TaPHT1;9-4B and its transcriptional regulator TaMYB4-7D contribute to phosphate uptake and plant growth in bread wheat

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Summary

- Efficient phosphate (Pi) uptake and utilisation are essential for promoting crop yield. However, the underlying molecular mechanism is still poorly understood in complex crop species such as hexaploid wheat. Here we report that TaPHT1;9-4B and its transcriptional regulator TaMYB4-7D function in Pi acquisition, translocation and plant growth in bread wheat.
- TaPHT1;9-4B, a high-affinity Pi transporter highly upregulated in roots by Pi deficiency, was identified using quantitative proteomics. Disruption of TaPHT1;9-4B function by BSVMV-VIGS or CRISPR editing impaired wheat tolerance to Pi deprivation, whereas transgenic expression of TaPHT1;9-4B in rice improved Pi uptake and plant growth. Using yeast-one-hybrid assay, we isolated TaMYB4-7D, a R2R3 MYB transcription factor that could activate TaPHT1;9-4B expression by binding to its promoter. Silencing TaMYB4-7D decreased TaPHT1;9-4B expression, Pi uptake and plant growth.
- Four promoter haplotypes were identified for TaPHT1;9-4B, with Hap3 showing significant positive associations with TaPHT1;9-4B transcript level, growth performance and phosphorus (P) content in wheat plants. A functional marker was therefore developed for tagging Hap3.
- Collectively, our data shed new light on the molecular mechanism controlling Pi acquisition and utilisation in bread wheat. TaPHT1;9-4B and TaMYB4-7D may aid further research towards the development of P efficient crop cultivars.

Introduction

Phosphorus (P) is one of the essential mineral nutrients required by plants. However, Pi concentration in soil solution is frequently below the critical level (<10 μM) needed by plants (Raghothama, 1999). To improve crop yields, millions of tons of Pi fertilisers are applied annually to agricultural fields worldwide. However, only 10–20% of the applied Pi is effectively absorbed by plants, while the remaining is lost, which not only increases agricultural costs but also causes environmental pollution (eutrophication of surface water) and acidification of soil (Zhang et al., 2018). To ameliorate the reliance of crop production on Pi fertilisers, it is essential to develop P efficient cultivars with enhanced Pi absorption and use efficiency (Grierson et al., 2011).

Plants have evolved sophisticated mechanisms to adapt to Pi fluctuations in growth environments. Under low-Pi conditions, various morphological, physiological and molecular changes, such as formation of finer roots and root hairs, secretion of more organic acids, phosphatases and phytases, and expression of Pi-starvation-induced (PSI) genes, are augmented (Heuer et al., 2017; Ajmera et al., 2019). These events have led to increased Pi availability in the rhizosphere and higher rate of Pi uptake by root cells. Central to Pi absorption is the function of the PHT1 family of high-affinity Pi transporters (Gu et al., 2016; Kopriva & Chu, 2018; Wang et al., 2018). Higher plants often express multiple PHT1 proteins, which play pivotal roles in plant adaptation to Pi-deficiency conditions because they are rapidly upregulated at low-Pi concentrations and account for nearly all Pi uptake from the soil and translocation within plants (Gu et al., 2016).

To date, nine and 13 PHT1s have been found in the model plants Arabidopsis thaliana and rice (Oryza sativa), respectively, many of which have been characterised in terms of spatial and temporal expression patterns, transport properties, and roles in Pi acquisition and plant growth and development (Gu et al., 2016; Młodzińska & Zboińska, 2016). Furthermore, substantial insights have been gained into the molecular mechanism regulating the transcript and protein levels of PHT1s in the two model species (Gu et al., 2016; Młodzińska & Zboińska, 2016; Wang et al., 2018a; Ajmera et al., 2019). Various transcriptional factors (TFs) have been found to regulate the transcript of PHT1s and many other PSI genes. For example, AtPHR1 and OsPHR1 to OsPHR4, which carry a MYB-coiled-coil (MYB-CC) domain in their
proteins, are key transcriptional activators of PSI genes in Arabidopsis and rice (Rubio et al., 2001; Zhou et al., 2008; Guo et al., 2015; Ruan et al., 2017). In addition, several MYB TFs possessing R2R3 or R3 domain have also been shown to contribute to PHT1 transcriptional regulation, such as OsMYB1, OsMYB2P-1, OsMYB4P and OsMYB5P in rice and CPC and ETC1 in Arabidopsis (Dai et al., 2012; Yang et al., 2014, 2018; Chen & Schmidt, 2015; Gu et al., 2017).

Bread wheat (Triticum aestivum L., BBAAADD, 2n = 6x = 42), the most widely cultivated staple food crop (Shewry & Hey, 2015), was evolved through two polyploidisation events; the first one involved the einkorn wheat T. urartu (AA, 2n = 2x = 14) and a species related Aegilops speltoides, which gave rise to a tetraploid species Triticum turgidum (BBAA); the second one occurred between tetraploid wheat and A. tauschii (DD, 2n = 2x = 14), resulting in allohexaploid wheat (Feldman & Levy, 2012). Therefore, the bread wheat genome carries three homoeologous subgenomes (A, B and D), with most genes having two to three homoeologs (IWGSC et al., 2018). Compared with A. thalidama and rice, bread wheat has considerably more PHT1 genes (Teng et al., 2017; Grün et al., 2018; Zhang et al., 2019). However, very few of them have been functionally analysed in depth (Secco et al., 2017). Yeast complementation assay has been used to demonstrate the Pi transport function of several TaPHT1s (Davies et al., 2002; Zeng et al., 2002; Guo et al., 2014; Teng et al., 2017). Positive correlations were found between TaPHT1 transcript levels and Pi accumulation in some studies (Teng et al., 2013, 2017; Aziz et al., 2014; Shukla et al., 2016; Deng et al., 2018; Grün et al., 2018; de Souza Campos et al., 2019). Consistent with these results, transgenic overexpression of two TaPHT1 genes have been found to enhance plant growth and Pi acquisition in bread wheat (Liu et al., 2013; Guo et al., 2014). Under low-Pi conditions, at least seven TaPHT1 genes are transcriptionally upregulated (Teng et al., 2017; Grün et al., 2018). But to date, it is still unknown if their proteins may accumulate in bread wheat roots, and little molecular insight has been gained into their transcriptional control in response to Pi supply status, although overexpression of two TFs, TaPHR-A1 and TaNFYA-B1, have been shown to stimulate the expression of several TaPHT1 genes in transgenic wheat plants (Wang et al., 2013; Qu et al., 2015).

Based on the information presented above, the main objectives of this work were to analyse active TaPHT1s in elite commercial bread wheat cultivars and to identify the TF regulating TaPHT1 transcription in wheat. Therefore, we examined Pi-deficiency responsive proteins (PDRPs) in bread wheat roots using iTRAQ (isobaric tagging for relative and absolute quantification)-based proteomic analysis. Analysis of PDRPs enabled us to find four different TaPHT1 proteins whose protein levels were significantly upregulated by Pi deficiency. Detailed analysis on one of the four TaPHT1s, that is TaPHT1:9-4B, led to the identification of TaMYB4-7D, a R2R3 type of MYB TF that could bind to the promoter region of TaPHT1:9-4B and promote its transcript level. Together, these results suggest that TaPHT1:9-4B and its transcriptional regulator TaMYB4-7D contribute positively to Pi uptake and wheat growth. An elite promoter haplotype of TaPHT1:9-4B was therefore mined for improving Pi uptake and use efficiencies in wheat.

Materials and Methods

Plant materials and growth conditions and oligonucleotide primers

The plant materials used in this study included 62 bread wheat cultivars, 11 tetraploid wheat accessions, and 28 diploid wheat relatives (Aegilops bicornis, A. longissima, A. sharonensis, A. speltoides and A. tauschii) (Supporting Information Table S1). Interestingly, cv Zhoumai 18, an elite bread wheat variety widely cultivated in China (Zhou et al., 2014), was used for proteomic analysis. Seeds were sterilised and germinated as detailed in Methods S1. Two-wk-old wheat seedlings with three leaves, at the autotrophic stage and sensitive to abiotic stresses (Li et al., 2017), were divided into two groups, one remained in Hoagland solution containing sufficient Pi (1 mM, as control), and the other was transferred to Pi-deficient Hoagland medium (0 mM Pi, with KH2PO4 replaced by KCl) (Secco et al., 2010). The nutrient solution was replenished every day for both groups. Determination of plant growth parameters, total P concentration and anthocyanin content are described in Methods S1. All oligonucleotide primers in this work are listed in Table S2.

Proteomic analysis and validation by parallel reaction monitoring (PRM)

Total proteins were extracted from the roots of the wheat seedlings grown on Pi-sufficient or Pi-deficient media for 8 d using the trichloroacetic acid/acetone method, and quantified using the Bradford protein assay (Bio-Rad, Hercules, CA, USA) (Wisniewski et al., 2009). Three biological replicates were executed, with at least six plants per replicate. The iTRAQ-based proteomics experiment is described in detail in Methods S1. A PRM experiment, conducted to validate the expression changes of 11 PDRPs found in iTRAQ analysis, was described in Methods S1.

Identification of PDRPs

Peptide data were searched against the protein database of Chinese Spring (CS) (IWGSC et al., 2018, REFSEQ v.1.0) using BLASTP (v.2.6) by applying an identity cut-off of 100% (McGinnis & Madden, 2004). To increase accuracy, the proteins specified by the A, B or D subgenomes were separately searched, and homoeologous loci encoding PDRPs were identified and illustrated (Fig. S1), followed by analysis of potential expression bias among the homoeologs (Hu et al., 2014) (Methods S1).

Functional analysis of TaPHT1:9-4B

Function analysis of TaPHT1:9-4B was conducted using yeast complementation assay, virus induced gene silencing (VIGS), CRISPR/Cas9-mediated genome editing and transgenic expression experiments. To perform the complementation assay, TaPHT1:9-4B coding sequence was cloned into the yeast expression vector Yp112A1NE and transformed into the Pi absorption defective yeast mutant MB192, as described previously (Bun-Ya
et al., 1991; Teng et al., 2017). Barley stripe mosaic virus induced VIGS (BSMV-VIGS) (Tufan et al., 2011), as well as pWMBX110-SpCas9-mediated genome editing (Ma et al., 2015; Liu et al., 2020), were used to investigate the effects of disrupting TaPHT1;9 function on Pi absorption and plant growth (Methods S1). Transgenic expression of TaPHT1;9-4B in japonica rice (cv Nipponbare) was carried out to assess the effects of overexpressing TaPHT1;9-4B on Pi uptake and plant growth as outlined in Methods S1.

Identification and functional investigation of TaMYB4-7D

Total RNAs (2 μg) isolated from the roots of wheat seedlings grown in Pi-deficient medium for 8 d were used to construct a cDNA library for yeast-one-hybrid (Y1H) assay to identify potential trans-factors binding to the promoter region of TaPHT1;9-4B, which was amplified from the cv Zhoumai 18. Screening this library led to the finding of the transcription factor TaMYB4-7D capable of binding to the TaPHT1;9-4B promoter. Details of the screening and related validation experiments are described in Methods S1. The transcriptional activation potency of TaMYB4-7D was tested in the yeast strain AH109 as described in the Yeast Handbook (Clontech, Palo Alto, CA, USA). Functional analysis of TaMYB4-7D by BSMV-VIGS is outlined in Methods S1.

Nucleotide diversity analysis of TaPHT1;9-4B and TaMYB4-7D

Genomic DNA samples, isolated from the 62 bread wheat cultivars (21 landraces and 41 modern cultivars) and 39 wheat relatives (Table S1), were used for nucleotide diversity (π) analysis (Methods S1).

Chromosomal assignment of genes, subcellular localisation of proteins, qRT-PCR assay, haplotype analysis and development of CAPS marker

Details of these experiments are provided in Methods S1.

Statistics

Numerical values were calculated as means ± standard deviation (SD), which were statistically analysed using either Student’s t-test or one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test installed in the SPSS statistics package (SPSS Inc., Chicago, IL, USA).

Results

Identification of PDRPs

After 8 d culture under Pi-deficiency (0 mM Pi, NP) conditions, wheat seedlings showed inhibited leaf growth but enhanced root length relative to those grown in the presence of 1 mM Pi (Fig. S2a). Quantitative analysis confirmed these phenotypic changes (Fig. S2b–e). Pi-deficiency treatment decreased P concentrations in root and shoot tissues (Fig. S2f,g). Because decrease in P concentration and change of plant growth were both observed at 8 d after Pi deficiency (Fig. S2), we therefore considered that the 8 d time point was suitable for studying the effects of Pi deficiency on wheat root proteome using the iTRAQ approach.

In total, 20 733 peptides were identified from 151 654 mass spectra, which corresponded to 6842 expressed proteins when searched against the NCBI nonredundant database (Dataset S1). Of them, 4306 were detected in all three biological replicates of both control and Pi-deficiency treatments (Dataset S1). In total, 323 PDRPs showed comparatively larger changes in their abundance (indicated by unique peptide LC-MS/MS reporter peak area), being upregulated ≥1.20-fold or downregulated ≤0.83-fold (Fig. 1a; Dataset S1). The PDRPs were functionally involved in many biological processes (e.g. protein metabolism, signal transduction and transport) (Dataset S1). Eleven PDRPs were selected for validation by a PRM experiment. A high consistency was found between the expression changes revealed using iTRAQ or PRM approaches for nine of the 11 examined proteins (Fig. S3; Dataset S2), therefore confirming the reliability of the iTRAQ dataset generated in this work.

Expression characteristics of PDRPs

Genomic distribution of PDRPs was studied using the CS genome assembly (Methods S1). Among the 20 733 peptides and 4306 PDRPs, 15 044 peptides and 4013 PDRPs were matched to the predicted proteins of CS (Dataset S3). Furthermore, 1015 PDRPs could be assigned to specific homoeologs in the A, B, or D subgenomes (Dataset S3); the remaining could not be assigned to specific subgenomes. Markedly, of the 1015 PDRPs, 738 (72.7%) were strongly affected by homoeolog expression bias, with each PDRP specified by only one of the two, three or more homoeologs; 62 (6.1%) were expressed from the single genes (located in A, B or D) without homoeologs in the other two subgenomes (Dataset S3). Among the single homoeolog-derived PDRPs (738+62), 241, 289 and 270 belonged to the A, B and D subgenomes, respectively (Fig. 1b; Dataset S3). The remaining 215 PDRPs, each specified by two or three homoeologs (Fig. 1b; Dataset S3), were not affected by homoeolog expression bias, with 65 of them showing unbalanced expression in one or two homoeologs (P<0.05) (Fig. 1b; Dataset S3). Clearly, single homoeolog-derived proteins dominated the 1015 PDRPs. Additional analysis showed that the 1131 PDRPs were distributed mainly on chromosomes 2B, 3B, 2A, 5B, 2D, 6D, 5D and 4B (Fig. 1c).

Primary structure, subcellular location and yeast complementation assay of TaPHT1;9-4B

Among the identified PDRPs, four high-affinity PHT1 proteins (TaPHT1;3-5B, TaPHT1;6-5B, TaPHT1;9-4B and TaPT2), each being specified by a single homoeolog, were significantly upregulated by Pi deficiency, with the average fold of induction
of TaPHT1;9-4B being relatively high (Fig. 1d). The high induction of TaPHT1;9-4B by Pi deficiency was also found in the PRM analysis (Fig. S3; Dataset S2). In the study by Teng et al. (2017), the transcripts of TaPHT1;3 and TaPHT1;6, named as TaPHT1.3 and TaPHT1.6, respectively, were detected in both wheat roots and shoots, whereas those of TaPHT1;9 (TaPHT1.9) were found predominantly in the roots by qRT-PCR. Amino acid sequence comparisons indicated that TaPHT1;3-5B, TaPHT1;6-5B and TaPHT1;9-4B were identical to TaPht1;4-Chr5BL, TaPht1;6-Chr5BL and TaPht1;1a-Chr4BL, respectively, in the wheat PHT1 nomenclature system proposed by Grün et al. (2018). Searching the genomic sequence of CS revealed that TaPHT1;3-5B, TaPHT1;6-5B and TaPHT1;9-4B corresponded to the chromosome loci Trac SC5B02G512000, Trac SC5B02G470100 and Trac SC4B02 G317000, respectively. TaPt2, renamed as TaPHT1.10-U by Teng et al. (2017) or TaPht1;2a-Chr4DL by Grün et al. (2018), corresponded to Trac SC4D02G313800 in the CS genome sequence; it has been shown to function as a high-affinity Pi transporter using yeast complementation assay and by analysing overexpression and RNAi transgenic wheat lines (Davies et al., 2002; Zeng et al., 2002; Guo et al., 2014). Because TaPHT1;9-4B was found expressed in wheat roots by both previous study (Teng et al., 2017) and our work (Figs 1d, S3), and yet its role in Pi transport and utilisation had not been well studied, we therefore focused on analysing the function of TaPHT1;9-4B in subsequent research.

Phylogenetic analysis with previously characterised Arabidopsis and rice PHTs, as listed in Table S3, showed that TaPHT1;9-4B and TaPt2 were tightly clustered with the three rice PHT1 proteins OsPHT1;1, OsPHT1;2 and OsPHT1;3 (Ai et al., 2009; Sun et al., 2012; Chang et al., 2019) (Fig. 2a). TaPHT1;3-5B was closely related to OsPHT1;8 (Jia et al., 2011), while TaPHT1;6-5B was aggregated with OsPHT1;6 and OsPHT1;7 (Ai et al., 2009) (Fig. 2a). TaPHT1;9-4B and TaPt2 were 99.04% identical, and they exhibited 78.69–80.04% identities to OsPHT1;1, OsPHT1;2 and OsPHT1;3 (Table S4). TaPHT1;3-5B was 87.59% identical to OsPHT1;8, while the identities of TaPHT1;6-5B to OsPHT1;6 and OsPHT1;7 were 77.16% and 75.19%, respectively (Table S4).

PCR amplification of TaPHT1;9 homologs using subgenome-specific primer pairs produced three amplicons in
wild-type (WT) CS but only two amplicons in the N4AT4D, N4BT4D and N4DT4B lines that lacked 4A, 4B and 4D chromosomes, respectively (Fig. 2b), confirming that TaPHT1;9 homoeologs are located on group 4 chromosomes. Consistent with this result, three TaPHT1;9 homoeologs are present on the 4A, 4B and 4D chromosomes of CS, respectively, with TaPHT1;9-4B physically mapped to the 606.81 Mb position on chromosome 4B.

TaPHT1;9-4B did not have intron in its coding region or the 5’ and 3’ untranslated regions; its coding sequence was 1566 bp, whose deduced protein contained 521 amino acids (Fig. S4). Consistent with the previous finding (Teng et al., 2017), we
observed that TaPHT1;9-4B was predominantly expressed in wheat root, with its transcript level dramatically upregulated by Pi-deficiency treatment (Fig. S5). In silico analysis predicted that the secondary structure of TaPHT1;9-4B contained 12 putative transmembrane (TM) domains (Fig. 2c). Expression of TaPHT1;9-4B-GFP fusion cistron under the control of CaMV35S promoter produced GFP fluorescence only at the plasma membrane (PM), suggesting that TaPHT1;9-4B was targeted to the PM (Fig. 2d), which was marked by the RFP fusion protein of a known hydrophobic and PM-located protein AtRCI2B (Medina et al., 2007).

Expression of TaPHT1;9-4B in the yeast strain MB192, which carries a mutation in the high-affinity Pi transporter PHO84 (Bun-Ya et al., 1991), restored its growth under low-Pi conditions (20 and 60 μM) (Fig. 2e). Quantitative growth curves demonstrated that TaPHT1;9-4B partially complemented the function of PHO84 (Fig. 2f). However, the functional complementation by TaPHT1;9-4B was affected by pH, with the highest degree of complementation observed at pH 6.0 (Fig. 2g).

Analysis of TaPHT1;9-4B using BSMV-VIGS in wheat and ectopic expression in rice

We analysed the effects of silencing TaPHT1;9 expression in bread wheat using BSMV-VIGS. A 256-bp cDNA fragment conserved among all three TaPHT1;9 homoeologs was used to construct the recombinant virus BSMV-TaPHT1;9 for eliciting VIGS (Methods S1; Fig. S6). BSMV-GFP was used as a control for VIGS. At 8 d after viral inoculation, BSMV-TaPHT1;9-infected leaves showed mild chlorosis, and TaPHT1;9 expression level was decreased by >62.0% (Fig. S7). Subsequently, BSMV-TaPHT1;9- and BSMV-GFP-infected seedlings were separately transferred to Hoagland solution containing different concentrations of Pi (0 μM, 50 μM or 1 mM) for 10 d. As shown in Fig. 3a–c, BSMV-TaPHT1;9-infected plants were negatively affected in morphology and growth parameters, including shoot and root length and dry weight, compared with the controls infected by BSMV-GFP under both Pi-replete (1 mM Pi) or Pi-deprived (0 or 50 μM Pi) conditions. P concentrations determined for the shoot and root samples of BSMV-TaPHT1;9-infected plants tended to be lower than those measured for the controls, but for the roots treated with 0 μM Pi, P concentration was higher for BSMV-TaPHT1;9-infected plants than those infected by BSMV-GFP (Fig. 3d). The transcript levels of TaPHT1;3 and TaPHT1;6 were upregulated in TaPHT1;9 silenced plants (Fig. S8), implying possible functional interaction between TaPHT1;9 and the two examined TaPHT1 genes. We also observed that TaIPS1.1 was upregulated in both TaPHT1;9 silenced plants and the controls under low-Pi or Pi-deficient conditions, with the scale of the upregulation being much larger in the former (Fig. S8). This finding was consistent with the fact that TaIPS1.1 expression was induced by low-Pi treatment (Teng et al., 2017). The higher induction of TaIPS1.1 in TaPHT1;9 silenced plants may be associated with a stronger Pi-deficiency stress in these individuals.

Furthermore, we ectopically expressed TaPHT1;9-4B coding sequence in rice using the ubiquitin gene promoter by Agrobacterium-mediated transformation. Independent T3 transgenic rice lines expressing TaPHT1;9-4B, identified by PCR analysis (Fig. S9), were analysed. In general, the two TaPHT1;9-4B overexpression lines (OE1 and OE3) exhibited improved growth parameters than WT control under either Pi-replete (300 μM Pi) or Pi-deprived (0 or 10 μM Pi) conditions (Fig. 3e–g). P concentrations in the root and shoot tissues of TaPHT1;9-4B overexpression plants were markedly higher than those of WT controls with the supply of 300 or 10 μM Pi (Fig. 3h). In the absence of external Pi, P concentration increased significantly in the shoots but decreased in the roots of the OE lines (Fig. 3h), suggesting that the overexpressed TaPHT1;9-4B enhanced Pi translocation from root to shoot tissues under Pi-deficiency conditions.

Verification of TaPHT1;9-4B function using CRISPR mutants in wheat

We developed three CRISPR mutants for TaPHT1;9-4B using a sgRNA targeting the coding region with the wheat cultivar Fielder (Fig. 4a). While the mutations in tapht1;9-4b-1 and -2 led to premature termination of protein sequence, removal of three nucleotides in tapht1;9-4b-3 resulted in one amino acid substitution (Y310C) and deletion of the serine residue at position of 311 (Figs 4b, S10). Through DNA sequencing, we verified that the 4A and 4D homoeologs of TaPHT1;9 were not edited (mutated) in tapht1;9-4b-1, -2 and -3. Subsequently, 2-wk-old CRISPR mutant and WT Fielder plants cultured under normal conditions were transferred to Pi-sufficient (1 mM), low-Pi (50 μM) or Pi-deficient (0 μM) media, and examined after a 3-wk culture. The three mutants were clearly weaker than the control (Figs 4c,d, S11a), and their shoot and root dry weights were significantly smaller than those of the control (Figs 4e, S11b). The shoot and root P concentrations of the three mutants were all significantly lower than those of the control in the presence of 1 mM or 50 μM Pi (Figs 4f, S11c). Under Pi-deficient conditions, the shoot P concentration was decreased in all three mutants relative to that of control, but the root P concentration behaved in an opposite manner (Fig. 4f), indicating that the mutation of TaPHT1;9-4B might hinder the translocation of Pi from root to shoot under Pi-deficiency conditions. These results verified the data obtained by analysing TaPHT1;9 silenced wheat plants (Fig. 3a–d), and confirmed the function of TaPHT1;9-4B in Pi uptake and translocation in wheat.

Identification and functional analysis of TaMYB4-7D

A Y1H assay was conducted using a 1513-bp fragment of TaPHT1;9-4B promoter as a bait to screen the cDNA library derived from Pi-deficient roots (Fig. S12). Of the 23 cDNA clones showing Y1H interactions, three represented the same gene coding for a R2R3-type MYB-like TF (Table S5). The coding sequence of this gene was 705 bp and yielded a protein of 234 amino acids upon conceptual translation (Fig. S13a), which
Fig. 3 Functional analysis of TaPHT1;9-4B using BSMV-VIGS in wheat and ectopic expression in rice. (a–d) Phenotype and growth parameters in BSMV-VIGS experiment. The wheat plants infected by BSMV-GFP or BSMV-TaPHT1;9, cultured under Pi-sufficient (1 mM), low-Pi (50 µM) or Pi-deficient (0 µM) Hoagland solutions for 10 d, were examined for overall growth morphology (a) and shoot and root related parameters including lengths (b), dry weights (c) and P concentrations (d). (e–h) Phenotype and growth parameters in transgenic rice experiment. Two TaPHT1;9-4B overexpressing transgenic rice lines (OE1 and OE3), cultured under Pi-sufficient (300 µM), low-Pi (10 µM) or Pi-deficient (0 µM) nutrient solutions for 21 d, were analysed for overall growth performance (e) and shoot and root related parameters including lengths (f), dry weights (g) and P concentrations (h). Bars, 5 cm. Data represent means ± SD of three biological replicates. Asterisks indicate statistically significant differences (*, P < 0.05; Student’s t-test).
was identical to TaMYB4 (GenBank accession AEG64799.1) previously found to be expressed in wheat stem and root tissues (Ma et al., 2011) (Fig. S13b). In CS genomic sequence, the gene (TraesCS7D02G272400) encoding TaMYB4 was located on chromosome 7D. This was validated by PCR analysis of CS and associated nulli-tetrasomic (NT) lines (Fig. S14a). Therefore, it was designated as TaMYB4-7D to facilitate further functional analysis. The TaMYB4-7D-GFP fusion protein was found only in the nucleus when transiently expressed in tobacco leaf cells (Fig. S14b). When TaMYB4-7D was expressed in yeast cells using the pGBKT vector, designed to test the transactivation activity of TFs (Zhu et al., 2015), the transformed cells showed growth on the synthetic defined medium lacking tryptophan and histidine (SD/-Trp/-His), and the yeast colonies turned blue when grown on SD/-Trp/-His medium containing X-α-Gal (Fig. S14c). These results indicated that TaMYB4-7D was likely to be a functional TF with transactivation activity.

TaPHT1;9-4B promoter carried four predicted MYB TF binding sites (MBSs, P1 to P4) (Figs 5a, S15). Y1H assay showed that the yeast cells harbouring this promoter (bait) and the pGADT7-TaMYB4 vector (prey) grew well on the SD/-Leu medium containing different concentrations (200, 300 and 500 μM) of Leu medium for 3 wk. Data represent means ± SD of three biological replicates. Asterisks indicate statistically significant differences (P < 0.05; Student’s t-test).
were investigated in 62 bread wheat cultivars, TaPHT1;9-4B. Nucleotide diversities in the promoter and coding regions of TaPHT1;9-4B were 2.07 × 10⁻² and 1.81 × 10⁻², respectively (Fig. S20). The genetic diversity of this gene showed a clear decline during the evolution from diploid to polyploid species.

The above results prompted us to investigate if the four promoter haplotypes may be associated with differences in TaPHT1;9-4B expression level and P accumulation in bread wheat cultivars. To test this possibility, we selected four modern bread wheat cultivars for each haplotype (Table S1), and cultured their seedlings under low-Pi (50 μM) conditions. Among the cultivars tested, the Hap3 promoter was associated with the highest TaPHT1;9-4B expression level, better growth performance with higher biomass, and elevated P content in whole plants (Figs 6d, e, S21a–c). However, P concentrations did not differ substantially among the promoter haplotypes (Fig. S21d), implying that the Hap3 type of cultivars could efficiently utilise P to promote plant growth.

To distinguish Hap3 from the other three haplotypes, we developed a CAPS marker based on the two SNPs at positions −799 (C/G) and −796 (C/G) (Fig. 6a). A 703-bp fragment was amplified from the wheat cultivars examined in this work using TaPHT1;9-4B-CAPS-799 F/R primers (Table S2), with the resulting sequence data described in Fig. S22. Digestion by Fnu4HI restriction endonuclease cleaved the ampiclons of Hap1, Hap2 and Hap4 into two fragments (457 and 246 bp), whereas no cleavage occurred to the ampiclons from Hap3 (Fig. 6f). By contrast, the orthologues of TaPHT1;9-4B in tetraploid wheat (TaPHT1;9-4B-Tt) and four S genome-containing Aegilops species (TaPHT1;9-4S) showed much higher genetic diversities. For TaPHT1;9-4B, the π values of promoter and coding regions were 5.13 × 10⁻³ and 2.73 × 10⁻³, respectively (Fig. S20). For TaPHT1;9-4S, the corresponding π values were 2.07 × 10⁻² and 1.81 × 10⁻² (Fig. S20). The genetic diversity of this gene showed a clear decline during the evolution from diploid to polyploid species.

The two SNPs (54 G/A and 1155 C/A) in the coding sequence of TaPHT1;9-4B did not cause amino acid change. We therefore focused on the nine SNPs in its promoter region. The nine SNPs formed four promoter haplotypes (Hap1–Hap4) among the cultivars analysed in this work (Fig. 6a). Compared with Hap1, Hap2, Hap3 and Hap4 contained six, two and one SNPs, respectively (Fig. 6a). The promoter activities of the four haplotypes were tested by their potency to drive GUS expression in germinating wheat grains. The results showed that the Hap3 promoter conferred the highest GUS signals, followed by Hap1 and Hap4 promoters, with the Hap2 promoter showing the lowest activity (Fig. 6b,c). Among the six SNPs of Hap2, one (−281) occurred in a putative MBS element (Fig. 6a), which might be responsible for its decreased promoter activity.

The above results prompted us to investigate if the four promoter haplotypes may be associated with differences in TaPHT1;9-4B expression level and P accumulation in bread wheat cultivars. To test this possibility, we selected four modern bread wheat cultivars for each haplotype (Table S1), and cultured their seedlings under low-Pi (50 μM) conditions. Among the cultivars tested, the Hap3 promoter was associated with the highest TaPHT1;9-4B expression level, better growth performance with higher biomass, and elevated P content in whole plants (Figs 6d, e, S21a–c). However, P concentrations did not differ substantially among the promoter haplotypes (Fig. S21d), implying that the Hap3 type of cultivars could efficiently utilise P to promote plant growth.

To distinguish Hap3 from the other three haplotypes, we developed a CAPS marker based on the two SNPs at positions −799 (C/G) and −796 (C/G) (Fig. 6a). A 703-bp fragment was amplified from the wheat cultivars examined in this work using TaPHT1;9-4B-CAPS-799 F/R primers (Table S2), with the resulting sequence data described in Fig. S22. Digestion by Fnu4HI restriction endonuclease cleaved the ampiclons of Hap1, Hap2 and Hap4 into two fragments (457 and 246 bp), whereas no cleavage occurred to the ampiclons from Hap3 (Fig. 6f). We also investigated the genetic diversity of TaMYB4-7D, which exhibited no polymorphism in either its promoter or coding regions in the 62 bread wheat cultivars examined.

Discussion

In this study, we characterised TaPHT1;9-4B and its transcriptional regulator TaMYB4-7D to increase the understanding of bread wheat PHT1s and to gain functional insight into these important proteins in crop plants.
Fig. 5 Functional analysis of TaMYB4-7D. (a) Four predicted MBSs (P1–P4) in TaPHT1;9-4B promoter region. (b) Y1H assay between TaPHT1;9-4B promoter and TaMYB4-7D, with positive interaction indicated by yeast cell growth in the presence of aureobasidin A (AbA). Pro/pGADT7 and Pro/pGADT7-TaMYB4-7D mark the yeast cells carrying TaPHT1;9-4B promoter with the empty pGADT7 vector or the construct pGADT7-TaMYB4-7D. (c) Diagrams of effector and reporter constructs used in dual-luciferase assay. (d) Ratios of LUC to REN activities obtained in the absence or presence of TaMYB4-7D. (e, f) Binding of TaMYB4-7D to the four putative MBSs in TaPHT1;9-4B promoter. Y1H assay, conducted as above, showed that P1–P4, but not their mutants (mP1–mP4) (e), interacted with TaPHT1;9-4B promoter (f). (g–j) Examination of TaMYB4 silenced wheat plants. The plants infected by BSMV-GFP or BSMV-TaMYB4, cultured under Pi-sufficient (1 mM), low-Pi (50 μM) or Pi-deficient (0 μM) Hoagland solutions for 10 d, were examined for shoot and root lengths (g), dry weights (h) and P concentrations (i), as well as TaPHT1;9 transcript levels in leaf and root tissues. Data represent means ± SD of three biological replicates. Asterisks indicate statistically significant differences (*, P < 0.05; Student’s t-test).
Fig. 6 Analysis of the molecular haplotypes of TaPHT1;9-4B promoter. (a) The four haplotypes (Hap1, Hap2, Hap3 and Hap4) of TaPHT1;9-4B promoter identified based on nine SNPs located in the 5’ proximal region (~1513 bp to +1 bp, relative to the translation start codon ATG). The two SNPs, that is G/A (54) and C/A (1155), in the coding sequence were also shown, although they did not cause amino acid changes. The four predicted MBSs were indicated by red diamonds. (b, c) Comparison of the activities of four promoter haplotypes based on their potency to drive GUS expression after being introduced into germinating wheat grains by particle bombardment. The 35S promoter was used as a positive control. Unbombarded WT grains were used as a negative control (CK) for GUS staining. Bar, 5 mm. (d, e) Transcript levels of TaPHT1;9-4B and P contents under low-Pi (50 μM) conditions in four sets of bread wheat cultivars (1–16, four lines per set, Supporting Information Table S1) with different TaPHT1;9-4B promoter haplotypes. Data represent means ± SD of three biological replicates. Different letters indicate statistically significant differences (P < 0.05, one-way ANOVA followed by Duncan’s multiple range test). (f) Development of a CAPS marker for TaPHT1;9-4B promoter haplotype Hap3 by digesting PCR amplicons with the restriction endonuclease Fnu4HI (arrow).
TaPHT1 proteins functioning in Pi uptake and translocation in bread wheat roots

It is well known that bread wheat carries a large and complex family of PHT1 genes (Teng et al., 2017; Grün et al., 2018). Here we identified four TaPHT1s (i.e. TaPHT1;3-5B, TaPHT1;6-5B, TaPHT1;9-4B and TaPT2) whose protein levels were significantly upregulated in the roots of an elite bread wheat cultivar by Pi deficiency using quantitative proteomic analysis. The presence of TaPT2 among the four Pi-deficiency upregulated TaPHT1s (Fig. 1d) is consistent with the important function of this transport in Pi uptake and plant growth in wheat (Davies et al., 2002; Zeng et al., 2002; Guo et al., 2014). More importantly, we conducted new functional analysis on TaPHT1;9-4B using a variety of genetic and molecular methods. Based on the data gathered in this and previous studies, several suggestions can be made on the TaPHT1 proteins functioning in Pi uptake and translocation in bread wheat.

First, TaPHT1;9-4B is an active high-affinity Pi transporter predominantly expressed in bread wheat roots, and is required for maintaining Pi uptake and wheat plant growth under both Pi-sufficient and Pi-limiting conditions. This is supported by its complementation of phy8 mutation in yeast cells, strong induction of TaPHT1;9 expression in the roots by decreased Pi supply, and substantial reductions in the growth of wheat shoots and roots when TaPHT1;9 function was disrupted by BSMV-VIGS editing. Furthermore, transgenic overexpression of TaPHT1;9-4B in rice improved plant growth and Pi accumulation under both Pi-sufficient and Pi-deprived conditions, therefore adding further evidence on the active role of TaPHT1;9-4B in mediating Pi uptake and promoting plant growth. It is worth noting that under Pi-deficiency conditions (0 μM Pi), disrupting TaPHT1;9 function by either BSMV-VIGS or CRISPR/Cas9 editing led to higher accumulation of P in the roots than in the shoots (Figs 3d, 4f), whereas the reverse was observed when TaPHT1;9-4B was overexpressed in rice (Fig. 3h). This indicates that TaPHT1;9-4B functions in Pi transport from root to shoot. In agreement with our results, previous research also showed that TaPHT1;9 (TaPhit1;1a) was mainly expressed in bread wheat roots and upregulated by low-Pi treatment (Teng et al., 2013, 2017; Grün et al., 2018), although in these studies no efforts were made to analyse the function of specific TaPHT1;9 homologs.

Second, TaPHT1;9-4B shows both functional similarity and difference with TaPT2. TaPT2 is also primarily expressed in bread wheat roots, partially complements phy8 mutation in yeast cells, and can improve (suppress) plant growth and Pi accumulation when overexpressed (silenced) (Davies et al., 2002; Zeng et al., 2002; Guo et al., 2014). Therefore, TaPHT1;9-4B and TaPT2 may act similarly in Pi uptake in bread wheat roots under low P conditions. This functional similarity is additionally supported by the high amino acid sequence identity between the two proteins (99.04% identical, Table S4) and tight clustering of the two proteins in phylogenetic tree. However, TaPHT1;9-4B functions under both Pi-sufficient and Pi-deprived conditions, whereas TaPT2 does not appear to do so because altering its expression in transgenic plants did not affect plant growth and Pi level when Pi supply was adequate (Guo et al., 2014).

Finally, multiple TaPHT1s are activated in the roots of Pi-starved bread wheat plants. In addition to TaPHT1;9-4B and TaPT2, the protein levels of TaPHT1;3-5B and TaPHT1;6-5B were also upregulated by Pi deficiency, indicating the function of multiple TaPHT1 proteins in Pi-starved wheat roots. Moreover, TaPHT1;3 and TaPHT1;6 may compensate for the function of TaPHT1;9 because they were upregulated in TaPHT1;9 silenced wheat plants (Fig. S8). Of the 13 OsPHT1 genes, eight were transcriptionally elevated in rice roots in response to decreased Pi supply (Secco et al., 2013). Among the nine AtPHT1 genes, eight were expressed in the roots (Ayadi et al., 2015; Młodzińska & Zboiriska, 2016). Therefore, the rich knowledge on rice and Arabidopsis PHT1 proteins may aid further functional analysis of the four TaPHT1s activated by Pi deprivation in wheat roots.

Regulation of TaPHT1;9-4B transcription by TaMYB4-7D

In bread wheat, only one MYB family TF, TaPHR1, has so far been demonstrated to upregulate a subset of PSI genes and to enhance Pi uptake and root growth when overexpressed in transgenic plants (Wang et al., 2013). Here we isolated TaMYB4-7D and generated several lines of evidence suggesting that TaMYB4-7D positively regulates the transcription of TaPHT1;9-4B in bread wheat. First, TaMYB4-7D was located in the nucleus and possessed transcription activation properties. Second, TaMYB4-7D could bind to the four MBSs in the promoter region of TaPHT1;9-4B in a cellular environment. Third, silencing TaMYB4-7D expression decreased wheat plant growth and Pi accumulation, which resembled highly the negative effects brought about by reducing the expression of TaPHT1;9-4B. Finally, the upregulation of TaPHT1;9-4B expression by low-Pi or Pi-deficiency treatment was strongly attenuated in TaMYB4-7D silenced wheat plants. Therefore, it is likely that TaMYB4-7D and TaPHT1;9-4B may form a functional module that takes an active part in Pi uptake in wheat, especially in the roots where TaPHT1;9-4B is predominantly expressed and significantly elevated by inadequate Pi supply. Other TFs, such as PHR and WRKY proteins, may also contribute to the control of TaPHT1;9-4B expression as P1BS and W-box, to which PHR and WRKY TFs bind, respectively, are present in the promoter region of many TaPHT1 genes, including TaPHT1;9 (TaPhit1;1a) homologs (Grün et al., 2018).

Two studies in rice suggest that the R2R3 MYB TFs OsMYB4P and OsMYB5P can positively regulate the transcription of multiple OsPHT1s (Yang et al., 2014, 2018). For OsMYB5P, direct binding of OsMYB5P to the promoter region of nine OsPHT1 genes was demonstrated using ChIP assay (Yang et al., 2018). In this context, it will be interesting to study if TaMYB4-7D may also regulate the expression of the other three Pi-deficiency upregulated TaPHT1s. In a preliminary analysis, MBSs were found to be present in the promoter region of TaPHT1;3-5B, TaPHT1;6-5B and TaPT2, and TaMYB4-7D could interact with, and activate, the promoter of the three genes.
(Figs S23, S24). These data reinforce the importance of TaMYB4-7D in the regulation of TaPHT1s.

Ma et al. (2011) showed that TaMYB4 (TaMYB4-7D) could repress the genes involved in lignin biosynthesis by binding to the AC-II cis-element, which suppressed lignin accumulation but stimulated flavonoid biosynthesis. P deficiency often leads to strong upregulation of flavonoid and anthocyanin productions in higher plants (Raghothama, 1999; Wang et al., 2018b). Under Pi-deprived conditions, the silencing of TaMYB4 inhibited efficient anthocyanin accumulation and the expression of several anthocyanin biosynthesis genes (Figs S18, S19). Therefore, we speculated that TaMYB4 may play a dual role under Pi-limiting conditions, that is promoting Pi uptake by increasing the expression of PHT1s and increasing flavonoid and anthocyanin biosynthesis by elevating the expression of anthocyanin biosynthesis genes but suppressing those involved in lignin accumulation.

Efficient identification of Pi-deficiency regulated wheat proteins using iTRAQ-based quantitative proteomics

The iTRAQ-based proteomic analysis is emerging as an efficient approach for uncovering the pathways and proteins functioning in plant responses to changing Pi supplies. In Arabidopsis, iTRAQ analysis permitted the quantification of >10 000 proteins, and clarified the involvement of chromatin reorganisation and redox homeostasis in the regulation of root growth (Suen et al., 2018), and identified flavonoid biosynthesis as the most significantly enhanced metabolic process, under Pi-starvation conditions (Wang et al., 2018b). In rice, iTRAQ analysis has facilitated the functional characterisation of two vacuolar phosphate efflux transporters (Xu et al., 2019), and the roles of multiple types of kinases, in Pi-starved plants (Yang et al., 2019). Here we used this approach and identified 4306 PDRPs with 1015 of them mapped to specific homoeologs in A, B or D subgenomes. These PDRPs may become a valuable resource for systematically dissecting the complex proteome changes required for bread wheat to adapt to low-Pi availability.

Markedly, the majority of the 1015 assigned PDRPs were the products of single homoeologs, with no expression detected for their remaining homoeologs in our iTRAQ analysis. Therefore, homoeolog expression bias may underpin a large proportion of the bread wheat proteome under Pi-deficiency conditions. A previous transcriptomic study on bread wheat plants infected by a fungal pathogen also reported that a high proportion of the biotic stress responsive gene expression resulted from single homoeologs from one subgenome alone (subgenomes A, B or D) (Powell et al., 2017). The finding of homoeolog expression bias in our work provides a new direction for studying mechanism underlying the dynamic gene expression patterns regulated by Pi availability in wheat.

Haplotype variation and potential value of TaPHT1;9-4B in improving Pi uptake and use efficiencies in bread wheat

By examining the promoter and coding regions of TaPHT1;9-4B and its orthologues in hexaploid and tetraploid wheat lines and diploid relatives, a dramatic decrease in nucleotide diversity was observed, suggesting that strong selection occurred on TaPHT1;9-4B during the evolution of polyploid wheat. Nevertheless, the promoter region of TaPHT1;9-4B displayed a moderate level of haplotype variation, with Hap3 showing significant positive associations with TaPHT1;9-4B transcript levels, growth performance and P content in bread wheat cultivars. Considering the active role of TaPHT1;9-4B in Pi acquisition as demonstrated by this work, it is necessary to test its potential value in improving wheat P acquisition and use efficiencies. The CAPS marker, CAPS-799, capable of distinguishing Hap3 from the other three haplotypes, may be used to transfer the Hap3 allele of TaPHT1;9-4B into the elite bread wheat background to evaluate its effects on P acquisition and plant growth. Alternatively, transgenic expression may be used to explore the breeding values of TaPHT1;9-4B and its transcriptional regulator TaMYB4-7D, as ectopic overexpression of certain PHT1 proteins and regulatory TFs has been shown to enhance P accumulation, root and shoot biomass and yield performance in crop plants (Liu et al., 2013; Wang et al., 2013; Guo et al., 2014; Yan et al., 2014; Zhang et al., 2014; Qu et al., 2015; Dai et al., 2016; Yang et al., 2016; Kopriva & Chu, 2018).

In summary, our work has uncovered four PHT1 proteins upregulated in bread wheat roots by Pi deficiency. The high-affinity transporter TaPHT1;9-4B and its transcriptional regulator TaMYB4-7D contributed to efficient Pi acquisition and plant growth under Pi-limiting conditions. This two genes may stimulate further research into the molecular mechanism controlling Pi homeostasis and utilisation in wheat, which together with the superior promoter haplotype of TaPHT1;9-4B may facilitate the development of Pi efficient wheat cultivars in the future.

Accession numbers

The amplified sequences of TaPHT1;9-4B homolog for diploids, and tetraploids have been submitted to GenBank (accession nos. MN043994–MN044002). Proteomic data were submitted to ProteomeXchange with the dataset identifier PXD003570.

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Author contributions

GZK conceived the project. PFW performed and analysed the experiments. GZL, GWL and DWW helped with data analysis. SSY helped with the preparation of wheat materials. CYW, YXX and TCG evaluated wheat cultivars. DWW, PFW and GZK wrote the manuscript.
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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Dataset S1** Identification and analysis of phosphate deficiency responsive proteins (PDRPs) in bread wheat roots using iTRAQ-based proteomics.

**Dataset S2** Quantification analysis of 11 Pi-deficiency responsive proteins (PDRPs) using PRM analysis in bread wheat roots.

**Dataset S3** Mapping PDRPs to the genomic loci of Chinese Spring.

**Fig. S1** Examples illustrating homoeolog mapping of proteomic peptides in this work.

**Fig. S2** Phenotype and growth parameters of wheat seedlings cultured under Pi-deficient conditions for 10 d.

**Fig. S3** Comparison of 11 PDRPs with respect to their expression changes induced by Pi deficiency revealed using iTRAQ or PRM approaches.

**Fig. S4** Nucleotide and deduced amino acid sequence of TaPHT1;9-4B.
Fig. S5 Transcript level of TaPHT1;9 in the root and shoot tissues of the wheat plants cultured under Pi-sufficient or -deficient media for 8 d.

Fig. S6 The coding sequences of TaPHT1;9 homoeologs (4A, 4B and 4D) in Chinese Spring.

Fig. S7 Functional analysis of TaPHT1;9 in bread wheat using BSMV-VIGS.

Fig. S8 Evaluation of the transcript levels of three TaPHT1 genes and TaIPS1.1 in the roots of the wheat plants infected by BSMV-GFP or BSMV-TaPHT1;9.

Fig. S9 Molecular identification of transgenic rice lines expressing TaPHT1;9-4B.

Fig. S10 The effects of nucleotide mutations in three CRISPR mutants on TaPHT1;9-4B protein.

Fig. S11 Phenotypes, dry weights and P concentrations of three CRISPR mutants and WT Fielder control cultured under Pi-sufficient conditions.

Fig. S12 Y1H screening using TaPHT1;9-4B promoter as bait.

Fig. S13 Sequences and phylogenetic tree of TaMYB4-7D.

Fig. S14 Chromosomal location, subcellular localisation and transcriptional activation activities of TaMYB4-7D.

Fig. S15 Sequence of TaPHT1;9-4B promoter.

Fig. S16 Transcript level of TaMYB4 in the root and shoot tissues of the wheat plants cultured under Pi-sufficient or -deficient media for 8 d.

Fig. S17 Analysis of the bread wheat plants with TaMYB4 expression silenced by BSMV-VIGS.

Fig. S18 Evaluation of foliar anthocyanin contents in the wheat plants infected by BSMV-GFP or BSMV-TaMYB4.

Fig. S19 Evaluation of the transcript levels of four anthocyanin biosynthesis genes in the leaves of BSMV-GFP- or BSMV-TaMYB4-infected wheat plants.

Fig. S20 Analysis of nucleotide diversity of PHT1;9-4B promoter and its genomic coding sequence in bread wheat and relatives.

Fig. S21 Phenotypes, dry weights, P contents and P concentrations of the 16 wheat varieties with different promoter haplotypes of TaPHT1;9-4B under low-Pi conditions.

Fig. S22 Nucleotide sequence comparison of the DNA fragments used to differentiate four TaPHT1;9-4B promoter haplotypes (Hap1 to Hap4).

Fig. S23 Nucleotide sequence of the promoter region of TaPHT1;3-5B, TaPHT1;6-5B and TaPT2.

Fig. S24 Binding of TaMYB4-7D to the promoter region of TaPHT1;3-5B, TaPHT1;6-5B and TaPT2.

Methods S1 Additional description of methods.

Table S1 Accessions of hexaploid bread wheat and its relative species used in this study.

Table S2 All primers used in this study.

Table S3 Accession numbers of PHT proteins for constructing phylogenetic tree of TaPHT1;9-4B.

Table S4 Identities of PHT proteins used for constructing the phylogenetic tree of TaPHT1;9-4B.

Table S5 Potential proteins interacting with the promoter of TaPHT1;9-4B obtained using Y1H screening.

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