Expression analysis of putative high-affinity phosphate transporters in Chinese winter wheats

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\textbf{ABSTRACT}

China’s soils tend to be phosphate deficient. Application of phosphorus fertilizers to the soil is yield and cost ineffective as much of the phosphate applied is rapidly locked-in and is inaccessible to the crop. Chinese Institutes have established intensive wheat breeding programmes to generate wheat varieties that produce adequate yields and grain quality in such soils. Three such wheat cultivars have been identified with good performance characteristics in the field. These three cultivars are thought to harbour chromosome translocations that may confer enhanced phosphate scavenging abilities to the plants. The isolation and study of the expression of high-affinity phosphate transporters in tissues of these wheats, in two of the donor wheatgrasses and in another widely planted Chinese wheat variety is presented and the first full-length sequence of a wheat phosphate transporter and partial clones of several other putative phosphate transporters are reported. Relative quantitative reverse-transcription – polymerase chain-reaction was used to demonstrate that different phosphate transporters have different expression patterns within a given variety and respond differently to phosphate deprivation. The significance of the genetic background for these findings and for the different phosphate acquisition properties of the wheats under study is discussed.

Key-words: Secale cereale; Thinopyrum elongatum; Thinopyrum intermedium; Triticum aestivum; China; Lovrin 10; wheatgrass; Xiaoyan 54.

\textbf{INTRODUCTION}

Wheat has been cultivated in China for several thousands of years. The crop is believed to have reached China from the near East by the second millennium BC (Yang & Smale 1997). China is now considered to be one of the world’s secondary centres of wheat diversity, and has abundant genetic resources including unique subspecies, local varieties and wild relatives (Baoqi, Aimin & Binjean 2001). The germplasm resource available to farmers is composed of Chinese local varieties (known for their high crossability with rye), improved cultivars or lines by Chinese wheat breeders and foreign germplasm introduced by Chinese scientists. To date a total of 40 540 accessions of wheat germplasm have been collected of which 13 233 belong to Chinese landraces (Li et al. 1998). All of the Chinese bread wheats (Triticum aestivum L.) are essentially hexaploids and are descendants of hybridized wild grass species (Harlan 1987).

China is the now the world’s largest wheat producer (115 million metric tonnes per year, with average yield superior to 4 t ha\textsuperscript{-1}). Winter wheat (autumn or winter sown with winter growth habit) accounts for nearly 90% of total production (Fig. 1); the rest is mostly low-quality spring wheat (sown March/April). Early maturity is one of the distinguishing features of the local Chinese facultative or ‘winter’ wheat cultivars (less than 252 d), a characteristic which is highly heritable and allows for planting of multiple crops. In order to preserve this early maturity characteristic many of the new cultivars incorporate landraces as parental material.

Predictions indicate that there will be a big surplus for phosphate (P) nutrient on farmlands of 2000 China (data obtained from Food and Agriculture Organization of the United Nations). Most of China’s soils in the main wheat-growing regions of the North are however, P deficient, having an average of 6–8 ppm available P from a total of 1230 ppm total P, because they are rich in clay and very alkaline (Fig. 1) (Lindert, Lu & Wu 1996). The efficiency of P fertilizer utilization by plants is therefore low (< 20%) on these soils as a high percentage of P ends up as fixed P.

Phosphate acquisition and translocation greatly influences the quality and yield of a crop such as wheat. In soils having low availability of P, it is important for the plant to have mechanisms for acquiring the P it needs from the soil. Young cereal plants in particular need a sufficient amount of P for the development of tillers and spikelets (Romer & Schilling 1986). In developing countries with soils with unfavourable P dynamics such as those found in Northern
China, considerable efforts have been made to select genotypes with a low P demand capable of producing adequate yields and grain quality under such conditions. During the period 1990–92, 500 varieties (or lines) were collected and assessed and 18 of these, displaying promising agronomic characteristics, were subjected to further screening. Wheat varieties were classified as ‘efficient’ if grain yields at low available soil P [8 ppm Olsen (Olsen et al. 1954)] were similar to those at high available soil P (20 ppm Olsen), irrespective of the potential yield ceiling of a variety (Li et al. 1994). Three efficient wheats, Xiaoyan 54, 81(85)-5-3-3-3 and Lovrin 10 (Fig. 2) were further characterized – all are thought to harbour chromosome translocations from alien species that may confer enhanced P scavenging ability to the plants.

In this study we have examined a representative sample of Chinese wheats from diverse genetic backgrounds:

Xiaoyan 54 By means of a translocation of common wheat (*Triticum aestivum* L.) with tall wheatgrass (*Thinopyrum elongatum*), Li and colleagues obtained a super large spike wheat called Xiaoyan 6 which has a grain fill period of approximately 40 d (Li 1992; Ma et al. 2001). Xiaoyan 6 exhibited a steady high-yielding potential (5250–6000 kg ha$^{-1}$) and has been cultivated for many years in the Yellow River and Huai River valleys of China (242 000 ha planted in 1995). It has a semi-dwarf character with high resistance to lodging, is resistant to stripe rust and scab and to the effect of China’s dry hot winds. Xiaoyan 54, which is derived from Xiaoyan 6, is more cold tolerant and therefore more suited to growing in the North. It is also more able to tolerate China’s dry winters. It is grown widely in Henan and Shaanxi province. Xiaoyan 54 has high quality protein content (18·25%) and gluten content (47%) and is good bread-
Figure 2. Field trials conducted at Changping Experimental Station, Institute of Genetics, Beijing. Top panel: furthest row: rye (*Secale cereale*) growing in soil plots containing 20 ppm Olson P (right-hand strip) and 8 ppm Olson P (left-hand strip). Middle row: Lovrin 10 growing under a similar P regime. Front row: Xiaoyan 54, again growing under high P and low P conditions. (a) and (b) Close-up of Jing 411 growing under high (20 ppm Olson) and low (8 ppm Olson) P conditions – note the reduction in growth and yield of Jing 411 in (b). (c) and (d) Close up of Xiaoyan 54 growing under similar conditions.
9 of the major facilitator superfamily of proteins and by analogy with other sequences are likely to function as H+/PO\textsubscript{4}\textsuperscript{2−} cotransporters. It is known that plants normally moderate their capacity to take up P to maintain the P concentration in their tissues within defined physiological limits (Mimura 1999). In most soils the concentration of available P in soil solution is several orders of magnitude lower than that in plant tissues, so expression of the high-affinity P transporters in the plant roots is up-regulated. Conversely, when the P concentration in the soil or growth medium is high, expression of the high-affinity P transporters is normally repressed (Muchhal & Raghothama 1999). It is thought that transcriptional control of the genes coding for these proteins is regulated by systemic signals that respond to the internal P status of the plant (Smith, Rae & Hawkesford 2000).

Here we examine the expression pattern of various putative high-affinity P transporters in the wheat described above to assess the extent of their contribution to the ability of the selected varieties to survive on low P soils. Also included in the study are the donor wheatgrasses Thinopyrum elongatum and Thinopyrum intermedium. Thinopyrum elongatum in particular is known to be especially tolerant of alkaline soils such as those found in Northern China.

MATERIALS AND METHODS

Plant material

Wheat varieties Xiaoyan 54, Lovrin 10 and Jing411, experimental line 81(85)-5-3-3-3 and the wheatgrasses Thinopyrum elongatum and Thinopyrum intermedium were obtained as seed from The State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics, Chinese Academy of Sciences, Beijing, China.

Growth of plant material

Wheat and wheatgrasses were surface-sterilized and germinated on filter paper. When the hypocotyls had emerged they were transferred to a thin layer of quartz saturated with 0·2 mM calcium sulphate solution. Once root growth was established (normally after 5 d) the plants were transferred to hydroponic culture in a growth cabinet kept at a constant 20 °C, with a relative humidity of 75%, a photon fluence rate of 280–300 µmol photons m\textsuperscript{−2} s\textsuperscript{−1} with a 16 h day/8 h night cycle. The plants were harvested approximately 10 d after transplanting.

Basal nutrient solution

A basal nutrient solution of the following composition (according to Bollons & Barraclough 1997) was used: 3 mM nitrogen, 3 mM potassium, 1·5 mM calcium, 0·3 mM magnesium, 0·3 mM sulphur, 100 µM iron, 50 µM boron, 10 µM manganese, 1 µM zinc, 1 µM copper and 0·5 µM molybdenum (supplied as Ca(NO\textsubscript{3})\textsubscript{2}, KCl, MgSO\textsubscript{4}, FeNa – EDTA, H\textsubscript{2}BO\textsubscript{3}, Mn, Zn and Cu SO\textsubscript{4}, and (NH\textsubscript{4})\textsubscript{2}MO\textsubscript{2}O\textsubscript{2}, respec-
Phosphorus treatments

For the first 5 d of hydroponic culture all plants were grown with adequate P (0·3 mM), supplied as KH$_2$PO$_4$. Seeds were then removed from all the young shoots. For P starvation plants were supplied with medium from which the 0·3 mM KH$_2$PO$_4$ had been omitted for 5 d prior to harvest.

Root hair preparation

Approximately 300 wheat seeds were surface-sterilized and plated on a square of nutrient-saturated muslin supported on a metal mesh. The mesh-supported seeds were placed over a layer made up of three sheets of Whatman no. 3 paper (Whatman plc, Maidstone, Kent, UK) soaked in nutrient solution containing 0·3 mM KH$_2$PO$_4$, in the bottom of a large plastic box under sterile conditions. Germination occurred at 22 °C, under constant darkness. On day 3 the mesh was lifted approximately 6 mm above the surface of the Whatman paper. This allowed the seedling root to grow vertically in the free space and resulted in maximal root hair formation (as described in Bucher et al. 1997). The mesh was similarly raised a few millimetres higher on each subsequent day. Around day 5, the seeds were carefully removed from the germinated seedlings. For P starvation, one box of seedlings was transferred to filter paper soaked in nutrient solution deprived of KH$_2$PO$_4$, following seed removal, and starved for 5 d. Roots were harvested on day 10. Root hairs were isolated from the recovered roots essentially as described by Bucher et al. (1997).

RNA isolation

Tissue was gently ground to a powder under liquid nitrogen and 0·2 g removed to a 2 mL tube containing 1·5 mL Trizol (Sigma Tri Reagent; SigmaAldrich Co. Ltd, Poole, Dorset, UK) and mixed for 30 s. After 5 min incubation at room temperature the suspension was centrifuged at 12 000 × g for 10 min and the supernatant was removed into a clean 2 mL tube. An equal volume of chloroform was added and the phases vortexed for 15 s and incubated for 5 min at room temperature. The phases were separated by centrifugation at 4 °C for 15 min at 12 000 × g and the aqueous layer removed to a clean 2 mL tube. An equal volume of chloroform/isoamylalcohol was added and the aqueous layer separated as before. A one-tenth volume of 3·0 M sodium acetate and 0·6 volume of isopropanol were added and after 10 min incubation at room temperature the RNA was pelleted by centrifugation at 12 000 × g at 4 °C for 10 min. The pellet was washed twice with 70% alcohol and air-dried. The RNA was subsequently re-suspended in 50 µL diethylene pyrocarbonate (DEPC)-treated water and stored at −80 °C until required.

Isolation of phosphate transporter sequences from wheat

Based on known high-affinity P transporter sequences, degenerate polymerase chain reaction (PCR) primers were designed to highly conserved regions identified by sequence alignments. A combination of one of two upstream primers (5′-TTY TTY CAN GAY GCN TAY GAY-3′ and 5′-GTT CCN GCC GGN TAY TGG TTY CAN GT-3′) and a downstream primer (5′-GGN CCR AAR TTN GCR AAR AA-3′) were chosen for PCR. These primers were used for individual PCR reactions at a concentration of approximately 300 pmol. The Chinese Spring wheat, root-specific cDNA library was a kind gift from David Lonsdale at the John Innes Centre, and was used as template for some PCR reactions. Alternatively, high-quality first-strand cDNA (generated from small quantities of total RNA isolated from roots and root hairs of Xiaoyan 54 and experimental line 81(85)-5-3-3) synthesized using a SMART$^{\text{TM}}$ cDNA synthesis kit (BD Clontech Ltd, UK), PCR buffer supplemented with 1·0 mM dNTPs and 7 ×10$^{8}$ pfu cDNA library, 2 U Taq polymerase (AmpliTag; Perkin Elmer Life Sciences Ltd, Cambridge, UK), RNA isolated from roots and root hairs of Xiaoyan 54 and experimental line 81(85)-5-3-3. Seeds were supplied with medium from which the 0·3 m KH$_2$PO$_4$ had been omitted for 5 d prior to harvest.

5′ and 3′ Rapid amplification of cDNA ends

For 5′-rapid amplification of cDNA ends (RACE) reactions first strand cDNA was synthesized using a gene-specific primer and Superscript II$^\text{®}$ RT (Invitrogen), following the manufacturers recommended protocol. After first strand cDNA synthesis the original mRNA template was removed by treatment with RNase H, and unincorporated dNTPs, primer and proteins were separated from the cDNA using a High Pure PCR product purification kit (Roche Diagnostics Ltd, Lewes, East Sussex, UK). A homopolymeric tail was then added to the 3′-end of the cDNA using terminal deoxynucleotidyl transferase TdT (Invitrogen) and dCTP. PCR amplification was accomplished using Taq DNA polymerase (Promega, Southampton, UK), a nested, gene specific primer and a deoxyinosine-containing dG-anchor.
primer (5'-GGC CAC GCG TCG ACT AGT ACG GGI IGG II GGG GGG-3'). For 3'-RACE reactions first strand cDNA synthesis was initiated at the poly(A) tail of mRNA using a dT adapter primer (5'-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT-3'). Following first strand cDNA synthesis, PCR amplification was performed using a gene-specific primer that annealed to a site located within the cDNA molecule, and an universal amplification primer (5'-GGC CAC GCG TCG ACT AGT AC-3') that targeted the mRNA of the cDNA complementary to the 3' end of the mRNA. The 5'- and 3'-RACE products were cloned using a TOP-TOA cloning kit and TOP10F' chemically competent E. coli (Invitrogen).

**Isolation of full-length clones**
Reverse transcriptase (RT)-PCR, using gene specific primers designed to full-length coding sequences, was performed on cDNA isolated from P-induced roots.

**Sequence analysis**
Sequencing was performed using a BigDye® Terminator Cycle Sequencing Kit and an ABI Prism 310 Genetic Analyzer (Perkin-Elmer). Sequence analysis was carried out with the University of Wisconsin GCG package (Version 10.2, Genetics Computer Group (GCG), Madison, WI, USA) and ExPaSy Translate Tool (Swiss Institute of Bio-Informatics, Geneva, Switzerland).

**Amino acid sequences**
Amino acid sequences of putative high-affinity P transporters were aligned using CLUSTAL X (Thompson, Higgins & Gibson 1994) followed by manual editing of the multiple sequence (msf) file. Alignments were displayed using ESPript [http://prodes.toulouse.inra.fr/ESPr ipt/cgi-bin/nph-ESPript.exe.cgi].

**Phylogenetic tree construction**
Phylogenetic analysis was carried out using PROTDIST, FITCH, CONSENSE, RETREE and Phylodendron (D.G. Gilbert, Biology Department, Indiana University) [http://iubio.bio.indiana.edu/tre eapp/treeprint-form.html]. The phylogenetic analysis was subjected to 100 bootstrap replicates.

**Relative quantitative reverse-transcription-PCR**
For P transporter sequences, first strand cDNA synthesis was performed on 1 µg total RNA as template using SuperScript II® RT (Invitrogen) and 5 pmol of antisense primer (5'-GGN CCR AAR TTN GCR AAR AA-3'), following the manufacturers recommended protocol. For 18S RNA reactions the antisense primer (5'-CAC TTC ACC GGA CCA TTC AAT CG-3') was used for first strand synthesis using 0.5 µg total RNA as template. PCR was carried out using one-twentieth volume of first strand cDNA, 2.5 U Tag DNA polymerase (Promega), PCR buffer supplemented with 1.5 mM MgCl2 (MBI Fermentas), and nucleotide concentrations as recommended by the supplier (Amersham Pharmacia Biotech Inc). Sense and antisense primers were used at a final concentration of 200 pmol. Reactions were performed on an Omnigen Thermal Cycler (Hybaid) programmed to give a temperature profile of 2 min at 94°C followed by 40 cycles of 30 s at 94°C; 30 s at 50–62°C (depending on the primer annealing temperatures); 1 min or 1 min 30 s at 72°C (depending on the length of product) and a final 5 min extension at 72°C. PCR products were analysed on a 1% (w/v) TAE-agarose gel, using a GeneRuler® 1 kb DNA ladder (MBI Fermentas). Gels were visualized using an Eagle-Eye II system (Stratagene, La Jolla, CA, USA).

**RESULTS**

**Growth of plant material in hydroponics**
Plant high-affinity P transporters have a pH optimum of between 5.0 and 6.0 (Schachtman, Reid & Ayl ing 1998) indicating a preferential uptake of H2PO4– (predominant in media buffered below pH 6.0), but the pH of soils found in the wheat-growing regions of China are ~7.5. None the less, hydroponic studies were carried out in media buffered at pH 5.7 because a further increase in alkalinity of the medium would lead to precipitation of P (and possibly other essential micronutrients). Furthermore, there is no evidence to suggest that the pH optimum of the P transport system in the Chinese wheat plants would be affected by an alkaline soil environment. It is more likely that as has previously been reported for corn roots (Sentecne & Grignon 1985), the rate of P uptake into the roots will correlate with the pH found within the 'micro' environment of the cell wall and not of the external medium.

During growth in hydroponics at pH 5.7 the root system of Jing 411 was seen to be markedly shorter in comparison with those of the three other wheat varieties under study (Fig. 3). This observation held true for both P-replete and P-starved plants. Shoot lengths appeared to be unaffected in Jing 411. All varieties showed a comparative increase in root length on being starved of P nutrient.

**Isolation of high-affinity phosphate transporter homologues from wheat and wheatgrasses**
Using degenerate primers coupled with PCR we obtained a number of putative high-affinity P transporter fragments from various wheats (Accessions AJ344240 – AJ344249) and wheatgrasses (Accessions AJ413955 – AJ413964), together with a number of different isoforms for each transporter (Fig. 4). The size of the cDNA fragments obtained ranged from the approximately 150 bp (50 aa) shown in Fig. 4 to approximately 1-1 kb (370 aa).

Comparisons of the predicted protein sequences by
Figure 3. Root lengths of phosphate sufficient (+P) and phosphate starved (−P) plants of Xiaoyan 54, 81(85)-5-3-3-3, Lovrin 10 and Jing 411 grown in hydroponic culture as described in the Materials and Methods section. Also shown is the mean of individual measurements done (with standard deviations) on four separate plants.

<table>
<thead>
<tr>
<th>Variety</th>
<th>+P Roots (cm)</th>
<th>+P Shoots (cm)</th>
<th>−P Roots (cm)</th>
<th>−P Shoots (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Xiaoyan 54</td>
<td>30.75 ± 1.71</td>
<td>22.52 ± 2.32</td>
<td>33.50 ± 1.29</td>
<td>21.93 ± 1.70</td>
</tr>
<tr>
<td>2) 81(85)-5-3-3-3</td>
<td>30.00 ± 4.08</td>
<td>20.13 ± 2.78</td>
<td>34.00 ± 2.31</td>
<td>19.32 ± 0.78</td>
</tr>
<tr>
<td>3) Lovrin 10</td>
<td>32.25 ± 4.35</td>
<td>24.25 ± 2.63</td>
<td>38.00 ± 5.66</td>
<td>24.25 ± 1.85</td>
</tr>
<tr>
<td>4) Jing 411</td>
<td>12.00 ± 4.58</td>
<td>23.17 ± 2.02</td>
<td>13.75 ± 2.50</td>
<td>21.88 ± 0.85</td>
</tr>
</tbody>
</table>

Figure 4. ESPript 2 alignment of high-affinity P transporter sequences obtained by PCR. The aligned sequences span the C-terminal end of TM8 through to the middle of TM10 of the protein. These fragments were obtained from a Chinese Spring wheat (CSW) root-specific cDNA library, from cDNAs isolated from roots or root-hairs (rh) of the Chinese wheats Xiaoyan 54 (X54) and 81(85)-5-3-3-3 (81–85), or genomic DNA isolated from *Thinopyrum elongatum* (The) and *Thinopyrum intermedium* (Thi).
CLUSTAL X alignments with other known high-affinity P transporters and subsequent phylogenetic analysis revealed the existence of three distinct clusters of putative high-affinity P transporters in higher plants (Fig. 5).

**Cluster I**

This cluster is made up entirely of putative P transporters isolated from cereals and wheatgrasses belonging to the family Poaceae. A total of four cDNA fragments coding for isoforms of either TaPT1 or TaPT2 were isolated from roots of line 81(85)-5-3-3-3. These four cDNAs had virtually identical amino acid sequences, but varied in their coding sequence (data is not shown). The wheat P transporters TaPT2 (Accession AJ344240, isolated from 81(85)-5-3-3-3), TaPT3 (AJ344243, isolated from Chinese Spring wheat) and TaPT4 (AJ344244, also isolated from Chinese Spring Wheat) have sequence identity in common with another Chinese Spring Wheat transporter CswPT1, rice OsPT1 and maize ZmPT1-4. 

**Figure 5.** Phylodendrogram showing the relationship between high-affinity P transporters isolated from dicots (*Arabidopsis thaliana* AtPT1 (Q96302), APT1 (Q96243), APT2 (Q96303), APT4 (O04381), APT5 (O50040), APT6 (Q9ZWT3), APT7 (Q9S735), APT8 (unassigned) and APT9 (Q9MIT0), *Catharanthus roseus* (periwinkle) PTT1 (O22055), *Lupinus albus* (white lupin) LaPT1 (Q9AR19) and LaPT2 (Q9AU00), *Lycopersicon esculentum* (tomato) LePT1 (O22548) and LePT2 (O22549), *Medicago truncatula* (Medic Barrel) MtPT1 (O22301) and MtPT2 (O22302), *Nicotiana tabacum* (tobacco) NtPT1 (Q9ST22), NtPT2 (Q9LS55), NtPT3 (Q9AYT2) and NtPT4 (Q9AYT1), *Sesbania rostrata* SrPT1 (Q9AVR0) and SrPT2 (Q9AVQ9), *Solanum tuberosum* (potato) StPT1 (Q43650), StPT2 (Q41479) and StPT3 (CAC87043)) and monocots (*Oryza sativa* (rice) OsPT (Q9DB85) and OsPT1 (Q9M562), *Triticum aestivum* (wheat) CswPT1 (Q9XEL6), *Zea mays* (maize sequences from patent No. WO9958657)) with sequences isolated in this study. Clones marked with an * are not full-length (* approximately 50 aa, ** 50–150 aa, *** 350–400 aa). The distribution and relationships of P transporter family members on the tree are virtually identical to the tree distribution obtained using an alignment made up exclusively of the short conserved 50 aa regions of all the proteins (as shown in Fig. 4), using the same parameters for phylodendrogram construction (data not shown). For *Arabidopsis* the nomenclature found in the database is often confusing with single clones being referred to by different names. For the purpose of this figure we have used the format AtPT1 = APT2 = PHT1; APT1 = PHT2; AtPT2 = PHT4; AtPT4 = PHT3; AtPT5 = PHT5; AtPT6 = PHT6. AtPT7, AtPT8 and AtPT9 were numbered according to database submission dates.
atumin (The) and Thinopyrum intermedium (Thi) are all found within this cluster. The-15 (Accession AJ413955), The-17 (AJ413956) and Thi-9 (AJ413963 and AJ413964) are similar to the wheat TaPT1 and TaPT2 sequences. The-19 (AJ413957), Thi-1 (AJ413958) and Thi-4 (AJ413961) are similar (but not identical) to CswPT1 and TaPT6. Thi-2 (AJ413959) and Thi-7 (AJ413962) have the closest sequence homology with TaPT3 and TaPT4. Thi-3 (AJ413960) is a unique sequence and is the most distantly related of all the Thionopyrum sequences characterized.

Cluster II

This cluster contains putative P transporter-like sequences from rice, wheat, maize, and Arabidopsis thaliana. The wheat sequence TaPT5·1 isolated from experimental line 81(85)-5-3-3-3 appears to be novel. Sequencing of a 1·2 kb fragment of this clone (spanning from the 3′-end of TMS1 to the middle of TMS10) revealed a cDNA (Accession AJ344245) bearing only 50% identity with known high-affinity P transporter sequences but having several conserved structural features, including the membrane-spanning regions, the large central loop and phosphorylation motif(s) in common with other P transporters, coupled to unique external loops. Outside, loops 1 and 3 are shortened by 6 and 14 amino acids, respectively, so the N-terminal half of the protein has very little structure exposed to the outside. Two strategically placed cysteine residues which form part of an unique 16 amino acid extension HDDEVCKVNTCQVARY on external loop4 of the TaPT5·1 protein may be capable of forming disulphide bonds. Maize ZmPT5, which is most closely related to TaPT5, has a GLN/ALA rich repeat at its C-terminus; such repeat sequences are often found to be involved in transcriptional activation or repression. This region of TaPT5·1 has not yet been characterized. TaPT5·2 (Accession AJ344246, also isolated from line 81(85)-5-3-3-3), is a closely related isoform of TaPT5·1 and has similar structural characteristics. The 16 amino acid extension HDDEVCKVNSCQGASY on external loop 4 is also highly conserved.

Cluster III

This cluster is made up almost entirely of known dicotyledon sequences, and includes putative high-affinity P transporters from the legumes Lupinus albus, Medicago trunculata and Sesbania rostrata (all members of the Fabaceae family), tomato, tobacco and potato (belonging to the Solanaceae), and Arabidopsis (a member of the Brassicaceae).

The root hair isolate TaPT7 (Accession AJ344248) isolated from Xiaoyan 54 is seen to be similar to LePT2 isolated from P-starved roots of tomato (Liu et al. 1998). A second parologue of this transporter was also isolated from root hairs of Xiaoyan 54 (not shown). TaPT8 (Accession AJ344249, also isolated from root hairs of Xiaoyan 54) appears to be a novel fragment, having an 8 amino acid extension KCGDSFCD containing two cysteine residues on external loop5 (see Fig. 4). It appears to be most closely related in evolutionary terms to potato SPT3.

Isolation of full-length clones of TaPT2 from Xiaoyan 54 and experimental line 81(85)-5-3-3-3

Full-length TaPT2 from Xiaoyan 54 (accession AJ344240) was isolated using 5′ and 3′ RACE (primers used shown in Table 1) and encodes a novel 525 amino acid protein (Fig. 6). Southern analysis using full-length TaPT2 as probe revealed two fragments, indicating at least two copies of this gene can be found in Xiaoyan 54 (data not shown). Two independent full-length cDNA clones were obtained from 81(85)-5-3-3-3 (accessions AJ344241 and AJ344242), using gene-specific primers and RT-PCR (see Table 1). Accession AJ344241 differed from the full-length TaPT2 coding sequence isolated from Xiaoyan 54 by only one amino acid (L119F), whereas accession AJ344342 had five amino acid substitutions (positions L119F, I185V, M228L, T321S, L323F), which are similar (but not identical) to those found in Tho-5. 1998). A second parologue of this transporter was also isolated from root hairs of Xiaoyan 54 (not shown). TaPT8 (Accession AJ344249, also isolated from root hairs of Xiaoyan 54) appears to be a novel fragment, having an 8 amino acid extension KCGDSFCD containing two cysteine residues on external loop5 (see Fig. 4). It appears to be most closely related in evolutionary terms to potato SPT3.

Expression of high-affinity phosphate transporters in Chinese wheats and wheathgrasses

Relative quantitative reverse-transcription (RQRT)-PCR was used to determine the expression of individual P transporters in each wheat variety relative to the constitutively expressed 18S rRNA (Figs 7 and 8).

Cluster I transporters

The P transporters TaPT2 and TaPT3 are most strongly expressed in the roots of all wheat varieties studied (Fig. 7).

Table 1. Gene specific primers used in 5′ and 3′ RACE and in RT-PCR reactions to isolate full-length clones of the putative high-affinity P transporter TaPT2

<table>
<thead>
<tr>
<th>Gene specific primer</th>
<th>Nested gene specific primer</th>
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<tbody>
<tr>
<td>5′- RACE 5′-GGN CCR AAR TTN GCR AAR AA-3′</td>
<td>5′-TCG CCG GCG GCC ATG GCG-3′</td>
</tr>
<tr>
<td>3′- RACE 5′-GCA TGT CTC TAG ACA CCA GTC GG-3′</td>
<td></td>
</tr>
<tr>
<td>RT-PCR 5′-TCG CCG GCG GCC ATG GCG-3′</td>
<td></td>
</tr>
<tr>
<td>5′-GCA TGT CTC TAG ACA CCA GTC GG-3′</td>
<td></td>
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Figure 6. Full-length sequence of TaPT2 cDNA (accession AJ344240) isolated from Xiaoyan 54, coding for a 525 amino acid protein. HMMTOP 2.0 (Tusnady & Simon 2001) predicts 12 trans-membrane spanning domains (underlined), cytoplasmically oriented N- and C-terminii and a large, internally oriented central loop.
TaPT6 expression in contrast is more uniformly distributed between roots and leaves in the three P-efficient varieties/lines under study.

Whereas Jing 411 only expresses TaPT2 in roots under P deprivation, the three selected varieties also show some expression under conditions of high external P. The expression of TaPT2 in roots of the efficient varieties does not therefore appear to be as tightly regulated by P supply. TaPT6 expression shows some increase with P-deprivation in the selected wheat varieties, especially in line 81(85)5-3-3-3. In Jing 411 there is a significant increase of expression of TaPT6 in roots (but not shoots) of P-starved plants, and the pattern of TaPT6 expression is virtually the same as that seen for TaPT2 in this variety.
Significantly different patterns of PT2 expression occurred in the wheatgrasses, where the wheatgrass orthologue(s) of \( \text{TaPT2} \) are expressed strongly only under conditions of high external P (Fig. 7). A significant down-regulation of wheatgrass \( \text{PT2} \) expression must therefore occur when the plants are transferred into P-deficient nutrient solutions. This is entirely contrary to what has been observed for plant species previously studied. This strongly suggests that wheatgrass roots may adopt a different strategy for P exploitation compared with roots of cereal wheats. Expression patterns of the wheatgrass orthologue(s) of \( \text{TaPT3} \) mimicked those found in the wheat varieties, and were consistently the same for all the plants studied. \( \text{TaPT6} \) orthologues were not expressed at detectable levels in the wheatgrasses under the conditions studied.

**Cluster II transporters**

\( \text{TaPT5} \) and \( \text{TaPT5·2} \) are detectable in the experimental line 81(85)-5-3-3-3, but not in Xiaoyan 54, Lovrin 10 and Jing 411. Interestingly the two homologues have distinctly different patterns of expression in 81(85)-5-3-3-3, \( \text{TaPT5} \cdot 1 \) mRNA being constitutively expressed at similar levels in both roots and leaves of both P-replete and P-starved plants, whereas \( \text{TaPT5·2} \) expression is only seen in roots of P-starved plants (Fig. 8). This suggests that the homologues may be under the control of different promoters, only one of which is responsive to internal or external P concentrations.

**Cluster III transporters**

Neither \( \text{TaPT7} \) nor \( \text{TaPT8} \) expression was detectable in roots or shoots of any of the wheat/wheatgrass varieties grown hydroponically (data not shown). This may be due to the fact that they may only be expressed in root hairs, which are not present on plant roots grown in hydroponics. However, a subsequent attempt at amplifying these transporters from P-starved root hair isolates was also unsuccessful. One possible explanation for the absence of these transporters in the plants studied may be the timing of induction of different high-affinity P transporters in response to P starvation, which may be transient and short-lived and therefore not picked up within the narrow time window of 4 d post-starvation looked at in this study.

### DISCUSSION

**Phosphate efficiency in the wheat lines under study**

Xiaoyan 54, Lovrin 10 and experimental line 81(85)-5-3-3-3 all appear to be able to grow in soil conditions having low available P without their yield being significantly affected. In contrast, Jing 411 suffers a major loss of yield when grown in the same soils. The mechanism(s) adopted by Xiaoyan 54 and Lovrin 10 to provide this yield efficiency is currently postulated to involve a better ability to cope with low availability of soil P at an early (critical) stage of growth, possibly through better utilization of P reserves found in the seed, or by being more efficient at acquiring P from the soil, or a combination of both. Wheat plants with high seed P reserves have previously been shown to be capable of accumulating more P from the soil, this enhanced uptake being attributed to better root system development (Zhu & Smith 2001). In the present study it

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**Table 2.** Primers used for individual RQRT-PCR reactions to determine the expression of individual P transporters in each wheat variety relative to the constitutively expressed 18S rRNA.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense primer</th>
<th>Anti-sense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S RNA</td>
<td>5′-GAT AAG ATC GGA AGG TTT ACC-3′</td>
<td>5′-CAC CAC CAC GAA CCC-3′</td>
</tr>
<tr>
<td>( \text{TaPT2} )</td>
<td>5′-TGG TCC TCG ACC TCA TCG GCC AC-3′</td>
<td>5′-AAC ATC TCT CTC ACT GTG GG-3′</td>
</tr>
<tr>
<td>( \text{TaPT3} )</td>
<td>5′-GGC TTT ATG ATA ACT GGC CC-3′</td>
<td>5′-GTC TTC CTC ATC GAC GTC G-3′</td>
</tr>
<tr>
<td>( \text{TaPT4} )</td>
<td>5′-GTC TGC GAC CTC ATC AGC CAA-3′</td>
<td>5′-GGC TTG ATG ATA ACT GGC CC-3′</td>
</tr>
<tr>
<td>( \text{TaPT5·1} )</td>
<td>5′-CGA CCC GAG CTC AGC CGC C-3′</td>
<td>5′-GTC TTC CTC ATC GAC GTC G-3′</td>
</tr>
<tr>
<td>( \text{TaPT5·2} )</td>
<td>5′-TGG TCC TCG ACC TCA TCG GCC AC-3′</td>
<td>5′-AAC ATC TCT CTC ACT GTG GG-3′</td>
</tr>
<tr>
<td>( \text{TaPT6} )</td>
<td>5′-GGC TTT ATG ATA ACT GGC CC-3′</td>
<td>5′-GTC TTC CTC ATC GAC GTC G-3′</td>
</tr>
<tr>
<td>( \text{TaPT7} )</td>
<td>5′-GCC TGT ACC TCT GCT GCC CTC-3′</td>
<td>5′-CAC CAC CAC GAA CCC-3′</td>
</tr>
<tr>
<td>( \text{TaPT8} )</td>
<td>5′-GTC TGC GAC CTC ATC AGC CAA-3′</td>
<td>5′-AAC ATC TCT CTC ACT GTG GG-3′</td>
</tr>
</tbody>
</table>

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**Figure 8.** Expression patterns of \( \text{TaPT5·1} \) and \( \text{TaPT5·2} \) in roots and leaves of experimental line 81(85)-5-3-3-3 relative to the constitutively expressed 18S rRNA. Conditions used were essentially as described in the legend to Fig. 7.
was noted that during growth in hydroponics the root system of Jing 411 was markedly shorter in comparison with those of the three other wheat varieties under study. A recent study with Jing 411 and Xiaoyan 54 demonstrated that the latter variety has higher root : shoot ratios, higher relative yields, and allocates higher percentages of assimilated carbon, soluble sugar and absorbed P to its roots under P deficiency so that it can maintain relatively high root-growth rates (Zou et al. 2002). This would result in an enhancement of the total surface area available for soil exploration and P acquisition. Xiaoyan 54 also releases more assimilated carbon as root exudates under P deficiency. It seems likely that shallow root architecture may also play a significant role in the ability of the efficient varieties to acquire P from the soil (Fu-Suo Zhang, personal comm.). Because soil P availability almost invariably decreases with soil depth, the shallow angle of root growth of P-deficient plants can be viewed as an adaptive response allowing increased P uptake (Bonser, Lynch & Snapp 1996; Nielsen et al. 1998; Ge, Rubio & Lynch 2000; Yan et al. 2001).

As well as good adaptation of root systems, another highly significant factor in responding to variable P availability in soils is the uptake rate per unit root (Egle et al. 2000). A dual uptake model for ions is generally evoked, characterized by the possession of a high-affinity transport system operating at low (μM) concentration and a low-affinity transporter functioning at high (mM) concentrations. In general, the low-affinity system appears to be expressed constitutively in plants, whereas the high-affinity uptake system is regulated by the availability of P. The results of many P uptake experiments confirm that plants adjust their P uptake based on their internal P status, particularly by increasing their maximum influx or rate of P absorption ($I_{\text{max}}$), whereas changes in $K_e$ and $C_{\text{m}}$ are of minor importance in this process. The capacity for P uptake by high-affinity P transporters can thus be enhanced either by increasing the driving force (e.g. protons or membrane potential) or by increasing the total number of high-affinity transporter molecules.

**Phosphate transporter expression in the wheat lines**

The existence of multiple high-affinity P transporters in plants, coupled to the size and complexity of the wheat means that conclusive analysis of the role of P transporters in uptake efficiency in wheat is a challenging undertaking. However in this study we have identified several putative wheat P transporters some of which are related to transporters previously isolated from maize, *Arabidopsis* or tomato, but others that are unique.

We have identified two transporters *TaPT2* and *TaPT3* that are potential homologues of the barley high-affinity P transporters *HvPT1*, *HvPT2* and *HvPT3* reported by Smith, Cybinski & Rae (1999) in both the wheats and wheatgrasses. Significantly, the expression of *TaPT2* in the roots of the efficient wheat varieties under study does not appear to be tightly regulated by P supply. In contrast, the expression of this transporter in barley, and in the Chinese wheat variety Jing411 (which is less tolerant to external changes in P), is P-starvation dependent. This difference in the expression pattern of *TaPT2* may be a reflection of the enhanced ability of the three varieties to generate adequate yields in low P soils, and may provide a clue to the P nutrition mechanism(s) adopted by these plants to maintain their yields. Intriguingly, it has recently been demonstrated in barley that Zn plays a specific role in the signal transduction pathway responsible for the regulation of genes encoding high-affinity P transporters in plant roots (Huang et al. 2000). Normally expression of *HvPT1* responds to the P status of the plant. It was however, observed that under conditions of Zn deficiency this tight control is lost, leading to very high accumulation of P in plants. This observation may give an important insight into the possible mechanism whereby plants may modify expression of their P transporters in response to external nutrient stressors.

In the wheatgrasses the expression of the *TaPT2* orthologues is quite different to that found in wheats, the transporters being strongly expressed only under conditions of relatively high external P. It is thought that *Thiinopyrum* plants (*elongatum* and *intermedium*) may respond to heterogeneously distributed P in the soil by enhancing uptake in localized, relatively P-rich patches. This would explain the expression pattern of the high-affinity P transporter *PT2* in roots of these plants. Such a strategy would require an extensive root system able to seek out such P-enriched patches. The three wheat lines under study may possibly have acquired a similar strategy superimposed on top of a P-deficiency responsive one. This would also explain the unusual expression patterns of *TaPT2* observed in these varieties. To add further complexity to the overall picture, expression patterns of *TaPT3* (and its orthologues) were consistently the same in roots of all the plants studied.

Although we have demonstrated that the wheatgrasses have P transporter sequences that are similar to those found in the wheats from China, none of the sequences isolated from the wheatgrasses are identical at the gene level to the Chinese wheat sequences. No transfer of P transporter genes between the wheatgrasses and the wheats under study can be inferred to have taken place during the various chromosome translocation events, based on this data. The possibility of such a gene transfer having occurred however, cannot be completely ruled out as our survey of the P transporters that occur in each species has not been exhaustive. The possibility still remains therefore that transfer of a P transporter gene(s) could contribute to P efficiency in the wheats harbouring alien inserts.

The root hair isolate *TaPT7* (from P-starved root hairs of Xiaoyan 54) is similar in sequence composition to *LePT2* isolated from tomato roots (Liu et al. 1998) and is likely to be a *LePT2* homologue. In tomato roots, *LePT2* expression is induced during P starvation, the *LePT2* transcripts being exclusively localized to rhizodermal cells. A second root hair isolate *TaPT8* (also from P-starved root hairs of Xiaoyan 54) has similarities to the potato P transporter...
A recent study has indicated preferential expression of \( StPT3 \) in root sectors where mycorrhizal structures are formed (Rausch et al. 2001). Expression of high-affinity \( P \) transporters in root hairs is a logical strategy as this would result in better rhizosphere exploitation and rapid \( P \) uptake. For wheat and rye it has been shown that root hairs can participate substantially in uptake of \( P \) from the soil, and can satisfy more than 60% of the plants \( P \) demand (Gahoonia & Nielsen 1996, 1998). This is not surprising because root hairs substantially extend the root surface area for uptake. No expression of \( TaPT7 \) or \( TapT8 \) in roots or roots hairs of wheat was detectable by RT-PCR within the time-window (4 d post-starvation) of this study.

Three sequences with unusual amino acid extensions were also identified. The sequences were similar in overall structure to known high-affinity \( P \) transporters. It is tempting to postulate that this sequence conservation would indicate a similar conservation of function and a role for these proteins in \( P \) uptake. This argument is strengthened by the observation that \( TaPT5-2 \) is induced by \( P \)-starvation in roots of experimental line 81(85)-5-3-3-3. The unusual amino acid extensions, all of which contained a CX4C motif, are predicted to be located outside of the membrane. Such exposed cysteine motifs may indicate a regulatory binding site on the proteins and a role for these transporters in \( P \) regulation. The presence of an 8 amino acid extension \( KCGDSFCD \) reminiscent of a zinc-binding domain on external loop 5 of \( TaPT8 \) and the isolation of this cDNA from root hairs indicates a possible role for this protein in environmental sensing. The constitutive expression of \( TaPT5-1 \) in leaves and roots and the induced expression of \( TaPT5-2 \) in \( P \)-starved roots may implicate the 16 amino acid extension \( HDDEVCKVN[T/S]CQ[V/G][A/R/S]Y \) (found on external loop 4 of the proteins) as being involved in internal \( P \) signalling within the plant. Further studies are clearly needed on these unusual proteins in order to understand the significance of the CX4C motifs in these amino acid extensions.

Some of the other as yet uncharacterized high-affinity transporters isolated in this study may participate in the intracellular movement of \( P \). Others may be involved in scavenging \( P \) that has leaked into the apoplast. Under natural conditions, plant growth rate is normally adjusted to the availability of the nutrient. Reduced rate of growth leads to redistribution of \( P \) within the plant, thus allowing plants to overcome brief periods of \( P \) starvation without a major effect on growth. Transfer of \( P \) from roots to shoots is reduced and \( P \) in older leaves is remobilized to younger growing leaves and other active sinks.

CONCLUSIONS

Wheat plants probably have several different morphological and physiological adaptations that enable them to acquire and more efficiently utilize \( P \) from sparingly soluble soil fractions. The role of high-affinity \( P \) transporters in these adaptation processes remains unclear, but the current study has clearly demonstrated interesting differences in the expression pattern of such transporters in the selected wheat varieties when grown in hydroponic culture. Further studies are needed to see whether these results can be extrapolated to the field. Unusual patterns of expression of at least one \( P \) transporter in the wheatgrasses \( Thinopyrum elongatum \) and \( Thinopyrum intermedium \), used as donors of chromosome fragments to the wheat’s Xiaoyan 54 and 81(85)-5-3-3-3, also warrant further study.

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