

A phosphate starvation response regulator *Ta-PHR1* is involved in phosphate signalling and increases grain yield in wheat

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- **Background and Aims** Phosphorus deficiency is a major limiting factor for crop yield worldwide. Previous studies revealed that *PHR1* and its homologues play a key role in regulating the phosphate starvation response in plants. However, the function of *PHR* homologues in common wheat (*Triticum aestivum*) is still not fully understood. The aim of the study was to characterize the function of *PHR1* genes in regulating phosphate signalling and plant growth in wheat.
- **Methods** Wheat transgenic lines over-expressing a wheat *PHR1* gene were generated and evaluated under phosphorus-deficient and -sufficient conditions in hydroponic culture, a soil pot trial and two field experiments.
- **Key Results** Three *PHR1* homologous genes *Ta-PHR1-A1*, *B1* and *D1* were isolated from wheat, and the function of *Ta-PHR1-A1* was analysed. The results showed that *Ta-PHR1-A1* transcriptionally activated the expression of *Ta-PHT1.2* in yeast cells. Over-expressing *Ta-PHR1-A1* in wheat upregulated a subset of phosphate starvation response genes, stimulated lateral branching and improved phosphorus uptake when the plants were grown in soil and in nutrient solution. The data from two field trials demonstrated that over-expressing *Ta-PHR1-A1* increased grain yield by increasing grain number per spike.
- **Conclusions** *Ta-PHR1* is involved in phosphate signalling in wheat, and was valuable in molecular breeding of crops, with improved phosphorus use efficiency and yield performance.

Key words: *Triticum aestivum*, *PHR1*, phosphate starvation response, phosphorus uptake, grain yield, lateral root.

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

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; Schunmann *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, *PTF1* (*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 (Muller *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_2O_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 to generate yeast expression plasmids pBD-*Ta-PHR1-A1-proTa-PHT1.2::GUS* and pBD-*proTa-PHT1.2::GUS*, respectively. These plasmids were then introduced into yeast strain YRG-2 alone or in pairs with pAD-GaL4-*Ta-PHR1-A1*. A filter lift assay of *LacZ* reporter 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 *Pst*I site of pAHC25 (Christensen *et al.*, 1992), resulting in a vector named *pUbi-pAHC25*. Then the *Ta-PHR1-A1* cDNA was

inserted into the *Not*I site of *pUbi-pAHC25*, resulting in the construct *pUbi::Ta-PHR1-pAHC25*. 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-pAHC25* in the chromosomes of the transgenic lines was detected by fluorescence *in situ* hybridization (FISH). The seeds of the wild type Shi4185 and the T₄ 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-pAHC25* as probe.

Hydroponic and soil pot experiments

The wild type and the T₄ 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 fluence 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-aerated 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 replications was used in both trials. Seeds were sown at the end 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 WinRHIZO software developed by Regent Instruments Canada Inc. (Ottawa, ON, Canada), and primary root length using 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 H_2SO_4 and H_2O_2 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).

Real-time PCR analysis

Total RNA was extracted using the TRIzol[®] reagent (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was synthesized from $2 \mu\text{g}$ of DNase I-treated total RNA using a PrimeScript[®] 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 $2^{-\Delta\Delta\text{Ct}}$. 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).

RESULTS

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 were mapped on chromosomes 7A, 4B and 4D by using a set of deletion lines of 'Chinese Spring' (Supplementary Data Fig. S1), and thus were named *Ta-PHR1-A1*, *Ta-PHR1-B1* and *Ta-PHR1-D1*, respectively. 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-PHR1*s 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

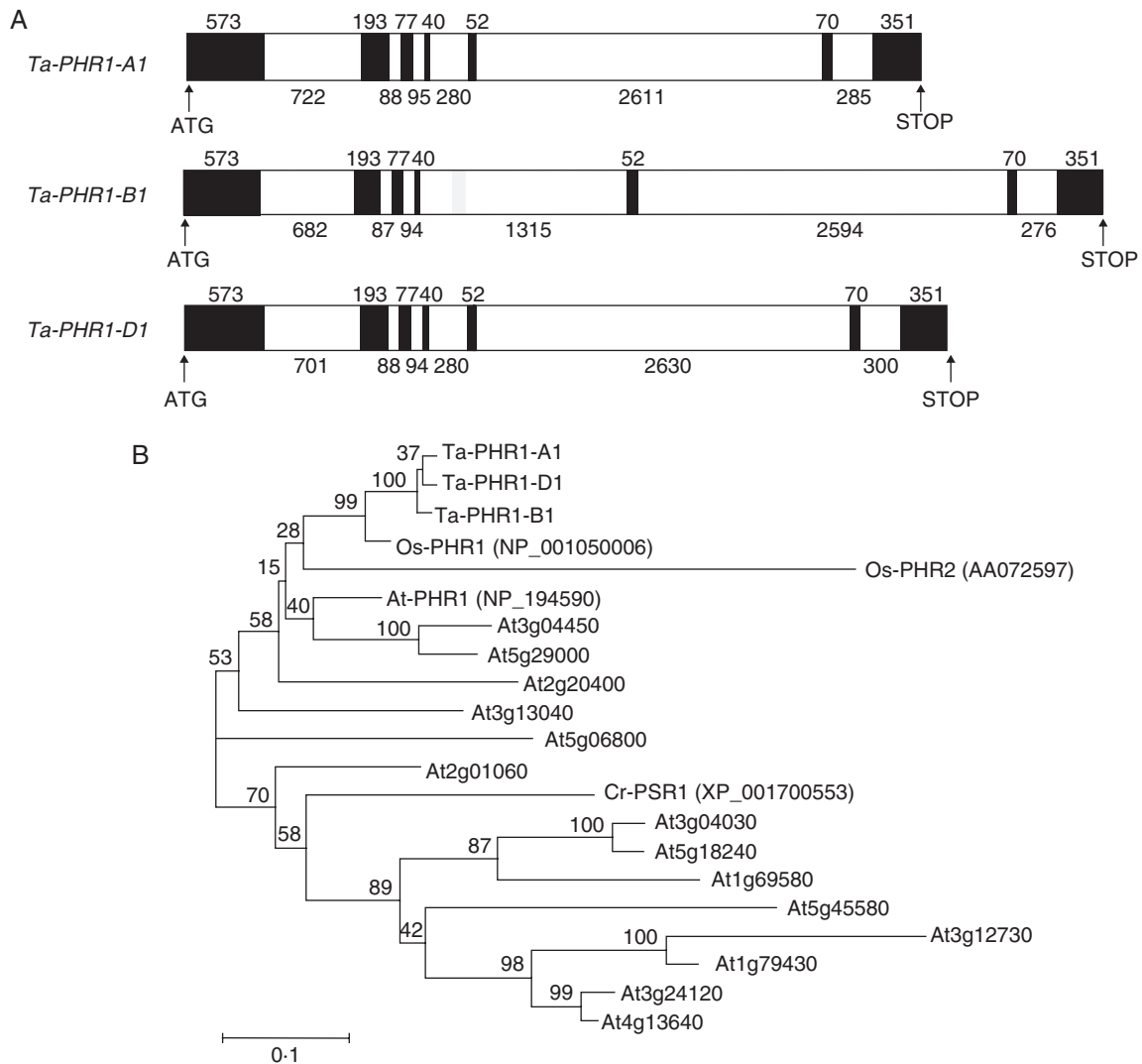


FIG. 1. Structures of the *Ta-PHR1* genes and phylogenetic analysis with other related MYB-CC proteins. (A) Structures of the *Ta-PHR1* genes. Exons are indicated as black boxes and introns as white boxes. Numbers indicate the length of each exon and intron. ATG and the stop codon are indicated by arrowheads. (B) Phylogenetic tree of the proteins sharing the MYB and predicted CC domains. The phylogenetic tree was constructed using the CLUSTAL_X (Thompson *et al.*, 1997) program and the Neighbor-Joining method. The numbers above the lines refer to bootstrap values (of 100 samples). Scale bar = 0.1 substitutions per site. Protein accession numbers are: *Ta-PHR1-A1*, KC218925; *Ta-PHR1-B1*, KC286910; *Ta-PHR1-D1*, KC286911; *Os-PHR1*, NP_001050006; *Os-PHR2*, AA072597; *At-PHR1*, NP_194590; and *CrPSR1*, XP_001700553.

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-PHR1*s 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

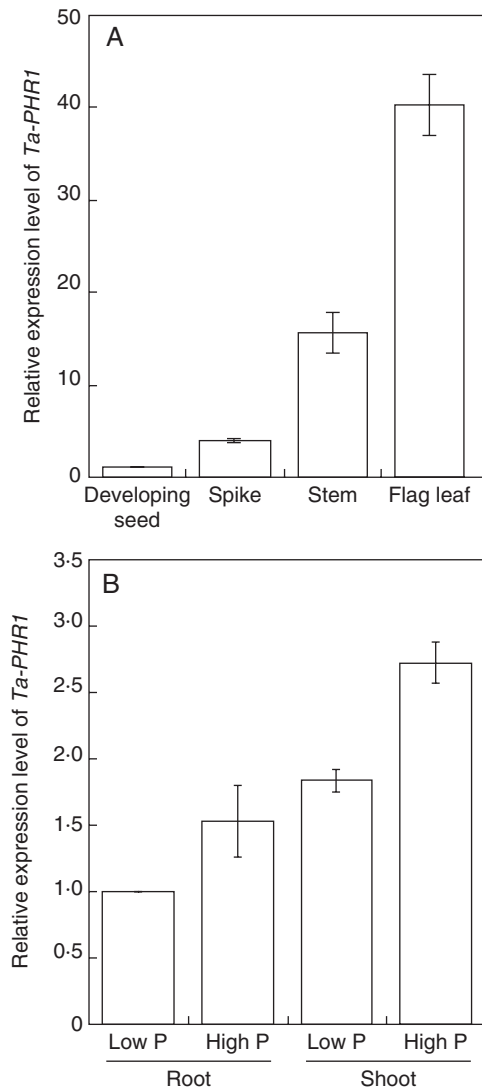


FIG. 2. Expression patterns of *Ta-PHR1*. (A) Expression of *Ta-PHR1* in different organs of Xiaoyan 54 plants 2 weeks after flowering. Data are the mean \pm s.e. of three replicates. (B) Expression level of *Ta-PHR1* in the shoots and roots under low and high P conditions. Xiaoyan 54 seeds were germinated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

the constructs *Ta-PHR1*-nYFP + *Ta-PHR1*-cYFP generated a strong YFP signal in the nuclei (Fig. 3A), whereas no YFP signal was observed in the nuclei of the protoplast cells transformed with pX-nYFP + pX-cYFP, pX-nYFP + *Ta-PHR1*-cYFP or *Ta-PHR1*-nYFP + pX-cYFP (Fig. 3A). These results clearly illustrated that *Ta-PHR1*-A1 formed a homodimer in plant cells, and the nuclear location of *Ta-PHR1* implied that it may function as a transcription factor.

We next examined whether *Ta-PHR1*-A1 homodimer activated the expression of a putative high-affinity Pi transporter *Ta-PHT1.2*, the promoter of which was previously predicted

to contain a putative PIBS element (Miao *et al.*, 2009). Histochemical assay showed that no GUS activity was detected in yeast strains containing the plasmids pAD-GAL4 + pBD-GAL4-*proTa-PHT1.2::GUS*, indicating that the expression cassette of *proTa-PHT1.2::GUS* cannot be activated by yeast transcription. Filter lift assays for GUS staining showed that strong blue colour appeared in the yeast cells containing plasmids pAD-*Ta-PHR1*-A1 + pBD-*Ta-PHR1*-A1-*proTa-PHT1.2::GUS*, whereas no blue colour was observed in the yeast cells with other plasmid combinations (Fig. 3B). In addition, higher GUS activity was detected in the yeast cells containing the plasmids pAD-*Ta-PHR1*-A1 + pBD-*Ta-PHR1*-A1-*proTa-PHT1.2::GUS* than other plasmid combinations (Fig. 3C). These results clearly showed that the interaction of *Ta-PHR1* with itself activated the expression of *GUS* driven by the *Ta-PHT1.2* promoter in yeast cells.

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

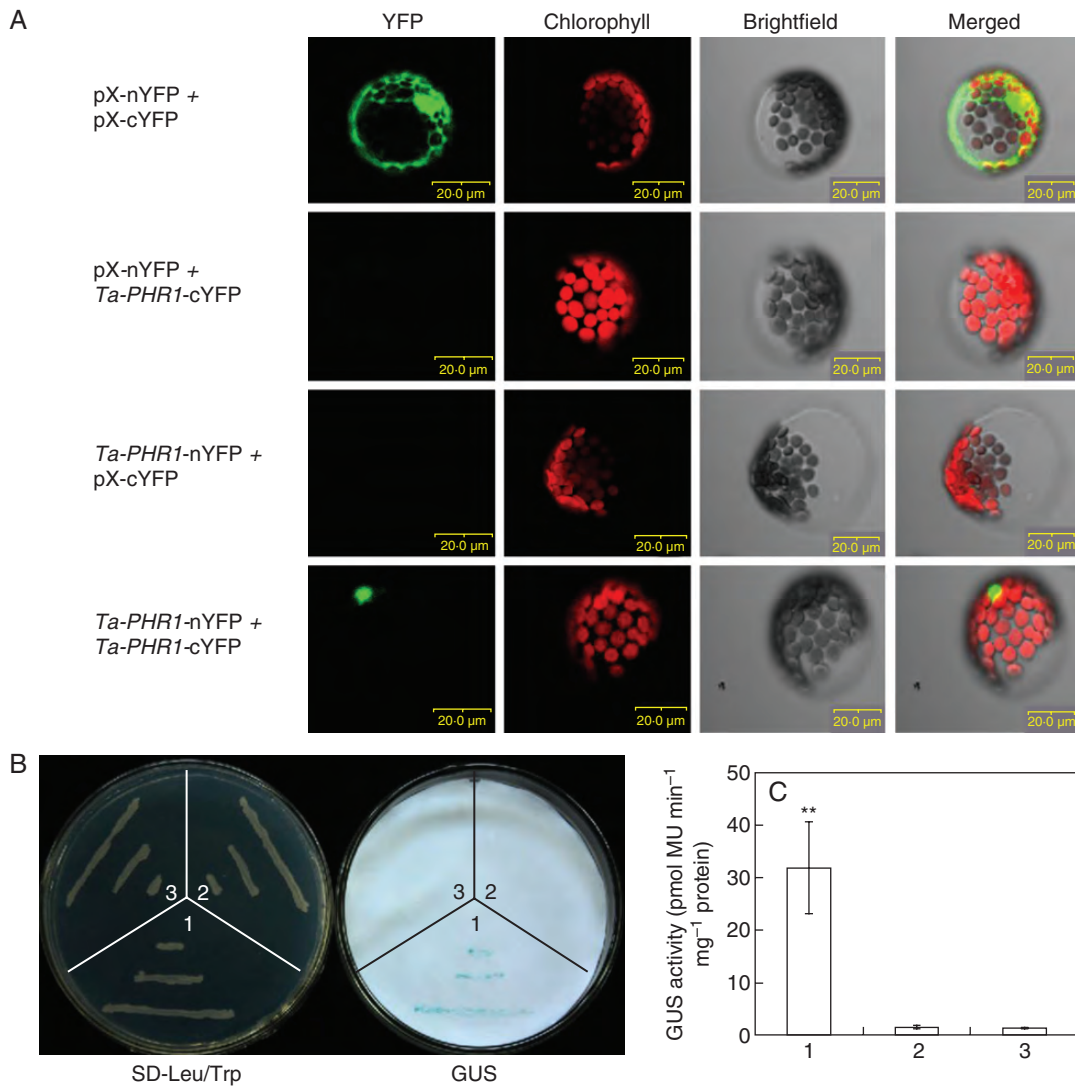


FIG. 3. *Ta-PHR1-A1* homodimer confers transcriptional activation of *Ta-PHT1.2*. (A) BiFC demonstrated that *Ta-PHR1-A1* formed a homodimer *in vivo*. Images were captured 20 h after transient expression under an Olympus confocal microscope. Scale bars = 20 μ m. (B) *Ta-PHR1-A1* activated the expression of the reporter gene *GUS* driven by the *Ta-PHT1.2* promoter. Left panel: the growth of the yeast cells on SD-Leu/Trp media. Right panel: *GUS* staining. 1, pAD-GAL4-*Ta-PHR1-A1* + pBD-*Ta-PHR1-A1-proTa-PHT1.2::GUS*; 2, pAD-GAL4-*Ta-PHR1-A1* + pBD-GAL4-*Ta-PHR1-A1*; 3, pAD-GAL4 + pBD-*proTa-PHT1.2::GUS*. (C) *GUS* activities of the yeast cells from (B); ** indicates that the difference was significant at the 0.01 level.

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,

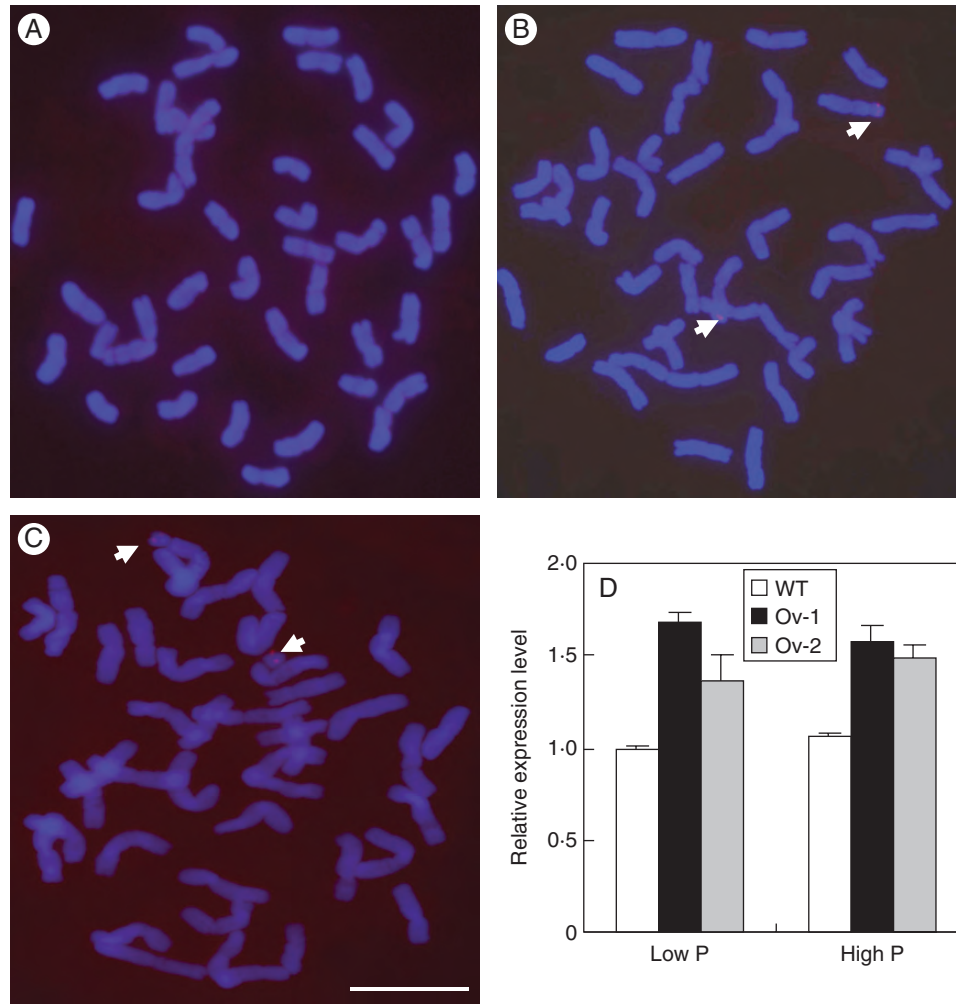


FIG. 4. Fluorescent *in situ* hybridization (FISH) and expression analysis of *Ta-PHR1-A1* in the wild type (WT) and its transgenic lines. (A–C) *pUBI::Ta-PHR1-A1* vector was used as the detection probe in FISH of the wild type (A) and the transgenic lines Ov-1 (B) and Ov-2 (C). Fluorescent signals are indicated by arrows. Scale bar = 10 μm . (D) Relative expression levels of *Ta-PHR1* in the WT and the transgenic lines Ov-1 and Ov-2 grown in nutrient solution. 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) for 7 d, respectively. Then the seedlings were collected for gene expression analysis. Data are the mean \pm s.e. of three biological replications.

2002). 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

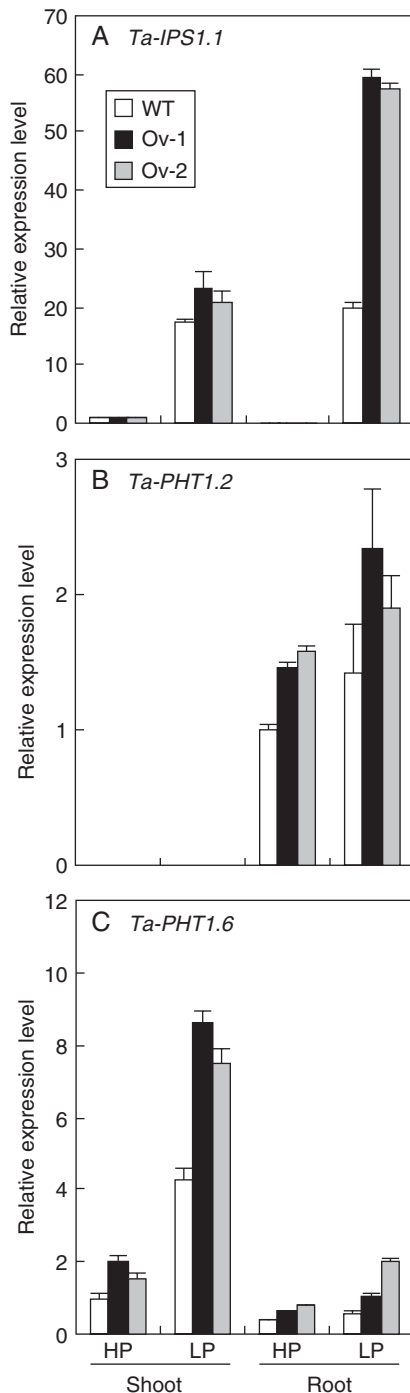


FIG. 5. Relative expression levels of (A) *Ta-IPS1.1*, (B) *Ta-PHT1.2* and (C) *Ta-PHT1.6* in the shoots and roots of the wild type (WT) and the *Ta-PHR1-A1* transgenic lines (Ov-1 and Ov-2). Seeds were germinated for 7 d, and then the germinated seeds with residual endosperm removed were transferred to nutrient solution containing 10 μM Pi (low P, LP) and 200 μM Pi (high P, HP), respectively. After the plants were grown in the low and high P nutrient solutions for 7 d, the shoots and roots were collected separately for gene expression analysis. Data were the mean \pm s.e. of three replications.

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).

Over-expression of *Ta-PHR1-A1* increases grain yield

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 in both $-P$ and $+P$ treatments, 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

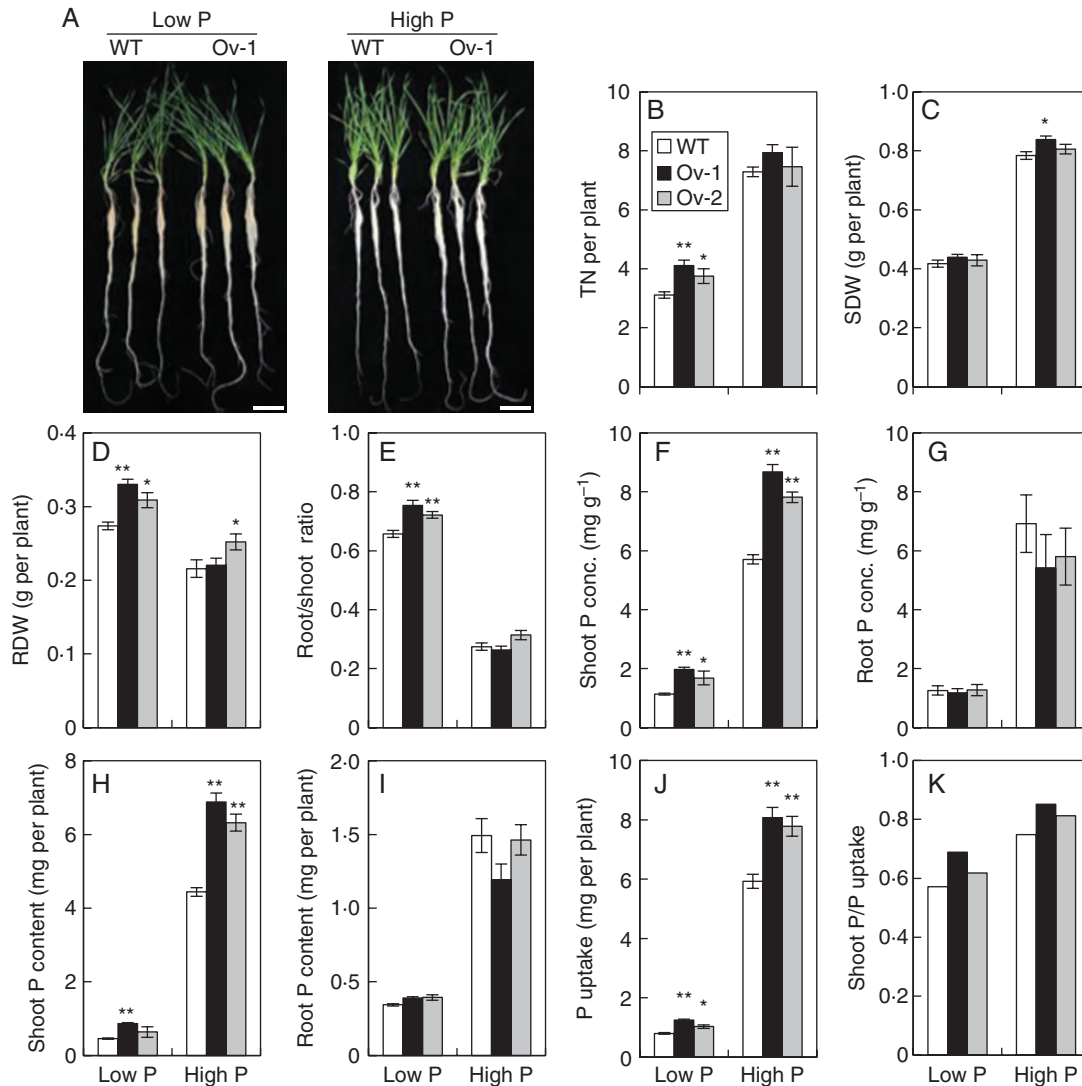


FIG. 6. Growth performance and P uptake of the wild type and the transgenic lines grown in hydroponic culture. Seeds were germinated for 7 d, and then the germinated seeds with residual endosperm removed were transferred to nutrient solution containing $10 \mu\text{M}$ Pi (low P) and $200 \mu\text{M}$ 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 \pm 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. (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.

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-IPSI.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. Previously the post-

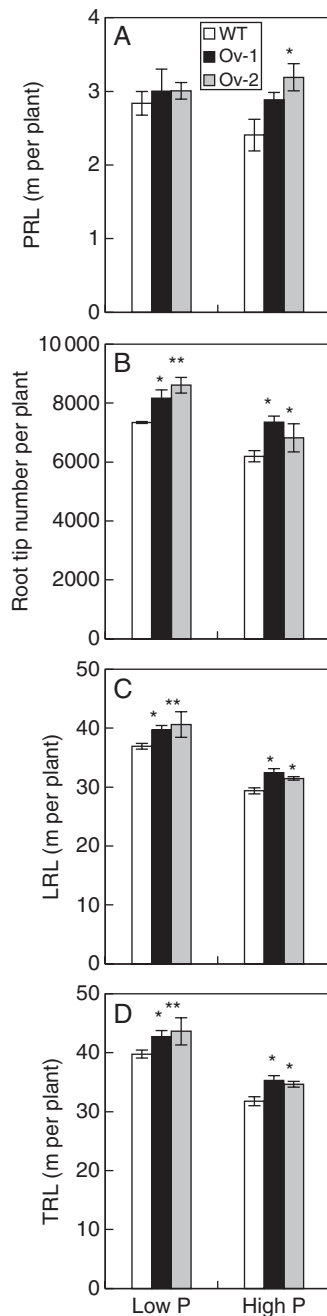


FIG. 7. Root morphological parameters of the wild type and the *Ta-PHR1* transgenic lines grown in hydroponic culture. The fresh root samples from the hydroponic culture (see Fig. 6) were scanned with an STD1600 scanner (Epsom, USA). The scan images were then used to measure root tip number (B) and total 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 \pm 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.

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 *PHR1* and its homologues previously. In arabidopsis, the functional loss of *PHR1* results in reduced growth of the shoot, irrespective of external Pi conditions (Nilsson et al., 2007). Over-expressing *PHR1* 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

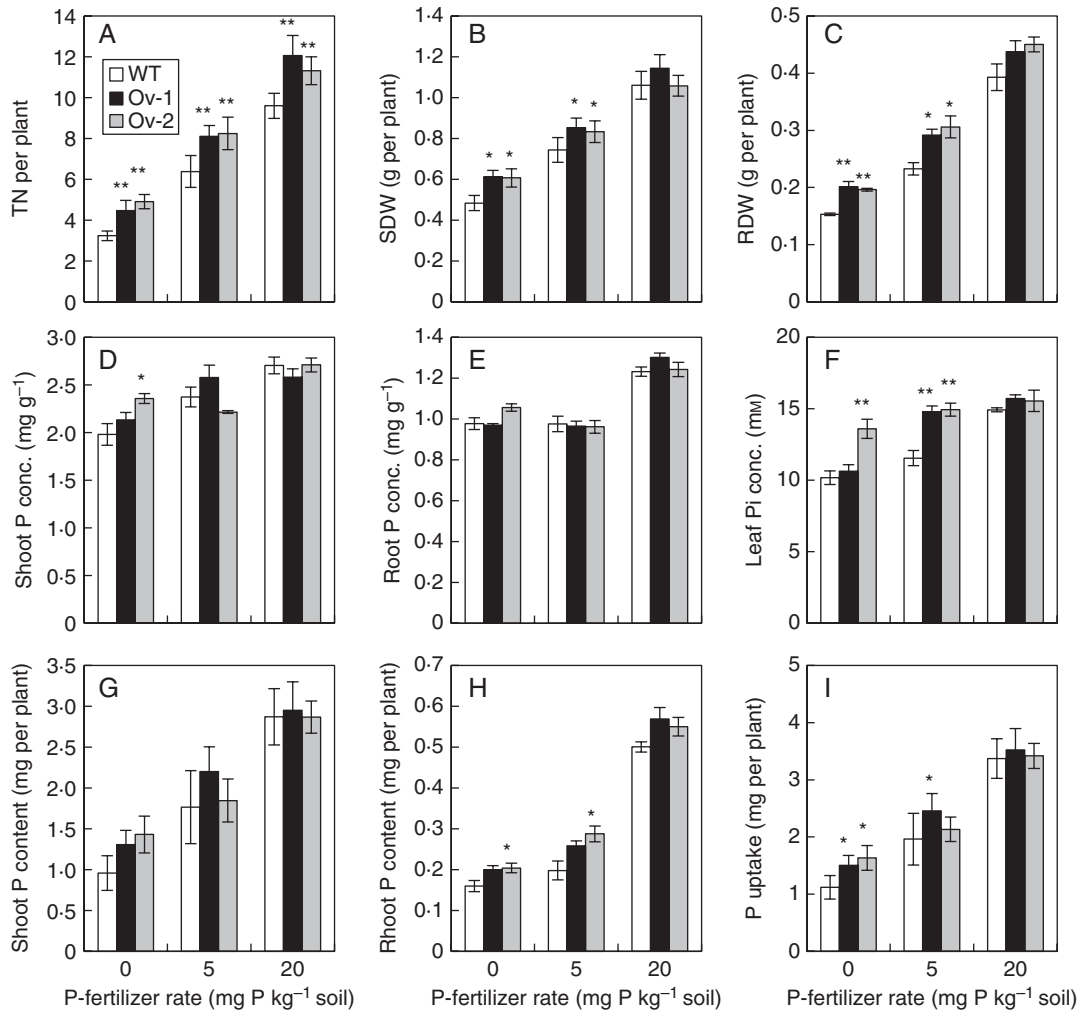


FIG. 8. Growth performance and P uptake of the wild type and the *Ta-PHR1* transgenic lines grown in the soil pot trial. The plants were grown in the soils supplied with 0 (P0), 5 (P5) and 20 (P20) mg P kg⁻¹ soil. Data are the mean \pm s.e. of four replications. * and ** indicate that the difference 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.

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

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