Title: Organ-specific phosphorus-allocation patterns and transcript profiles linked to phosphorus efficiency in two contrasting wheat genotypes

Running title: Molecular determinants of P efficiency in wheat

Authors:
Tariq Aziz¹,³, Patrick M. Finnegan¹,², Hans Lambers¹,² and Ricarda Jost¹* 

Affiliations:
¹School of Plant Biology, The University of Western Australia, Crawley (Perth), Western Australia, Australia
²The UWA Institute of Agriculture, The University of Western Australia, Crawley (Perth), Western Australia, Australia
³Institute of Soil & Environmental Sciences, University of Agriculture, Faisalabad, Pakistan

Corresponding Author

*Email: ricarda.jost@uwa.edu.au. Fax: +61 8 6488 1108. Phone: +61 8 6488 2205.
Summary statement:

Many cultivars of various crop species differing in their P efficiency have been described at a physiological level. However, relatively little is known about the underlying molecular mechanisms of the associated traits. This study shows that determinants of P efficiency in wheat include the ratio of free phosphate to organic P compounds as well as differential transcript accumulation of genes involved in phosphate transport, de novo phospholipid synthesis as well as starch degradation in plant organs. In the more efficient wheat cultivar Chinese 80-55, these molecular adjustments lead to a higher root biomass irrespective of external P availability, higher phosphate concentration and content in P-limited roots and stems as well as higher organic P levels in all tissues under well-fertilised conditions.
Abstract:

Recent studies have identified genotypic variation in phosphorus (P) efficiency, but rarely have the underlying mechanisms been described at the molecular level. We demonstrate that the highly P-efficient wheat (*Triticum aestivum* L.) cultivar Chinese 80-55 maintains higher inorganic phosphate (P$_i$) concentrations in all organs upon P$_i$ withdrawal in combination with higher P$_i$ acquisition in the presence of P$_i$ when compared to the less-efficient cultivar Machete. These findings correlated with differential organ-specific expression of P$_i$ transporters *TaPHT1;2, TaPHT1;5, TaPHT1;8, TaPHT2;1* and H$^+$-ATPase *TaHa1*. Observed transcript level differences between the cultivars suggest that higher *de novo* phospholipid biosynthetic activities in P$_i$-limited elongating basal leaf sections are another crucial adaptation in Chinese 80-55 for sustaining growth upon P$_i$ withdrawal. These activities may be supported through enhanced breakdown of starch in Chinese 80-55 stems as suggested by higher *TaGPho1* transcript levels. Chinese 80-55 fine roots on the other hand show strong suppression of transcripts involved in glycolysis, transcriptional regulation and ribosomal activities. Our work reveals major differences in the way the two contrasting cultivars allocate P$_i$ and organic P compounds between source and sink tissues and in the acclimation of their metabolism to changes in P$_i$ availability.

Keywords: nutrients/other, genetic variation, transcriptome, phosphate transport, glycolysis, phosphocholine, transcription and translation
Phosphorus (P) deficiency can be a major nutritional disorder limiting crop growth (Vance, Uhde-Stone & Allan, 2003). P efficiency is commonly defined as the biomass produced by plants grown at low P$_i$ supply relative to that of plants grown with an adequate P$_i$ supply (Hammond et al., 2009; Ma, Rengel & Siddique, 2011; Siddiqi & Glass, 1981). High P efficiency can be achieved through a combination of a) releasing and acquiring more P$_i$ from insoluble complexes and organic pools in the soil, b) exploring larger volumes of soil, c) optimizing the metabolic use of P or d) re-directing resources to growing organs (Aziz et al., 2011a; Richardson et al., 2011). Architectural adaptations that increase the root surface area and increase the root-to-shoot ratio will increase P$_i$ acquisition. These types of adaptations include increased root-hair length and density, lateral root elongation and root branching as well as the formation of cluster roots in some species (Lambers et al., 2006; Lopez-Bucio, Cruz-Ramirez & Herrera-Estrella, 2003; Lynch & Brown, 2001). Most species also form mycorrhizal associations to increase P absorption (Bucher, 2007). These changes in root structure are often accompanied by increased release of protons, carboxylates (Neumann et al., 1999; Vance et al., 2003), nucleases and phosphatases (Jungk, Seeling & Gerke, 1993; Tran, Hurley & Plaxton, 2010) and higher P$_i$ uptake capacity mediated by high-affinity P$_i$ transporter (PHT) proteins (Bucher, Rausch & Daram, 2001). Other physiological and molecular adaptations that help to improve the internal P-utilisation efficiency (PUE) of a plant include accelerated leaf senescence (Crafts-Brandner, 1992), reduced shoot growth rate (Ericsson, 1995; Fredeen, Rao & Terry, 1989) and changes in metabolic pathways involving P$_i$, such as primary carbon (Ciereszko & Kleczkowski, 2005; Plaxton & Tran, 2011) and phospholipid metabolism (Andersson et al., 2005; Cheng et al., 2011; Essigmann et al., 1998). Increased internal P$_i$ recycling and release from vacuolar storage pools also lead to a higher internal PUE (Marchive et al., 2009; Tran et al., 2010).
Most of the adaptations associated with higher P efficiency are the result of the induction or suppression of specific genes. Numerous molecular studies have highlighted the importance of the regulation of gene expression to optimise root morphology, P\textsubscript{i} uptake and metabolic adjustments for improving the P efficiency of crop plants (Hammond et al., 2003; Lopez-Bucio et al., 2000; May et al., 2011; Vance et al., 2003). They have led to the identification of a large array of genes and signalling components that have a role in adjusting growth and metabolism to altered P\textsubscript{i} availability in plants (Chiou & Lin, 2011; Peret et al., 2011; Rouached, Arpat & Poirier, 2010; Yang & Finnegan, 2010). High- and low-affinity P\textsubscript{i} transporters play a pivotal role in a number of these acclimation processes. PHT1 proteins are located in the plasma membrane, while PHT2, PHT3 and PHT4 proteins are located in plastids, mitochondria and endomembranes, respectively. Some PHT1 proteins are abundant in the root epidermis, facilitating the uptake of P\textsubscript{i} that is released from organic matter and soil particles against a steep concentration gradient (Miller, Shen & Xu, 2009; Nussaume et al., 2011). PHT proteins of all four families have also been identified in aboveground vegetative and reproductive organs indicating that they not only facilitate P\textsubscript{i} uptake from the rhizosphere, but are also responsible for P\textsubscript{i} (re-) mobilisation within cells and throughout the plant (Guo et al., 2008; Nagarajan et al., 2011; Rausch et al., 2004).

The regulation of PHT proteins and genes has been studied mostly in Arabidopsis thaliana (Bayle et al., 2011; Misson et al., 2004; Nagarajan et al., 2011), but there is still little information about the precise biochemical and physiological function of each individual transporter. There are a number of reports on PHT genes from monocotyledons including wheat (Davies et al., 2002; Guo et al., 2013; Liu et al., 2013; Miao et al., 2009), barley (Huang et al., 2011; Preuss et al., 2010; Rae et al., 2003; Schuenmann et al., 2004), Brachypodium distachyon (Hong et al., 2012), maize (Nagy et al., 2006; Takabatake et al., 1999) and rice (Jia et al., 2011; Liu et al., 2011; Paszkowski et al., 2002). Despite wheat
being a major cereal crop around the world, genomic sequence information is still incomplete and there are only about ten entries for cDNA sequences encoding full-length PHT open-reading frames in public databases with scarce detail about their specific functions and regulation (see GenBank and TriFLDB at http://trifldb.psc.riken.jp/index.pl). Davies and co-workers (2002) studied the expression of seven expressed sequence tags encoding putative P\(_i\) transporters in three cultivars of winter wheat. The PHT genes TaPT2 to TaPT8, and their closely related homologs, all had different expression patterns across the varieties and also across roots and leaves within each variety. Tittarelli et al. (2007) showed that TaPT2.1 promoter activity increased under P\(_i\) deficiency. The promoter of the closely related TaPT2 (also called TaPHT1.2) isoform was induced in roots of P-deficient plants, and more strongly activated in the roots of P-efficient plants than in those of P-inefficient genotypes irrespective of P\(_i\) supply (Miao et al., 2009). Guo et al. (2013) found that TaPHT2;1 encoding a plastid-localised transporter was strongly expressed in leaves and further induced in P\(_i\)-starved plants with an underlying diurnal expression pattern. Glassop, Smith & Smith (2005) found that another PHT gene, TaPT1, was induced specifically upon root colonisation with three different AM fungi. The differential regulation of these PHT genes in response to P\(_i\) availability could be essential for redirecting the flux of P\(_i\) to sink tissues when P\(_i\) is limiting for growth.

In *Arabidopsis*, P\(_i\) itself is considered to be one of the potential local signals for altering the expression of genes involved in acclimation responses to altered P\(_i\) supply (Abel, 2011; Rouached et al., 2010; Thibaud et al., 2010). These studies have investigated either whole seedlings or root and shoot organs. We hypothesise that P remobilisation within tissues or between organs could be a major determinant of PUE for plants grown at low P\(_i\) availability. Genes involved in this process could respond to other systemic signals such as sugars or P-containing metabolites. Very little is known about the expression patterns of these genes in
different young (sink) and mature (source) plant organs or about their effect on P remobilisation and translocation when $P_i$ deficiency occurs at later stages of plant development. Wheat is an excellent model system to study these effects due to its well-defined gradient of developmentally distinct cell populations along the axis of its expanding leaves (Dean & Leech, 1982; Tobin et al., 1989). We studied the expression of 17 genes, including those encoding PHT proteins and enzymes involved in the metabolic adjustments to $P_i$ limitation, in different sections of young and mature leaves as well as in stems, fine roots and mature roots of two wheat genotypes contrasting in their P efficiency. The genotypes were selected on the basis of their relative growth, $P_i$ acquisition and PUE in the presence of relatively insoluble iron phosphate as the sole P supply (Osborne & Rengel, 2002a).

**Materials and Methods**

**Plant Material**

Seeds of wheat cultivars Machete and Chinese 80-55 were kindly provided by Zed Rengel, School of Earth and Environment, The University of Western Australia. Plants were grown in a temperature-controlled glasshouse at The University of Western Australia. The average daily minimum and maximum temperatures were 14 and 22°C, respectively, the average maximum light intensity at 1 pm was 1600 PAR (70% transmission) with sunrise at 6.30 am and sunset at 6 pm. Relative humidity varied from 24% (day) to 80% (night). Climate data for the growth period (3 March to 16 April 2011; *i.e.*, autumn) are available from the Australian Bureau of Meteorology ([www.bom.gov.au](http://www.bom.gov.au)). Seeds were sown in washed river sand moistened with distilled water in polyethylene foil-lined metal trays. Ten days after germination the root systems of seedlings were gently washed in distilled water to remove sand and uniformly-sized seedlings were transferred to 3.5 l continuously aerated nutrient solution in white plastic pots with four plants per pot supported by foam plugs in the holes of
the lid. The composition of the full-strength nutrient solution (pH 5.8) was 0.625 mM
$\text{NH}_4\text{NO}_3$, 3.75 mM KNO$_3$, 1.5 mM CaSO$_4$, 0.4 mM MgSO$_4$, 50 $\mu$M Fe-EDTA, 50 $\mu$M KCl,
25 $\mu$M H$_3$BO$_3$, 2 $\mu$M MnSO$_4$, 2 $\mu$M ZnSO$_4$, 0.5 $\mu$M CuSO$_4$, 0.5 $\mu$M Na$_2$MoO$_4$. The plants
were grown for 15 days after transplanting (DAT) with adequate P$_i$ supply (0.2 mM
KH$_2$PO$_4$). The nutrient solution was replaced on alternate days to ensure a continuous supply
of nutrients. The pH of the solutions was monitored daily prior to their exchange, and
deviated by no more than 0.5 ± 0.02 units. On 15 DAT, 12 plants from three pots of each
genotype were harvested and the rest of the pots were divided into two groups, receiving
nutrient solution either with (0.2 mM P$_i$) or without added P$_i$. The nutrient solution was
replaced daily to ensure a continuous supply of nutrients. Plants were harvested commencing
three days from the start of treatment, on 18, 23 and 33 DAT.

During each harvest, roots were washed on site with distilled water to remove the nutrient
solution. Leaves were divided into young expanding (top) and mature (lower) leaves. Each
leaf was dissected into top (2 – 3 cm), middle (remaining length of leaf blade) and basal (2 –
3 cm) sections (see Supplemental Fig. 1). Roots were divided into the main (primary) root
and fine (lateral) roots. All samples were divided into two aliquots: Material from one plant
was immediately frozen in liquid nitrogen and stored at -80°C for mRNA extraction and free
P$_i$ analysis, while material from the other three plants was dried at 70°C for 7 d and stored
under desiccation until dry weights were recorded prior to total P analysis.

**Free and total P analyses**

Free P$_i$ concentrations were determined using 70 mg of frozen tissue homogenised in 700 µl
of 1 % (v/v) acetic acid in a tissue homogeniser (Precellys-24, Bertin Technologies, Aix-en-
Provence, France) using two ceramic beads (2 mm dia.) per 2 ml tube and default settings.
Homogenates were clarified twice by centrifugation at 10,000 g for 20 min at 4°C. P$_i$ was
determined in the cleared extracts using the ammonium molybdate method of Ames (1966) scaled down to be used in a microtitre plate assay measuring the absorbance at 620 nm. Total P was determined from approximately 100 mg ground oven-dried plant material digested with 3 ml of HNO$_3$ and 1 ml of HClO$_4$ at 160°C in a method modified from Zasoski & Burau (1977). The ash of the digested material was resuspended in 10 ml of deionised water (MilliQ) and an aliquot was used to determine P$_i$ as described.

**Primer Design**

Using the NCBI database, we mined about 40 UniGene EST datasets for individually annotated transcript loci from wheat genes encoding candidate PHT proteins, the regulatory RNA IPS1 as well as MYB and bHLH transcription factors, ribosomal proteins and metabolic enzymes. Out of these, 17 sets (Supplemental Table S1) were selected for expression profiling. For primer design, we assumed that each UniGene set contained EST sequences derived from each of the three wheat genomes (A, B and D), as individual UniGene alignments showed three distinct EST clusters. We were unable at this time to predict the individual contribution of each locus to the overall transcript pool in the different tissues and across treatments. Thus, we designed real-time RT-PCR primer pairs spanning moderately conserved consensus transcript regions to detect transcripts generated from all three gene copies while still being able to distinguish between different isoforms (Supplemental Table S1).

**Quantitative RT-PCR**

The mRNA was extracted from 70 mg of homogenised, frozen tissue using magnetic oligo(dT)$_{25}$-coated beads (Dynabeads, Life Technologies, Mulgrave, Australia) according to Jost, Berkowitz & Masle (2007). Fifty units of M-MLV reverse transcriptase (RNase H Minus, Point Mutant, Promega) and 4 units of RNase inhibitor (RNasin, Promega) were used
in each cDNA synthesis. Real-time PCR and Ct value determination were carried out using a
real-time thermocycler (ABI 7500 FAST Sequence Detection System, Life Technologies,
Mulgrave, Australia) and accompanying software according to the manufacturer’s
instructions. Reactions contained 2.5 μl of bead-bound cDNA (0.5 ng of mRNA equivalent),
2.5 μl of primer mix (1.2 μM each, Supplementary Table 1), and 5 μl PCR master mix (2x
ABI Power SYBR® Green PCR Master Mix, Life Technologies, Mulgrave, Australia). After
each run a melting curve analysis was performed to verify target-specific product
amplification. PCR efficiencies of individual primer pairs were determined using the LinReg
algorithm version 11.1 (Ruijter et al., 2009) (Supplemental Table S1). The wheat TaAPT1
gene was used as a reference gene (Jost et al., 2009).

Statistical Analysis

The data were statistically analysed by two-way ANOVA (GenStat, 12th Edition). Tukey’s
multiple comparison or Duncan’s multiple range tests were used to separate means.

Results

Starting seed material

Osborne & Rengel (2002a) screened 99 wheat cultivars in a glasshouse on either P-deficient
or P-sufficient supplies of poorly accessible iron phosphate as the sole P source. Only one of
these cultivars, Machete, was considered P inefficient based on three out of four selection
criteria: shoot dry weight (DW), P-deficient over P-sufficient shoot DW ratio and PUE. By
contrast, cultivars Chinese 80-55, Westonia and Wawht2147 were considered P efficient,
with each cultivar scoring highly in two out of the four criteria. Chinese 80-55 was the only
cultivar that showed both high shoot DW in the absence of an external P supply and a high
shoot DW ratio for P-deficient over P-sufficient plants, while at the same time displaying a
large root system. The two contrasting cultivars used in the present study, Chinese 80-55 and Machete, differed in seed weight and P content. The weight of 1000 seeds was 46 g for Chinese 80-55 and 31 g for Machete. Seed total P concentrations were 126 ± 6 and 93 ± 7 (SE) µmol P g\(^{-1}\) seed in Chinese 80-55 and Machete, respectively.

**Genotypes differ in their root but not shoot biomass allocation**

After pre-growth with an adequate P\(_i\) supply (15 DAT), Chinese 80-55 produced more root biomass than Machete, both when P\(_i\) supply was maintained and when it was withdrawn (Fig. 1). This difference was observed at all harvests, except for P\(_i\)-limited plants at 18 DAT (i.e. three days without P\(_i\) supply), and was greatest at the end of the experiment, 33 DAT. P\(_i\) withdrawal reduced shoot biomass accumulation by about 50 % in both cultivars by 33 DAT. Chinese 80-55 produced larger shoots than Machete in both treatments by 23 DAT. The fact that this trend was not apparent at the final harvest may indicate that Chinese 80-55 plants were no longer being maintained in a well-fertilised condition by the daily doses of 0.2 mM P\(_i\) due to their size. The root-to-shoot ratio was significantly higher for Chinese 80-55 than for Machete at the final harvest (35 % higher for both P\(_i\) treatments, Fig. 1). It was also significantly higher in all plants after 18 days of P\(_i\) withdrawal (33 DAT) compared to that at full nutrition (45 % increase, Fig. 1 and Supplemental Table S2). Whole plant biomass accumulation over the P\(_i\)-withdrawal period was 5.2 ± 0.2 g for Machete and 7.8 ± 0.5 g for Chinese 80-55. However, there was no genotype-dependent difference in relative growth rate under either P\(_i\) supply (Fig. 1). The relative growth rate of both cultivars decreased significantly when P\(_i\) was withheld from the solution (20 % reduction for Machete and 15% for Chinese 80-55 compared with 0.2mM P\(_i\) treatment at 33 DAT, Supplemental Table S2).

**Genotype-dependent variation in P distribution in response to P\(_i\) supply**
At the start of the experiment, the total P (Pₜ) concentration in young and mature leaf sections ranged from 258 to 403 µmol P g⁻¹ DM, respectively, and was similar across the leaf blade (data not shown) and between genotypes (Fig. 2). The total P accumulation at the start of the experiment (15 DAT) was 139 ± 8 µmol for Machete seedlings and 192 ± 10 µmol for Chinese 80-55 seedlings. When Pᵢ was supplied, Pₜ concentrations were similar in the aerial parts of both cultivars and between harvests with a slight decrease observed in all aboveground parts at 33 DAT. There was a trend towards higher Pᵢ concentrations in fine and mature roots of Machete relative to those in Chinese 80-55, most likely the result of dilution effects due to sustained root growth in the latter.

Withholding Pᵢ from the solution drastically reduced Pᵢ concentrations in all organs of both genotypes. At the beginning of the experiment (15 DAT) and at 18 DAT, Pᵢ concentrations were similar in both cultivars; however, at 33 DAT, the Pᵢ concentration was higher in all organs of the more P efficient Chinese 80-55 compared to those of Machete (Fig. 2). The differences in Pᵢ concentrations in stems, as well as fine and mature roots, were nearly two-fold and highly significant. This result is interesting, as Chinese 80-55 also featured a larger root biomass compared to Machete (Fig. 1). At the end of the experiment, the total P content of the plants following Pᵢ withdrawal was very low for Machete (69 ± 1 µmol) and much higher for Chinese 80-55 (246 ± 24 µmol). This resulted in a net loss of P during this period for Machete (-68 ± 1 µmol P per plant), while Chinese 80-55 plants were able to maintain their P content over the withdrawal period with an insignificant P gain of 54 ± 21 µmol.

Throughout the experiment, Pᵢ-limited Chinese 80-55 contained more P in all organs analysed than Machete, resulting in P content differences of 84 ± 10 µmole P in shoots, 81 ± 13 µmole P in fine roots and 11 ± 3 µmole P in mature roots at 33 DAT (see Supplemental Table S5). Since the seed P content differed by only 3 µmole P seed⁻¹, this must reflect a higher Pᵢ-
uptake during the first 15 days in hydroponic culture. The little P that was available inside the
plant without continued P$_i$ supply was much more efficiently redistributed by Chinese 80-55
with relatively higher P$_i$ content in elongating basal leaf sections and particularly fine roots
than in more mature parts, such as leaf tips and mature roots, while P concentration and also
P content declined more evenly across all organs of Machete. When considering the total
plant P content at 15 DAT, the re-use efficiency of Chinese 80-55 (0.041 ± 0.002 g units
µmol$^{-1}$ P) was higher than that of Machete (0.038 ± 0.002 g units µmol$^{-1}$ P). This
interpretation changes, however, when considering the total P accumulation after 18 days of
P$_i$ withdrawal with Machete having a 2-fold higher re-use efficiency than Chinese 80-55
(0.075 ± 0.004 g units µmol$^{-1}$ P and 0.032 ± 0.001 g units µmol$^{-1}$ P, respectively), due to the
fact that Machete lost P over the experimental period without any growth penalty.

While free P$_i$ concentrations across different plant parts were comparable between cultivars at
the beginning of the experiment (15 DAT), they varied significantly in different parts of roots
and leaves at both P$_i$ supplies (Fig. 3). At the final harvest, fine and mature roots, as well as
all three mature leaf sections of P$_i$-sufficient Machete had significantly higher P$_i$
concentrations than those of P$_i$-sufficient Chinese 80-55, while P$_i$ concentrations in young
leaves and stems from P$_i$-sufficient plants were similar between the two cultivars (Fig. 3, grey
bars). The lack of P$_i$ in the nutrient solution had the opposite effect, with higher P$_i$
concentrations found in all Chinese 80-55 organs, with significant variation between cultivars
found in mature roots as well as the base and middle sections of young leaves. Withholding P$_i$
from Chinese 80-55 reduced the internal P$_i$ concentrations by 75 to 86 % in young leaves,
mature leaves, stems and roots. In Machete, withholding P$_i$ had a stronger effect, lowering the
P$_i$ concentrations by more than 90% in each of these organs. There was a significant
interaction between treatments and genotypes in determining the P$_i$ concentration for most
organs, apart from stems and the upper two thirds of young leaves (Supplemental Table S3).
Along the leaf developmental gradient of P_\textsubscript{i}-limited plants, P_\textsubscript{i} concentrations were generally 15% lower in the basal section of the mature leaf sheath. Young P_\textsubscript{i}-limited Machete leaves showed a similar trend (21% lower P_\textsubscript{i} concentrations at the base), while their counterparts in Chinese 80-55 showed a more even P_\textsubscript{i} distribution. In leaves of P_\textsubscript{i}-sufficient plants, P_\textsubscript{i} concentrations in the basal sections were generally higher than those in the photoautotrophic parts. Overall, the differences observed in P_\textsubscript{i} concentration at 33 DAT between the cultivars grown under P_\textsubscript{i}-limited conditions could be attributed almost exclusively to a change in free P_\textsubscript{i} concentration.

Despite the relatively minor differences in P_\textsubscript{i} concentrations between the two cultivars, they varied widely in their organic P (P_\textsubscript{o}) concentrations (P_\textsubscript{o} = P_\textsubscript{i} – P_\textsubscript{i}, Fig. 3) in response to P_\textsubscript{i} supply: At the beginning of the experiment (15 DAT) there was no significant difference between cultivars, but at 33 DAT, P_\textsubscript{o} concentrations were at least 2-fold higher in all P_\textsubscript{i}-sufficient Chinese 80-55 organs compared to those from Machete, with more than a 4-fold difference in the photoautotrophic parts of mature leaves. Since the root DM of Chinese 80-55 is greater than that of Machete (Fig. 1), the approx. 2-fold higher P_\textsubscript{o} concentration in Chinese 80-55 roots resulted in a much higher P_\textsubscript{o} content for this plant organ compared to Machete.

Withholding P_\textsubscript{i} from the nutrient solution resulted in a significant decrease in P_\textsubscript{o} concentration in all organs of both genotypes. It dropped to between 27 to 12% of the P_\textsubscript{o} concentration found in organs of P_\textsubscript{i}-supplied Machete plants, and much more dramatically to between 7 to 3% in the corresponding Chinese 80-55 organs. This is in stark contrast to the inverse relationship observed for P_\textsubscript{i} (Fig. 3, grey bars). As a result, the P_\textsubscript{o} concentration was relatively higher in Machete in various P_\textsubscript{i}-limited organs, particularly in basal parts of young leaves, where it was 2-fold higher than in Chinese 80-55.
Genotype-dependent variation in P$_i$-responsiveness of *PHT* and *IPS1* transcripts

The relative responsiveness of the transcript pools for a panel of P$_i$-responsive genes was examined in the two contrasting wheat cultivars. A first screen of the transcript pools was done on material harvested at 23 and 33 DAT, pooling sub-samples from three biological replicates. The trends for transcript levels were the same in organs harvested 23 and 33 DAT, with much stronger relative changes in transcript amounts observed for most of the P$_i$-responsive genes tested at 33 DAT, *i.e.* after 18 days of P$_i$ deprivation (data not shown). Transcript analysis was therefore conducted on individual biological replicates from the latter time-point relative to *TaAPT1* as the reference transcript.

The *TaPHT3;1* gene that encodes a putative mitochondrial P$_i$ transporter was highly expressed (*i.e.* at a similar level as the *TaAPT1* reference gene) in all organs examined and was only weakly responsive to the external P$_i$ supply in P$_i$-limited mature roots, stems and mature leaves of both cultivars (Fig. 4). Moderate genotypic variation was observed in fine roots, stems and mature leaf bases only. The *TaPHT2;1* gene that encodes a putative plastid P$_i$ transporter was expressed at a similar level as *TaPHT3;1* in leaves and stems, but transcripts were barely detectable in roots (the detection limit for our assay was a theoretical 40 – ΔCt value of 22 ± 2). *TaPHT2;1* transcript abundance was higher in most plant parts of P$_i$-sufficient plants from both cultivars, particularly in the upper two thirds of young and mature leaves of Chinese 80-55. Interestingly, stems from P$_i$-limited Chinese 80-55 had 14-fold higher *PHT2;1* transcript levels than those from Machete, while the opposite trend was observed in mature roots of P$_i$-sufficient plants. Genotypic differences were also observed in fine roots at both external P$_i$ supplies with *PHT2;1* being 15-fold more abundant in the Machete cultivar.
The plasma-membrane PHT1 transporter genes *TapHT1;2*, *TaPHT1;5* and *TaPHT1;8* were strongly induced under P$_i$ limiting conditions in both cultivars in most plant parts, apart from fine roots. *TaPHT1;5* transcripts were also not P$_i$-responsive in the tips and bases of mature leaves, while in the middle section of mature leaves they were induced more than 500-fold in both cultivars upon withholding P$_i$. Expression patterns overall were very similar between cultivars, but *TaPHT1;5* transcripts were much more abundant in young basal leaf sections of P$_i$-supplied Chinese 80-55 compared to Machete. *TaPHT1;8* was most strongly induced by P$_i$ deprivation of all the PHT genes tested and its expression patterns differed significantly between cultivars. Its transcripts were less abundant in fine roots, young leaf tips, as well as young and mature leaf bases, of P$_i$-limited Chinese 80-55 compared to Machete. However, *TaPHT1;8* transcripts were more abundant in mature roots, young leaf bases and mature leaf middle sections of P$_i$-sufficient Chinese 80-55 and more strongly suppressed in young leaf tips and stems compared to P$_i$-sufficient Machete. Compared to the other two PHT1 genes tested, *TaPHT1;2* transcript abundance was only weakly responsive to external P$_i$ supply in most plant parts, but showed the most pronounced differences in expression patterns between cultivars: While being induced more than 750-fold in young leaf middle sections of P$_i$-limited Chinese 80-55, it was not P$_i$-responsive in this plant part in Machete. At the same time, basal sections of young and mature leaves from Machete showed P$_i$-dependent accumulation of *TaPHT1;2* transcripts, while the abundance of these transcripts was not P$_i$-responsive in the mature leaf base of Chinese 80-55 and was even strongly suppressed in the young leaf base of P$_i$-limited Chinese 80-55. The same was true for fine roots, with Machete showing a strong induction of *TaPHT1;2* transcript levels upon P$_i$ withdrawal, while they were constitutively and highly expressed in Chinese 80-55.

The two wheat orthologs for one of the first marker genes described for low plant P status, the *Induced by Phosphate Starvation 1* (*IPS1*) gene (Liu, Muchhal & Raghothama, 1997),
TaIPS1.1/1.2, were strongly induced across P\textsubscript{i}-limited plant parts (e.g., between 39-fold in fine roots and about 1900-fold in mature roots of the Chinese 80-55 cultivar), with no qualitative differences between cultivars. In Chinese 80-55, IPS1 induction was 50% weaker in P\textsubscript{i}-limited fine roots and its suppression was 2-fold less in P\textsubscript{i}-sufficient middle sections of young leaves compared to those in Machete.

**Genes involved in phospholipid, primary carbon and energy metabolism show genotype-dependent expression in young organs**

We also analysed the transcript pools of genes involved in primary metabolism, *i.e.* *de novo* phospholipid synthesis (phosphoethanolamine N-methyltransferase isoforms, *TaPEAMT1* and *TaPEAMT2*), carbon metabolism (α-1,4-glucan phosphorylase, *TaGPho1*; fructose 1,6-bisphosphate aldolase, *TaALD*; cytosolic malate dehydrogenase, *TaMDH*; UDP-glucose dehydrogenase, *TaUGD*), and generation of a proton-motive force (plasma-membrane H\textsuperscript{+}-ATPase, *TaHa1*). *TaPEAMT1* and *TaPEAMT2* transcripts were highly abundant in both genotypes in all tissues. However, expression varied significantly between genotypes and P\textsubscript{i} levels (Fig. 5). Under P\textsubscript{i} deficiency, *TaPEAMT1* was suppressed in young and mature leaf tips, as well as stem tissue of both genotypes. Transcript amounts were also lower in the middle sections of both young and mature leaves in Chinese 80-55, but not in Machete. Interestingly, expression of this gene appeared to be suppressed or unaltered rather than induced in P\textsubscript{i}-sufficient basal sections of young and mature leaves in Chinese 80-55, resulting in higher *TaPEAMT1* transcript amounts in the corresponding P\textsubscript{i}-sufficient Machete tissues. In roots, *TaPEAMT1* was expressed at both P\textsubscript{i} levels, with suppression under P\textsubscript{i} deficiency only occurring in mature roots of Machete.

*TaPEAMT2* was expressed in all portions of the leaves and roots, but its expression was generally higher when plants were limited by P\textsubscript{i}. There was no clear distinction between
cultivars in their *TaPEAMT2*-transcript profiles, apart from these being somewhat more P$_i$-responsive in mature roots and less responsive in fine roots and mid sections of young leaves in Chinese 80-55 than in those of Machete. The young leaf bases of P$_i$-fertilised Chinese 80-55 had less of these transcripts than the corresponding Machete tissue.

*TaGPhol* was expressed in green tissues of both genotypes, irrespective of their P status with little expression in roots (Fig. 5). In stems of both cultivars, these transcript levels increased upon withholding P$_i$. While the increase in *TaGPhol* transcripts in response to withholding P$_i$ was less for Chinese 80-55 (3-fold) than for Machete (16-fold), the base level was 64-fold higher in P$_i$-supplied Chinese 80-55. In Machete, *TaGPhol* transcript abundance increased in young leaves upon withholding P$_i$, but decreased in mature leaves of these plants. In contrast, in Chinese 80-55 *TaGPhol* was expressed independently of the external P$_i$ supply in young leaves, while being strongly induced in the basal section of mature leaf blades from P$_i$-limited plants.

*TaALD* transcript abundance was induced under P$_i$ deficiency in most tissues of both genotypes; however this induction was lacking in the basal sections and tips of mature Machete leaves (Fig. 5). Compared to Machete, the abundance of *TaALD* transcripts was lower in fine roots of Chinese 80-55, irrespective of plant P status, while *TaALD* transcripts were more abundant in the mature roots of P$_i$-fed plants.

Transcripts from *TaMDH* were overall more abundant in P$_i$-deficient than in P$_i$-fed leaves and stems of both cultivars, apart from leaf tips (Fig. 5). Under P$_i$ supply, Chinese 80-55 had 50% lower levels of *TaMDH* transcripts in leaf bases, but significantly more of these transcripts in stems than Machete did under both P$_i$ regimes. *TaMDH* transcript amounts were lower in P$_i$-deficient fine roots of both cultivars, with significantly lower levels in Chinese 80-55 under both P$_i$ supplies.
A trend in transcript abundance similar to that for *TaMDH* was also observed for *TaUGD*, with a more pronounced induction in most leaf tissues of *P*$_{_i}$-deficient plants (Fig. 5). Transcript profiles in fine roots and stems of both cultivars were nearly identical to those of *TaMDH*. But in mature roots, *TaUGD* transcript amounts were higher in Chinese 80-55 than in Machete in both *P*$_{_i}$-limited and *P*$_{_i}$-sufficient plants.

Apart from young leaf tips, *TaHa1* transcript abundance was highly variable with *P*$_{_i}$ supply and between genotypes (Fig. 6). Transcripts in mature roots were not responsive to external *P*$_{_i}$ supply, but were more abundant in Chinese 80-55 than in Machete. Upon withholding *P*$_{_i}$, *TaHa1* transcript abundance in fine roots was reduced in both cultivars, with Chinese 80-55 having 20% of the transcripts of Machete under both *P*$_{_i}$ conditions. In stems, *TaHa1* transcript amounts were significantly higher upon withholding *P*$_{_i}$ in both cultivars, although the transcript amounts when *P*$_{_i}$ was available were much higher in Chinese 80-55. Mature leaves of *P*$_{_i}$-sufficient Machete showed similar or higher *TaHa1* transcript amounts than those of *P*$_{_i}$-limited plants. This trend was completely reversed in mature and young leaf bases from *P*$_{_i}$-fertilised Chinese 80-55 with both tissues having a much lower abundance of *TaHa1* transcripts than the corresponding Machete leaf parts. Transcript levels upon withholding *P*$_{_i}$ were similar in these sink tissues between cultivars. Middle sections of young leaves from *P*$_{_i}$-sufficient Chinese 80-55 had much higher *TaHa1* transcript amounts than those in Machete, but had lower amounts under *P*$_{_i}$-limiting conditions. In contrast to Chinese 80-55, Machete *TaHa1* expression was highly responsive to *P*$_{_i}$ in this tissue.

Genes involved in transcription and translation are strongly suppressed in fine roots and young leaf tissues of cultivar Chinese 80-55

The transcript levels of genes encoding transcription factors *TaMYB76* and *TaPTF1* were significantly lower in fine roots of *P*$_{_i}$-deficient than of *P*$_{_i}$-sufficient plants from both cultivars,
with Chinese 80-55 having the lowest amounts (Fig. 6). Pₐ-deficiency-dependent suppression of TaPTF1, but not TaMYB76, also took place in mature Machete roots, while transcripts for neither gene responded to Pᵢ availability in mature roots of Chinese 80-55. Machete had higher mRNA levels for both transcription factor genes in young leaf bases from Pᵢ-limited plants, while only TaMYB76 was induced in Chinese 80-55. The opposite was true for mature leaf bases under Pᵢ-limited conditions, where TaMYB76 transcript levels were not responsive to Pᵢ in Chinese 80-55, while transcript levels were reduced for TaMYB76 in Machete and for TaPTF1 in both cultivars. In young leaf tips of Pᵢ-limited Chinese 80-55, TaMYB76 and TaPTF1 transcript amounts were lower than in those of Machete, but were higher in Pᵢ-fed Chinese 80-55 than in those of Machete. Stems of Chinese 80-55 had higher amounts of both TaMYB76 and TaPTF1 transcripts than Machete had, with more pronounced genotypic differences under Pᵢ-sufficient conditions.

Transcripts from genes encoding subunits 17 and 18a of the large 70S ribosomal subunit were less abundant for Chinese 80-55 compared to Machete in all sink tissues examined, irrespective of the external Pᵢ supply (Fig. 6). In Machete, and to a lesser extent Chinese 80-55, transcripts from these genes were induced in young leaves and mature roots and suppressed to varying extents in fine roots by withholding Pᵢ.

**Discussion**

We have determined organ-specific P-accumulation patterns and transcript-expression profiles of 17 marker genes in response to Pᵢ availability in two wheat cultivars with contrasting P efficiency (Osborne & Rengel, 2002a,b). We found marked differences in the P-allocation patterns between different plant organs as well as transcript expression patterns that are potential molecular determinants for improved PUE in the absence of external Pᵢ supply. The most striking differences in responses between cultivars Chinese 80-55 (high
PUE) and Machete (low PUE) were observed in fine roots, stems and basal sections of leaves, suggesting that the underlying molecular explanation for a higher PUE in Chinese 80-55 over Machete may lie in differences in P metabolism in these plant parts, or in the Pi supply to these tissues (Fig. 7). Young leaf bases could therefore be excellent indicators for a P-efficiency traits in a plant, with contrasting expression patterns observed for PHT1 transcripts TaPHT1;2/1;5 and TaPHT1;8 (less strongly suppressed in the presence of external Pi, less strongly induced in the absence of Pi) as well as TaPEAMT1/2 (reduced in the presence of Pi) and TaGPho1 (slightly increased under Pi fertilisation, strongly reduced upon Pi withdrawal). We propose that determining Pi and Po accumulation in leaf bases or stems in combination with monitoring key marker genes such as PHT2;1, GPho1, Hal, PTF1 and L18a could greatly advance efforts to select high-PUE genotypes, not only in wheat, but also in other monocot crops.

**P, and Po allocation patterns in sink tissues correlate well with PUE**

The 15-day pre-treatment period in nutrient solution containing Pi allowed the seedlings to accumulate sufficient P to sustain growth for the length of the experiment. This is reflected in similar tissue Pi concentrations between the cultivars at 15 DAT, despite differences in their initial seed P content. At the final harvest, the Pi concentration in all tissues of both genotypes without continued Pi supply were lower than the critical concentration for growth, as determined by Hoppo, Elliott & Reuter (1999), with Chinese 80-55 being able to maintain higher Pi content in all organs, especially in young sink tissues. The average relative growth rates of Pi-limited plants were positively correlated with plant Pi content.

Under P-limiting conditions, plants translocate relatively more photosynthate to the roots to produce more root biomass at the expense of shoot growth (Aziz et al., 2011b; Bates & Lynch, 2001; Lynch & Brown, 2001). The low-PUE Machete responded just as well with
respect to increasing the root-to-shoot ratio as the high-PUE Chinese 80-55 during the first 8 days (23 DAT) of withholding P$_i$. In fact, the root-to-shoot ratio for Chinese 80-55 decreased between 15 to 18 DAT, while it increased for Machete. Surprisingly, the two cultivars did not differ much in their shoot biomass production at either P supply rate. They also showed only relatively small differences in their P re-use efficiencies when compared with the total P accumulated at the beginning of the experiment. This is contrary to earlier findings by Osborne and Rengel (2000a), where the average shoot biomass of 37-day old plants was about 25% of that in our study. This difference is largely explained by the fact that our plants were pre-grown in hydroponics in the presence of a readily available P source before P was withheld, while Osborne and Rengel (2000a) grew plants in soil supplemented with a poorly available P source. Despite these differences, we have confirmed the ability of Chinese 80-55 to invest more in root biomass (Osborne & Rengel, 2002a,b). Under field conditions, larger root systems often lead to higher grain yield (Elliott et al., 1997; Horst, Abdou & Wiesler, 1996; Manske et al., 2000; Osborne & Rengel, 2002a), thus highlighting the importance of ‘root breeding’ efforts that will help to produce healthier plants that can explore larger soil volumes to access more nutrients and water (Wang, Yan & Liao, 2010).

The root and shoot growth that was observed when P$_i$ was withheld from the plants was supported by re-mobilisation of P absorbed during the pre-treatment phase. Both cultivars severely depleted their P$_o$ pools within 18 days (33 DAT). Chinese 80-55 appeared to be more efficient than Machete at reallocating P$_i$ under P-deficient conditions, providing more P$_i$ to all organs, particularly young leaves, stems and mature roots. It was surprising to see that both cultivars maintained high levels of free P$_i$ in their organs, even when P$_i$ was withheld from the nutrient solution. The Chinese 80-55 cultivar in particular accumulated substantial amounts of P$_i$ in all organs without continued P$_i$ supply. It is unclear whether this was a sign of increased turnover of organic compounds for internal remobilisation, a hypothesis
supported by the higher capacity of Chinese 80-55 to synthesise organic P compounds under well-fertilised conditions, or whether this indicates that the plants were unable to maintain conditions that favour the synthesis of organic P compounds. In both cases, the severe decline in overall tissue P content in Machete may have been due to P loss from the roots due to diminished cell membrane integrity under P deficiency (Graham, Leonard & Menge, 1981). This effect would have been exacerbated by the frequent replacement of the nutrient solution during the experiment. Phospholipids are an important structural component of cell membranes. However, they are rapidly turned over (Andersson et al., 2005; Russo et al., 2007), and under P deficiency their breakdown is associated with increased membrane leakage (Ratnayake, Leonard & Menge, 1978).

Differences in growth between the two cultivars when supplied with P, were strongly correlated with significant differences in P concentrations across all organs. Both cultivars had the same P concentration in shoots, while Chinese 80-55 roots had a lower P concentration, but higher P content, than Machete roots. The differences in P pools indicate a more efficient utilisation of P by Chinese 80-55 and / or better P remobilisation out of vacuolar storage, given that the P concentrations in the different organs of Chinese 80-55 were generally lower under P fertilisation and the P-to-P ratio was always higher than in Machete. These differences were most pronounced in the photosynthetically active mature leaves as well as in roots, indicating that Chinese 80-55 is able to allocate more P to support photosynthesis and root growth under P-sufficient growth conditions.

Genotype-dependent expression of TaPHT2;1, TaPHT1;2, TaPHT1;8 and TaHal across organs correlates with P allocation profiles

Given that P-allocation patterns at least partially depend on PHT proteins, it was interesting to see pronounced differences in the way that some of these transporters were regulated in
response to P\textsubscript{i} availability in different tissues in the two cultivars. Expression of the plastid-localised \textit{TaPHT2;1} was very high in leaves and lower in roots of both genotypes, but it was not strictly correlated with P\textsubscript{i} supply. Guo \textit{et al.} (2013) demonstrated that reduced expression of this transporter in transgenic wheat lines resulted in reduced photosynthetic activity as well as reduced plant P\textsubscript{i} content under both sufficient and limited P\textsubscript{i} supply and suggested its association with systemic signalling networks. The \textit{TaPHT2;1} expression profile observed in this study is consistent with the notion that Chinese 80-55 is better at assimilating P\textsubscript{i} into organic P compounds under P\textsubscript{i} supply. In contrast to \textit{TaPHT2;1}, \textit{TaPHT1;8}, which likely encodes a plasma-membrane-localised transporter, is strongly induced in stem and leaf tissues of the P\textsubscript{i}-limited wheat seedlings and might be involved in re-mobilisation of P\textsubscript{i}. Its structural similarity to OsPT8 from rice (Supplemental Fig. 2) suggests it could be a high-affinity P\textsubscript{i} transporter. OsPT8 seems to be primarily involved in P\textsubscript{i} uptake by the root (Jia \textit{et al.}, 2011; Wu \textit{et al.}, 2011). Interestingly, the root-specific expression of the closely related \textit{HvPHT1;3} and \textit{HvPHT1;4} transporters in barley (Supplemental Fig. 2) has been negatively correlated with PUE in four barley genotypes (Huang \textit{et al.}, 2011). This might also be the case in wheat, given that \textit{TaPHT1;8} expression is mostly lower in Chinese 80-55 than in Machete across organs from P\textsubscript{i}-limited plants. Yet, in Chinese 80-55 \textit{TaPHT1;8} expression in roots and basal sections of young leaves is not down-regulated by P\textsubscript{i} supply to the same extent as that in Machete. This observation, together with a very strong accumulation of transcripts from \textit{TaPHT1;2}, and to a lesser extent from \textit{TaPHT1;5}, in these plant parts of P\textsubscript{i}-fed Chinese 80-55 could account for the greater P\textsubscript{i} uptake reported for this cultivar by Osborne and Rengel (2002 a,b) and the more effective allocation of P\textsubscript{i} to sink organs such as young leaves found in this study.

\textit{TaPHT1;5} is a close ortholog to \textit{HvPHT1;6} (Supplemental Fig. 2), a low-affinity P\textsubscript{i} transporter expressed in both root and shoot phloem tissues of barley (Rae \textit{et al.}, 2003). Here,
TaPHT1;5 was induced to a similar extent in both cultivars by withholding Pᵢ and only showed significantly higher expression in basal sections of young leaves of Pᵢ- sufficient Chinese 80-55. HvPHT1;6 has also been associated with Pᵢ remobilisation and PUE in barley and can mediate concentration-dependent Pᵢ efflux in vitro (Huang et al., 2011; Preuss et al., 2010). It is also interesting to note that HvIPS1 expression profiles in the study by Huang et al. (2011) were very similar to those of HvPHT1;3 and HvPHT1;6 and, therefore, were negatively correlated with PUE, while HvIPS2 expression was induced in all four Pᵢ-limited genotypes. Here, we used a primer pair that simultaneously detects both TaIPS1.1 and TaIPS1.2 transcripts. It is quite remarkable to see that this well-known marker for Pᵢ starvation (Franco-Zorrilla et al., 2007; Huang et al., 2011) was induced to a similar extent in both wheat cultivars, while all the other candidate genes examined showed differential responses. This indicates that TaIPS1.1/2 might respond to the external lack of Pᵢ, while most of our selected target genes respond to internal Pᵢ or Pₒ pools that are regulated differently between the two cultivars.

TaPHT1;2 is structurally similar to two high-affinity Pᵢ transporters in barley roots, HvPHT1;1 and HvPHT1;2 (Preuss, Huang & Tyerman, 2011; Rae et al., 2003; Schuenmann et al., 2004) as well as to HvPHT1;8, which shows induction after colonisation with arbuscular mycorrhizal fungi (Christophersen, Smith & Smith, 2009; Glassop et al., 2005) (Supplemental Fig. 2). Apart from its distinct genotype-dependent expression profiles in primary and secondary roots, TaPHT1;2 expression was also differently regulated in aerial tissues of both cultivars. TaPHT1;2 is thus a good candidate for promoting more efficient use of Pᵢ in sink tissues, as well as increasing Pᵢ influx into photosynthetically active tissues when Pᵢ supply is restricted. It has to be noted, however, that higher expression of Pᵢ-starvation inducible TaPHT1;2 (and to a lesser extent TaPHT1;8) in heterotrophic leaf tissues in Machete correlated with higher Pₒ concentrations in Pᵢ-limited leaves and could therefore
contribute to the less efficient P use in this cultivar. Constitutive expression of *TaPHT1;5* and *TaPHT1;8* in basal sections of young leaf blades versus their induction upon P\textsubscript{i} withdrawal in the upper two thirds of leaves could on the other hand account for the more even P\textsubscript{i} distribution observed across the blade of young Chinese 80-55 leaves. Any P\textsubscript{i} in the xylem stream not taken up by cells in the basal section will be available further along the blade. Differential uptake into cells may be the key to changing P\textsubscript{i}-allocation patterns within the plant, although little is known about the regulation of P\textsubscript{i} efflux from cells.

There are very few reports on the role of individual H\textsuperscript{+}-ATPase isoforms in supporting the uptake of P\textsubscript{i} or other anions into cells. In this study, transcripts encoding plasma-membrane-localised H\textsuperscript{+}-ATPase TaHa1 accumulated in stems and basal leaf sections under P\textsubscript{i}-limiting conditions in both cultivars. In P\textsubscript{i}-sufficient plants, transcript amounts were differentially regulated between the two cultivars. This suggests that in Chinese 80-55 a strong proton-motive force across the plasma membrane might improve P\textsubscript{i} import into photosynthetically active tissues and thus its conversion into P\textsubscript{o}. Strong suppression of *TaHal* transcript accumulation in Chinese 80-55 leaf bases could indicate that these tissues have less capacity to take up anions and rely more heavily on photosynthate produced further along the leaf blade. Overall, *TaHal* is the most genotypically variable marker tested in this work.

*De novo* phospholipid biosynthesis is not suppressed in elongating P\textsubscript{i}-limited leaf sections of the more P-efficient Chinese 80-55

Among the P\textsubscript{i}-conserving metabolic adjustments in P\textsubscript{i}-deficient plants is the synthesis of galactolipids and sulfolipids to replace phospholipids (Haertel & Benning, 2000; Yu, Xu & Benning, 2002). Consistent with its proposed role in *de novo* phospholipid biosynthesis, *TaPEAMT1* transcripts, encoding one of two phosphoethanolamine N-methyltransferase isoforms in wheat, were suppressed by withholding P\textsubscript{i} and induced by supplying P\textsubscript{i} in stems.
and photosynthetically active parts of young and mature leaves of both cultivars, as were the *Arabidopsis* orthologs *AtNMT1* and *AtNMT3* (Mueller *et al.*, 2007). Unexpectedly, *TaPEAMT1* expression was maintained in the basal section of mature leaves and even increased in the elongation zone of *P*$_i$-limited young leaves of the high-PUE Chinese 80-55. This suggests that Chinese 80-55 may preserve phospholipid levels in these tissues during *P*$_i$ limitation, a critical trait to sustain cell division and cell elongation (Cruz-Ramirez *et al.*, 2004). Given the different enzymatic properties of *TaPEAMT1* and *TaPEAMT2* (Jost *et al.*, 2009), it is not surprising that *TaPEAMT2* transcript levels are induced in the absence of external *P*$_i$ supply in both wheat cultivars.

**P-efficient Chinese 80-55 may mobilise starch reserves more efficiently in response to *P*$_i$ availability to sustain growth**

Another strategy used to conserve *P*$_i$ during *P*$_i$ deficiency is to remodel carbon metabolism, by-passing *P*$_i$-requiring reactions (Ciereszko & Kleczkowski, 2005; Loreti *et al.*, 2001; Plaxton & Tran, 2011). In wheat, transcripts for *TaGPho1*, encoding the plastid enzyme that breaks down transient starch (Tiwari & Kumar, 2012), were hardly detectable in root tissues and were moderately abundant in both young and mature leaves of both cultivars at both *P*-supply levels, which is consistent with earlier findings (Schupp & Ziegler, 2004). Surprisingly, *TaGPho1* transcripts accumulated in a genotype-dependent manner in young leaf tissues and stems in response to the external *P*$_i$ supply. In the presence of *P*$_i$, the more *P*-efficient Chinese 80-55 may be able to mobilise transitory starch more effectively from stems that act as starch repositories and promote anabolic reactions in the chloroplast. At the same time phosphorolytic starch breakdown may be low in basal mature leaf sections in this cultivar, perhaps to increase hydrolytic starch degradation at night. Under *P*$_i$ limitation, breakdown of transitory starch could also be higher in stems of Chinese 80-55, most likely supplying carbon for growth and respiration, including the alternative nonphosphorylating
pathway (Kondracka & Rychter, 1997). Unlike Chinese 80-55, P_limited Machete accumulated higher \textit{TaGPho1} transcript amounts in basal sections of young leaves which may explain the rapid decline in free P_i content and the higher P_o concentration in these plant parts.

In stems and leaves, the accumulation of transcripts for \textit{TaALD}, encoding glycolytic aldolase that supports both glycolysis and glycolytic bypass reactions (Plaxton & Tran, 2011), was enhanced in the absence of P_i in both cultivars. In roots, however, \textit{TaALD} expression was genotype dependent. This suggests that suppression of glycolytic activities in fine roots might increase PUE in Chinese 80-55 irrespective of the external P_i availability. In this context, it is important to note that \textit{TaALD} transcript accumulation is not suppressed in mature roots of P_i-sufficient Chinese 80-55 as is seen in Machete. The potentially higher glycolytic activity in mature roots together with increased starch break-down in stems and suppression of glycolysis in fine roots themselves could perhaps support the increased production of fine roots in this cultivar irrespective of the external P_i availability. The wheat isoform of cytosolic malate dehydrogenase (MDH) studied here shows very similar expression patterns to \textit{TaALD}, but in basal sections of mature leaves and stems its expression profile mirrored that of \textit{TaGPho1}, supporting the view that tight control of starch breakdown is important in these tissues for enhanced PUE. The transcript profile across tissues and treatments of \textit{TaUGD}, a key gene involved in the synthesis of structural polysaccharides (Klinghammer & Tenhaken, 2007), was similar to that of \textit{TaALD} and \textit{TaMDH}, suggesting a possible transcriptional co-regulation and that some of the carbon accumulating under P_i-limiting conditions may be invested in structural components other than starch. Together, the genotype-dependent metabolic adjustments that may be occurring in elongating leaf sections, and particularly in stems and roots suggest that carbon allocation between these organs could be crucial for optimising PUE in wheat.
Transcription factors TaMYB76 and TaPTF1 are co-expressed with other genotypic markers in stems and fine roots

Targeting transcription factors to achieve favourable expression patterns of down-stream targets may have potential to enhance PUE. In fine roots, the expression profiles of *TaPHT2;1, TaPHT3;1, TaHa1*, two ribosomal protein subunit genes (*TaL117, TaL18a*), *TaALD, TaMDH* and *TaUGD* across treatments and cultivars overlapped with those of the two transcription factor genes *TaMYB76* and *TaPTF1*. This co-expression indicates the presence of an organ-specific transcriptional network involving an unidentified transcription factor that may act as a master switch controlling a set of genes involved in determining PUE.

In stems, there is a strong correlation between the expression profiles of the two transcription factors and *TaGPho1* as well as, to a lesser extent, *TaPHT2;1, TaPHT3;1, TaMDH* and *TaUGD*. Li et al. (2011) demonstrated that overexpression of ZmPTF1 in maize enhanced soluble sugar levels in roots but decreased them in leaves. This was matched by corresponding expression patterns of genes involved in sucrose catabolism. The contrasting expression pattern between fine roots and stems could indicate that expression of *TaPTF1* and *TaMYB76* is controlled by tissue-specific repressor(s) or enhancer(s). In the other organs examined, more complex interactions seem to drive P,

Reducing ribosome numbers in sink tissues could be important for improving PUE

The genes encoding large ribosomal subunits TaL117 and TaL18a showed consistently lower transcript amounts in all growing organs in the P-use-efficient Chinese 80-55 compared to the less efficient Machete. This could indicate that *de novo* ribosome biosynthesis in these tissues is adversely affecting PUE (Veneklaas et al., 2012). In bacteria, it has only recently been demonstrated that the ‘global physiological state’ of the cell, *i.e.* ribosome numbers and RNA polymerase concentrations as well as free amino acid and nucleotide pools, coordinates gene
expression during growth transitions and that transcription factors mediate complementary secondary responses (Berthoumieux et al., 2013). In plants, ribosome loading with mRNA has been identified as a critical factor in mounting a successful stress response (Kawaguchi et al., 2004; Reynoso et al., 2013). In our case, the transcript abundance for TaL117 and TaL18a in roots and basal sections of young leaves seems to be co-regulated with TaPTF1 and TaMYB76 expression. It remains to be seen how these two levels of control affect the ability of a plant to adjust to changing P$_i$ availability. The fact that the P$_i$ responsiveness of these genes differs greatly between cultivars suggests that there is yet another layer of control (e.g., a transcription factor or an epigenetic regulator) that is determining the relative PUE of the cultivars. Unravelling the nature of this master switch would provide a valuable target for future plant breeding efforts.

Concluding remarks

Wheat cultivar Chinese 80-55 produced more root biomass and contained more P than Machete under the two P$_i$ regimes indicating better P$_i$ acquisition when P was present, and an overall more efficient P utilisation. This is most likely achieved through greater P remobilisation and higher P-assimilation rates supported by differential expression of PHT, H$^+$-ATPase, phospholipid- and carbon-metabolism genes between source and sink tissues. This differential P and biomass partitioning is accompanied by changes in the transcript levels of transcription factor and ribosomal protein genes that may support the metabolic adjustments in Chinese 80-55 that lead to a greater PUE compared to Machete.

QTL studies in wheat have shown that PUE is a complex, polygenic trait (Guo et al., 2012; Su et al., 2009; Zhang et al., 2013). In-depth transcript and metabolite profiling of cultivars differing in PUE will be necessary to unravel these complex relationships. Our study reveals that careful selection of the plant organ to be used in these studies is also crucially important.
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The *xipotl* mutant of Arabidopsis reveals a critical role for phospholipid metabolism in root system development and epidermal cell integrity. *Plant Cell*, 16, 2020-34.


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**Figure legends**

**Figure 1:** Shoot dry matter (DM), root DM, root-to-shoot ratio and relative growth rate (RGR) of two contrasting wheat genotypes, Machete (black bars) and Chinese 80-55 (grey bars), at four harvest times (mean ± SE, n = 3). Significant differences according to Tukey’s HSD at p < 0.05 for genotypes at a given treatment are shown (*). The plants were grown in hydroponics with 0.2 mM P\(_i\) for 15 d before being either harvested or divided into two groups that received either no (0 mM) or 0.2 mM P\(_i\). Subsequent harvests were made at 18, 23 and 33 days after transplanting.

**Figure 2:** Total phosphorus concentration (P\(_t\)) in leaves, stems and roots of the wheat cultivars Machete (black bars) and Chinese 80-55 (grey bars) at four harvest times (mean ± SE, n = 3). Significant differences determined by 2-way ANOVA with Tukey’s HSD at p < 0.05 for genotypes at a given harvest are shown (*). The plants were grown as described in the legend to Figure 1. Since no differences were observed between different sections of young and mature leaves, average P\(_t\) concentrations are shown (see Supplemental Table S3 for more details on the statistical analyses).

**Figure 3:** Organic and inorganic phosphorus concentrations in leaves, stems and roots of two cultivars at the beginning of the experiment (15 DAT) and at the final harvest (33 DAT) (mean ± SE, n = 3). Statistically significant differences determined by Tukey’s HSD at p < 0.05 for genotypes at a given treatment are indicated (*); organic P (black bars) was
calculated as the difference between $P_t$ and inorganic $P$ (grey bars) fractions. The plants were grown as described in the legend to Figure 1.

**Figure 4:** Relative transcript abundance from five $P(H)T$ transporter and microRNA antagonist $IPS1/2$ genes ($IPS1$) in various plant parts from two contrasting wheat genotypes (mean ± SE, n = 3). Significant differences determined by Tukey’s HSD at $p < 0.05$ for genotypes at a given treatment are indicated (*). The plants were grown as described in the legend to Figure 1, which included being transferred to hydroponic medium containing either 0.2 mM $P_i$ (dark bars) and no $P_i$ (light bars) for 18 days. Data are presented as 40-$\Delta C_t$ expression values according to Bari et al. (2006). This is a log2 scale, where each unit represents a 2-fold change in transcript abundance. Given that $\Delta C_t$ values represent the difference in PCR threshold cycle (Ct) number between a given target and the reference transcript $TaAPT1$, a value of 40 equals the expression level of $TaAPT1$. The number of 40 is an arbitrarily chosen value, considering that the PCR is terminated after 40 cycles.

**Figure 5:** Relative transcript levels for genes involved in phospholipid ($P(EA)MT1$, $P(EA)MT2$) and carbon ($GPho1$, $ALD$, $MDH$, $UGD$) metabolism in various plant parts of the contrasting wheat genotypes Machete and Chinese 80-55 (mean ± SE, n = 3). Significant differences determined by Tukey’s HSD at $p < 0.05$ for genotypes at a given treatment are indicated (*). The plants were grown as described in the legend to Figure 1, which included being transferred to hydroponic medium containing either 0.2 mM $P_i$ (dark bars) and no $P_i$ (light bars) for 18 days. Data are presented as 40-$\Delta C_t$ expression values according to Bari et al. (2006). For details see the legend in Figure 4.
Figure 6: Relative transcript levels for gene products involved in proton pumping (Hαl H+\textsuperscript+-ATPase), translation (L117, L18a) and transcription (MYB76, TaPTF1) in various tissues of the contrasting wheat genotypes Machete and Chinese 80-55 (mean ± SE, n = 3). Significant differences determined by Tukey’s HSD at p < 0.05 for genotypes at a given treatment are indicated (*). The plants were grown as described in the legend to Figure 1, which included being transferred to hydroponic medium containing either 0.2 mM P\textsubscript{i} (dark bars) and no P\textsubscript{i} (light bars) for 18 days. Data are presented as 40-ΔCt expression values according to Bari et al. (2006). For details see the legend in Figure 4.

Figure 7: Summary of the metabolic consequences hypothesized to arise from the observed differences in transcript abundance and P fractions in the more P-efficient cultivar Chinese 80-55 compared to the less efficient Machete. The MapMan tool (Usadel et al., 2005) was used to highlight leaf segment- and organ level-differences in individual transcript abundance (expressed as log2 expression ratios of Chinese 80-55 (C) over Machete (M)) as well as ratios of inorganic P concentrations (1) and organic P concentrations (2) under +P and –P conditions, respectively. Only values that were significantly different between the two cultivars in at least one condition are shown. Horizontal arrows indicate the P\textsubscript{i}-uptake capacity of different Chinese 80-55 plant parts compared to Machete under the two P\textsubscript{i} supplies. Vertical arrows indicate anticipated changes in the levels of starch (S), phosphocholine (PC) or large ribosomal subunits (R) as a consequence of changes in transcript abundance for associated enzymes and proteins. The transcripts are shown in the same order as in Figures 4–6, with a = TaPHT1;2, b = TaPHT1;5, c = TaPHT1;8, d = TaPHT2;1, e = TaPHT3;1, f = TaIPS1, g = TaPEAMT1, h = TaPEAMT2, i = TaGPho1, j = TaPHT4;2.
TaALD, k = TaMDH, l = TaUGD, m = TaHa1, n = TaMYB76, o = TaPTF1, p = TaL117, q = TaL18a.
Supplemental Data

Supplemental Figure 1: A. Habit of wheat seedlings of cultivars Machete and Chinese 80-55 at 18 days after transfer into nutrient solution lacking $P_i$ (left) or supplemented with 0.2 mM $P_i$ (right). B. Schematic diagram of the leaf tissues harvested for this study. Tillers of wheat seedlings of both cultivars at 33 DAT (Zadok’s stage 30+, fully tillering or at the start of jointing) had two to six fully expanded leaves (termed mature leaves) and two to four expanding leaves (termed young leaves). Each leaf was divided into three sections according to the diagram on the right adapted from Li et al. (2010)

Supplemental Figure 2: Phylogenetic tree showing the relative position of the ten known wheat PHT protein sequences (in red) with respect to other monocot PHT protein sequences as well as Arabidopsis and yeast orthologs. Transcripts encoding the TaPHT proteins marked with an asterisk were analysed in this study. Amino acid sequences were assembled using ClustalX 2.1 and the tree was constructed with MEGA 5.10 using the Maximum Likelihood Method and Phylogeny Test with 1000 bootstrap replications (Larkin et al., 2007; Tamura et al., 2011). The wheat protein sequences used were TaPHT1;1 (CAH25730), TaPHT1;2 (AAP49822), (Liu et al., 2013), TaPHT1;5 (AAD26146, extended to full-length by a BLAST search using Geneious version R6.1), TaPHT1;8 (AK333026 translation), TaPHT1;11 (BAM62783), TaPHT2;1 (AAP49821), TaPHT3;1 (AK332510 translation), TaPHT4;1 (AK336028 translation) and TaPHT4;2 (AK331635 translation). Arabidopsis PHT sequences were named according to Nussaume et al. (2011); the nomenclature for the predicted Brachypodium distachyon PHT sequences followed the one in NCBI (http://www.ncbi.nlm.nih.gov); barley PHT sequences were named according to Schuenmann
et al. (2004) and Huang et al. (2011); rice PHT sequences were derived from Tanaka et al. (2008), Liu et al. (2011) and Paszkowski et al. (2002).

**Supplemental Table S1: Primers used in this study.**

**Supplemental Table S2:** Significance of P levels, genotypes and their interaction on growth parameters of two wheat genotypes, Machete and Chinese 80-55, at p < 0.05. SDM = shoot dry matter, RDM = root dry matter, RSR = root-to-shoot ratio and RGR = relative growth rate.

**Supplemental Table S3:** Significance of P levels, genotypes and their interaction on P fractions (Pᵢ = inorganic P, Pₒ = organic P, Pₜ = total P) at third harvest in various plant parts of two wheat genotypes, Machete and Chinese 80-55, at p < 0.05. YLA = young leaf tip, YLB = young leaf middle section, YLC = young leaf base, MLA = mature leaf tip, MLB = mature leaf middle section, MLC = mature leaf base, FR = fine roots and MR = mature roots.

**Supplemental Table S4:** Significance of P levels, genotypes and their interaction on relative expression of 16 wheat genes across plant organs in two wheat genotypes, Machete and Chinese 80-55, at final harvest (18 days after transfer to nutrient solution with or without Pᵢ), at p < 0.05.
Supplemental Table S5: Total P content of individual plant organs of the two cultivars across treatments and at different harvesting time points. YLA = young leaf tip, YLB = young leaf middle, YLC = young leaf base, MLA = mature leaf tip, MLB = mature leaf middle, MLC = mature leaf base, FR = fine roots, MR = mature roots.