A phosphate starvation response regulator Ta-PHRI is involved in phosphate signalling and increases grain yield in wheat

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INTRODUCTION

The world is facing an immense challenge to increase food productivity for its growing population. As phosphorus (P) fertilizers are essential for growing our food, annual consumption of P fertilizers has increased rapidly in the past decades and is expected to increase by 50–100% by 2050 (Cordell et al., 2009). However, the majority of P applied to soil is immobilized and becomes unavailable for plants; the recovery of applied P by crops in a growing season is often low (Schachtman et al., 1998; Vance et al., 2003). Furthermore, P fertilizer is mainly made from the non-renewable phosphate rock, which is expected to run out in the near future (Cordell et al., 2009). Therefore, systematic approaches need to be made to use soil P and fertilizer P sustainably and efficiently, including development of P-efficient crop varieties (Lynch, 2011).

It is well documented that genetic differences in P use efficiency exist among plant species and genotypes within a species, indicating that it is possible to improve P use efficiency of plants through a genetic approach. Conventional selection methods have been shown to be successful in breeding crops with improved P use efficiency. For example, the CIMMYT (International Maize and Wheat Improvement Centre) wheat programme has selected wheat cultivars that can efficiently acquire P under low P conditions, but also respond to P applications (Manske et al., 2000). As P has low mobility and high fixation in soils (Schachtman et al., 1998), selecting root traits with an increased root–soil contact area and ability to mobilize unavailable P is crucial in improving P use efficiency (Manske et al., 2000). However, these root traits are hidden in the soil; therefore, root breeding through conventional phenotypic selection is time consuming and destructive. It has been shown that quantitative trait loci (QTLs) for root morphology and architecture are linked to those for P acquisition efficiency in soybean (Glycine max), maize (Zea mays) and wheat (Triticum aestivum) (Zhu et al., 2005; Liang et al., 2010; Y. Z. Ren et al., 2012). This suggests that marker-assisted selection will facilitate efficient selection for root traits in root breeding for P use efficiency.

Besides conventional breeding and marker-assisted selection, gene modification has also successfully improved P use efficiency of crops. In response to P deficiency, plants have evolved a wide range of morphological, physiological and biochemical strategies to improve P mobilization and uptake from the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
soil (Raghothama, 1999; Cheng et al., 2011; Plaxton and Tran, 2011). Alterations in root architecture are important to enable plants to explore new Pi resources in the soil more efficiently. Under P-deficient conditions, plants increase their root hair length and density, and lateral or adventitious root number and length (Lopez-Bucio et al., 2002; Lynch, 2011). Recently, a β-expansin gene, Gm-EXPB2, has been shown to regulate root architecture responses to Pi starvation in soybean, and over-expressing Gm-EXPB2 significantly improves P uptake and growth of soybean (Guo et al., 2011). Since most of the soil P presents in sparingly soluble forms which can be mobilized by root-secreted protons (H\(^+\)), low molecular weight organic anions and phosphatase enzymes (Cordell et al., 2009), stimulating root secretions through gene modification (e.g. over-expressing phosphatase) has been shown to be effective in enhancing Pi acquisition and growth of crops (Tian et al., 2012). For instance, over-expressing a wheat malate transporter (Ta-ALMT1) was shown to enhance P uptake and grain yield of barley (Hordeum vulgare) grown on an acid soil (Delhaize et al., 2009). The phosphate transport gene family PHT1 is pivotal for Pi uptake from soil and the transportation of Pi from root to shoot (Chiou et al., 2001; Schumann et al., 2004; Nagarajan et al., 2011). Over-expression of the PHT1 gene facilitates Pi uptake in transgenic rice plants, and even results in excess P accumulation toxic to plant development (Jia et al., 2011); however, it does not function in increasing Pi uptake in transgenic barley plants grown in nutrient solution and soil (Rae et al., 2004).

The complex gene network responding to Pi starvation is mediated by a number of regulators (Schachtman and Shin, 2007; Nilsson et al., 2010; Chiou and Lin, et al., 2011; Hammond and White, 2011). Over-expression of a transcription factor, PFT1 (Pi starvation induced transcription factor 1), enhances P uptake and yield under P-deficient conditions in both rice and maize (Yi et al., 2005; Li et al., 2011), showing the importance of transcription factors in improving P use efficiency of crops. In the signalling network in sensing P availability, the MYB-CC (coiled-coil) type transcription factor PHR1 plays a central role (Chiou and Lin et al., 2011). PHR1 was first reported to regulate a small subset of Pi starvation-inducible genes in arabidopsis by binding as a dimer to the cis-element P1BS (GNATATNC) in the promoter region of its downstream gene (Rubio et al., 2001). Defective mutation in At-PHR1 impairs a broad range of Pi starvation responses, including the responsiveness of various Pi starvation-inducible genes, root morphology and metabolic responses (Rubio et al., 2001). Recent genome-wide analysis revealed that the majority of Pi starvation-inducible genes contain the P1BS element, and hence the PHR1 gene has been considered to play a more important role than had been previously reported (Mueller et al., 2007; Nilsson et al., 2007, 2010). At-PHR1 and its homologues PHL1 in arabidopsis and Os-PHR2 in rice regulate a set of Pi starvation-inducible genes, including those involving Pi uptake and redistribution (Rubio et al., 2001; Nilsson et al., 2007; Zhou et al., 2008; Bustos et al., 2010). Over-expression of PHR1 in arabidopsis results in strongly increased Pi uptake irrespective of P regime, providing a useful approach for molecular breeding of plants towards more efficient Pi uptake and assimilation (Nilsson et al., 2007). However, enhanced Pi uptake by over-expressing PHR1 does not essentially improve plant growth. For example, over-expression of Os-PHR2 in rice dramatically increases Pi uptake under a range of P supply levels, but it greatly inhibits growth and yield of rice (Zhou et al., 2008). The growth retardation mediated by Os-PHR2 may be caused by toxicity of excessive Pi accumulation in shoots, or by some unknown genetic factor(s) regulated by Os-PHR2 (Wu and Xu, 2010).

Wheat is one of the most important food crops in the world. Worldwide wheat production annually consumes 6-5 Mt of P\(_2\)O\(_5\) (approx. 40 % of P used by cereal crops), much higher than used by other cereals, including rice and maize (FAO, 2006). Therefore, improving P use efficiency of wheat is important in sustainable use of P resources. To breed wheat with improved P use efficiency, understanding the Pi signalling network in wheat is important. Presently, only a few Pi starvation-inducible genes have been cloned and their expression patterns have been analysed, including various PHT1 genes (Davies et al., 2002; Glassop et al., 2005; Tittarelli et al., 2007), thus the Pi signalling network in wheat remains largely unknown. Although improving P use efficiency via transgenic modification has been reported in a number of crops (Ramaekers et al., 2010; Tian et al., 2012), studies of transgenic wheat with improved P use efficiency are still lacking. Here, we isolated three wheat PHR1 genes (Ta-PHR1-A1, B1 and D1) with homology to At-PHR1, and characterized the function of Ta-PHR1-A1 in regulating the Pi starvation response. We found that Ta-PHR1-A1 is involved in Pi signalling in wheat and, when over-expressed, it increased P uptake and grain yield of wheat. Our research promotes the understanding of Pi signalling in wheat, and provides valuable gene resource for breeding wheat with improved P use efficiency and yield.

MATERIALS AND METHODS

Gene cloning

A dbEST search was performed on the web pages of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/nucest/) for the At-PHR1 protein sequence (NP_194590). Primers (Ta-PHR1CF and Ta-PHR1CR, Supplementary Data Table S1) were designed according to identified expressed sequence tags (ESTs) containing At-PHR1 orthologues to amplify specific fragments from a wheat cDNA template. Full-length cDNAs were amplified from wheat variety Xiaoyan 54, designated as Ta-PHR1. Thereafter, three full-length genomic DNA sequences of Ta-PHR1 were isolated by screening a bacterial artificial chromosome (BAC) library of Xiaoyan 54. A set of nullisomic-tetrasomic and deletion lines of *Triticum aestivum* ‘Chinese Spring’ were used for determining the chromosomal localization of the Ta-PHR1 genes.

Gene alignment and phylogenetic analysis

Gene structures of Ta-PHR1 genes were assembled by alignment of cDNAs with genomic sequences via the Megalign
program (DNAstar, Madison, WI, USA). Multiple sequence alignment of the MYB and the predicted CC conserved domains was carried out using the ClustalX 1.81 program (Thompson et al., 1997) with default multiple alignment parameters, and viewed by GeneDoc 3.2 with default BLOSUM score. The phylogenetic analysis was performed by the Neighbor-Joining method. The phylogenetic tree was constructed using PHYLIP version 3.5c (http://bioweb.pasteur.fr) with a bootstrap analysis of 100 resampling replications as described previously (Zhou et al., 2008). The protein sequences for alignment were extracted from NCBI.

**Transient analysis of Ta-PHR1-A1 protein–protein interaction in arabidopsis protoplasts**

The coding sequence of Ta-PHR1-A1 was amplified with gene-specific primers (Ta-PHR1_BiFC_F and Ta-PHR1_p101-CFP_R, Supplementary Data Table S1) and was cloned into pX-nYFP and pX-cYFP vectors using the Gateway-compatible vector cloning system (Gampala et al., 2007) to generate constructs of BiFC-Ta-PHR1-A1-nYFP and BiFC-Ta-PHR1-A1-cYFP, respectively. The arabidopsis protoplast isolation and transformation were carried out as described elsewhere (Yuan et al., 2008). A confocal laser scanning microscope (Olympus FV500, Japan) was used to detect the fluorescence signals at an excitation wavelength of 488 nm and emission wavelength of 506–538 nm, and the autofluorescence of chloroplasts was observed at an excitation wavelength of 488 nm and emission wavelength of 664–696 nm.

**Analysis of Ta-PHR1-A1 regulation of its target gene Ta-PHT1.2 in yeast cells**

Yeast two-hybrid analysis was performed according to the protocol of the Hybri-Zap two-hybrid system as described by the manufacturer (Stratagene, La Jolla, CA, USA). The coding sequence of Ta-PHR1-A1 was integrated into pAD-GAL4 and pBD-GAL4 to generate the plasmids pAD-GAL4-Ta-PHR1-A1 and pBD-Gal4-Ta-PHR1-A1, respectively.

The promoter of Ta-PHT1.2 (proTa-PHT1.2) was isolated from Xiaoyan 54 using the PCR primer pair proTa-PHT1.2FK and proTa-PHT1.2RS (Supplementary Data Table S1), and was then replaced by the 2× 35S promoter of the pJIT166 plasmid. The β-glucuronidase (GUS) expression cassette (proTa-PHT1.2::GUS::TCaMV) was cut out from the pJIT166 derivatives, and was integrated into pBD-Gal4-Ta-PHR1-A1 and pBD-Gal4-Ta-PHR1-A1, respectively. The GUS gene activity and total protein and MU quantification were performed as previously described (Yuan et al., 2008).

**Generation of transgenic wheat plants with Ta-PHR1-A1**

In order to generate the vector for wheat transformation, the maize Ubiquitin promoter (pUbi) was subcloned into the PstI site of pAH25 (Christensen et al., 1992), resulting in a vector named pUbi-pAH25. Then the Ta-PHR1-A1 cDNA was inserted into the NorI site of pUbi-pAH25, resulting in the construct pUbi::Ta-PHR1-pAH25. The construct was transformed into the immature embryos of wheat variety Shi4185 via the particle bombardment method (Becker et al., 1994). Shi4185 is a commercial winter wheat variety released in 1999, and was used as the control variety in the National Wheat Trial in the North of Huanghuai Region until 2011.

The insertion of pUbi::Ta-PHR1-pAH25 in the chromosomes of the transgenic lines was detected by fluorescence in situ hybridization (FISH). The seeds of the wild type Shi4185 and the Ta transgenic lines Ov-1 and Ov-2 were germinated at 20°C for 3 d. The root tips were treated with nitrous oxide for 2 h and then treated with 90% acetic acid on ice for 30 min. The treated root tips were stored in 70% ethanol at −20°C for future analysis by FISH. FISH was performed according to the methods described previously (Kato et al., 2004; Han et al., 2006), by using the linearized pUbi::Ta-PHR1-pAH25 as probe.

**Hydroponic and soil pot experiments**

The wild type and the Ta transgenic lines Ov-1 and Ov-2 were used in the hydroponic culture, soil pot experiment and field trials. The hydroponic culture was carried out in a growth chamber which was set at 20 ± 1°C, 50–70% relative humidity and a photon flux rate of 300 μmol photons m⁻² s⁻¹ with a 16 h day/8 h night cycle. Two treatments were used, the Pi-sufficient nutrient solution (high P) contained 200 μM KH₂PO₄ as described previously (Y. Z. Ren et al., 2012), and the Pi-deficient nutrient solution (low P) contained 10 μM KH₂PO₄ and an additional 190 μM KCl. Seeds were germinated in saturated CaSO₄ solution for 7 d at 20°C, and then the germinated seeds with residual endosperm removed were transferred to plastic pots containing 1 L of nutrient solution. The nutrient solution was refreshed every day and its pH was adjusted to 6.0 with dilute HCl and KOH before refreshing. The pots each contained three plants and were air-germinated and placed randomly in the growth chamber. For evaluating plant growth and P uptake (HC trial 1), the plants were grown in the high and low P nutrient solutions for 21 d, and then the shoots and roots were collected separately to measure dry weight, root morphological parameters and total P concentration. For detection of gene expression (HC trial 2), the plants were grown in the high and low P nutrient solutions for 7 d, and then the shoots and roots were collected separately for future analysis.

The soil pot trial was conducted using a calcareous soil with an Olsen-P level of 5.9 mg kg⁻¹. The soil was collected from the experimental station of the Institute of Genetics and Developmental Biology (IGDB), Chinese Academy of Sciences in Beijing. The trial was carried out from 2 March to 30 April in 2009 (60 d) and consisted of three P supply levels each with four replicates: P0 (0 mg P kg⁻¹ soil), P5 (5 mg P kg⁻¹ soil) and P20 (20 mg P kg⁻¹ soil) as potassium dihydrogen phosphate. All three treatments were provided with 75 mg kg⁻¹ of N (as urea) and 25 mg kg⁻¹ of K (as potassium chloride). The fertilizers were thoroughly mixed with the soil before sowing. The seeds of the wild type and the transgenic lines were germinated at 25°C for 2 d. Six germinated seedlings were planted in each plastic pot containing 3 kg of air-
dried soil, and were thinned to three plants per pot at the three-leaf stage. The pots were placed randomly outdoors, and their positions were changed every week. The aerial parts of the plants were harvested for determination of tiller number, shoot dry weight, Pi concentration and total P concentration. The roots were collected to measure root dry weight and total P concentration.

Field trials

Two field trials were conducted in the 2010–2011 growing season. The first trial was located in the experimental station of the IGDB in Beijing, and the second trial was located in the experimental station of Shijiazhuang Academy of Agricultural and Forestry Sciences in Xinji, Hebei province.

Both trials received two P treatments, the +P treatment received no P and the +P treatment received 13.5 g m⁻² of P as calcium superphosphate before sowing. In both treatments, 7.4 g m⁻² of K were applied in the form of potassium chloride before sowing, and 18.0 g m⁻² of N in the form of urea were applied: 12 g m⁻² before sowing and 6 g m⁻² at the stem elongation stage. A random block design with six positions were changed every week. The aerial parts of the plants were harvested in the middle of September, and plants were harvested in the middle of the following June. Both trials had a seed rate of 150 seeds m⁻². The Beijing field trial had two 1.5 m long rows with a row spacing of 23 cm in each replication, and the Hebei field trial had three 3 m long rows with a row spacing of 23 cm in each replication. Total biomass yield per plant, grain yield per plant, spike number per plant and grain number of the primary spike were recorded on 30 representative plants. Thousand-grain weight was determined according to the dry weight of 500 dried grains. The total P concentrations in the straw and grain in the Beijing field trial were measured.

Root parameters measurements

Root morphological parameters were measured in HC trial 1. The root systems were scanned with an STD1600 scanner (Epson, Long Beach, CA, USA). The scan images were then used to measure root tip number and total root length with SigmaScan Pro 5 software (Systat Software Inc., Chicago, IL, USA). Total lateral root length was calculated by subtracting the primary root length from the total root length.

Measurements of total P and Pi content in plant samples

Plant samples were dried at 80 °C to determine dry weight. The dried samples were milled and subsequently digested with concentrated H₂SO₄ and H₂O₂ for determination of total P using the molybdate-blue colorimetric method (Murphy and Riley, 1962). To measure the Pi concentration in the leaf, the sap of the fresh youngest expanded leaf was extracted by squeezing leaves in an Eppendorf tube. Then the Pi concentration in the sap was determined using the molybdate-blue colorimetric method (Murphy and Riley, 1962).

RESULTS

Real-time PCR analysis

Total RNA was extracted using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was synthesized from 2 μg of DNase I-treated total RNA using a PrimerScript® RT reagent Kit (TaKaRa, Dalian, China), according to the manufacturer’s instructions. Real-time PCR was performed by using the SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China) on a Mastercycler ep realplex machine (Eppendorf, Hamburg, Germany). Triplicate quantitative assays were performed on each cDNA sample. The relative quantification of each sample was determined by normalization to the amount of Ta-Actin cDNA detected in the same sample. The relative expression level was calculated by the formula 2⁻ΔΔCt. The PCR primers are given in Supplementary Data Table S1.

Statistical analysis of data

One-way analysis of variance (ANOVA) was performed by SAS statistical software (SAS Institute Inc., Cary, NC, USA).

Accession numbers

Sequence data of Ta-PHR1 genes can be found in the EMBL/GenBank data libraries under the following accession numbers: Ta-PHR1-A1 (KC218925), Ta-PHR1-B1 (KC286910), Ta-PHR1-D1 (KC286911).

Cloning and characterization of Ta-PHR1 genes

By using the protein sequences of At-PHR1 (NP_194590) and Cr-PSR1 (XP_001700553) as queries, we identified possible PHR1 ESTs in wheat through a dbEST search in the NCBI database. According to the EST sequences, we isolated a full-length cDNA sequence, named Ta-PHR1, from wheat variety Xiaoyan 54. Then Ta-PHR1 was used to design PCR primers to screen the Xiaoyan 54 BAC library, and finally three full-length genomic DNA sequences containing Ta-PHR1 homologues were obtained. These three genes had the same gene structures with identical exon sizes and similar intron sizes, except that there was a 1035 bp miniature inverted transposable element (MITE) insertion in intron 4 of Ta-PHR1-B1 (Fig. 1A). Their deduced protein sequences each had 451 amino acids, and contained a conserved MYB DNA-binding domain and a predicted CC domain (Supplementary Data Fig. S2), as has been shown for At-PHR1, Cr-PSR1 and Os-PHRs (Rubio et al., 2001; Zhou et al., 2008). Phylogenetic analysis revealed that the Ta-PHR1s belonged to the same subgroup with Os-PHR1, Os-PHR2 and At-PHR1, and were more closely related to Os-PHR1 (Fig. 1B).

Expression patterns of Ta-PHR1

As Ta-PHR1-A1, B1 and D1 were highly similar in their nucleotide sequences, gene-specific primers could not be designed and therefore here the relative expression levels

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reflected the transcript abundances of all three genes. We first investigated the spatial expression pattern of Ta-PHR1 in the above-ground part of wheat plants at the grain filling stage (2 weeks after flowering), and found that Ta-PHR1 was expressed most strongly in the flag leaf, moderately in the stem and weakly in the spike and developing seed (Fig. 2A). We then analysed the response of Ta-PHR1 to P supply levels in the seedlings of Xiaoyan 54 grown in nutrient solutions containing 200 μM Pi (high P) and 10 μM Pi (low P) for 7 d in HC trial 2. Compared with the high P treatment, the low P treatment slightly downregulated Ta-PHR1 in the shoots and upregulated Ta-PHR1 in the roots (Fig. 2B). At the seedling stage under our experimental conditions, Ta-PHR1 was expressed more abundantly in the shoots than in the roots in both high and low P conditions (Fig. 2B).

Ta-PHR1-A1 homodimer confers transcriptional activation of Ta-PHT1.2 in yeast cells

As Ta-PHR1s were predicted to contain a CC domain (Supplementary Data Fig. S2), a fold usually involved in protein–protein interactions, we used the bimolecular fluorescence complementation (BiFC) assay system described in Gampala et al. (2007) to detect Ta-PHR1 homodimer in arabidopsis protoplast cells. The coding sequence of Ta-PHR1-A1 was cloned in-frame into pX-nYFP and pX-cYFP vectors to generate the constructs Ta-PHR1-nYFP and Ta-PHR1-cYFP, respectively. When protoplast cells were transformed with the empty vectors pX-nYFP + pX-cYFP, a yellow fluorescent protein (YFP) signal was produced throughout the cytoplasm (Fig. 3A), indicating that the BiFC system worked well under our experimental conditions. Protoplast cells transformed with
taining 200 mM Pi for 7 d, and then the seedlings were grown in a nutrient solution containing different organs of Xiaoyan 54 plants 2 weeks after flowering. Data are the mean ± s.e. of three replicates. (B) Expression level of Ta-PHR1 in the shoots and roots under low and high P conditions. Xiaoyan 54 seeds were germinated for 7 d and then the seedlings were grown in a nutrient solution containing 200 μM Pi for 7 d. Thereafter, the seedlings were divided into two groups. These two groups were grown in nutrient solution containing 200 μM Pi (high P) and 10 μM Pi (low P), respectively. After 7 d, the roots and shoots were harvested for gene expression analysis. Data are the mean ± s.e. of three replicates.

Fig. 2. Expression patterns of Ta-PHR1. (A) Expression of Ta-PHR1 in different organs of Xiaoyan 54 plants 2 weeks after flowering. Data are the mean ± s.e. of three replicates. (B) Expression level of Ta-PHR1 in the shoots and roots under low and high P conditions. Xiaoyan 54 seeds were germinated for 7 d and then the seedlings were grown in a nutrient solution containing 200 μM Pi for 7 d. Thereafter, the seedlings were divided into two groups. These two groups were grown in nutrient solution containing 200 μM Pi (high P) and 10 μM Pi (low P), respectively. After 7 d, the roots and shoots were harvested for gene expression analysis. Data are the mean ± s.e. of three replicates.

Over-expression of Ta-PHR1-A1 upregulates Ta-PHT1 and Ta-IPS1.1

In order to investigate the function of Ta-PHR1, we developed Ta-PHR1-A1 transgenic wheat plants using maize ubiquitin promoter-controlled constructs. FISH analysis using the pUbi::Ta-PHR1-pACH25 probe showed that there was no fluorescence signal on the chromosomes of the wild type (Fig. 4A), but there were fluorescence signals on a pair of chromosomes of the two independent transgenic lines (designated as Ov-1 and Ov-2) (Fig. 4B, C), indicating that pUbi::Ta-PHR1-A1 was successfully introduced into the genome of the wild type. These two transgenic lines expressed Ta-PHR1 more strongly than the wild type under both low P and high P conditions in HC trial 2, indicating that Ta-PHR1-A1 was over-expressed in the transgenic lines (Fig. 4D).

The members of the MT4/TPS1 family have been used as molecular indicators of plant Pi status (Shin et al., 2006), and have been found to be regulated by PHR1 and its rice homologue Os-PHR2 (Rubio et al., 2001; Zhou et al., 2008). To determine whether this is the same case in wheat, we checked the expression of Ta-IPS1.1 and Ta-IPS1.2, members of the MT4/TPS1 family, in the wild type and the Ta-PHR1-A1 transgenic lines in a hydroponic trial (HC trial 2). In the wild type, Ta-IPS1.1 and Ta-IPS1.2 were strongly upregulated by the low P treatment in both the roots and shoots (Fig. 5A; Supplementary Data S3A), indicating that both the roots and shoots in the low P treatment were P starved. By comparing the expression levels of Ta-IPS1.1 and Ta-IPS1.2 between the wild type and transgenic lines, we found that the over-expression of Ta-PHR1-A1 mainly affected the expression of Ta-IPS1.1 under low P conditions. In the low P treatment, the mRNA levels of Ta-IPS1.1 in the transgenic lines Ov-1 and Ov-2 were slightly higher in the shoots, and about 2-fold higher in the roots than those in the wild type (Fig. 5A). No significant difference was found in the expression of Ta-IPS1.2 between the wild type and transgenic lines under either high P or low P conditions (Supplementary Data Fig. S3A).

As has been shown above, Ta-PHR1-A1 activated the expression of GUS driven by the Ta-PHT1.2 promoter in yeast cells (Fig. 3C). Therefore, we examined the expression of
the Ta-PHT1.2 gene in the wild type and transgenic lines. As has been shown previously (Davies et al., 2002), Ta-PHT1.2 was specifically expressed in the roots, and was upregulated by the low P treatment in both the wild type and transgenic lines (Fig. 5B). Compared with the wild type, the two transgenic lines had higher expression levels of Ta-PHT1.2 in the roots under both high and low P conditions (Fig. 5B), indicating that over-expression of Ta-PHR1 up-regulated Ta-PHT1.2.

We also investigated the expression levels of Ta-PHT1.6, a PHT1 gene mainly expressed in the shoots. Ta-PHT1.6 had detectable expression in both the shoots and the roots, and was upregulated by the low P treatment in the shoots (Fig. 5C). Compared with the wild-type plants, the Ta-PHR1-A1 transgenic plants had higher Ta-PHT1.6 mRNA levels in the shoots and roots under both high and low P conditions, and particularly in the shoots under low P conditions (Fig. 5C).

Besides the above-mentioned Pi starvation-inducible genes, we also analysed other Pi starvation-inducible genes, including Ta-PHF1 homologous to arabidopsis and rice PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (PHF1) (Gonzalez et al., 2005; Chen et al., 2011), Ta-PHO2, homologous to arabidopsis At-PHO2/UBC24 (Bari et al., 2006), Ta-SPX3 homologous to the rice SPX domain gene Os-SPX3 (Wang et al., 2009), Ta-PAP16 encoding a purple acid phosphatase homologous to arabidopsis PAP16 (Li et al., 2002), Ta-SQD2 encoding sulfolipid synthase homologous to arabidopsis SQD2 (Yu et al., 2002), and Ta-MGD2 encoding a UDP-galactose-dependent digalactosyldiacylglycerol synthase homologous to arabidopsis MGD2 (Kelly and Dörmann,
The expression of these genes did not show obvious difference between the wild type and transgenic lines (Supplementary Data Fig. S3).

**Over-expression of Ta-PHR1-A1 promotes shoot development and root branching of wheat seedlings**

We investigated the effects of over-expressing Ta-PHR1-A1 on the growth of wheat seedlings using both hydroponic culture (HC trial 1) and a soil pot trial. In HC trial 1, the low P treatment reduced tiller number and shoot dry weight, and increased root dry weight and the root/shoot ratio of both the wild type and transgenic lines, compared with the high P treatment (Fig. 6A–E), indicating that the wheat plants grown in the low P treatment were subjected to P-deficient stress. In the low P treatment, both of the transgenic lines had a significantly higher tiller number, root dry weight and root/shoot ratio than the wild type; however, only one of the transgenic lines had significantly higher shoot dry weight and root dry weight than the wild type in the high P treatment (Fig. 6B–E).

As over-expressing Ta-PHR1-A1 increased root dry weight, we then analysed how Ta-PHR1-A1 affected root morphology. Over-expression of Ta-PHR1-A1 did not always increase primary root length, as only the transgenic line Ov-2 had a higher primary root length than the wild type in the high P treatment (Fig. 7A). The two transgenic lines had a higher root tip number, lateral root length and total root length than the wild type under both high and low P conditions (Fig. 7B–D). These results suggested that over-expressing Ta-PHR1-A1 mainly increased lateral root branching.

The effects of over-expressing Ta-PHR1-A1 on increasing wheat seedling growth were also observed when the wheat plants were grown in soil. In the pot trial, the two transgenic lines had significantly higher tiller number than the wild type at all the three P fertilizer rates (Fig. 8A), and
significantly higher shoot dry weight and root dry weight than the wild type in the P0 (0 mg P kg\(^{-1}\) soil) and P5 (5 mg P kg\(^{-1}\) soil) treatments (Fig. 8B, C).

Two field trials were carried out in Beijing and Hebei province to investigate the effects of over-expressing Ta-PHR1 on the yield traits (Table 1). We observed positive effects of over-expressing Ta-PHR1 on grain yield, especially in the +P treatment, in that the transgenic line Ov-1 had a significantly higher grain yield than the wild type in Beijing and Hebei, and the transgenic line Ov-2 had a significantly higher grain yield than the wild type in Hebei. We further analysed the effects of over-expressing Ta-PHR1 on the yield components. As shown in Table 1, over-expressing Ta-PHR1 significantly increased grain number per spike in the +P treatment in both Beijing and Hebei, and in the –P treatment (for Ov-2 only) in Hebei, but did not significantly affect spike number per plant and 1000-grain weight (Table 1).

Over-expression of Ta-PHR1-A1 improves P uptake

We analysed P uptake by wheat seedlings grown in nutrient solution in HC trial 1, and in the soils in the pot trial. In HC trial 1, both of the transgenic lines had significantly higher total P concentration and P content in the shoots, and significantly higher P uptake than the wild type under both high and low P conditions (Fig. 6F, H, J), but had a similar total P concentration and P content in the roots to the wild type (Fig. 6G, I). These results indicated that over-expressing Ta-PHR1-A1 increased P uptake, and promoted P translocation from roots to shoots as reflected by the ratios of shoot P content over P uptake (Fig. 6K). In the pot trial, only the transgenic line Ov-2 was found to have an increased total P concentration in the shoots at the P-fertilizer rate P0, and P content in the roots at the P-fertilizer rates P0 and P5 (Fig. 8D, H). However, one or both of the transgenic lines were found to have an increased leaf Pi concentration (Fig. 8F) and P uptake at the P-fertilizer rates P0 and P5 (Fig. 8I).

In the field trial in Beijing, we analysed the total P concentration in the grains and straw. In the –P treatment, both of the transgenic lines had a higher total P concentration and P content in the grains and straw, and higher P accumulation in the above-ground parts; in the +P treatment, only the transgenic line Ov-1 was found to have increased grain P content and P accumulation in the above-ground parts (Table 1). Over-expressing Ta-PHR1-A1 did not significantly affect the distribution of P in the grains (P harvest index; Table 1).

DISCUSSION

Ta-PHR1-A1 is involved in Pi starvation signalling in wheat

By screening a common wheat BAC library, we cloned three PHR1 homologous genes Ta-PHR1-A1, Ta-PHR1-B1 and Ta-PHR1-D1 which are located on chromosomes 7A, 4B and 4D, respectively (Supplementary Data Fig. S1). The location of Ta-PHR1-A1 on chromosome 7A may be caused by structural rearrangement between chromosome arms 7AL and 4BS (Devos et al., 1995). These three genes had the same gene structures with identical exon sizes and similar intron sizes (Fig. 1A), and their deduced protein sequences showed high similarities to each other (Fig. 1B). The high
similarity in gene sequence, structure and chromosomal location indicates that Ta-PHR1-A1, B1 and D1 were homologous alleles. The deduced proteins of these three genes contained a conserved MYB DNA-binding domain and a predicted CC domain (Supplementary Data Fig. S2), as have been shown for At-PHR1, At-PHL1 and Os-PHR1/2 (Rubio et al., 2001; Zhou et al., 2008; Bustos et al., 2010).

At-PHR1 and its homologues At-PHL1 in arabidopsis and Os-PHR2 in rice regulate a set of Pi starvation-inducible genes (Rubio et al., 2001; Nilsson et al., 2007; Zhou et al., 2008; Bustos et al., 2010), reflecting the key role of PHR1 in Pi starvation signalling. The direct targets of PHR1 are found to be greatly enriched in P1BS containing Pi starvation-inducible genes (Bustos et al., 2010). Our current study showed that Ta-PHR1-A1 formed a homodimer (Fig. 3A), and conferred transcriptional activation of Ta-PHT1.2 (Fig. 3B, C), whose promoter contains a putative P1BS element (Miao et al., 2009). This result demonstrated that Ta-PHR1-A1 was a functional transcriptional factor in regulating Pi starvation-inducible genes. Over-expression of Ta-PHR1-A1 in wheat upregulated Ta-PHT1.2, and other Pi starvation-inducible genes, including Ta-IPS1.1 and Ta-PHT1.6 (Fig. 5), further confirming the role of Ta-PHR1-A1 in Pi signalling in wheat. As the expression of Ta-PHR1, like that of At-PHR1 and Os-PHR2, was not dramatically regulated by Pi supply levels (Fig. 2B), it is assumed that Ta-PHR1s might be subjected to post-translational regulation in Pi starvation signalling in wheat.

**Fig. 6.** Growth performance and P uptake of the wild type and the transgenic lines grown in hydroponic culture. Seeds were germinated for 7 d, and then the germinated seeds with residual endosperm removed were transferred to nutrient solution containing 10 μM Pi (low P) and 200 μM Pi (high P), respectively. After the plants were grown in the low and high P nutrient solutions for 21 d, the shoots and roots were collected separately for further analysis. Data were the mean ± s.e. of four replications. * and ** indicate that the difference between the means of the wild type and the transgenic line Ov-1 under low and high P conditions. (A) The shoot and root phenotypes of the wild type and the transgenic line Ov-1 under low and high P conditions. (B) TN, tiller number. (C) SDW, shoot dry weight. (D) RDW, root dry weight. (E) Root/shoot dry weight ratio. (F) Shoot P conc., shoot total P concentration. (G) Root P conc., root total P concentration. (H) Shoot P content, total P accumulated in the shoot. (I) Root P content, total P accumulated in the root. (J) P uptake, total P accumulated in the root and shoot. (K) Shoot P/P uptake, ratio of shoot P content over P uptake.
translational regulation on PHR1 protein has been shown in arabidopsis, in which At-PHR1 protein can be sumoylated by SUMO E3 ligase SIZ1 (Miura et al., 2005). Over-expression of Ta-PHR1-A1 did not obviously alter the expression levels of Ta-IPS1.2, Ta-PHF1, Ta-PHO2, TaSPX3, Ta-PAP16, Ta-SQD2 and Ta-MGD2 (Supplementary Data Fig. S3), suggesting that transcriptional factors other than Ta-PHR1-A1 may function in Pi signalling in wheat. In each genome of arabidopsis and rice, there are at least two PHR1 homologues that differentially regulate Pi starvation-inducible genes (Rubio et al., 2001; Zhou et al., 2008; Bustos et al., 2010). For example, the rice Pi starvation-inducible genes Os-IPS1, Os-IPS2, Os-PAP16 and Os-SQD2 are regulated by Os-PHR2, but not by Os-PHR1 (Zhou et al., 2008).

Over-expression of Ta-PHR1-A1 promotes wheat growth and grain yield

Over-expressing Ta-PHR1-A1 enhanced wheat seedling development when grown in the soil or in nutrient solution. At the seedling stage in the pot trial, over-expression of Ta-PHR1-A1 promoted tiller number regardless of P supply levels (Fig. 8A), and increased shoot dry weight under P-deficient conditions (P-fertilizer rates P0 and P5) (Fig. 8B). When the plants were grown in the nutrient solution (HC trial 1), the effects of over-expressing Ta-PHR1-A1 on increasing tiller number and shoot dry weight were also observed (Fig. 6B, C). However, over-expressing Ta-PHR1-A1 did not increase average tiller size as reflected by average shoot dry weight per tiller (shoot dry weight/tiller number). We calculated shoot dry weight/tiller number values according to the data of tiller number and shoot dry weight in the pot trial (Fig. 8A, B), and found that shoot dry weight/tiller number was lower for the transgenic lines than for the wild type at all the three P-fertilizer rates. For example, shoot dry weight/tiller number was 0.150 for the wild type, and was 0.137 and 0.124 for Ov-1 and Ov-2 at the P-fertilizer rate P0, respectively. The smaller tiller size of the transgenic lines may partially explain the results of the two field trials in that over-expressing Ta-PHR1-A1 did not increase spike number per plant (Table 1), as a small tiller size may not favour its development into a productive spike.

Over-expressing Ta-PHR1-A1 also improved grain yield of wheat under field conditions. In the field trials carried out at two different sites (Beijing and Hebei), over-expressing Ta-PHR1-A1 increased grain yield under P-sufficient conditions, and also showed positive effects on grain yield under P-deficient conditions (Table 1). Compared with the wild type, the increased grain yields of the transgenic lines were due to the effects of over-expressing Ta-PHR1-A1 on increasing grain number per spike, but not spike number per plant and 1000-grain weight (Table 1). Taken together, our research demonstrated the positive regulation by Ta-PHR1-A1 on wheat growth and yield performance. The regulation of plant growth has been reported for PHRI and its homologues previously. In arabidopsis, the functional loss of PHRI results in reduced growth of the shoot, irrespective of external Pi conditions (Nilsson et al., 2007). Over-expressing PHRI and its homologues has been reported to inhibit growth in rice and Brassica napus; the growth retardation may be caused by excessive Pi accumulation in the shoots of the transgenic lines (Zhou et al., 2008; F. Ren et al., 2012). In these two studies on rice and B. napus, OsPHR2 and BnPHR1 were upregulated by several fold, and consequently the shoots of the transgenic

Fig. 7. Root morphological parameters of the wild type and the Ta-PHRI transgenic lines grown in hydroponic culture. The fresh root samples from the hydroponic culture (see Fig. 6) were scanned with an STD1600 scanner (Epsom, USA). The scan images were then used to measure root tip number (B) and total root length (TRL, D) with WinRHIZO software. Primary root length (PRL, A) was measured by using SigmaScan Pro 5 software (Systat Software Inc.). Total lateral root length (LRL, C) was calculated by subtracting the primary root length from the total root length. Data were the mean ± s.e. of four replications. * and ** indicate that the difference between the means of the wild type and the transgenic line was significant at P < 0.05 and P < 0.01, respectively.

expression of the wild type and the transgenic line was significant at four replications. * and ** indicate that the difference between the means of the wild type and the transgenic line was significant at four replications.
lines accumulated several fold higher Pi concentrations than those of the wild type. When we over-expressed Ta-PHR1-A1 in arabidopsis, we also observed that the transgenic lines accumulated a much higher Pi concentration in the shoot, and had less shoot biomass than the wild type (unpublished data). Therefore, the increasing effect of over-expressing Ta-PHR1-A1 on wheat growth was possibly due to the fact that the expression levels of Ta-PHR1 were only moderately upregulated (about 50% higher) in the transgenic lines (Fig. 4D), and the leaf Pi concentrations in the transgenic lines were also moderately increased (about 30% higher, Fig. 8F).

**Over-expression of Ta-PHR1-A1 increases lateral root branching**

In response to low availability of P in the rhizosphere, plants have developed highly specialized morphological adaptive mechanisms to increase the ability of their root systems to utilize Pi from soils; these include increased root/shoot ratio, enhanced adventitious rooting and lateral root branching, and greater root hair density (George et al., 2011). Besides its role in regulating the expression of Pi starvation-inducible genes, PHR1 and its homologues have been found to regulate root growth. In arabidopsis, the functional loss of PHR1 reduces root growth, regardless of external Pi conditions (Nilsson et al., 2007). A more detailed study showed that the phr1 mutant has decreased root hair length, while over-expressing PHR1 increases root hair length under P-deficient conditions (Bustos et al., 2010). In rice, over-expression of Os-PHR2 has also been found to increase root hair length and density, and to enhance the elongation of the primary root and adventitious roots under P-deficient conditions (Zhou et al., 2008). To the best of our knowledge, the effect of PHR1 and its homologues on lateral root development is not yet known. In the current study, the Ta-PHR1-A1 transgenic plants had higher root dry weight than the wild type under

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**Fig. 8. Growth performance and P uptake of the wild type and the Ta-PHR1 transgenic lines grown in the soil pot trial.** The plants were grown in the soils supplied with 0 (P0), 5 (P5) and 20 (P20) mg P kg⁻¹ soil. Data are the mean ± s.e. of four replications. * and ** indicate that the difference between the means of the wild type and the transgenic lines was significant at P < 0.05 and P < 0.01, respectively. (A) TN/plant, tiller number per plant. (B) SDW, shoot dry weight per plant. (C) RDW, root dry weight per plant. (D) Shoot P conc., shoot total P concentration. (E) Root P conc., root total P concentration. (F) Leaf Pi conc., inorganic phosphate (Pi) concentration in the fresh youngest expanded leaf. (G) Shoot P content, total P accumulated in the shoot. (H) Root P content, total P accumulated in the root. (I) P uptake, total P accumulated in the whole plant.
Table 1. Agronomic traits and P uptake of the wild type and its Ta-PHR1-A1 transgenic lines under −P and +P conditions in the field trials in Beijing and Hebei province

| Trait                        | −P (mean ± s.e.) | +P (mean ± s.e.) |
|------------------------------|------------------|------------------|
|                              | WT   | Ov-1 | Ov-2 | WT   | Ov-1 | Ov-2 |
| Beijing                      |      |      |      |      |      |      |
| Grain yield (g per plant)    | 5.4 ± 0.1 5.6 ± 0.3 5.5 ± 0.2 | 6.1 ± 0.2 7.1 ± 0.2* 6.4 ± 0.2 |
| Grain number per spike       | 48.4 ± 0.9 49.1 ± 1.0 50.2 ± 0.8 | 50.4 ± 0.8 54.3 ± 0.8* 52.4 ± 0.6* |
| Spike number per plant       | 3.8 ± 0.1 4.0 ± 0.3 3.9 ± 0.4 | 4.1 ± 0.1 4.3 ± 0.1 4.1 ± 0.2 |
| 1000-grain weight (g)        | 40.8 ± 0.4 41.1 ± 0.6 41.9 ± 0.7 | 43.4 ± 0.4 43.0 ± 0.7 42.7 ± 0.5 |
| Grain total P concentration (mg g⁻¹) | 4.14 ± 0.05 4.82 ± 0.12* 4.92 ± 0.14* | 5.35 ± 0.05 5.61 ± 0.08 5.52 ± 0.09 |
| Grain P content (mg per plant) | 22.4 ± 0.9 27.0 ± 1.2* 27.1 ± 1.3* | 32.6 ± 0.7 39.8 ± 1.6* 35.3 ± 1.1 |
| Straw total P concentration (mg g⁻¹) | 0.35 ± 0.01 0.47 ± 0.02* 0.46 ± 0.01* | 0.63 ± 0.02 0.63 ± 0.01 0.54 ± 0.02 |
| Straw P content (mg per plant) | 2.2 ± 0.13 2.8 ± 0.1 2.9 ± 0.2* | 4.4 ± 0.1 4.2 ± 0.1 3.8 ± 0.1 |
| P uptake (mg per plant)      | 24.6 ± 1.0 29.8 ± 1.3* 30.0 ± 1.3* | 37.0 ± 0.7 44.0 ± 1.6* 39.1 ± 1.3 |
| Phosphate harvest index      | 0.91 ± 0.01 0.91 ± 0.01 0.90 ± 0.01 | 0.88 ± 0.02 0.90 ± 0.01 0.90 ± 0.01 |
| Hebei Province               |      |      |      |      |      |      |
| Grain yield (g per plant)    | 5.3 ± 0.3 5.8 ± 0.2 6.2 ± 0.3 | 6.1 ± 0.3 7.7 ± 0.6* 7.5 ± 0.6* |
| Grain number per spike       | 50.1 ± 2.0 51.4 ± 1.2 56.7 ± 0.6* | 47.9 ± 0.9 54.4 ± 1.6* 54.1 ± 1.8* |
| Spike number per plant       | 2.2 ± 0.2 2.4 ± 0.0 2.3 ± 0.1 | 2.7 ± 0.2 3.0 ± 0.2 3.0 ± 0.3 |
| 1000-grain weight (g)        | 47.0 ± 0.8 46.2 ± 0.3 45.6 ± 0.5 | 44.9 ± 0.5 44.3 ± 0.4 44.1 ± 0.8 |

In both trials, the −P treatment involved the application of no P, and the +P treatment 13.5 g m⁻² of P as calcium superphosphate before sowing. Data are the mean ± s.e. of six replications. *Indicates that the difference between the means of the wild type and transgenic lines was significant at P < 0.05.

P-deficient conditions in the pot trial (Fig. 8C). When grown in the nutrient solution in HC trial 1, the Ta-PHR1-A1 transgenic lines also had a higher root dry weight than the wild type under low P conditions, and also under high P conditions (Fig. 6D), and an increased root/shoot ratio under low P condition (Fig. 6E), indicating that over-expressing Ta-PHR1-A1 increased root response to P deficiency, and further confirming the roles of Ta-PHR1-A1 in Pi signalling. More detailed analysis demonstrated that over-expressing Ta-PHR1-A1 increased root tip number (Fig. 7B), lateral root length (Fig. 7C) and, consequently, total root length, under both low and high P conditions (Fig. 7D). These results supported the positive regulation by Ta-PHR1-A1 of lateral root branching. However, over-expressing Ta-PHR1-A1 did not constitutively increase primary root length, as only the transgenic line Ov-2 had a longer primary root length than the wild type under high P conditions (Fig. 7A). Taking these results together, At-PHR1, Os-PHR2 and Ta-PHR1-A1 differentially regulate root morphology, although they all are involved in Pi signalling.

Over-expression of Ta-PHR1-A1 increases P uptake

Over-expressing Ta-PHR1-A1 increased P uptake when the plants were grown either in the nutrient solution (Fig. 6) or in the soil (Fig. 8, Table 1). However, the increasing effects were more pronounced in the nutrient solution than in the soil; this phenomenon was possibly due to the low mobility of Pi in the soil (Schachtman et al., 1998). In the hydroponic culture (HC trial 1), over-expressing Ta-PHR1-A1 increased shoot total P concentration (Fig. 6F), shoot P content (Fig. 6H) and P uptake under both low and high P conditions (Fig. 6J), but not root total P concentration (Fig. 6G) and root P content (Fig. 6I), resulting in a greater distribution of P from roots to shoots in the transgenic lines than in the wild type (Fig. 6K). The increased P distributions in shoots have also been observed in At-PHR1 and Os-PHR2 over-expressing lines (Nilsson et al., 2007; Zhou et al., 2008). Although over-expressing Ta-PHR1-A1 increased the distribution of P from roots to shoots at the seedling stage in the hydroponic culture, it did not increase the distribution of P from shoots to grains, as the P harvest index showed no difference between the transgenic lines and the wild type in the field trial in Beijing (Table 1). When the plants were grown in soil in the pot trial and the field trial in Beijing, increases of P uptake mediated by over-expressing Ta-PHR1-A1 were mainly detected under P-deficient conditions (Fig. 8I, Table 1), this was possible because over-expression of Ta-PHR1-A1 increased root dry weight under P-deficient conditions (Fig. 8C). Enhanced root development has been reported by many researchers to increase the ability of the root system to utilize P from soils (for a review, see George et al., 2011).

The increased P uptake and distribution in the shoots in the transgenic lines may also be due to the fact that over-expressing Ta-PHR1-A1 upregulated the expression of Ta-PHT1.2 and 1.6 (Fig. 5B, C). The PHT1 type of Pi transporters mainly function in Pi acquisition from soils and translocation from roots to other parts of plants (Bucher, 2007). Ta-PHT1.2 encodes a functional high-affinity Pi transporter (Zeng et al., 2002), and is predominantly expressed in the roots (Fig. 5B), suggesting its role in P uptake. Ta-PHT1.6 is expressed in both shoots and roots under high P conditions, and more abundantly in the shoots of P-starved plants (Fig. 5C), implying that Ta-PHT1.6 may play a role in mediating the distribution of P from roots to shoots. The upregulation of PHT1 transporters by over-expressing At-PHR1 and Os-PHR2 has been reported in arabidopsis and rice (Nilsson et al., 2007; Zhou et al., 2008), respectively, and was found to be responsible for most of the Os-PHR2-mediated accumulation of excess shoot Pi (Liu et al., 2010). As information on...
the sequence, function and expression of PHT1 genes in wheat remains largely unknown, further detailed research on PHT1 genes and their regulation by Ta-PHR1-A1 will help us to better understand the mechanism of the control of Ta-PHR1-A1 in P uptake and re-distribution within wheat plants.

In summary, we cloned three PHR1 genes on chromosomes 7A, 4B and 4D in wheat. The three Ta-PHR1 genes are highly similar in sequence, and share the same gene structures. Our data showed that Ta-PHR1-A1 was involved in Pi signalling in wheat, and over-expressing Ta-PHR1-A1 in wheat upregulated Pi starvation-inducible genes, stimulated later root growth and hence improved P uptake. Over-expressing Ta-PHR1-A1 also improved shoot growth and grain yield. The positive regulation by Ta-PHR1-A1 of P uptake and plant growth will provide a valuable approach to engineer crops with improved P use efficiency and yield performance.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: chromosome locations of Ta-PHR1-A1, B1 and D1. Figure S2: alignment of Ta-PHR1 protein sequences. Figure S3: relative expression levels of phosphate starvation-inducible genes in the wild type and its Ta-PHR1 transgenic lines under low and high P conditions in hydroponic culture. Table S1: primers for RT–PCR or real-time RT–PCR.

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