$g$^{-1}$ RNA and s are presented. There are significant differences in the expression of HvPHO2-5 and HvPHO2-3 for interactions of P rate × genotype ($P = 0.006$).

Clipper and Pallas under low-P conditions (Fig. 2, B and C). This shows that greater PUE could have an adverse effect on PAE due to a concomitant increase in carbohydrate partitioning into roots, indicated by higher root-shoot ratios (Fig. 1C).

Expression Levels of the Genes Related to High-Affinity Pi Transporters in P-Acquisition-Inefficient Genotypes May Not Be the Limiting Factor
in Pi retranslocation and regulated by the miR399-PHO2 signaling pathway (Wang et al., 2009; Liu et al., 2010). In addition, the expression of HvPHT1;3 in roots mimics that of HvPHT1;6 (Fig. 6). These data suggest that HvPHT1;3 may play a role in Pi remobilization in roots similar to HvPHT1;6. Higher expression of HvPHT1;6 and HvPHT1;3 is associated with higher PUE (Figs. 2C and 6). PUE has been proposed to be a bottleneck for further improvement of P efficiency in modern crop cultivars (Wang et al., 2010). Interestingly, the P-acquisition-inefficient genotype Sahara is a landrace, whereas P-acquisition-efficient Clipper is a modern cultivar. Higher remobilization of internal P to metabolically active tissues such as growing root tips and shoot meristems under low-P conditions could promote root growth for access to unexplored P resources in soil while maintaining shoot growth. This would lead to higher PAE. However, higher remobilization of internal P could be associated with higher carbohydrate partitioning into roots, as indicated by higher root-shoot ratios in the P-inefficient genotypes (Fig. 1C). A higher root-shoot ratio is also found in P-inefficient wheat genotypes (Liao et al., 2008). Reduced shoot growth would decrease the production of carbohydrates and consequently have an adverse effect on PAE. Therefore, optimization of Pi remobilization may be necessary for high PAE and high biomass production.

Closely related orthologs (BdPHT1;3/BdPHT1;6 and TaPHT1;3/TaPHT1;6) of HvPHT1;3 and HvPHT1;6 were present in both Brachypodium and wheat (Supplemental Fig. S1), suggesting that the low-affinity Pi transporters are conserved within Brachypodium and the Triticeae lineage. Understanding the function and regulation of HvPHT1;3 and HvPHT1;6 could contribute to further improvement of PAE in both barley and other winter cereals.

Correlation in the Expression of HvPHT1;3 and HvPHT1;6 with HvIPS1

The 24-nucleotide motifs are conserved in HvIPS1 and HvIPS2 (Fig. 7, A and B), and both genes were highly responsive to P deficiency (Fig. 4). However, genetic variation in gene expression was observed only for HvIPS1. The expression of HvIPS1 is highly correlated with the expression of HvPHT1;3 and HvPHT1;6 in the four barley genotypes tested under low-P conditions (Figs. 4 and 6), suggesting that HvIPS1 plays a unique role in Pi remobilization.

The conserved motifs are more variable in two barley IPS genes than those of Arabidopsis (Franco-Zorrilla et al., 2007). The calculated MFE values of RNA duplexes formed between HvIPS1 or HvIPS2 and known HvmiR399s (Fig. 7, A and B) suggest that HvIPS1 is more effective in sequestering HvmiR399s than HvIPS2. Consequently, HvIPS1 could be more effective in the protection of HvPHO2 against HvmiR399-guided cleavage. In contrast to Arabidopsis, MFE values predicted for RNA duplexes formed between 24-nucleotide motifs of AtIPS genes and AtmiR399s were similar among five AtIPS genes (data not shown). This suggests that mRNA of all five AtIPS genes may have comparable effectiveness in sequestering AtmiR399s as that shown for AtIPS1 (Franco-Zorrilla et al., 2007). The transcript levels of the miR399 target gene HvPHO2 were generally higher in the low-P conditions (Fig. 8) and were parallel to the transcript levels of HvIPS1 to some extent (Fig. 4), suggesting that the miR399-PHO2 signaling pathway is conserved in barley. AtPHO2 has been shown to be involved in the regulation of PHT1 genes such as AtPHT1;8 and AtPHT1;9 in Arabidopsis (Aung et al., 2006; Bari et al., 2006) and OsPHT1;2 and OsPHT1;8 in rice (Wang et al., 2009). Therefore, it is likely that HvPHT1;6 and HvPHT1;1 are also subject to the regulation of the miR399-PHO2 signaling pathway.

Notably, there are some differences in the transcript levels of PHO2 among Arabidopsis, rice, and barley. The transcript levels of HvPHO2 in roots are generally higher when the transcript levels of HvIPS1 reach the highest values in severely P-deficient plants except for Sahara (Fig. 8), whereas the expression of AtPHO2 in roots is decreased with an increase of AtIPS genes (Aung et al., 2006; Bari et al., 2006; Franco-Zorrilla et al., 2007). There is no change in the transcript levels of OsPHO2 in low-P conditions relative to high-P conditions when OsIPS1 and OsIPS2 are increased (Zhou et al., 2008). miR399-guided cleavage can decrease transcript levels of PHO2, while IPS transcripts can protect HvPHO2 mRNA against miR399-guided degradation (Franco-Zorrilla et al., 2007). A number of factors could have effects on the transcript levels of PHO2, including (1) PHO2 transcription rates under different P conditions, (2) PHO2 mRNA turnover rate, (3) the abundance of different members of the miR399 family (Lin et al., 2008), (4) the abundance of transcripts from different IPS genes, which vary in the conserved 24-nucleotide motif (Hou et al., 2005; Franco-Zorrilla et al., 2007) and the formation of incomplete double-stranded RNA complexes with different stability (Fig. 7, A and B), and (5) four to five complementarity sites for miR399 present in the 5’ UTR of PHO mRNA (Franco-Zorrilla et al., 2007), since different members of the miR399 family may have preference for these sites (Allen et al., 2005). The observed differences in the transcript levels of PHO2 between the three species could be due to any combination of these factors or other factors. Further studies are obviously required to elucidate the difference. Despite the differences in the transcript levels of PHO2 among these three species, the correlation in the expression levels between IPS and PHT1 genes related to Pi retranslocation is consistent across the three species. In addition, although the expression levels of HvPHT1;3 and HvPHT1;6 are highly correlated with those of HvIPS1 (Figs. 4 and 6), the correlation in the expression between HvPHT1;3/HvPHT1;6 and HvPHO2 is not as tight as that with HvIPS1, especially in Sahara at P7.5 (Figs. 6 and 8), suggesting that additional mechanisms may be involved in the regulation of PHO2 and downstream PHT1 genes.
in Pi remobilization. There are other regulators involved in Pi signaling (Lin et al., 2009, 2010). It has been shown that OsSPX1 and OsPHR2 are involved in the regulation of low-affinity Pi transporters such as OsPHT1;2 (Wang et al., 2009; Liu et al., 2010). Sugars as a systemic signal can also regulate the expression of Pi transporters (Hammond and White, 2008). Therefore, the transcript levels of HvPHT1;3 and HvPHT1;6 could be modulated by additional regulators. A further analysis of barley orthologs of OsSPX1 and OsPHR2 could shed light on the regulation of HvPHT1;3 and HvPHT1;6 via the miR399-PHO2 pathway.

In summary, Pi retranslocation within the plant is an important mechanism in plant adaptation to variable Pi supply. Low-affinity Pi transporters are responsible for Pi retranslocation within the plant. The higher expression of the low-affinity Pi transporter genes, HvPHT1;6/HvPHT1;3, could lead to greater PUE, but the concomitant increase in carbohydrate partitioning into roots could have an adverse effect on carbohydrate production and PAE. Therefore, optimization of PUE in plants may be required for high PAE and high yield in low-input agriculture systems.

MATERIALS AND METHODS

Plant Growth and P Analysis

Four barley (Hordeum vulgare) genotypes, Pallas, brb (a mutant of Pallas), Clipper, and Sahara, previously shown to differ in P efficiency (Zhu et al., 2002; Gahoonia and Nielsen, 2003) were used in our experiments. All seeds were multiplied in a potting mix for similar nutrient contents. The weight of seeds used in the experiment was similar, in the range of 52 to 54 mg seed⁻¹ for three genotypes (Pallas, brb, and Clipper), but smaller for Sahara (45 mg seed⁻¹). The P content of Pallas, brb, Clipper, and Sahara was 259, 212, 285, and 193 µg P seed⁻¹, respectively.

Siliceous sand was used in the experiments. Basal nutrients and plant growth conditions were as described by Genc et al. (2007). Calcium carbonate powder (0.5%, w/v) was added to the sand to simulate calcareous sandy soil, and to reduce P availability (Ryan et al., 1985; Westermann, 1992). Three P rates (7.5, 22.5, and 75 mg P kg⁻¹ sand as KH₂PO₄) were used in the experiments for severely P-deficient, moderately P-deficient, and P-adequate plants, respectively.

Plants were harvested at 16 d after seed imbibition. At harvest, shoots of two plants in each pot were cut above the soil surface, and the fresh weight of each plant was recorded. The shoots of one plant were oven dried for shoot dry weight and nutrient analysis, and the shoots of the other plant were frozen in liquid nitrogen for transcript analysis. Shoot P was determined with inductively coupled plasma emission spectrometry (Zarcinas et al., 1987). The roots of two plants in each pot were washed free of sand particles and separated. The excess water in roots was removed with tissue paper, and the fresh weight of roots was recorded. The roots of one plant were frozen immediately in liquid nitrogen for transcript analysis, and the roots of the other plant were used for measurements of root morphology.

Identification of Two New Paralogs of HvPHT1;1

A 388-bp probe derived from the coding sequence of HvPHT1;1 (GenBank accession no. AV188394: 945–1332 bp) was used for screening a Haruna Nijo BAC library (Saisho et al., 2007). Two unique BAC clones were isolated, one containing HvPHT1;10 and the other containing HvPHT1;1, HvPHT1;9, and HvPHT1;2. The two BAC clones were sequenced, and contigs were assembled for HvPHT1;9 and HvPHT1;10.

Real-Time Quantitative RT-PCR

RNA isolation and real-time quantitative RT-PCR analysis of transcripts were conducted as described by Preuss et al. (2011). Briefly, total RNA from roots was prepared using Trizol reagent according to the manufacturer’s instructions (Invitrogen) and treated with DNase I (Ambion). Then, RNA integrity was checked on an agarose gel. Two micrograms of total RNA from roots was used to synthesize cDNA with SuperScript III reverse transcriptase (Invitrogen). The transcript levels of four control genes (barley a-tubulin, heat shock protein 70, glycaldehyde-3-phosphate dehydrogenase, and cyclophilin) were determined for all DNA samples, and the most similar three of these four genes were used as normalization controls. Normalization was carried out as described by Vandesompele et al. (2002) and Burton et al. (2008) for the transcript levels of all root cDNA samples. Four biological replicates were used for transcript analysis. Three technical replicates were conducted for each cDNA sample. The normalized copies µg⁻¹ RNA were used to represent transcript levels. The primer sequences for all genes determined are listed in Supplemental Table S1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic tree of PHT1 Pi transporters in Arabidopsis, rice, Brachypodium, barley, and wheat.

Supplemental Figure S2. PCR amplification of genomic DNA of four barley genotypes using HvPHT1;9-specific primers.

Supplemental Table S1. Primer sequences used for quantitative, real-time RT-PCR analysis of transcript levels.

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Genetic Variation in PHT1 and IPS Expression