Running title:
Genetic variation in PHT1 and IPS expression

Corresponding author:
Chun Y. Huang
Australian Centre for Plant Functional Genomics
The University of Adelaide, Waite Campus
PMB1, Glen Osmond.
South Australia, 5064
Australia

Tel: +61 8 8303 7168
FAX: +61 8 8303 7102
Email: chunyuan.huang@adelaide.edu.au

Journal Research Area:
Environmental Stress and Adaptation
Manuscript title:

Phosphate utilization efficiency correlates with expression of low-affinity phosphate transporters and non-coding RNA, *IPS1* in barley

Chun Y Huang¹, Neil Shirley¹, Yusuf Genc², Bujun Shi¹ and Peter Langridge¹

¹ Australian Centre for Plant Functional Genomics, The University of Adelaide, PMB1, Glen Osmond, South Australia, 5064, Australia
² School of Agriculture, Wine and Food, The University of Adelaide, South Australia, 5005, Australia
Footnotes: This work was supported by the Grains Research and Development Corporation (CYH, NS, BJS, PL), the Australian Research Council (CYH, NS, BJS, PL), South Australian Government (CYH, NS, BJS, PL), and the University of Adelaide (CYH, NS, YG, BJS, PL).

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Chun Y Huang (chunyuan.huang@adelaide.edu.au).

*Corresponding author; e-mail chunyuan.huang@adelaide.edu.au; fax 61-8-8303-7102
ABSTRACT

Genetic variation in phosphorus (P) efficiency exists among wheat and barley genotypes, but underlying mechanisms for the variation remain elusive. High- and low-affinity phosphate (P_i) 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 P_i transporters in four barley genotypes differing in PAE. Results showed that there was no clear pattern in the expression of four paralogs of the high-affinity P_i transporter HvPHT1;1 among the four barley genotypes, but the expression of a low-affinity P_i transporter, HvPHT1;6 and its close homolog HvHPT1;3 was correlated with the genotypes differing in PUE. Interestingly, the expression of HvPHT1;6 and HvPHT1;3 was correlated with the expression of HvIPS1 (P starvation-inducible, non-coding RNA), but not with HvIPS2, suggesting that HvIPS1 plays a distinct role in the regulation of the low affinity P_i 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 P_i transporter expression may assist further improvement of PAE for low-input agriculture systems.
INTRODUCTION

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 (P_i) fertilizers are applied to achieve high crop yields. Wheat and barley production uses approximately 46% of the P fertilizers applied to cereals (FAO Fertilizer and Plant Nutrition Bulletin 17, http://www.fao.org). Extensive fertilization of P leads to rapid depletion of non-renewable 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_i 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; Ismail et al., 2007; Ramaekers et al., 2010), but PUE can influence PAE. P supply to plants is often fluctuating, and therefore P remobilization within the plant is critical for the 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 including denser and longer root hairs (Bates and Lynch, 2001), 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; and Liao et al., 2008) is not clear (Manske et al., 2001; Wang et al., 2010).

\( \text{PHT1} \) P\(_i\) transporters play a critical role in \( \text{P}_i \) acquisition from soil solution and \( \text{P}_i \) remobilization within the plant. Nine members of the \( \text{PHT1} \) gene family have been found in the \textit{Arabidopsis} genome (Mudge et al., 2002) and thirteen in rice (Paszkowski et al., 2002). Functional characterization shows that some of the PHT1 members, such as AtPHT1;1, AtPHT1;4 (Shin et al., 2004) and OsPHT1;6 (Ai et al., 2009), are high-affinity transporters while others are low-affinity transporters, such as OsPHT1;2 (Ai et al., 2009). Eight \( \text{PHT1} \) genes have been described in barley (Smith et al., 1999). The promoter::reporter fusions of \( \text{HvPHT1;1} \) and \( \text{HvPHT1;2} \) show that reporters are expressed in root hairs, epidermal and cortex cells, and vascular tissues of roots (Schunmann et al., 2004; Glassop et al., 2005). Recently it was confirmed that HvPHT1;1 is a high-affinity \( \text{P}_i \) transporter with a \( K_m \) value of 1.9 \( \mu \text{M} \) when expressed in \textit{Xenopus laevis} oocytes (Preuss et al., 2011), suggesting that HvPHT1;1 is involved in \( \text{P}_i \) acquisition. \( \text{HvPHT1;6} \) is expressed in both shoots and roots (Huang et al., 2008), and localized in the leaf phloem tissue (Rae et al., 2003). It displays unsaturated uptake in a millimolar range of \( \text{P}_i \) concentrations when expressed in oocytes (Preuss et al., 2010), suggesting that HvPHT1;6 is involved in \( \text{P}_i \) retranslocation. \( \text{HvPHT1;7} \) is specifically expressed in green anthers (Druka et al., 2006), and \( \text{HvPHT1;8} \) is a mycorrhiza-specific gene (Glassop et al., 2005). \( \text{HvPHT1;3} \) and \( \text{HvPHT1;4} \) are almost identical in nucleotide sequence. \( \text{HvPHT1;3} \) is specifically...
expressed in roots (Smith et al., 1999). However, the functions of *HvPHT1;3* and *HvPHT1;4* in P homeostasis are not clear. Only one wheat *PHT1* gene, *TaPHT1;2D1* has been characterized so far (Miao et al., 2009) although several *TaPHT1* genes are reported (Davies et al., 2002; Miao et al., 2009). Whole genomic sequences of barley and wheat are not yet available, which has hindered progress towards functional characterization of *PHT1* genes and their regulation in barley and wheat.

P acquisition and translocation are highly regulated processes. Transcriptional activation of P transporter genes is believed to be the major control point in P transporter activity (Raghothama, 1999; Franco-Zorrilla et al., 2004). *IPS* (Induced by Phosphate Starvation) genes have been shown to be involved in P homeostasis (Shin et al., 2006; Franco-Zorrilla et al., 2007; Doerner, 2008; Hammond and White, 2008; Liu et al., 2010). They are a class of non-protein coding RNA. Overall sequence identities among *IPS* genes are relatively low, but 22-24 nucleotide sequences are highly conserved among plant species (Hou et al., 2005; Shin et al., 2006). *IPS* genes are expressed primarily in vascular tissues of roots and shoots (Hou et al., 2005; Shin et al., 2006). Five *IPS* genes are found in the *Arabidopsis* genome (Franco-Zorrilla et al., 2007), and two *IPS* genes are present in monocotyledonous species such as rice, maize and barley (Hou et al., 2005). The *Arabidopsis* loss-of-function mutant *at4*, shows high P accumulation in shoots (Shin et al., 2006). Inversely, overexpression of *AtIPS1* and *At4* decreases P accumulation in shoots (Franco-Zorrilla et al., 2007), indicating that *IPS* genes are involved in P remobilization and translocation.

MiRNAs represent a class of non-coding small RNAs that generally function as post-transcriptional negative regulators through base pairing to nearly
complementary sequences in target mRNAs (Jones-Rhoades et al., 2006). MiR399 is the first miRNA found to be involved in P\textsubscript{i} 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 an ubiquitin E2 conjugase, regulates P\textsubscript{i} homeostasis in the plant (Aung et al., 2006; Bari et al., 2006; Mallory and Bouche, 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 expression of AtPHT1;8 and AtPHT1;9, and controls remobilization and translocation of P\textsubscript{i} via an unknown process (Aung et al., 2006; Bari et al., 2006; Chiou et al., 2006; Doerner, 2008).

Considerable 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 P\textsubscript{i} transporters, and the relationships between the expression of P\textsubscript{i} transporters and PAE or PUE (Ramaekers et al., 2010). To address this issue, we use barley (Hordeum vulgare L.), a diploid relative of hexaploid wheat (Triticum aestivum L.) as a model system for winter cereals to study the functions of PHT1 genes involved in acquisition and remobilization of P\textsubscript{i} (Preuss et al., 2010; Preuss et al., 2011). In this report, we show that differences in expression levels of four paralogs of the high-affinity P\textsubscript{i} transporter gene, HvPHT1;1, are not apparent among four barley genotypes differing in PAE, but there are a large difference in the expression of the low-affinity P\textsubscript{i} transporters,
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 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; Bates and Lynch, 2001; Lambers et al., 2006). 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 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 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 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 \textit{brb} and Sahara, and shoot dry weight of Clipper was significantly higher than that of Sahara (Fig. 1A).

Root growth on a fresh weight (FW) basis was reduced by the low rates of P supply, but the reduction was much less than for shoot growth (Fig. 1A,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 three other genotypes, but no differences were found among \textit{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 \textit{brb}, but not between Clipper and Sahara.

An increase in root:shoot ratio is often observed in the 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 FW basis increased as P supply decreased in all genotypes (Fig. 1C). At low P supply (P7.5), root:shoot ratios of \textit{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 \textit{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 4000 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 Clipper remained higher than the other genotypes, and Pallas had a significantly higher P concentration than either brb or Sahara. When P supply further decreased to P7.5, P concentrations of shoots further reduced in all four genotypes (Fig. 2A). The shoot P concentration of Pallas remained higher than that of 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 brb or Sahara, but no difference between Pallas and Clipper. These results indicate that Pallas and Clipper are efficient in P acquisition, and brb and Sahara are inefficient in P acquisition.

When biomass production per unit P in shoots of four genotypes was calculated for P utilization efficiency (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 (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. This suggests that high remobilization of P occurs simultaneously with enhanced carbohydrate partitioning into roots.

**Identification of Additional HvPHT1;1 Paralogs in the Barley Genome**

Two paralogs of HvPHT1;1 genes have been described by Smith et al. (1999). We isolated two additional 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 HvPHT1;1. The first gene encoding a protein was identical to HvPHT1;1 (Fig. 3A), but differed in the 3′ untranslated region (UTR). We designated this new HvPHT1 gene as HvPHT1;9 (Genbank accession number: AM904733). The transcript of HvPHT1;9 had a 56-nucleotide deletion in the 3′UTR compared with that of HvPHT1;1 (Fig. 3B). The second new HvPHT1;1 paralog is similar to HvPHT1;2, and is designated as HvPHT1;10 (GenBank accession: FN392213). The protein sequence of HvPHT1;10 differs only by four amino acid residues from HvPHT1;2 (Fig. 3A).

Database searches identified an ortholog of HvPHT1;9 in wheat (T. aestivum L.) (Genbank accession number: BJ277773), which also has a 30 nucleotide deletion in the 3′UTR compared with that of TaPHT1;1 (Genbank accession number:
CD871730). No *Brachypodium* sequences which are similar to either *HvPHT1;1* or *HvPHT1;9* were identified (Fig. S1).

Database searches using the coding sequences of *HvPHT1;2* and *HvPHT1;10* identified four orthologs in wheat and one ortholog in *Brachypodium*. All five orthologs of HvPHT1;2/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 number: AJ344240) was used to represent TaPHT1;2 in the phylogenetic analysis of PHT1 proteins (Fig. S1). The phylogenetic tree showed that protein sequences of HvPHT1;1/HvPHT1;9, HvPHT1;2, HvPHT1;10, TaPHT1;1/TaPHT1;9, TaPHT1;2 and BdPHT1;2 form a closely related clade (Fig. S1), suggesting that HvPHT1;1/HvPHT1;9 and TaPHT1;1/TaPHT1;9 originated from HvPHT1;10/ HvPHT1;2 and TaPHT1;2.

**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 *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 a 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 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 genotype, Pallas and Clipper and the P-acquisition-inefficient, *brb* and Sahara, especially at the moderately low P supply (P22.5), but this is not seen for *HvIPS2* expression.
Expression of Four *HvPHT1;1* Paralogs in Four Barley Genotypes

At P75, a moderate level of *HvPHT1;1* transcripts (3 to 12 × 10⁶ 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 three other genotypes. The transcript level of *HvPHT1;1* in *brb* was significantly higher than that in Pallas, but was similar to that in Clipper. When P supply decreased to P7.5, the transcript levels of *HvPHT1;1* further increased in all four genotypes (Fig. 5A). The transcript level of *HvPHT1;1* in Sahara remained higher than three other genotypes, but there was no significant difference among Pallas, *brb* and Clipper (Fig. 5).

Much lower levels of *HvPHT1;9* transcripts (0.4 to 2.3 × 10⁶ 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 at P22.5 and P7.5 increased the transcript levels of *HvPHT1;9* in Pallas, *brb* and Clipper, but the transcript levels 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 significant 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: 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 (Fig. 2S). 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 \(9 \times 10^6\) normalized copies \(\mu g^{-1}\) 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 of 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 \(HvPHT1;2\) 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 a much higher transcript level 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 \(HvPHT1;10\) in Clipper and Sahara were at least twice as much as those of \(HvPHT1;2\) at all three rates of P. There was no difference in the transcript level of \(HvPHT1;10\) between Clipper and Sahara across three rates of P supply (Fig. 5).

The transcript levels of the four \(HvPHT1;1\) paralogs reveal that all four genes are highly responsive to P deficiency. Although there were genotypic differences in
the expression of the four *HvPHT1;1* paralogs, the expression pattern was not apparent between two P-acquisition-inefficient genotypes, and two P-acquisition-efficient genotypes.

**Expression of *HvPHT1;6* and *HvPHT1;3* in Four Barley Genotypes**

At sufficient P supply (P75), the transcript levels of *HvPHT1;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 *HvPHT1;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 *HvPHT1;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 *HvPHT1;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 *HvPHT1;6* in Pallas and Clipper was also increased at P7.5, but remained lower than 3.0 × 10^6 normalized copies μg\(^{-1}\) RNA.

*HvPHT1;3* protein has the same number of amino acid residues as *HvPHT1;6*. *HvPHT1;3* protein is more similar to *HvPHT1;6* (77%) than the four *HvPHT1;1* paralogs (70% identity). The transcript levels of *HvPHT1;3* almost mimicked those of *HvPHT1;6* (Fig. 6). A large difference in the expression of *HvPHT1;6* and *HvPHT1;3* was evident between the P-acquisition-efficient and P-acquisition-inefficient genotypes (Fig. 6). Interestingly, the difference in the expression of *HvPHT1;6* and
HvPHT1;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 df=30, respectively, P<0.001), but not with that of HvIPS2. The P-acquisition-inefficient genotypes, brb and Sahara had much higher expression levels of HvPHT1;3, HvPHT1;6 and HvIPS1 in roots under the low P conditions than those of the P-acquisition-efficient genotypes, Pallas and Clipper (Fig. 4 and Fig. 6).

Stability of RNA Duplexes Formed between Conserved 24-Nucleotide Motifs of HvIPS1 or HvIPS2 and HvmiR399s

The conserved 24 nucleotide motif of HvIPS1 differs from that of HvIPS2 by six nucleotides in the 11th to 16th nucleotide positions, which correspond to the 10th to 14th nucleotides positions of HvmiR399s from the 5´ end (Fig. 7A,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 HvIPS2 and HvmiR399s (Fig. 7A,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 of more negative than -30.9 kcal mole⁻¹ (Fig. 7A,C). In contrast, the 24-nucleotide motif of HvIPS2 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 of less negative than -27.1 kcal mole⁻¹ except for HvmiR399d.
A higher MFE value was predicted in the RNA duplex formed between HvIPS1 or HvIPS2 and HvmiR399d (Fig. 7A,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 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 4000 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 three other genotypes (Fig. 8). When P supply reduced to P22.5, the transcript levels of HvPHO2 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) was 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 indicate 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 when 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 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, Clipper and Pallas, under low P conditions (Fig. 2B,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 P$_i$ Transporters in P-Acquisition-Inefficient Genotypes May Not Be the Limiting Factor

All four paralogs of the high-affinity P$_i$ 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* (Fig. 5, Fig. 3B and Fig. S2). Our recent work revealed that HvPHT1;1 is a high-affinity P$_i$ transporter with a $K_m$ value of 1.9 µM when expressed in *Xenopus* oocytes (Preuss et al., 2011). Higher transcript abundance of *HvPHT1;1* is found in the root hair zone than that in the root tip (Preuss et al., 2011). The expression pattern of *HvPHT1;1* in root tissues overlaps with *HvPHT1;2* (Schunmann 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 (Fig. S1), suggesting that the *PHT1* genes involved in P acquisition are conserved in *Brachypodium* and *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 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 P$_i$ 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 P$_i$ signaling initiated by plant low P status (Fig. 2A and Fig. 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 P$_i$ signal (Yang and Finnegan, 2010) may be impaired due to absence of root hairs in brb.

**Expression Levels of the Genes Related to Low-Affinity P$_i$ Transporters is Correlated with PUE**

*HvPHT1;6* is a low-affinity P$_i$ transporter (Preuss et al., 2010). It is expressed in both shoots and roots (Huang et al., 2008), and specially in phloem tissues of the leaf (Rae et al., 2003), suggesting that *HvPHT1;6* plays a role in P$_i$ remobilization in the whole plant. The protein sequence of HvPHT1;3 is more similar to HvPHT1;6 than the HvPHT1;1 paralogs. HvPHT1;3 is grouped to the clade containing OsPHT1;8, BdPHT1;3 and TaPHT1;3 with a high bootstrap value (Fig. S1). OsPHT1;8 and the low-affinity P$_i$ transporter OsPHT1;2 have been shown to be
involved in Pi retranslocation and regulated by the miR399-PHO2 signaling pathway (Wang et al., 2009; Liu et al., 2010). In addition, the expression of \textit{HvPHT1;3} in roots mimics that of \textit{HvPHT1;6} (Fig. 6). These data suggests that \textit{HvPHT1;3} may play a role in Pi remobilization in roots similar to \textit{HvPHT1;6}. Higher expression of \textit{HvPHT1;6} and \textit{HvPHT1;3} is associated with higher PUE (Fig. 2C and Fig. 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, 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 production of carbohydrates, and therefore have an adverse effect on PAE. Therefore, optimization of P, remobilization may be necessary for high PAE and high biomass production.

Closely related orthologs (BdPHT1;3, BdPHT1;6, and TaPHT1;3, and TaPHT1;6) of \textit{HvPHT1;3} and \textit{HvPHT1;6} were present in both \textit{Brachypodium} and wheat (Fig. S1), suggesting that the low-affinity P, transporters are conserved within \textit{Brachypodium} and \textit{Triticeae} lineage. Understanding the function and regulation of \textit{HvPHT1;3} and \textit{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. 7A,B), and both genes were highly responsive to P deficiency (Fig. 4). However, genetic variation in the 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 (Fig. 4 and Fig. 6), suggesting that HvIPS1 plays a unique role in P$_i$ remobilization.

The conserved motifs are more variable in two barley IPS genes than those of Arabidopsis (Franco-Zorrilla et al., 2007). The calculated minimum free energy (MFE) values of RNA duplexes formed between HvIPS1 or HvIPS2 and known HvmiR399s (Fig. 7A,B) suggests 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 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;3 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 is generally higher when the transcript levels of HvIPS1 reach the highest values in severely P-deficient plants except for Sahara (Fig. 8), whereas 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) abundance of different members of the miR399 family (Lin et al., 2008), (4) abundance of transcripts from different members of IPS genes, which vary in the conserved 24-nt motif (Hou et al., 2005; Franco-Zorrilla et al., 2007), and formation of incomplete double stranded RNA complexes with different stability (Fig. 7A,B), and (5) 4-5 complementarity sites for miR399 present in 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 combinations of these factors or other factors. Further studies are obviously required to elucidate the difference. Despite of the
differences in the transcript levels of PHO2 among 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 (Fig. 4 and Fig. 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 (Fig. 6 and Fig. 8), suggesting that additional mechanisms may be involved in the regulation of PHO2 and down-stream PHT1 genes in Pi remobilization. There are other regulators involved in P signaling (Lin et al., 2009; Lin et al., 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 adaption to variable P 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* L.) genotypes, Pallas, *brb* (a mutant of Pallas), Clipper and Sahara previously shown to differ in P efficiency (Gahoonia and Nielsen 2003; Zhu et al. 2002) were used in present experiments. All seeds were multiplied in a potting mix for similar nutrient contents. The weight of seeds used in the experiment was similar in a range of 52 - 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 experiment. Basal nutrients and plant growth conditions were as described by Genc et al (2007). Calcium carbonate powder (0.5% w/w) was added in 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.

Plants were harvested at 16 days after seed imbibition. At harvest, shoots of two plants in each pot were cut above soil surface, and 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
spectrometer (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 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 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: AY188394, 945 to 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, Austin, TX, USA). Then RNA integrity was checked on an agarose gel. Two micrograms of total RNA from roots was used to synthesize cDNA with SuperScriptTM III reverse transcriptase (Invitrogen). The transcript levels of four control genes (barley a-tubulin, heat shock protein 70, glyceraldehyde-3-phosphate dehydrogenase and cyclophilin) were
determined for all cDNA samples, and the most similar three of these four genes were used as normalization controls. Normalization was carried out as described by Vanesompele 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 RNA⁻¹ were used to represent transcript levels. The primer sequences for all genes determined are listed in Table S1.

**Minimum Free Energy of RNA Duplexes**

The RNAhybrid software (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) was used with default settings to calculate minimum free energy of RNA duplexes formed between HvIPS genes and HvmiR399s (Rehmsmeier et al., 2004).

**Statistical Analysis**

The experimental set up was a completely randomised block design with four replications. Data of plant growth, P nutrition and transcripts were analyzed using the Genstat Statistical Program (version 11.1, VSN International Ltd). The logarithm transformed data were used in analysis when treatment effects were larger than 10 times. The Least Significant Difference (LSD) at $P=0.05$ was used for comparisons of means.
ACKNOWLEDGEMENTS

We are grateful to Dr Julie Hayes for valuable comments on the manuscript, Dr Iver Jakobsen for providing the seeds of Pallas and brb, and John Toubia, Margaret Pallotta, and Hui Zhou for technical assistance.

LITERATURE CITED


carbon and nitrogen metabolism in phosphate-deficient barley plants


FIGURE LEGEND

**Figure 1.** Shoot dry weight and root fresh weight of four barley genotypes. (A) Shoots 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 as P7.5, P22.5 and P75. Plants were harvested at the 16th day after imbibition. Means and standard errors of four replicates are presented. There are significant differences in shoot dry weight and root:shoot ratio for interactions of P rates \(\times\) genotypes \((P<0.001)\). Vertical lines indicate least significant difference (LSD0.05) for shoot dry weight and root:shoot ratio. There are no significant differences in root fresh weight for interactions of P rates \(\times\) genotypes \((P=0.25)\).

**Figure 2.** P concentration, P content and P utilization efficiency of shoots in four barley genotypes. (A) P concentrations of shoots. (B) P content of shoots. (C) P use efficiency of shoots. 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 as P7.5, P22.5 and P75. Plants were harvested at the 16th day after imbibition. Means and standard errors of four replicates are presented. There are significant differences in P concentrations and PUE for interactions of P rates \(\times\) genotypes \((P<0.009)\). Vertical lines indicate least significant difference (LSD0.05) for P concentrations and PUE. There are no significant differences in shoot P content for interactions of P rates \(\times\) genotypes \((P=0.39)\).
Figure 3. Alignment of four HvPHT1 proteins and partial nucleotide sequences of HvPHT1;1 and HvPHT1;9. (A) Alignment of four HvPHT1 proteins. Amino acid residues which differ among four HvPHT1 proteins are shown, and the residue numbers are indicated above. (B) Alignment of nucleotide sequences of HvPHT1;1 and HvPHT1;9 in the 3’UTR. The nucleotide deletion in HvPHT1;9 is shown as x, and specific primers for quantitative RT-PCR of HvPHT1;1 and HvPHT1;9 are underlined. Accession numbers are as follows: AY188394 (HvPHT1;1), AY187020 (HvPHT1;2), AM904733 (HvPHT1;9) and FN392213 (HvPHT1;10).

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 as P7.5, P22.5 and P75. Plants were harvested at the 16th day after seed imbibition. Quantitative real-time RT-PCR was used to determine transcript levels in roots. The means (n=4) of normalized copies per µg RNA and standard errors are presented. Logarithm 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 rates × genotypes (P<0.001), but not for 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 as P7.5, P22.5 and P75. Plants were harvested at the 16th day 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 µg RNA and standard errors are presented. Logarithm 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 rates × genotypes (*P*<0.001 for all three genes), but not for *HvPHT1;10* (*P*=0.49).

**Figure 6.** Transcript levels of *HvPHT1;6* and *HvPHT1;3* 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 as P7.5, P22.5 and P75. Plants were harvested at the 16th day 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 µg RNA and standard errors are presented. Logarithm transformed data were used in the statistical analysis for comparisons of means. There are significant differences in the expression of *HvPHT1;6* and *HvPHT1;3* for interactions of P rates × genotypes (*P*<0.001).

**Figure 7.** Nucleotide sequence alignment of conserved motifs of *HvIPS1* and *HvIPS2* with known *HvmiR399* members and minimum free energy (MFE) of RNA duplexes. (A) Alignment of the conserved motif of *HvIPS1* with known *HvmiR399* members. (B) Alignment of the conserved motif of *HvIPS2* with known *HvmiR399*. (C) An example for structure of the RNA duplex formed between *HvIPS1* in red and *HvmiR399b* in green. (D) An example for structure of the RNA duplex formed between *HvIPS2* in red and *HvmiR399b* in green. MFE and the structure of RNA duplexes were obtained with RNAhybrid software (see Materials and Methods).
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 as P7.5, P22.5 and P75. Plants were harvested at the 16\(^{th}\) day 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 µg RNA and standard errors are presented. There are significant differences in the expression of *HvPHO2-5* and *HvPHO2-3* for interactions of P rates × genotypes (\(P=0.006\)).