Identification and expression profiling of Pht1 phosphate transporters in wheat in controlled environments and in the field

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INTRODUCTION

Inorganic phosphate (Pi) acquisition in plants is mediated by plasma membrane-localised phosphate transporter (Pht) proteins, which belong to the major facilitator superfamily (MFS), and function as Pi/H⁺ symporters within high- and low-affinity ranges (Rae et al. 2003; Raghothama 2005; Liu et al. 2011; Nussaume et al. 2011). The transporter genes have been classified into four families (Pht1–4) with distinct molecular structures, localisation and functions (Smith et al. 1999; Liu et al. 2011; Guo et al. 2013; Shukla et al. 2016). The transporters are involved in initial root Pi acquisition as well as Pi translocation throughout the plant. Some Pht1 transporters are expressed in the root, predominantly in root tips and root hairs (Schümann et al. 2004). Such root-expressed Pht1 transporters have been identified in a broad range of different plant species including wheat, indicating an involvement in initial root Pi uptake from the soil solution (Liu et al. 1998; Smith et al. 1999; Davies et al. 2002; Mudge et al. 2002; Nagy et al. 2006; Wang et al. 2013). The establishment of arbuscular mycorrhiza (AM) symbiosis is a well-known adaptation strategy of plants to increase Pi accessibility in low Pi environments (Tarařdár & Marschner 1994; Koide & Kabir 2000), and the expression pattern of some Pht1 transporters is closely related to root AM colonisation in rice, wheat, Brachypodium and Medicago (Harrison et al. 2002; Paszkowski et al. 2002; Glassop et al. 2005; Hong et al. 2012).

In addition to initial root Pi uptake, the large diversity of expression profiles in plant tissues indicates that Pht1 transporters are also involved in Pi translocation as well as Pi remobilisation, in the aerial parts of the plant, especially during generative growth. In barley, weak expression of root-expressed HvPht1;2 and HvPht1;1 has been reported in leaves (Schümann et al. 2004), whereas HvPht1;6 was strongly expressed in both lower stem leaves and in flag leaves. In rice, OsPht1;1 was expressed abundantly in epidermal root cells and in stele cells of leaves, and weakly in spikelets and emerging buds (Sun et al. 2012). Pht1 transporter expression was observed in panicles and flag leaves of rice (Liu et al. 2011) and in flowers of soybean (Qin et al. 2012).

Phosphate starvation responses influence root Pi uptake mechanisms, as well as Pi partitioning between roots and aerial tissues, via altered Pht1 transporter expression. Increased Pht1 transporter expression was reported in plants during a short period of Pi starvation or upon mycorrhizal infection (Smith et al. 1999; Rae et al. 2003; Calderón-Vázquez et al. 2008; Ai et al. 2009; Miao et al. 2009; Huang et al. 2011; Qin et al. 2012). In Pi-starved maize plants, Pht expression is present in roots and leaves, and one of those Phts, ZmPht1;6, is expressed in roots independent of the Pi supply, which were colonised by...
symbiotic mycorrhizal fungi. In the leaves ZmPht1;6 is induced under Pi depletion (Nagy et al. 2006). In rice, the expression of the low affinity Pht2 transporter is regulated by a MYB-domain transcription factor, OsPHR2, influencing P translocation from root to shoot (Zhou et al. 2008). Furthermore, the rice high-affinity OsPHT8 transporter plays important roles in both the acquisition of Pi from the external environment, and in the translocation of Pi within plants (Jia et al. 2011). Overexpression of the MYB transcription factor PHR1 in wheat led to increased Pht1.2 and Pht1.6 expression, with a promotion of shoot development and root branching as well as increased grain yield (Wang et al. 2013).

The Pht1 family members in wheat (TaPht1) may be important targets for enhancing low P tolerance and P acquisition efficiency/P utilisation efficiency in agronomic systems, however the lack of wheat genome sequence information has hindered investigation of this approach (Davies et al. 2002; Huang et al. 2011). The aim of this study was to identify all putative TaPht1 family members using genomic sequence information and homologies with model plants and other cereal species, and to compare the wheat Pht1 expression profiles in relation to P nutrition under controlled environment hydroponic cultures with expression patterns observed in field-grown wheat plants.

**MATERIAL AND METHODS**

Identification of wheat TaPht1 genes and phylogenetic analysis

Putative members of the Pht1 gene family in *Triticum aestivum* were identified based on Pht1 gene sequences from barley (*HvPht1*; Rae et al. 2003), *Brachypodium distachyon* (*BradiPht1*; Hong et al. 2012) and rice (*OsPht1*; Paszkowski et al. 2002) by BLAST analysis with a cut-off threshold of 0.0001–0.01 (Altschul et al. 1990) using available wheat genome databases on the EnsemblePlant (Kersey et al. 2016), PLAZA3.0 (Proost et al. 2015) and the Rothamsted decypher web platform [wheat databases: IWGSC (2014), TGAC (TGACv1 genome assembly of *Triticum aestivum* cv. Chinese Spring, generated by The Genome Analysis Centre, Norwich, as part of the BBSRC-funded project, Triticaceae Genomics for Sustainable Agriculture) and other wheat varieties (opendata.earlham.ac.uk/Triticum_aestivum/EL/v1 www.earlham.ac.uk/sequencing-wheat-genome)].

The transcription start sites, including the 5'-non-coding regions, could be verified from published sequence information data only for *TaPht1;2* on chromosome 4BL, and for *TaPht1;4* on chromosome 5BL (Kawaura et al. 2009). The ATG start sites of the remaining TaPht1s were verified by alignment analysis of DNA as well as amino acid sequences. There may be additional introns in the 5'-non-coding regions, which were ignored in this analysis (see Supplemental genomic sequences). Putative *TaPht1* sequences from genomic databases were allocated by multiple sequence alignment to those identified in this study (Table S5). The sequence similarities and phylogenetic relationships of the wheat homologous Pht1s to *Brachypodium*, rice (*Oryza sativa* ssp. japonica, MonocotPLAZA 3.0 gene locus names; Proost et al. 2015) and barley Pht1s were analysed based on the coding DNA as well as the protein sequences. Multiple sequence alignments were generated using ClustalX version 2.0 (Larkin et al. 2007). MEGA6 (version 6.06; Tamura et al. 2013) was used for the calculation of phylogenetic trees (the neighbour-joining method; Saitou & Nei 1987). The bootstrap consensus tree (expressed as percentages) inferred from 1000 replicates was taken to represent the evolutionary relationships (Felsenstein 1985). The evolutionary distances were computed for both cDNA and amino acid sequences using the p-distance method (Nei & Kumar 2000). Due to the inconsistent numbering of the Pht1 transporters in different plant genomes, the TaPht1 genes were, in part, classified according to the barley nomenclature, when putative homologues could be identified.

Identification of putative transcription factor binding sites in the promoter region of wheat Pht1 genes

In addition to the coding regions, 5'-non-coding and promoter regions up to 2000 bases upstream from the ATG start codons of all wheat Pht1 genes were identified from the genomic databases (Supplemental genomic sequences).

The locations of the putative MYB transcription factor PHR1 promoter element binding site PIBS (GNATATNC), the WRKY transcription factor binding W-box (C/T TGAC C/T) site and the bHLH transcription factor binding helix-loop-helix promoter element (CA[G/T][CA][TG]; Rubio et al. 2001; Zhou et al. 2008; Lin et al. 2009; Wang et al. 2014) in the promoter/5'-non-coding regions were identified by pair-wise nucleotide alignment (Table S4, EMBOSS Needle-Needleman-Wunsch alignment algorithm to find the optimum alignment (including gaps) of two sequences along their entire length; McWilliam et al. 2013; Li et al. 2015).

Plant material

For hydroponic experiments in controlled environments, seeds of *T. aestivum* cv. Hereward were surface-sterilised for 10 min in a 1% perchlorate solution, rinsed with sterile water and germinated for 5 days on sterile water-soaked paper tissue. Seedlings were transferred to a single 1-1 aerated hydroponic culture pot (one plant per pot) in a controlled environment chamber (228 Fitotron growth cabinet; SANYO-Gallenkamp, UK): 12-h day length, 70% humidity, 20 °C, photon flux rate 500 µmol photons m⁻² s⁻¹ (40 28W/20 49W fluorescent tubes in an air-cooled light box); night conditions were 16 °C and 80% humidity. Light intensity was measured with a SKP 200 light meter (Skye, UK). The Letcombe nutrient solution (Drew & Saker 1984) was modified for wheat (5 mm KNO₃, 2 mm NaNO₃, 1.5 mm Ca(NO₃)₂, 1 mm MgSO₄, 1 mm KH₂PO₄, 25 µm FeEDTA, 9.2 µm H₂BO₃, 5 µM KCl, 3.6 µM MnCl₂, 770 nm ZnCl₂, 160 nm CuCl₂, 16 nm Na₂MoO₄, pH 5.8). The nutrient solution was exchanged three times per week. Pi starvation was initiated after 6 days of growth by replacing 1 mm KH₂PO₄ with 1 mm KCl. Roots and shoots were harvested (in triplicate) 0, 3, 6, 9 and 12 days after onset of the Pi depletion. The entire root of each plant was rinsed in deionised water, dried briefly on paper towels before freezing in liquid nitrogen.

Field-grown *T. aestivum* cv. Hereward was harvested in 2011/2012 from sections 0 and 1 (representing continuous wheat plots, drilled in autumn 2010/2011) of the 'Broadbalk'...
field experiment (Rothamsted Research 2006; Watts et al. 2006), with low or high soil Pi availability (Table 2). The soil is a flinty, silty clay loam (Luvisol) with a clay content of 25–35% and with calcareous layers below 2-m depth in the sampling area (Watts et al. 2006). Soil analyses and yield data were provided by the long-term experiments National Capability (http://www.rothamsted.ac.uk/long-term-experiments-nationall-capability; eRA data 2010–2013). Five replicates of root and shoot tissues were sampled at five growth stages (Zadoks et al. 1974): tillering (GS25), stem elongation (GS32), early and late booting (GS45/49), anthesis (GS65) and ripening (GS75; 20 days post-anthesis) from plots receiving no Pi fertiliser (plot 20-0/20-1 ‘Pi-starved’) and a control plot (35 kg Pi ha⁻¹ applied as triple superphosphate), which was likely to yield Pi-replete plants (plot 09-0/09-1 ‘Pi-supplied’). Roots were excava-
ted with a fork-like spade, rinsed with deionised water, dried briefly on paper towel before freezing in liquid nitrogen and being stored at −80 °C. The shoot tissues were kept on ice and separated into either full shoot samples for chemical analysis or samples of different plant parts, which were frozen with liquid nitrogen.

All frozen tissue samples were ground with a mortar and pestle in liquid nitrogen (glumes and leaves) or with a SPEX SamplePrep 6870 Freezer/Mill (Metuchen, USA) (roots, ears, rachis and grain). Aliquots were stored at −80 °C for RNA extraction. The remaining shoot material was used for chemical analysis.

Chemical Analysis

Oven-dried shoot material (72 h/80 °C) was milled (ZM 200 Retsch mill, Haan, Germany) and stored in glass vials at room temperature in the dark. Mineral element concentra-
tions were determined with Inductively Coupled Plasma-Atomic Emission Spectrometry (Perkin Elmer LAS, Seer Green, UK) as described in Shimachi et al. (2010). Aliquots of 250 mg were digested for 2 h at room temperature with 5 ml 70% nitric/70% perchloric acid mixture (15:85 v/v) in 25 ml glass vials. The vials were then placed into a tempera-
ture-controlled carbonate heating block with a heating regime: 60 °C for 3 h, 100 °C for 1 h, 120 °C for 1 h, 175 °C for 2 h and cooled to room temperature. After adding 5 ml 25% HCl, the block was reheated to 80 °C for 1 h. Subsequently, 20 ml deionised H₂O was added, heated for another 30 min at 80 °C and removed from the heating block to cool. Deionised H₂O was added to give a final vol-
ume of 20 ml.

Total plant RNA isolation, cDNA synthesis and sequence analysis

Total RNA was isolated from homogenised plant material using a modified protocol from Verwoerd et al. (1989) involving an additional phenol-chloroform-isomyl alcohol extraction and DNase treatment (Promega, Madison, WI, USA). Integrity of the RNA and absence of genomic DNA was con-
firmed on a 1.5% (w/v) agarose gel. cDNA was synthesised in a total reaction volume of 20 μl using 2 μg total RNA, 1 μl 10 mM dT-adapter primer and 1 h synthesis time according to the standard protocol for Superscript III Reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Determination of transcript abundance by quantitative real-time PCR

Real-time quantitative PCR (q-PCR) was performed in a 25 μl reaction volume for each sample containing 1 μl of cDNA, 12.5 μl SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, Gillingham, UK), 0.025 μl ROX reference dye, 250 mM, 150 mM or 100 mM of gene-specific primer pairs (Table S1; Applied Biosystems 7500 Fast Real-time PCR Sys-
tem, AB7500 Software 2.0.5). Wheat genes mostly exist as a trio of A, B and D homoeoloci in the hexaploid wheat genome, and each homoeolocus may contribute differentially to wheat phenotypes. The intention of the expression profile analysis presented was to identify the total expression profile of all three potential homoeoloci of TaPht1 transporters, based on the available sequence information. The gene specific TaPht1 primer combinations cover all three genomes (ABD) for most expressed TaPht1 genes. Exceptions are TaPht1;6 (AB genomes) for which the alignment of D genome contigs was ambiguous, and TaPht1;7 (BD genomes) for which sequence information was only available from the D and B genome (Table S1). The amplification efficiency (E = 10⁻¹⁻¹S − 1) was determined for each primer pair (Table S1), using ten-fold cDNA dilution series in triplicate (n = 3) for different tissues and treatments as well as the LinRegPCR package (Ramakers et al. 2003; Tuomi et al. 2010). Due to the similarity between TaPht1 gene sequences, locations for efficient primer combina-
tions were limited, with a complete lack of primer combina-
tions for efficient expression analysis in green leaf and shoot material. Primer concentrations were adjusted to improve the PCR efficiency and considered acceptable within a range of 85% to 115%. Efficiency testing included melting curve analy-

A 500 bp cDNA fragment covering the expression ampli-
con of each TaPht1 gene analysed was amplified (Mastercycler® gradient; Eppendorf Scientific, Stevenage, UK) using a gene specific primer pair (Table S5) and RedTaq ready PCR-mix (Sigma-Aldrich, Gillingham, UK). These amplified DNA fragments were cloned into the pGEM-Teasy vector system (Promega, Southampton, UK) and sequenced (MWG-Eurofins, Ebersberg, Germany). Sequences were submitted to the EMBL database (Table S5). Due to the low level of tran-
scripts, cDNA fragments for TaPht1;9 and TaPht1;14 were not cloned. For TaPht1;3 and TaPht1;4 no suitable real-time q-PCR primer combinations for expression analyses were found. The real-time qPCR regime largely followed the SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, St Louis, MI, USA) standard protocol in a 25 μl reaction but used appropriate annealing temperatures for 20 s (Table S1) before the extension for 40 s at 60 °C. Blank water controls as well as melting curve analysis was performed with each real-time PCR as a standard control. Furthermore, standard dilution series of each TaPht1 plasmid-PCR fragment in triplicate were included. The replicate CT-values per time point/develop-
mental stage were normalised using a reference gene (the heterogenous nuclear ribonucleoprotein Q (hnRNP Q), Ta.10105.1.S1; Long et al. 2010) expression. The heteroge-
nous nuclear ribonucleoprotein exhibited the most stable constitutive expression under control as well as P starvation conditions among the reference genes tested (data not
shown). The amount of each amplicon was quantified with respect to the standard curve of the individual TaPht1 standard as mRNA copy number per μl cDNA.

**Statistical analysis**

Data were analysed using GenStat (16th edition, VSN International, Hemel Hempstead, UK). Expression data and P tissue concentrations in hydroponics experiments were analysed by two-way ANOVA with a factorial treatment structure (Fig. 3). Mineral concentration in wheat shoots from the field (Fig. S2, Table S2) were analysed by two-way ANOVA ($P \leq 0.05$). When residuals were not normally distributed, data were log$_{10}$-transformed (Table S3 for field expression analysis).

**RESULTS**

The wheat phosphate transporter 1 family

A maximum of 16 wheat A-genome and 14 B- and D-genome TaPht1 genes were identified, representing 16 phylogenetically distinct TaPht1 transporters (Figs 1, 2, S1, Tables S1, S5). A trio of A, B and D homoeoloci were verified for 14 TaPht1 genes (Table S5, Fig. S1, Data S1) on chromosomes 1, 2, 4, 5, 6 and 7. For TaPht1;11, homoeoloci were detected on the long arm of the A-genome chromosome 4 and on the short arm of the B- and D-genome chromosomes 4 (Table S5). The homoeoloci for TaPht1;3, TaPht1;4 and TaPht1;14 appeared to be on different chromosomes. TaPht1;3 and TaPht1;4 are neighbouring genes with one locus on the long arm of the B- and D-genomes of chromosome 5, and one on the long arm of chromosome 4 AL. The TaPht1;14 homologous genes are located on the long arm of chromosomes 1BL and 4AL, as well as on the short arm of chromosome 7DS. Only one locus on the short arm of the A-genome chromosome 2 was found for TaPht1;13 and similarly on the long arm of chromosome 4A for TaPht1;1b. There are homologous sequences present in the incomplete draft genome sequence database of *Triticum uratu*, the hexaploid wheat A-genome progenitor (TaPht1;13, PLANT_T.urartu_wgs|scaffold12793; TaPht1;1b, PLANT_T.urartu_wgs|scaffold1959), but none have been identified in the *Aegilops tauschii* genome (data not shown). Eleven of the wheat TaPht1s per genome are intronless in their coding DNA regions. Only a single intron is present in each of all homoeoloci of TaPht1;9, TaPht1;10, TaPht1;11, TaPht1;12 and TaPht1;14 (Supplemental wheat TaPht1 genomic sequences).

Several of the 16 phylogenetically distinct TaPht1 transporters share a high degree of homology in their coding DNA as well as amino acid sequences. The sequence similarity data, presented in Fig. S1 and Table 1 for all A-genome TaPht1s, except for the B-genome TaPht1;12, are representative for all three genomes. The A-genome TaPht1;12 on chromosome 2AS is truncated by missing part of the 3'-coding region, and has an insertion in the 5'-region leading to a non-functional gene. Furthermore, the genomic region of TaPht1;14 on chromosome 1BL is not fully sequenced and only a partial coding sequence is available containing a deletion. TaPht1;1a, TaPht1;1b, TaPht1;2a and TaPht1;2b share a high degree of identity of >95% for DNA and >98% for amino acid sequences (Table 1), representing very close paralogous genes. These four transporters showed 65% to 73% sequence identity to TaPht1;13, TaPht1;4, TaPht1;5, TaPht1;6 and TaPht1;7, and a lower degree of homology with all other TaPht1 transporters, notably with TaPht1;10 (Table 1). The protein and coding DNA sequence of TaPht1;3 and TaPht1;4 from the A-genome were both 99% identical. TaPht1;10 did not show a high sequence identity to any of the other TaPht1 transporters. TaPht1;9, TaPht1;12 and TaPht1;14 also exhibited low sequence identity to all other transporters, except to each other (~60%). TaPht1;3 and TaPht1;4 were most similar to TaPht1;5, TaPht1;6, TaPht1;7 and TaPht1;13 with 67–82% homology.

Table 1. Homology (%) of translated transcripts of A-genome phosphate transporters (with the exception of TaPht1;12 from the B-genome) in wheat. The data were generated with the ClustalX version 2.0 software for alignments used for phylogenetic analysis. Nucleotide sequence identities (bold) and protein sequence identities (italics). The degree of shading highlights the level of sequence identities.
TaPht1;12 and TaPht1;13 shared 50–60% homology with TaPht1;14 and TaPht1;9 (Table 1). Reverse Blast analysis of the low homology Pht1 genes, particularly of TaPht1;9, TaPht1;10 and Pht1;11, to the NCBI protein database confirmed all identified genes to be members of the Pht1 gene family.

Based on the phylogenetic analysis of the coding DNA and protein sequences, cereal Pht1 genes can be subdivided into several clusters (Figs 1, 2). There are some discrepancies between DNA and protein analysis leading to some variation in homologous and/or orthologous relationships depending on which sequence information is utilised. The phylogeny confirms the close relationship of nearly all homoeologous wheat TaPht1 genes except TaPht1;3, TaPht1;4, TaPht1;1a, TaPht1;1b, TaPht1;2a and TaPht1;2b. An estimation of the homologous relationship between the TaPht1;1a/b and Pht1;2a/b type genes could not be determined, even when including part of the 5’-non-coding/promoter and 3’-non-coding regions (Fig. S1). The only clear difference between the TaPht1;1a and TaPht1;1b type to the TaPht1;2a and TaPht1;2b type is a 12 base elongation of the 3’-end leading to TaPht1;2a and TaPht1;2b proteins which were four amino acids longer.

Based on the DNA coding sequences, the A-genome

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**Fig. 1.** Phylogenetic relationship of coding nucleotide sequence of the phosphate transporter family 1 in cereals. A neighbour joining (Saitou & Nei 1987) unrooted tree was generated for Brachypodium (Bradi), rice (Os), barley (Hv) and wheat (Ta) Pht1 transporters with MEGA6 (version 6.06) software; Tamura et al. 2013), from a multiple alignment (ClustalX version 2.0) using the coding nucleotide sequences. The bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the p-distance method (Nei & Kumar 2000). The analysis involved 76 nucleotide sequences. All positions containing gaps and missing data were eliminated. Different protein clusters and subclusters are shaded. The different phylogenetic tree branch clusters and subclusters are shaded.
TaPht1;1a/b and TaPht1;2a/b type genes were found in a separate sub-cluster, distinct from the B- and D-genome TaPht1;1a/b and TaPht1;2a/b genes, which also show a homologous relationship to the three barley Pht1 genes (Fig. 1). The high similarity in their coding regions, and partial similarity in the 5’-/3’-non-coding regions, suggests two recent duplication events in the A-, B- and D-genome common ancestor leading to the paralogous Pht1;1 and Pht1;2 type genes, with further duplication to paralogous Pht1;1a/b and Pht1;2a/b type genes.

TaPht1;1b is only present in the A-genome chromosome 4 but not in the B and D genome. This A-genome-specific duplication, or B/D-genome gene loss, is confirmed by the presence of TaPht1;1a in the genome of the A-genome progenitor, *Triticum urartu*, but not in the D-genome progenitor of *Aegilops tauschii*. Based on coding DNA sequence, TaPht1;1a/b and TaPht1;2a/b comprise a cluster with four phylogenetically closely related rice genes, three barley Pht1 genes and one *Brachypodium* Pht1 gene (Fig. 1). The separate sub-cluster of the
Brachypodium Pht, and the four rice Phts does not suggest any clear homologous or orthologous relationships to the TaPht1;1a/b and TaPht1;2a/b wheat genes (Fig. 1). This is also seen for the protein sequence-based phylogeny. The two barley proteins, HvPht1;2 and HvPht1;10, seem to be orthologous to the D-genome TaPht1;2a and TaPht1;2b, whilst HvPht1;1 is orthologous to the A-genome TaPht1;2b.

A second DNA-based cluster is composed of the four wheat TaPht1s, TaPht1;9, TaPht1;12, TaPht1;13 and TaPht1;14. No barley gene have been identified homologous to those wheat genes and there is no rice gene homologous to TaPht1;12 and TaPht1;14, with one Brachypodium gene homologous only to TaPht1;12. One rice and one Brachypodium gene are homologous to TaPht1;9. Two rice and Brachypodium genes are homologous to TaPht1;13 (Fig. 1). The A-genome TaPht1;13 protein sequence is much more closely related to the homoeologous TaPht1;14 proteins, building up a common cluster together with the TaPht1;9 homoeologous proteins (Fig. 2). The Brachypodium Pht1;11 can be confirmed to be orthologous to TaPht1;9. The rice Pht1;4 and Pht1;5 protein sequences are more closely related to the Pht1;9 proteins, and the rice Pht1;13 protein has a close relationship to the TaPht1;1 and TaPht1;2 cluster, with an orthologous relationship to the Brachypodium Pht1;4 protein (Fig. 2).

A third cluster, comprising the coding DNAs of TaPht1;3, TaPht1;4, TaPht1;6 and TaPht1;7, may be further subdivided into three sub-clusters. Two Brachypodium genes and one barley and rice gene are homologous to TaPht1;6; another Brachypodium, a barley and a rice gene are more homologous to TaPht1;7. There is only one Brachypodium and no rice genes which are homologous to TaPht1;3 and TaPht1;4 (Fig. 1). The proteins of TaPht1;3, TaPht1;4 and TaPht1;7 are found in one large branch cluster, including TaPht1;5, TaPht1;8 and TaPht1;11 and TaPht1;12, but the latter are found in different clusters based on the phylogeny of the coding DNA.

With some exceptions, the closely related orthologous barley, rice and Brachypodium Pht1 genes could be verified by common DNA and protein relationships. For Brachypodium Pht1;8 and Pht1;13, as well as for the rice Pht1;6, there were no closely related wheat homologues.

The very high sequence similarity between TaPht1;3 and TaPht1;4, in their coding, as well as partially in their non-coding and promoter regions, as also seen for TaPht1;1a/b and TaPht1;2a/b type genes, is suggestive of gene duplication. Interestingly the paralogous genes are phylogenetic more closely related than the homoeologous genes (Figs 1, 2). The coding DNA sequences of TaPht1;8 and TaPht1;10 are closely related in another cluster. Both Pht1s have orthologous genes in barley and Brachypodium, but the two rice genes, OsPht1;9 and OsPht1;10 seem to be more closely related to TaPht1;10. This is confirmed by the protein phylogeny in which TaPht1;10 is in a separate cluster together with the Brachypodium and rice transporters, including Brachypodium Pht1;10, which is shifted from the coding DNA similarity to TaPht1;13, to a closer protein relationship to TaPht1;10.

The TaPht1 gene expression in roots of hydroponically grown wheat seedlings

The real-time PCR gene expression analysis is the sum of all three homeocli of each TaPht1 homoeologous subfamily, and therefore provides information on the overall expression pattern. The high sequence similarities and/or sequence limitations restricted the generation of oligonucleotide primers able to differentiate between the homeocli. Due to the very high sequence similarity, including in the non-coding regions, between both TaPht1;1a and TaPht1;1b and also TaPht1;2a and TaPht1;2b, no differentiation of gene expression of the A and B genome types was possible; this prevented identification of the most strongly regulated genes in the homeologous subfamily by PCR. Transcripts of most wheat homeocli were identified, however, by different RNA-Seq experiments (Table S1).

Plants grown under Pi starvation in hydroponics had significantly lower total shoot P concentrations than plants grown in the presence of Pi (Fig. 3). No root expression of TaPht1;3, TaPht1;4, Pht1;7, Pht1;9, Pht1;13 and Pht1;14 was detected in hydroponically grown plants. At day 0 under Pi supply, the strongest expression in the root was seen for TaPht1;2a/b, with a three- to eight-fold higher transcript abundance compared to most of the other expressed TaPht1 genes. The transcript abundance of TaPht1;11, with just 0.28 cDNA copies per 0.1 μg total RNA, was particularly low. Pi deprivation led to three expression patterns. A quick response with up-regulated transcript levels after 3 days of Pi starvation for TaPht1;1a/b and TaPht1;2a/b, which further increased at day 6 to a maximum level 27–50-fold higher compared to day 0, and then declined again by day 12 (Fig. 3). A late Pi starvation response was seen for TaPht1;6, TaPht1;10 and Pht1;11. A Pi starvation-related increase of the transcript level of TaPht1;10 was not seen before day 6 and for TaPht1;6 and TaPht1;10, not before day 12, with an eight- to 15-fold higher expression compared to day 0 (Fig. 3). Finally, TaPht1;5 and TaPht1;8 transcript abundances were influenced more by age than Pi starvation, although a higher transcript level of TaPht1;8 was observed in Pi-starved roots at day 12, along with TaPht1;6 and TaPht1;11 (Fig. 3).

The TaPht1 transcript abundance in Pi-starved field-grown wheat at different developmental stages

Wheat plants grown on P-depleted plots with a substantially reduced soil Olsen P content lead to drastically reduced straw and grain yields in comparison to P-fertilised control plots (Table 2). From tillering until maturity, Pi concentrations in shoot and ear tissues of wheat plants grown on non-Pi fertilised plots were significantly lower than in plants grown on the Pi fertilised control plots (Fig. S2). Shoot P concentrations decreased with plant development, but increased in ears. Other elements such as shoot Mo and Zn concentrations exhibited the same patterns as shoot P concentrations, whereas shoot K concentrations increased in Pi-starved plants until booting stage (Fig. S2).

The transcript abundances of the TaPht genes in roots of wheat plants grown in Pi-fertilised field plots were similar to those grown in hydroponic culture. Transcript abundance in field-derived plant material was verified for TaPht1;1a/b, TaPht1;2a/b, TaPht1;5, TaPht1;6, TaPht1;7, TaPht1;8, TaPht1;10 and TaPht1;11. A weak trend was observed in which TaPht1 expression was highest at early growth stages and at maturity, and least during booting and anthesis (Fig. 4). For example, TaPht1;1a/b transcript abundance increased in roots
at the beginning of stem elongation, decreased at booting and anthesis, and increased again during ripening (Fig. 4). *TaPht1;1a/b* was also expressed in ears, glumes, grains and in the rachis (Fig. 4). Root *TaPht1;6* transcript abundance also increased under Pi starvation at tillering, stem elongation and anthesis (Fig. 4). *TaPht1;6* was the only *TaPht1* transporter with high transcript abundance during Pi starvation in the ear and particularly in the rachis (Fig. 4). *TaPht1;7* transcript abundance was very low in all tissues; an influence of Pi starvation was only seen with increased transcript abundance in ear tissues at late booting stage (Fig. 4).

In contrast to the hydroponic study, *TaPht1;2a/b, TaPht1;5* and *TaPht1;8* transcript abundances in roots showed no response to Pi availability (Fig. 5). *TaPht1;2a/b* transcript abundance in the root was highest at tillering and elongation, decreased at booting and increased again at maturity (Fig. 5). Root *TaPht1;5* gene expression was highest at tillering and milk ripening, lowest at elongation and at an intermediate level in ear tissues (Fig. 5). *TaPht1;8* transcript abundance followed the same pattern as *TaPht1;2a/b* transcript levels but was more abundant in the grain at ripening (Fig. 4).

Both *TaPht1;10* and *TaPht1;11* transcript abundances were much lower in shoot tissues compared to the roots (Fig. 4). In contrast to *TaPht1;1* and *TaPht1;6*, Pi starvation increased transcript abundances of *TaPht1;10* and *TaPht1;11* in the root throughout development (Fig. 4). *TaPht1;10* and *TaPht1;11* transcript abundances followed a similar pattern to other root-expressed *TaPht1* transporters, being highest at tillering,
decreasing after elongation and increasing again at milk and grain ripening stages (Fig. 4). However, TaPht1;10 and TaPht1;11 transcript abundances were highest at maturity but decreased in the grain during Pi starvation, similarly to TaPht1;6 (Fig. 4).

Putative transcription factor binding sites in the promoter region of selected wheat Pht1 genes

The putative MYB transcription factor PHR1 promoter element binding site, P1BS-Box (GNATATNC), the WRKY and bHLH transcription factor W-box (C/T TGAC C/T) and the helix-loop-helix (CA[G/T][C/A]TG) factor have been reported to be involved in the regulation of the Pi starvation response (Rubio et al. 2001; Zhou et al. 2008; Lin et al. 2009; Wang et al. 2014). A similar location for putative transcription factor binding sites in the promoter regions of all three homoeologous genes may be an indication of the importance of these cis-elements for the regulation of gene expression. A rapid early or a late response of the root gene expression of the TaPht1 genes implicates differences in their regulation, which may relate with the presence or absence as well as the position of those regulatory promoter elements. The locations of those elements in all available promoter/5′-non-coding regions of the wheat Pht1 genes were analysed and are indicated as upstream of the ATG start codon.

In the promoter/5′-non-coding regions of TaPht1;1a, the locations of two P1BS boxes and two W-boxes are conserved in the 750 to 1 upstream region for all three homoeologous genes (Fig. 5). Although there is 100% protein and 98% coding DNA identity between the A-genome TaPht1;1a and TaPht1;1b, the promoter/5′-non-coding regions share only 44% identity. One P1BS box is present at position 437 as in TaPht1;1a, but two additional P1BS boxes are located more 5′-distally and there is no proximity to a W-box, as found for TaPht1;1a. No bHLH-binding element is present in the TaPht1;1b promoter region.

The promoter/5′-non-coding region of TaPht1;2a contains W-boxes in similar positions to TaPht1;1a, apart from the A-genome gene promoter, in which the second W-box is located further 5′-upstream. The TaPht1;2a promoter/5′-non-coding region contains only one P1BS box in a similar location to that found in TaPht1;1a, which in the A-genome, is also shifted as with the W-box (Fig. 5). Interestingly, the distance between the first P1BS box to the second W-box in the TaPht1;1a promoter/5′-non-coding region is similar to the TaPht1;2a promoter P1BS-box distance to the W-box, for all three homoeologues. Additionally, the TaPht1;2a promoter/5′-non-coding region contains a bHLH-binding site at the 200 bp upstream region in all homoeologous promoter/5′-non-coding regions, with a similar distance to the first W-box, which is absent in the TaPht1;1a promoter. Further P1BS-boxes, W-boxes and bHLH-binding sites are present upstream for all three TaPht1;1a and TaPht1;2a genes but with a random and non-specific distribution in the homoeologous promoter/5′-non-coding regions (Fig. 5).

Interestingly the promoter/5′-non-coding region of the homeologous TaPht1;2b has a similar pattern of P1BS-boxes and W-boxes as found for TaPht1;1a, with a similar distance between the boxes and to each other. In the B-genome promoter, this combination is shifted slightly to 5′. Several bHLH elements with random non-specific distribution are present in the 1000–2000 bp non-coding region.
upstream region in all three promoters. There are no common bHLH-binding sites in the 200 bp upstream region as found in the TaPht1;2a promoter. The common region 200 bHLH-binding site in the TaPht1;2a promoter may allow additional regulation by an pHLH transcription factor.

A specific pattern of the three cis-elements was found in the promoter/5'-non-coding region of the homoeologous genes of TaPht1;11. In the 750 to 1 region two P1BS-boxes, as well as two W-boxes and bHLH-binding sites, are in nearly identical positions (Fig. 5). The second upstream bHLH-binding site is partially overlapping with the first W-box. A P1BS-box at a similar position was also found in the 350 to 300 promoter/5'-non-coding region of the homeologues of TaPht1;6. Four or five W-boxes are present further downstream in all homoeologous promoters/5'-non-coding regions, but in more distinct locations without any specific pattern (Fig. 5). Although TaPht1;10 also responded to P starvation in a delayed manner, no uniform distribution of the three transcription factor binding elements was present (Fig. 5). The promoter/5'-non-coding regions of the homoeologous TaPht1;5 and TaPht1;8 genes contain only randomly distributed cis-elements with no specific patterns, with exception of a similar position (~650 bp) of a W-box in the A- and D-genome promoters of TaPht1;8. The B-genome promoter of TaPht1;8 contains two W-boxes, each approximately 150 nucleotides upstream and downstream relative to the single W-box found in the A- and D-genome.
promoter (Fig. 5). For those genes, no root transcript could be verified. Only TaPht1;3, TaPht1;4, TaPht1;7; TaPht1;9 and TaPht1;14 had similar positions for at least one of the binding sites in the homoeologous promoter/5′-non-coding regions. A bHLH-binding site was present in the 230 to 302 bp region but no W-boxes or PIBS-boxes were found in any of the three homoeologous TaPht1;4 promoter/5′-non-coding regions. The TaPht1;7 homeolocti promoter/5′-coding regions had a nearly identical position of the PIBS-box at position 164 to 172, and no or randomly distribution of the W-box and bHLH-binding sites. Two conserved bHLH-binding sites were present in the 211–363 regions of all homoeologous TaPht1;9 promoter/5′-non-coding regions. Two conserved bHLH-bind-
ing sites were also present in similar positions in the A- and D-
genome promoter/5′-non-coding region of TaPht1;14. The corresponding sequence region of the B-genome TaPht1;14 was not available in the genome databases.

DISCUSSION

A complex phylogeny of the wheat Pht1 gene family

In dicotyledonous genomes, the size of the Pht1 gene family seems to be highly variable with just five Pht1 genes in Vitis vinifera, and up to 28 Pht1 genes in Brassica rapa (Proost et al. 2015). In the cereal genomes analysed, the Pht1 gene family does not show high variation in the number of Pht1 genes. In contrast to rice, maize (not shown) and Brachypodium genomes, which contain all 13 Pht1 genes, 16 Pht1 genes were identified in the wheat A-genome and 14 Pht1 genes in the B- and D-genomes. The comparison of the cereal Pht1 gene sequences allowed a phylogenetic separation into different phylogenetic distinct gene clusters. The differences found in wheat compared to rice, barley and Brachypodium, indicate a complexity of the cereal Pht1 gene family which may be partly explained by additional gene duplication and/or loss of genes during evolution, as seen for the rice or barley or wheat Pht1 in different clusters. The different gene compositions for the Pht1 gene families in the different grass species may be explained by different evolutionary development influencing the genomes of the different grass species. The grass genomes differ in size, ploidy level and chromosome number. Two polyploidisation events (Feldman et al. 1995) led to the hexaploid wheat genome (AABBDD; 2n = 42) of ~16000 Mbp size. The diploid rice (2n = 24) and Brachypodium distachyon (2n = 10) genomes (~430 Mbp and ~272 Mbp) are much smaller than the wheat genome. Although in general the gene order in the nuclear genomes of all grasses has been preserved, genomic rearrangements, duplication and polyploidisation events during the evolution of the different grass species has led to differences in gene copy number. The occurrence of homoeologous Pht1 genes on different chromosomes or different chromosome arms, as found for TaPht1;3 and Pht1;11, suggest rearrangements between chromosome 4 and 5, and in the A-genome between the short and long arm of chromosome 4. In comparison to its progenitors, many low copy DNA sequences seem to have been deleted in hexaploid wheat since the polyploidisation event (Feldman et al. 1997). Recent complete genome data on wheat chromosome 3B indicated that gene duplication played a major role in the recent evolution of wheat chromosome 3B (Glover et al. 2015). On chromosomes 11 and 12 of rice, recent segmental duplications, including large ongoing individual gene duplications, have been identified (Jiang et al. 2007). Furthermore at least ten duplicated regions, which represent 67.5% of the genome, were identified in wheat. Salse et al. (2008) suggested an ancient duplication of the diploid wheat genomes before their hybridisation into polyploid wheat. Those differences can be seen in the phylogenetic relationship between wheat, barley, rice and Brachypodium in relation to DNA and amino acid sequences. A homologous gene (or homologue) is a gene inherited in two species by a common ancestor. While homologous genes can be similar in sequence, similar sequences are not necessarily homologous. Orthologous are homologous genes where a gene diverges after a speciation event, but the gene and its main function are conserved. Orthologous genes are generally assumed to retain equivalent functions in different organisms and to share other key properties (Gabaldón & Koonin 2013). The homologous phylogenetic closely related DNA sequence similarities found does not always explain the orthologous relationship in relation to the function in the individual cereal species. The parallel analysis of the protein and DNA sequences provided information about the orthologous relationships: the differences of the clustering for the wheat TaPht1 homeologous subfamilies may be explained by differences in the paralogous relationships, which are seen more clearly using the DNA sequences, rather than on homologous similarity based on amino acid sequences.

Regulation of TaPht1 transcript abundances by mineral nutrition

Phosphate availability regulates root system architecture as well as many transcriptional, biochemical and physiological processes. One response to Pi deficiency is the increase of Pi-uptake capacity, as shown by Ullrich-Eberius et al. 1981; facilitated by the reported regulation of the Pht1 genes at the transcriptional as well as the post-transcriptional levels. This study compared two contrasting experimental systems to further understand the influence of Pi deficiency on wheat Pht1 gene expression. In many studies, Pi deficiency often leads to rapid increased steady-state expression of multiple Pht1 genes. For example, Pht1 transporter expression in tomato increased within 24 h of Pi starvation and decreased again after 24 h of Pi resupply (Liu et al. 1998). A more rapid response of Pht1 gene expression to Pi deprivation was found in Arabidopsis (Misson et al. 2005), suggesting a finely coordinated response to Pi availability. In the artificial hydroponic growth system used here, there was a rapid induction of some wheat Pht1 transporters, whereas others exhibit a delayed but equally strong induction to Pi starvation or no response at all. Similar observations were made for Pht1 transporters in maize and other plant species (Nagy et al. 2006; Morcuende et al. 2007; Lapis-Gaza et al. 2014). The pre-culture of the young wheat seedlings in the high Pi hydroponic experimental setup may have allowed the plants to store P for remobilisation. In contrast the quick reduction of shoot P concentrations resulted in a rapid up-regulation of Pi transport to attempt to maintain the Pi content in the plant. A rapid up-regulation of Pi transport suggests the importance of the transporters in initial Pi root uptake to enable a maximum Pi uptake under limited conditions or for increased Pi translocation to support upper
plant parts. There are rice Pht1 genes in the same cluster of the Pht1 gene family which are putative orthologous to the rapidly and strongly P1 starvation up-regulated wheat TaPht1;1 and TaPht1;2 genes. The phylogenetically closest, OsPht1;2, has been characterised as a low affinity P1 transporter with expression up-regulated by P1 starvation, and localised throughout the stele in primary roots and lateral roots, but not in epidermal and cortical cells of Pi-deprived roots (Ai et al. 2009). In contrast, rice OsPht1;1 and OsPht1;8 are constitutively expressed and not influenced by P1 starvation. RNA interference experiments have revealed the importance of both transporters for P1 uptake, and OsPht1;8 has been characterised as high-affinity P1 transporter. The characteristics of all three rice genes suggest an involvement in P1 uptake as well as in translocation (Jia et al. 2011; Sun et al. 2012). The expression patterns of TaPht1;1 and TaPht1;2 indicate similar functions in wheat.

The rice genes OsPht1;9 and OsPht1;10, orthologous to wheat TaPht1;10, and OsPht1;6, orthologous to TaPht1;6, have been shown to be important for P1 uptake as well as translocation, and are all up-regulated by P1 starvation, shown under long-term P1 starvation. Similarly, to TaPht1;6, the rice OsPht1;6 showed a late up-regulation at 14 days of P1 starvation (Ai et al. 2009; Wang et al. 2014). Differential Pht1 regulation in the P1 starvation response is an important consideration for determining which Pht1 transporters may be potential targets for P1 efficiency crop improvement.

Several regulators of P1 homeostasis in plants have been identified (Lin et al. 2009). The MYB transcription factor PHR1 activates several Pi starvation-induced genes by binding to the P1BS promoter element (Rubio et al. 2001; Zhou et al. 2008). Furthermore, WRKY, bHLH as well as the C2H2 zinc finger transcription factor have been reported to be involved in the regulation of P1 starvation response (Lin et al. 2009; Wang et al. 2014). Miao et al. (2009) identified P1BS elements (GNATATNC), WRKY and bHLH binding factors in the W-box (C/T TGAC C/T) and helix-loop-helix (CA[ G/T][ C/A][ T/G]) binding elements in the TaPht1;2a promoter. The expression patterns of TaPht1;1a/b and TaPht1;2a/b were identical in relation to P1 starvation in hydroponic cultures. Promoter analysis of TaPht1;1a revealed P1BS and W-box binding elements in similar regions as found in the TaPht1;2a and TaPht1;2b promoter (Fig. 5, Table S4). Overexpression of the wheat PHR-MYB transcription factor in transgenic wheat increased expression of TaPht1;2a/b (Wang et al. 2013; the primer used did not distinguish between the homeologues), suggesting regulation of TaPht1;2a/b as well as TaPht1;1a by the same molecular regulators. The rice OsPht1;1 promoter is lacking the P1BS element, and Sun et al. (2012) suggested that OsPht1;1 may not be regulated by PHR. In contrast, the promoter region of the phylogenetically closely related OsPht1;2, which is also up-regulated by P starvation, has two W-boxes as well as two bHLH-binding sites (data not shown) like TaPht1;2, implying a similar regulation. Wang et al. (2014) identified a WRKY transcription factor able to activate Arabidopsis Pht1;1 in response to Pi starvation. In the promoter region of TaPht1;6 and TaPht1;11, which showed a delayed Pi starvation response, there is at least one P1BS element in a similar position as compared to TaPht1;1a and TaPht1;2a/b, but the positions of the W-box elements are not in the same proximity to the W-box as found for TaPht1;1a and TaPht1;2a/b (Fig. 5, Table S4). The overexpression of TaPHR in wheat increased the expression of TaPht1;6 (Wang et al. 2013), revealing the importance of the MYB transcription factor for the regulation of TaPht1;6. The rice OsPht1;6 promoter contains also two W-boxes very close to each other (20 nucleotides, data not shown) which are also present in comparable location in the promoter of the B- and D-genome TaPht1;6 genes just three nucleotides from each other explain similar late Pi starvation response. In addition, the delayed Pi starvation response found in the hydroponic starvation experiment indicated involvement of other or additional regulators, in contrast to TaPht1;1a and TaPht1;2a/b. All Pht1;11 promoter regions contain two helix-loop-helix binding sites in nearly identical positions and partly overlapping the W-box binding site. This would enable regulation by bHLH transcription factors, in contrast to TaPht1;6, with either no or completely different locations in their respective promoter regions (Fig. 5, Table S4). A P1BS-box is present in the promoter region of the orthologous rice OsPht1;9 but not of OsPht1;10, although both genes are strongly induced by Pi starvation. TaPht1;10 gene expression was also strongly up-regulated by Pi starvation from day 6 onwards, and there is no common pattern of W-box and bHLH binding sites and a complete absence of a P1BS-box in all three homeologous promoter regions of TaPht1;10 (Fig. 5, Table S4), suggesting different regulation. Transcriptome analysis in Arabidopsis identified more than 40 Pi-responsive transcription factor genes (Misson et al. 2005; Morcuende et al. 2007). Some transcription factors were responsive in the short term and others during medium term or late Pi deficiency, suggesting specific sets are involved in regulating early and late responses of plants to Pi deficiency (Misson et al. 2005), and explaining the need for different sets of regulatory binding domains in the promoter region of Pht1 transporter genes. Root gene expression could not be verified for all wheat TaPht1 genes under normal and Pi starvation conditions. For some homeologous gene subfamilies, at least one of the described transcription factor binding sites is present in a similar promoter location. In addition to the regulation by P availability, tissue and cellular specific regulation must be considered responsible for the complex gene regulation.

There is a lack of studies investigating gene expression in agronomic systems in relation to nutrient use. Shimami et al. (2010) demonstrated in some tissues, including roots, of field-grown wheat that the expression of some sulphate transporter genes was up-regulated in sulphur-deficient plots, as also described for hydroponically cultured plants (Buchner et al. 2010). Teng et al. (2013) described high up-regulation of TaPht1;1, and TaPht1;2 under high Pi fertilisation, whereas TaPht1;8 expression decreased with increasing Pi supply, probably due to reduced mycorrhiza infection under such conditions. No information (accession numbers, protein or nucleotide sequences), except for primer sequences, were provided. Alignment of the primer sequences verified the specificity for the TaPht1;1, TaPht1;2 and TaPht1;8 genes. In contrast to the artificial hydroponics the soil P availability is much more complex for a growing plant. The efficiency of P use by plants from soil and fertiliser sources is often poor despite many soils containing a relatively large amount of total P that is not all available to plants. The low P soil mobility and P availability to the plants demands different requirements for regulation of the P1 uptake system. Additionally, the root structure of...
soil-grown and hydroponically grown plants differs substantially, which will affect the control of any nutrient uptake.

The transcript pattern of the wheat *Pht1* genes exhibits a more complex regulation in field-derived wheat plant material compared to hydroponic culture systems. *TaPht1* transcript patterns in field-grown roots exhibited high expression levels at early vegetative growth stages. These high transcript levels coincided with a growth stage when Pi requirement was high (Römer & Schilling 1986). Expression and possibly therefore requirements decreased during the booting stage (Fig. 4). However higher Pi fertilisation during later growth stages increased thousand-grain weight in wheat (Römer & Schilling 1986). In addition, Sun et al. (2012) observed a considerable increase in Pi transport into the shoot during grain filling by *OsPht1;1* overexpression in rice. The same increase was also seen for the root expression of several *TaPht1* transporter genes at anthesis and post-anthesis, including substantial post-anthesis expression in glumes and grains, indicating continued Pi uptake from the soil solution and delivery to the grain during grain development and ripening (Fig. 4).

Transcripts of root-expressed *TaPht1* transporters were also found in wheat ear (spike) tissues. In contrast to *TaPht1;5* homologues such as *HvPht1;5* (Rae et al. 2003; Huang et al. 2011) or *OsPht1;12* (Paszkowski et al. 2002; Fig. 2), *TaPht1;5* was weakly expressed in root and ear tissues (Fig. 4). Specific *Pht1* transporters are preferentially expressed in green and mature anthers, for instance in *Arabidopsis* (Mudge et al. 2006), maize (Nagy et al. 2006) and barley (Druka et al. 2006). Therefore, it is likely that high transcript abundance of *TaPht1;7* at growth stage 48 in ear tissues (Fig. 4) was coincident with anther or pollen development at booting, with very weak expression in all tissues at other developmental stages, as also found for the rice homologue *OsPht1;7* (Paszkowski et al. 2002; Fig. 2). *TaPht1;6* transcripts were present in all tissues analysed. This agrees with the expression pattern described for the rice homologous *OsPht1;6* (Ai et al. 2009). RNAi knockout of *OsPht1;6* decreased Pi uptake as well as Pi transport within the plant. Promoter-GUS expression was seen in almost all tissues and cell types, except for the epidermis of younger primary roots (Ai et al. 2009). The general expression patterns of *TaPht1;6* and *TaPht1;8*, with expression in all tissues, including developing wheat grains, suggests a similar function in wheat, playing a broad role in Pi uptake, translocation and internal transport throughout the plant.

A significant influence of Pi fertilisation on *Pht1* transcript abundance in field-grown wheat was only found for two *Pht1* genes and restricted mainly to root tissues. The gene expression of *TaPht1;10* and *TaPht1;11* was significant up-regulated in roots of Pi-deficient-grown wheat plants, however *TaPht1;1* and *TaPht1;2* and *TaPht1;6* gene expression patterns were not significantly changed in contrast to the hydroponic Pi starvation experiment. In the experimental field set up, the wheat plants were exposed to long term Pi deficiency, with a drastically reduced soil Olsen P concentration (Table 2). This contrasts with the Pi starvation applied in the hydroponic system in which the plants under high sufficient Pi content are suddenly Pi-depleted. In the hydroponic experiment, the *TaPht1;1* and *TaPht1;2* up-regulation induced by Pi starvation was reversed by day 12 after reaching a peak at day 6. This implies a different regulation under long-term deficiencies as found in the field experiment, and this may be an indication that some regulators are restricted to short-term Pi starvation responses. In *Arabidopsis* the induction of 47 of the 80 genes presumed to be associated with transcriptional regulation under Pi deficiency was more pronounced during long-term Pi deprivation, and only a small number of transcription factor genes overlapped during different stages of Pi deficiency (Misson et al. 2005). The differences in the promoter regions, particularly for *TaPht1;10*,...
suggests involvement of different regulatory factors acting as long-term Pi starvation regulators for which the corresponding cis-element still need to be identified. Sisaphaitthong et al. (2012) and Duan et al. (2015) reported an up-regulation of wheat Pht1 genes referred to as Pht1;10 and Pht1;11 and Pht1;12 by arbuscular mycorrhizal fungi. Our phylogenetic analysis revealed those three Pht1 genes as the homoeologous genes of TaPht1;11 in the A-, B- and D-genomes. Whether the up-regulation of TaPht1;11 in roots grown in Pi-deficient field plots is related to increased AM colonisation needs to be verified, but the up-regulation by Pi starvation under hydroponic AM-free culture implies a non-AM related regulation. An induction by AM colonisation, including localisation in root cortical cell containing mycorrhizal cells, was also reported for TaPht1;8 (Glaslop et al. 2005). In our experiments, we did not see a general increase in the transcript amount of TaPht1;8 in roots derived from field or hydroponic culture.

CONCLUSION
In conclusion, this study identified 16 A- and 14 B- and D-genome genes of the Pht1 gene family in wheat. The phylogeny implies partly similar orthologous genes to other cereals but also many differences, which may be explained by evolutionary changes in the genome structure. Plant growth and development require different Pi transport processes (Fig. 6), which need to be regulated depending on demand and Pi availability. The two contrasting experimental set-ups identified different regulatory patterns for the Pht1 genes. The hydroponic experiment defines a general pattern of regulation of Pht1 gene expression in relation to P deficiencies whereby the long-term field experiment takes higher complexity into account. Our results suggest differentiation between short-term and long-term regulation of gene expression in relation to Pi deficiency. Short-term regulation enables a rapid response to Pi-limited conditions to enable rapid Pi uptake and translocation. Long-term Pi deficiency establishes a long-term adaptation to Pi deficiency to allow survival and reproduction (Fig. 6). This process requires an up-regulation of specific Pht1 genes to enable P homeostasis. In general, the expression patterns of some TaPht1 genes are important for Pi acquisition, whilst others are likely to be required for Pi translocation from vegetative to generative organs, which seems to be only partly regulated by external Pi availability.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the supporting information tab for this article:
Table S1. Primer sequences used for real-time qPCR analysis of TaPht1 transporter expression: Amplicon size (bp), primer concentration (mM) and appropriate annealing temperature (°C).
Table S2. Statistical properties (SED, size (bp), primer concentration (mM) and appropriate annealing temperature (°C).
Table S3. Statistical properties for TaPht1 qRT-PCR expression profiling at Broadbalk field trial 2012 (Fig. 4).
Table S4. Location of putative transcription factor cis-regulatory elements in the promoter regions of wheat Pht1 genes. Distances indicated are upstream of the ATG start codon.
Table S5. Gene names, accession numbers and related references, chromosome and genome localisation for previously published and identified TaPht1 gene transporter sequences. Primer sequences used for partial TaPht1 cDNA-PCR cloning (average product size: 500 to 550 bp) including without references accession numbers. Accessions are direct unpublished data submissions.
Figure S1. Phylogenetic relationship of the wheat phosphate transporter family 1.
Figure S2. Nutritional status of field-grown wheat at Broadbalk in 2012.
Data S1. Wheat Pht1 genomic sequences. List of all available full-length or partial genomic nucleotides sequences of identified wheat Pht1 phosphate transporter genes, including max 2000 nucleotides of the 5’-non-coding/promoter and 400 nucleotides of the 3’-non-genomic non-coding regions (lowercase). TGAC genomic annotation and IWGSC chromosome scaffold number are given. Coding sequence nucleotides are in uppercase. Intron sequences are lowercase/italic. The identified cloned partial transcripts (Table S5) are highlighted in bold and verified 5’- and 3’-non-coding sequences are grey-shaded including accessions.

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Wheat phosphate transporters

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