

Isolation and comparative analysis of the wheat *TaPT2* promoter: identification *in silico* of new putative regulatory motifs conserved between monocots and dicots

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Abstract

Phosphorus deficiency is one of the major nutrient stresses affecting plant growth. Plants respond to phosphate (Pi) deficiency through multiple strategies, including the synthesis of high-affinity Pi transporters. In this study, the expression pattern of one putative wheat high-affinity phosphate transporter, *TaPT2*, was examined in roots and leaves under Pi-deficient conditions. *TaPT2* transcript levels increased in roots of Pi-starved plants. A 579 bp fragment of the *TaPT2* promoter is sufficient to drive the expression of the GUS reporter gene specifically in roots of Pi-deprived wheat. This *TaPT2* promoter fragment was also able to drive expression of the GUS reporter gene in transgenic *Arabidopsis thaliana*, under similar growth conditions. Conserved regions and candidate regulatory motifs were detected by comparing this promoter with Pi transporter promoters from barley, rice, and *Arabidopsis*. Altogether, these results indicate that there are conserved *cis*-acting elements and *trans*-acting factors that enable the *TaPT2* promoter to be regulated in a tissue-specific and Pi-dependent fashion in both monocots and dicots.

Key words: *Arabidopsis thaliana*, high affinity phosphate transporter, promoter, *Triticum aestivum*.

Introduction

Phosphorus is involved in several key biological processes such as metabolism, respiration, photosynthesis, enzyme regulation, and signalling. Phosphorus is also a structural component of many macromolecules (Eastmond and Graham, 2003; Kruger and von Schaewen, 2003; Meijer and Munnik, 2003; Fernie *et al.*, 2004; Chen *et al.*, 2005; Peñaloza *et al.*, 2005). The bioavailability of phosphate (Pi) in the soil is a worldwide problem for plant growth and increased productivity. Pi is often found in nature associated with metals, which decreases its bioavailability (Ticconi and Abel, 2004). A common agricultural solution to the problem of Pi bioavailability is to enrich the soil with bioavailable Pi fertilizers. However, only about 1% of the Pi applied to the soil is used by plants. Additionally, most of the Pi fertilizers come from phosphoric rocks, a raw material that is expected to be depleted by the end of the century at the present rate of use (Raghothama, 1999).

Plants must maintain intracellular Pi levels at a millimolar range, even when soil Pi concentrations are at a micromolar level (Reisenauer, 1966). To cope with low soil Pi, plants have developed strategies for overcoming Pi starvation. Such strategies involve changes in root architecture (Lopez-Bucio *et al.*, 2003), association with mycorrhiza (Karandashov *et al.*, 2004; Karandashov and Bucher, 2005), expression and secretion of acid phosphatases (Haran *et al.*, 2000), secretion of organic acids

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(Diatloff *et al.*, 2004), induction of microRNAs (Fujii *et al.*, 2005), and synthesis of high-affinity phosphate transporters (Karthikeyan *et al.*, 2002).

The molecular mechanisms that coordinate this global Pi deficiency response are not well understood. However, it is known to be transcriptionally regulated (Raghothama, 1999). Furthermore, Affymetrix experiments suggest that specific sets of transcription factors are involved in regulating the early and late responses to Pi deficiency in *Arabidopsis* (Misson *et al.*, 2005).

One of the best-conserved adaptations of plants to low Pi supply is the induced expression of high-affinity Pi transporter genes. These are known as Pht1 transporters to distinguish them from the low-affinity Pi transporters which are in the Pht2 family. Structurally, the Pht1 transporters are integral membrane proteins with two groups of six membrane-spanning segments linked by a hydrophilic region (Persson *et al.*, 2003). In *Arabidopsis thaliana*, Pht1 transporters are divided into nine groups, several of which are expressed exclusively in roots during phosphate starvation (Mudge *et al.*, 2002).

There is a high level of conservation among the high-affinity Pi transporters of different plants, suggesting that their function has been maintained throughout evolution. However, not all the high-affinity Pi transporters are transcriptionally regulated in the same way. Some are expressed in the presence or absence of Pi, in different tissues, in different cell types, or preferentially expressed in mycorrhizal associations, possibly suggesting an evolutionary divergence of their physiological function. Part of this divergence could be associated with the regulatory non-coding regions of these transporters.

Although the function and regulation of these high-affinity Pi transporters have been well studied in model dicot plants such as *Arabidopsis* (Daram *et al.*, 1998; Mukatira *et al.*, 2001; Misson *et al.*, 2004), they are not well characterized in cereals and other monocots.

Based on its contribution to the food industry, wheat is one of the major worldwide crops and is the main annual crop in Chile (www.odepa.gob.cl). In Chile, however, wheat is usually grown in acidic soils with low Pi bioavailability. This situation is a major problem because young wheat plants need Pi for the development of tillers and spikelets (Romer and Schilling, 1986). To date, several entries for Pi transporters in wheat can be found in public databases, but their function and mechanisms of regulation have not been studied in detail. Davies *et al.* (2002) isolated eight different high-affinity Pi transporters from wheat root cDNA libraries and they analysed the expression levels of some of them in different wheat varieties. They observed that different transporters have different expression patterns within the same variety and also that the same transporter could be expressed differentially in different varieties.

In the present work, the expression pattern of one of these putative high-affinity Pi transporters, *TaPT2*

(GeneBank accession number AJ344241), in wheat (Dalcuhue variety) is examined under phosphate-deficient conditions. A 579 bp fragment was isolated upstream of the first ATG codon of this gene, and it was demonstrated that this sequence can specifically drive the expression of the β -glucuronidase (GUS) reporter gene in wheat roots under Pi-deficient conditions. Interestingly, the isolated promoter can carry out the same regulation in *Arabidopsis thaliana*, suggesting that it contains conserved *trans*-acting factors and *cis*-acting elements that enable this promoter to be regulated in a tissue-specific and Pi-dependent fashion in both dicots and monocots. Bioinformatically, four highly conserved motifs were identified in both the wheat *TaPT2* promoter and the Pi transporter promoters from monocots and *Arabidopsis* that could explain their conserved heterologous regulation.

Materials and methods

Plant growth, transformation, and Pi treatments

Wild-type and transgenic *Arabidopsis thaliana* (ecotype Columbia) were grown in a mixture of soil and vermiculite (3:1) in a growth chamber with a 16 h light cycle ($140 \mu\text{E m}^{-2} \text{s}^{-1}$) at 22 °C. Alternatively, seeds were surface sterilized as described in Gonzalez *et al.* (2006) and grown under the same conditions as soil-grown plants in plates with Murashige–Skoog medium containing 0.8% agar, 0.1% sucrose, and 50 mg l^{-1} kanamycin for transgenic lines.

Transgenic *Arabidopsis* were obtained using the *Agrobacterium tumefaciens*-mediated floral dip method (Clough and Bent, 1998). *Triticum aestivum*, variety Bobwhite, was transformed with the *TaPT2* promoter–*uidA* fusion using biobalistics (Pelligrinineschi *et al.*, 2000) at INIA-Carillanca.

Treatments in the presence of phosphate or under phosphate-deficient conditions were performed to determine the effects on the wheat *TaPT2* transcript levels and the activity of the *TaPT2* promoter–*uidA* fusion. Treatments were performed as described by Peñaloza *et al.* (2002), with the following modifications: *Arabidopsis* and wheat seeds were germinated in 10 ml flasks placed on a shaker and maintained for 5 d in a nutritive solution containing bioavailable Pi [$1 \text{ mM NH}_4\text{NO}_3$, 3 mM KNO_3 , $2 \text{ mM Ca(NO}_3)_2$, 1 mM MgSO_4 , $0.07 \mu\text{M (NH}_4)_6\text{Mo}_7\text{O}_{24}$, $22 \mu\text{M H}_3\text{BO}_3$, $1.6 \mu\text{M ZnSO}_4$, $0.4 \mu\text{M CuSO}_4$, $2 \mu\text{M MnSO}_4$, $20 \mu\text{M Fe EDTA}$, $0.1 \text{ mM KH}_2\text{PO}_4$]. After 5 d, the plants were washed with sterile distilled water and grown in the presence and absence of bioavailable Pi (in 10 ml flasks placed on a shaker). These solutions are identical to the germination solution except that the solution without Pi (Pi-deficient solution) does not contain KH_2PO_4 . The pH was adjusted daily to 5.8. Solutions were maintained sterile and changed every 4 d.

Total RNA extraction

Between 50 mg and 100 mg of tissue was ground to a fine powder in liquid nitrogen. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The final pellet was dissolved in RNase-free water and stored at $-80 \text{ }^\circ\text{C}$. RNA quality and quantity were determined as described by Meisel *et al.* (2005).

Northern blot and reverse transcriptase (RT)-PCR

Northern blot experiments were carried out as described by Silva *et al.* (1999). Total RNA (20 μg), extracted from whole roots or

leaves, was fractionated by electrophoresis in an agarose gel containing 22% formaldehyde and capillary transferred to a nylon membrane (Hybond N⁺; Amersham Biosciences) using 10× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate). The RNA was UV-cross-linked to the nylon membrane using a Spectroliner TS-312A transilluminator. The membrane was prehybridized for 2 h in prehybridization solution [5× SSC, 50 mM sodium phosphate, pH 6.8, 5× Denhart solution (1× Denhart solution: 0.02% Ficoll; 0.02% PVP, 0.02% BSA), 1 mM EDTA, pH 8.0, 1% SDS, 10 g ml⁻¹ salmon sperm DNA]. The membrane was hybridized with the ³²P-labelled probe in hybridization solution (identical to prehybridization solution except that it contains 0.1% SDS) at 65 °C for 16 h. The membrane was subsequently washed with 5× SSC, 0.1% SDS for 15 min at 65 °C and for 30 min with 2× SSC, 0.1% SDS at 65 °C. Radioactivity was detected by exposing the membrane to an autoradiography film for 24 h.

All DNA ³²P-radiolabelled probes were obtained by PCR using the Fermentas Hexalabel kit. The primers used to prepare *TaPT2* and Actin probes are shown in Table 1. An 18S rRNA EST of *Arabidopsis* cloned in a pBluescript vector (H8B10T7), kindly donated by L. Holuigue, was used as a template to prepare a probe against 18S rRNA using the SK and KS primers. The probes were purified using the CONCERT Matrix Gel Extraction System (GIBCO BRL).

RT-PCR experiments were carried out using total RNA extracted from whole roots or leaves. First strand cDNA was synthesized from 2.5 µg of the DNase-treated RNA in a final volume of 20 µl. The reaction mix was prepared using the RevertAid H Minus M-MuLV reverse transcriptase kit (Fermentas, Vilnius, Lithuania) using anchored oligo(dT) of 18-mers according to the manufacturer's instructions. The primers used to amplify the internal regions of *TaPT2* (12F-PT2 and 553R-PT2) are shown in Table 1. The conditions for the PCR are as follows: cDNAs were diluted 100 times and 1 µl of each dilution was used as a template. The reactions were performed in a final volume of 20 µl, containing 0.5 µM of primers, 0.2 mM dNTPs, 2.0 mM MgCl₂, 5 U of *Taq* polymerase, and buffer. The conditions of the PCR were: 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 60 °C for 3 min, 72 °C for 2 min; 72 °C for 5 min.

Cloning of the *TaPT2* promoter

The wheat genomic DNA (*Triticum aestivum* variety Dalcahue) was extracted as described by Das *et al.* (1990) except that 100–300 mg of 3-week-old wheat leaves were used. The Universal Genome Walker™ Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) was used to isolate a 579 bp fragment of the *TaPT2* promoter. Ten 6 bp-recognizing and blunt end-forming restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II, *Stu*I, *Ssp*I, *Sma*I, *Msc*I, *Sca*I, *Eco*105I, and *Hpa*I) were used to digest the isolated genomic DNA. DNA fragments containing the adaptors at both ends were used as templates for amplifying the *TaPT2* promoter region. PT2-GSP1 and PT2-GSP2 specific primers were designed to isolate the promoter (Table 1 and Fig. 2). The first PCR, using AP1 and PT2-GSP1 primers, was

performed using Synergy DNA polymerase (Genecraft, Münster, Germany). The second PCR, using AP2 and PT2-GSP2 primers, was performed using the Advantage Genomic Polymerase Mix (Clontech). The amplified fragment, corresponding to the *Stu*I library, was cloned in the pGEM-T vector (Promega, Madison, WI, USA). The *TaPT2* promoter sequence was amplified using the AP2 and PT2-*Bam*HI primers (Table 1), cloned into the pGEM-T vector, and re-sequenced. The fragment was then released from the pGEM-T with a *Bam*HI-*Sal*I sequential digestion, isolated, and transcriptionally fused to the *uidA* reporter gene in the promoterless binary vector pBI101.1 (Jefferson *et al.*, 1987). The binary vector was introduced into *Agrobacterium tumefaciens* (GV3101) for *Arabidopsis* transformation as described earlier.

Bioinformatics analyses

A phylogenetic tree analysis was performed among *TaPT2* and other plant and fungal Pi transporters (see Supplementary data available at *JXB* online). To identify predicted conserved motifs, approximately 600 bp upstream of the predicted start, codons were analysed using the YMF 3.0 program (Blanchette and Sinha, 2001; <http://wingless.cs.washington.edu/YMF/YMFWeb/YMFInput.pl>). Only statistically significant motifs (*Z* score value >6.5) were selected (Sinha and Tompa, 2002). The motifs identified by this method were also found using the programs Motif Sampler (Thijs *et al.*, 2002) and MEME (Bailey and Elkan, 1995). Nucleotide entry codes (GenBank accession number or MIPS code for *Arabidopsis*) and references for each sequence analysed are: HvPT1, HvPT2, and HvPT4 to HvPT8 (AF543197, AY187019, AY187024, AY187021, AF543198, AY187022 and AY187023, respectively) (Schünmann *et al.*, 2004a; Glassop *et al.*, 2005); AtPT1 to AtPT9 (At5g43350, At5g43370, At5g43360, At2g38940, At2g32830, At5g43340, At3g54700, At1g20860 and At1g76430, respectively) (Mudge *et al.*, 2002); OsPT1 to OsPT13 (AF536961, AF536962, AF536963, AF536964, AF536965, AF536966, AF536967, AF536968, AF536969, AF536970, AF536971, AF536972 and AF536973, respectively) (Paszowski *et al.*, 2002). The 600 bp 5' proximal to the ATG codon of the OsPT1 to OsPT13 were obtained from the publicly available BAC sequences.

Analysis of putative transcription factor-binding sites was carried out using the PLACE database (<http://www.dna.affrc.go.jp/htdocs/PLACE/>) (Higo *et al.*, 1999).

Note: the plant phosphate transporter genes used in this work have been referred to using simplified nomenclature. According to the Commission on Plant Gene Nomenclature (<http://mbclserver.rutgers.edu/CPGN/Guide.html>), the official name for *TaPT2* is *TR1ae;Pht1;2*; for *TaPHT1MYC*, *TR1ae;Pht1;myc*; for HvPT1 to HvPT8, *HORvu;Pht1;1* to *HORvu;Pht1;8*; for OsPT1 to OsPT13, *ORYsa;Pht1;1* to *ORYsa;Pht1;13*; for AtPT1 to AtPT9, *ARATH;Pht1;1* to *ARATH;Pht1;9*; for AtPht2, *ARATH;Pht2;1*; for LePT1, LePT2, and LePT4, *LYCes;Pht1;1*, *LYCes;Pht1;2*, and *LYCes;Pht1;4*; for MtPT1 to MtPT4, *MEDtr;Pht1;1* to *MEDtr;Pht1;4*; for StPT3 and StPT4, *SOLtu;Pht1;3* and *SOLtu;Pht1;4*; for Zm PT6, *ZEAm;Pht1;6*.

Table 1. Primers used in this study

Primer	Sequence (5' → 3')	Method
PT2-GSP1	AGCCGAAGAAGAGCTGGCCGCAAGTGT	Genome Walker
PT2-GSP2	CTCGTTGAGGGCAGGGTCGGTGTAGTA	
PT2- <i>Bam</i> HI	GAGCTGTTTCAGTCGGGATCCCCGCC	Subcloning of the <i>TaPT2</i> promoter
12F-PT2	ACAGCTCAACGTGTTG	
553R-PT2	TGATCGTGACAATAGT	RT-PCR, probes for northern blot
ActinF	CACACTTTCTACAATGAGCT	
ActinR	GCAGTGATCTCTTGTCTCAT	

GUS activity measurement

Histochemical staining for GUS activity was performed as described by Meisel and Lam (1996) and Jefferson *et al.* (1987), with the following modifications. Transgenic wheat and *Arabidopsis* seedlings with different Pi treatment times were used directly for vacuum infiltration in 50 mM NaH₂PO₄, pH 7.0, 0.1 mM X-Gluc, 10 mM EDTA, 0.1% Triton X-100. Samples were incubated at 37 °C until sufficient staining had developed (24–72 h). Plant material was then fixed in 4% formaldehyde, 4% acetic acid, 28.5% ethanol for 30 min. Subsequent incubation in 70% ethanol for 1 h, 100% ethanol for 1 h, 70% ethanol for 1 h, followed by incubation in distilled water, was performed to remove chlorophyll from the plant material.

Fluorometric GUS assays were performed as described by Jefferson *et al.* (1987). The wheat and *Arabidopsis* root and leaf tissues were ground in the presence of liquid nitrogen in a mortar, and transferred to a microtube. One millilitre of the extraction buffer [50 mM NaH₂PO₄, pH 7.0, 1 mM EDTA, 0.1% Triton X-100, 0.1% (w/v) sodium laurylsarcosine, and 5 mM dithiothreitol] was added and mixed. After centrifuging for 10 min at 12 000 g at 4 °C, the supernatant was transferred to a microtube. The fluorogenic reaction was carried out in 2 ml volume containing 1 mM 4-methyl umbelliferyl glucuronide in an extraction buffer supplemented with a 50 µl aliquot of the protein extract supernatants. The protein quantity of the sample extracts was determined using BSA (bovine serum albumin) as a standard, according to Bradford (1976).

Results

Analysis of the TaPT2 protein sequence

The predicted TaPT2 protein sequence (GeneBank accession number CAC69856) comprises 525 amino acids. As was predicted by the TMPred program (Hofmann and Stoffel, 1993) and like other Pht1 family members, TaPT2 contains 12 conserved potential membrane-spanning domains arranged in a 6 + 6 configuration around a large low-conserved central cytoplasmic loop (data not shown). Furthermore, the TaPT2 protein sequence contains high-affinity transporter-conserved sequence motifs such as phosphorylation sites for protein kinase C and casein kinase II, as well as a potential site for *N*-glycosylation (data not shown; Smith *et al.*, 2000).

An unrooted phylogenetic tree shows that TaPT2 is clustered with the high-affinity Pi monocot transporters (rice and barley) not associated with mycorrhizas that are expressed in roots during Pi starvation (HvPT1, HvPT2, and OsPT2) (Paszkowski *et al.*, 2002; Rae *et al.*, 2003; Schünmann *et al.*, 2004a) (see Supplementary Fig. S1 at *JXB* online). The TaPT2 amino acid sequence is highly conserved with the *Hordeum vulgare* orthologue (98% identity with HvPT1 and 96% identity with HvPT2; data not shown).

TaPT2 expression in Dalcahue wheat variety in the presence or absence of bioavailable Pi

The expression of TaPT2 in wheat (Dalcahue variety) was first evaluated by northern blot analyses. Total RNA was

extracted from leaves and roots of plants grown for 7 d in the presence of Pi or under Pi-starvation conditions. In the presence of 0.1 mM Pi, no TaPT2 transcripts were observed in leaf extracts whereas a weak band was observed in roots (Fig. 1A). In the absence of Pi, TaPT2 transcripts were detected at high levels in roots but not in leaves. Since there is a high level of sequence conservation between TaPT2 and other Pi transporters (TaPT1 has a 93% sequence identity at the nucleotide level to TaPT1; data not shown) which may result in cross-hybridization, the northern blot results were confirmed by RT-PCR using specific primers for TaPT2 (Fig. 1B). As described for the wheat variety Jing411 (Davies *et al.*, 2002), the expression of TaPT2 is induced specifically in Pi-deprived roots of the Dalcahue wheat variety.

Identification and analysis of the TaPT2 promoter region

To gain further insights into the regulatory mechanism of TaPT2 gene expression, the promoter region of TaPT2 was isolated using the Genome Walker technique (Siebert *et al.*, 1995). A single 579 bp fragment, upstream of the start codon was isolated and cloned (Fig. 2). Using the PLACE algorithm (Higo *et al.*, 1999) some known *cis*

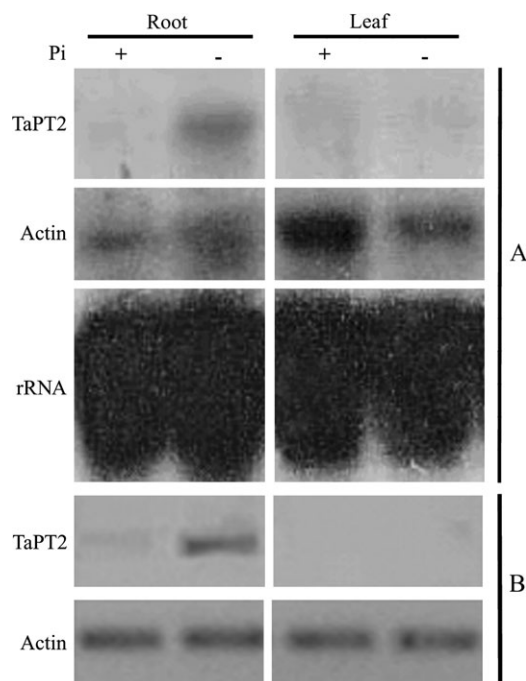


Fig. 1. Effect of Pi starvation on the expression level of TaPT2 in wheat roots and leaves. Total RNA was extracted from wheat plants grown for 7 d in the presence of 0.1 mM of Pi (+) or in the absence of Pi (-). (A) Northern blot showing the expression of TaPT2. Actin transcript levels and 18S ribosomal RNA were used as controls. (B) RT-PCR assay showing the root Pi-starved expression of TaPT2. The RT-PCR was performed with primers specific for TaPT2. These primers do not hybridize with TaPT1. Actin-specific primers were also used as a control.

regulatory motifs in this sequence were identified. One motif contains the sequence ATATT (rootmotiftapox1). This motif, identified previously in the promoter regions of the *roID* gene (*Agrobacterium rhizogenes* Ri plasmid) and the *TTG2* gene (*Arabidopsis thaliana*), appears to be associated with root gene expression (Elmayan and Tepfer, 1995; Johnson *et al.*, 2002). One base pair variants of the motifs associated with transcriptional responses to Pi starvation, P1BS (GNATATNC; Rubio *et al.*, 2001) and the PHO-like element [G(G/T/A)(C/T/A)GTGG; Mukatira *et al.*, 2001], two that are different from the consensus P1BS at the 3' end, and one that is different from the PHO-like element at the last base of the 5' end (Fig. 2), were found.

To identify new conserved motifs in the *TaPT2* promoter, sequences approximately 600 bp immediately upstream of the translation start site of 13 rice phosphate transporters, seven barley phosphate transporters, and nine *Arabidopsis* phosphate transporters were compared with the wheat *TaPT2* promoter sequence. Using the program YMF3.0 (Blanchette and Sinha, 2001), four statistically significant motifs were identified in the promoter regions of these phosphate transporters: motif 1 = SGCCGGCS (Z score 16.32), motif 2 = CTATNTATA (Z score 8.57), motif 3 = ATAAGTC (Z score 7.86), and motif 4 = ATATRCA (Z score 6.69) (Fig. 3).

In agreement with the low divergence observed between the *TaPT2*, *HvPT1*, and *HvPT2* transporters (Fig. S1 available at JXB online), a highly conserved

promoter region was found among these three genes. As shown in Fig. 3, the *TaPT2* promoter shares a 116 bp region with 77% identity to the *HvPT1* promoter (sequence alignment marked with one asterisk) and 255 bp with 78% identity to the *HvPT2* promoter (sequence alignment marked with two asterisks). Three of the four different motifs identified (motifs 1, 2, and 4) are present within this highly conserved promoter region (Fig. 3). One of these motifs is found exclusively in the promoters of monocot transporter genes (motif 1: SGCCGGCS). Motifs 2, 3, and 4 were identified in the promoters of phosphate transporter genes from both monocots and dicots (*Arabidopsis*). Notably, all four motifs are absent in the promoters of transporters expressed specifically in shoots and mycorrhiza: *HvPT6*, *HvPT8*, and *OsPT11*, respectively (Paszowski *et al.*, 2002; Rae *et al.*, 2003; Glassop *et al.*, 2005). Furthermore, motif 4 (ATATRCA) is similar to the P1BS motif (GNATATNC). Only one motif 2 (CTATNTATA) site was found in the promoter of the flower-specific transporter *AtPT6* (Mudge *et al.*, 2002).

The isolated *TaPT2* promoter is sufficient to drive Pi-deficient expression of *GUS* in the roots of transgenic wheat

To determine if the *TaPT2* 579 bp promoter fragment that was isolated is sufficient to drive Pi-deficiency expression in the roots of wheat, this fragment was cloned upstream of the *uidA* (*GUS*) gene in the binary plasmid pBI101.1 and transgenic wheat (Bobwhite variety) was generated.

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-579 CGGCCCGGGCTGGTCTTGTGTTGGTTCATAAGTCTAAGACTTTTTTGTAGTCCCAACTTATAAGTCCCAAGTCCCTAAAAGTCCCTACATGTTTGG
-482 TTCCCGAGACTTATAAGTCTCTATAAGGCC ATATT ACAACTATAAGTCCCTATAAGTCCCTCTTGAGAGTCTTATTCCATAAAGTCACAAATGTCCTCA
-385 CTTTAAGTCCCTATAAGTCCCTCCTGTTCGGTTTATAGTACAGACTTATAGGGACTTATTTAAGTCCCTAGACCAATAAATCTCTGGAAACAAACACCC
-288 TCTTAATCCTTCTAATAATAGAAATGTACCATCCGTTGACTCTGC GTATATTT TGTGAAGAACTATCGTGATAATGTACAATCCGTTGTATTTACAA
-191 GCGATGCCGT GCATATGA TATTCTATAGTTGCAACTAGCAATGTTAGATTGGCATACCAC AATAT ATCCTTACCTGTATGTTTCACCCGGAATTA
-94 GCCTATATATACGTCCTAGA AGCGTGG AGCAGCAGCACACAACAAGAGAGAACCAGAAGAAGTACAGAAGTTTAGCCGAGAGATCGCCGGCCGCGCATG*
  3 GCGACTGA ACAGCTCAACGTTGA AAGCACTCGATGTTGCCAAGACGCAACTGTACCATTTC AAGGCGGTCGTGATCGCCGGCATGGGTTCTTCA
100 CCGAGCCCTACGACCTCTTCGTCATCGCCCTCGTCACCAAGTGTGCGGGCGCATCTACTACACCGACCCCTGCCCTCAACGAG CCGGCCACCTCCC
197 GGCAACGCTGTCCGGCCGGTGAACGGCTGGCCCTATGCGGC ACACTTGCCGGCCAGCTCTTCTTCCGGTGGCTCGGGACAAGCTCGGGCCGCAA
294 AGCGTCTACGGCTTACGCTCATCCTCATGGTCTCTGCTCCATCGCGTCCGGGCTCTCGTTCGTTGGACACGAGGCAAGGCGTAAATGGGGACGCTAT
391 GTTCTCTCCGCTTCTGGCTTGGCTTGGCTCGGCGTCGGCGGCGACTATCCTCTGAGCGCCACCATCATGTCCGAATATGCTAACAAAGAACCCCGGCAC
488 CTTTATCGCCGCTGTGTTTGGCATGCAGGGTTTGGCATCCTATTGTT ACTATGTGCAGATCA TCGTCTCGTCCGATTCCGACATGCATTCCT
585 GCACCCGCACTTACATTTGACGCCCGCGCTCCATTGGCCCGGAGGCGGACTACGTGTGGCGCATCATCGTCATGTTCCGGACCATCCCGCCCGCCC
682 TGACCTACTACTGGCGTATGAAGATGCCGAAACTGCGCGGTACACAGCACTCATCGCCGGCAACGAAGCAAGCCACATCAGACATGTCCAAGT
779 GCTCAACAAGGAGATCTCAGAGGAGAATGTGCAGGGTGAGCGTGCCACTGGTGATACTTGGGGCTCTTCTCGCGACAGTTTATGAAGCCACCGGG
876 GTGCACTTGCTAGCGACCACAAGCACTTGGTTCCTGCTCGATGTGGCTTCTACAGCCAGAACCCTGTTCCAAAAGGACATCTTACCAAGATCGGGT
973 GGATCCCGCCGGCAAGACTATGAATGCATTGGAGGAGTTGTACCGCATCGCCCGCCCAAGCGCTCATCGGCTCTGCGGCACCGTGCCTCGGCTA
1070 CTGGTTCACCGTCCGCTTCATCGACATTATTTGGAGGTTTGGATCCAGCTCATGGGATTCACCATGATGACCATTTTCATGTCGCAATCGCCATA
1167 CTTTACGACTACTTGGTGAAGCCAGGGCACACACCGGCTTCGTGCTGCTACGGGCTCACTTTCTTCTCGCCAACTTCGGCCCCAACAGCACAA
1264 CTTTCATGTCGCGAGAGATCTTCCTCGAGGCTCCGGTCCAGATGCCACGGTATCTCTGCGGCTACCGGTAAGGCGGGCCGATCATCGGCGG
1361 GTTCCGGTTTCTGTATGCGTCGACAGGACAGAAGCCCGGACCGGCTACTCACGGGGATCGCGATCGCAGACGCACTTTTGTGCTCGCAGGC
1458 ACAAACTTCTGGGCTGCTCTTTTCTTTGTTGGTGCCAGAGTCTAAGGGCAAGTTCGTTGAGGAGCTCTCAAGGAGAAGCTCGCGCAGCAGACA
1555 CCATTGTTCCGACTGGTGTCTAG**

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Fig. 2. Sequence of the *TaPT2* (Dalcuhue wheat variety) promoter and open reading frame. The promoter sequence (obtained from the TaPT2–GSP1 primer) was fused virtually to the coding sequence of *TaPT2* (cDNA). The start and stop codons are highlighted with one and two asterisks, respectively. Primer sequences used in the Genome Walker experiment, TaPT2–GSP1 and TaPT2–GSP2, are in italics. Primers used in the RT-PCR assay, 12F–PT2 and 553R–PT2, are underlined. *Cis*-acting elements identified bioinformatically using the PLACE algorithm (Higo *et al.*, 1999) are shown in grey boxes: ATATT and AATAT are rootmotiftapox1 motifs; GTATATTT and GCATATGA, are P1BS-like motifs (consensus: GNATATNC); AGCGTGG is a PHO-like motif [consensus: C(G,T,A)(C,T,A)GTGG].

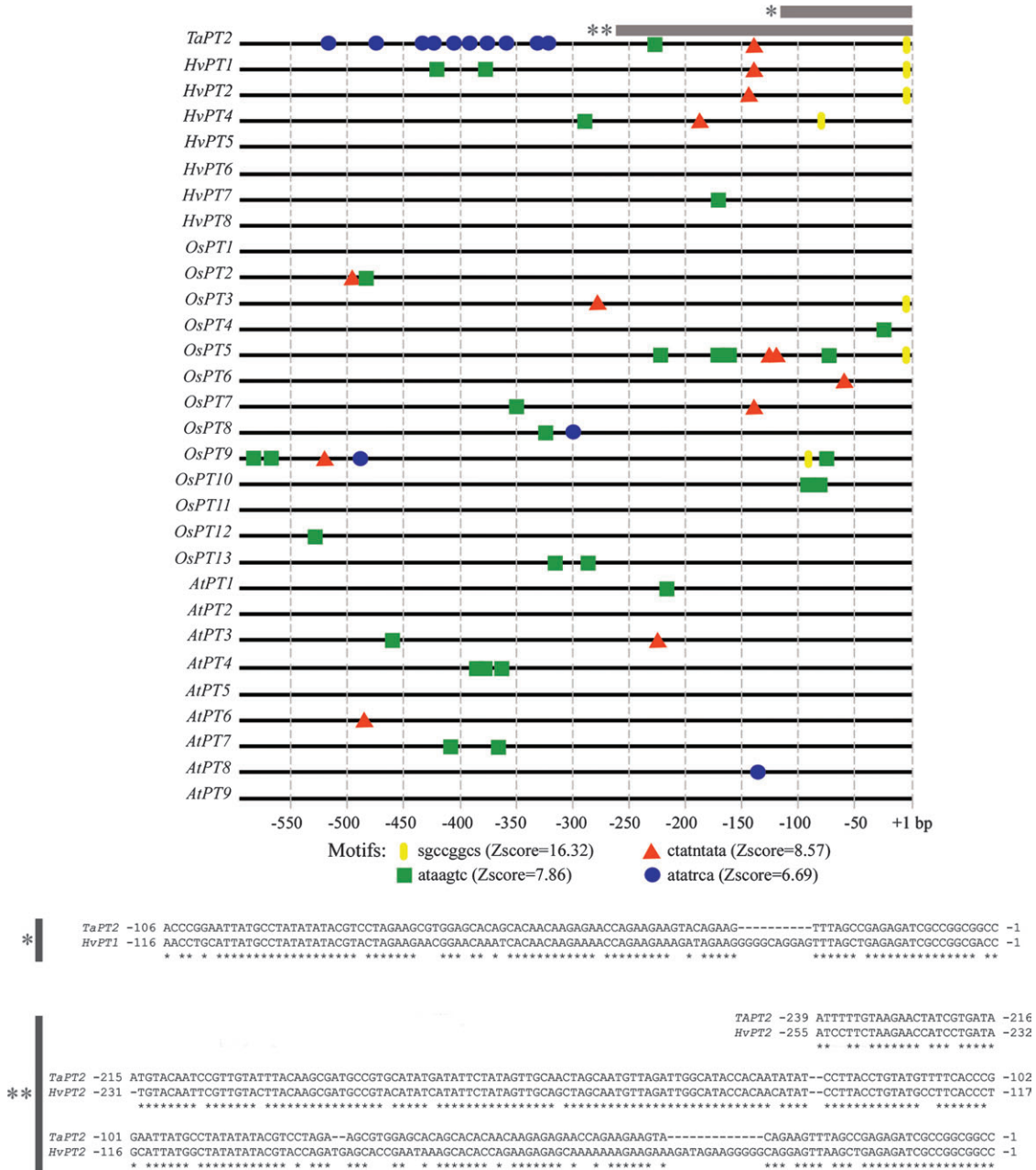


Fig. 3. Putative *cis*-regulatory elements identified in wheat *TaPT2* and other high-affinity Pi transporter promoters from barley, rice, and *Arabidopsis*. Promoter sequences of Pht1 family members from barley, rice, and *Arabidopsis* (*HvPTn*, *OsPTn*, and *AtPTn*, respectively) are shown as horizontal lines. The relative positions of the putative *cis*-regulatory elements identified by the YMF 3.0 program are marked within each promoter sequence with symbols specific for each type of motif. Four significantly conserved motifs were identified: motif 1 (SGCCGGCS) represented by a yellow vertical line; motif 2 (CTATNTATA) represented by a red triangle; motif 3 (ATAAGTC) represented by a green square; motif 4 (ATATRCA) represented by a blue circle. The degenerate bases allowed in the motifs are S (C or G), N (any nucleotide), and R (purine, A, or G). The levels of statistical significance of each motif are represented by the Z score. Alignment and localization of the conserved regulatory regions between the *TaPT2* promoter and the promoters *HvPT1* and *HvPT2* (one and two asterisks, respectively) are shown in the lower portion of the figure. Note that none of the four motifs identified was detected in *HvPT 5, 6, and 8*, *OsPT 1 and 11*, or *AtPT 2, 5, and 9*.

Three independent transgenic lines that weakly expressed GUS activity were obtained. The GUS activity of the best T3 homozygous line (line with the highest level of GUS expression) was analyzed fluorimetrically and histochemically in plants grown in the presence or absence of Pi (Fig. 4). As observed for *TaPT2* expression in the

Dalcahue variety (Fig. 1), the isolated *TaPT2* promoter triggered GUS expression in roots in the absence of Pi. These results suggest that the isolated 579 bp *TaPT2* promoter contains *cis* elements that are able to regulate root-specific expression under Pi-deficient conditions, such as P1BS or PHO-like motifs.

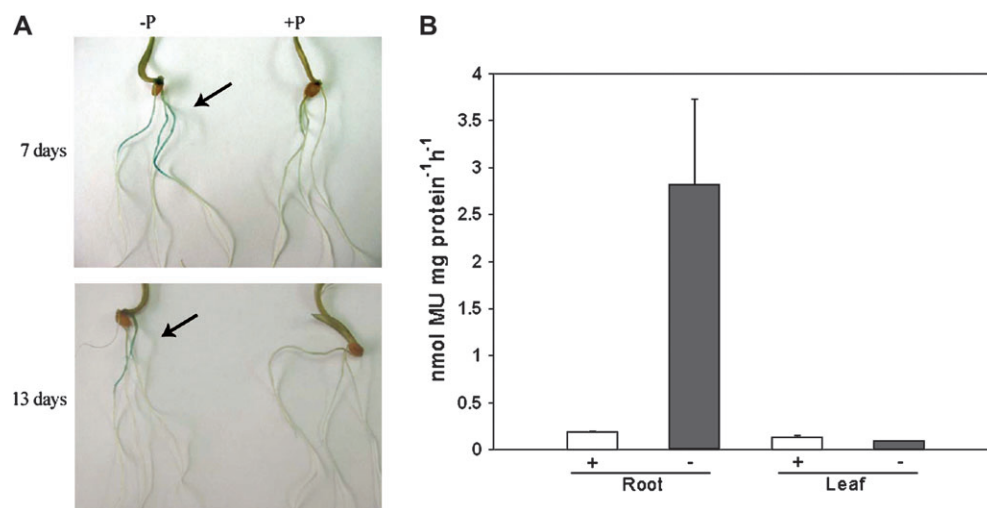


Fig. 4. GUS activity in transgenic wheat containing the *TaPT2* promoter-*uidA* fusion. (A) Histochemical localization of GUS activity. GUS activity was detected exclusively in roots after 7 d and 13 d of Pi starvation (arrows). (B) Fluorometric determination of GUS activity. Plants were grown for 7 d in the presence of 0.1 mM Pi and then transferred to culture medium without Pi for 13 d. Significant levels of GUS activity were detected in the roots of Pi-deprived plants. Bars represent standard deviation, $n=3$.

Isolated TaPT2 promoter drives Pi deficiency-specific expression of GUS in roots of transgenic Arabidopsis thaliana

The conservation of two of the putative regulatory motifs in the *Arabidopsis thaliana* high-affinity Pi transporter promoters, identified with the different prediction programs, suggests a probable Pi-deficiency induction of the *TaPT2* promoter in this dicot. To test whether the Pi-deficient induction of this promoter is conserved between monocots and dicots, *Arabidopsis* transgenic plants were generated using the same promoter-GUS construct used to transform wheat. GUS activity of seven homozygous T3 independent lines was analysed histochemically in the presence and absence of Pi. Results of the best transgenic line obtained (G3R, line with the highest level of GUS expression) are shown in Fig. 5. Interestingly, as was observed with the expression of *TaPT2* in the wheat variety Dalcahue (Fig. 1), as well as the activity of the *TaPT2* promoter fused to GUS in transgenic wheat (Fig. 4), the isolated *TaPT2* promoter also triggered GUS expression specifically in the roots of Pi-deprived *Arabidopsis* plants. These results demonstrate that the regulatory elements present in the isolated *TaPT2* promoter are sufficient to induce root-specific Pi-deficiency expression in both monocots and dicots. Additionally, this suggests that there is also conservation between monocots and dicots of the *trans*-acting factors that interact with these *cis*-acting elements.

Discussion

In this work, it was demonstrated that the promoter region of the wheat high-affinity Pi transporter, *TaPT2*, contains

regions sufficient to up-regulate gene expression in Pi-deprived roots of both monocot and dicot species.

Davies *et al.* (2002) have demonstrated previously that the *TaPT2* gene is expressed primarily in roots of Pi-deprived plants of the Chinese wheat Jing411, a variety that has low tolerance to Pi deficiency. This change in *TaPT2* gene expression in response to Pi deficiency was not, however, detected in three other varieties tolerant of the changes in external Pi conditions (Davies *et al.*, 2002).

In the present study it was demonstrated that a wheat cultivar of Chilean origin (cv. Dalcahue) also shows alterations in the expression levels of *TaPT2* under Pi-deficient conditions (Fig. 1). Dalcahue is a spring variety from the south of Chile that is tolerant of acidic soil and tolerant of low Pi levels (<http://www.inia.cl/semillas/trigosrecomendacion.htm>). This finding suggests that the differential expression of *TaPT2* in roots under different Pi conditions may not necessarily be associated with tolerance under low Pi conditions, as pointed out by Davies *et al.* (2002). It is possible that the different *TaPT2* expression levels in different wheat varieties could be due to differences in the regulatory sequences that are present in their promoters. A full-length *TaPT2* cDNA from the wheat variety Xiaoyan 54 (GenBank accession number AJ344248) contains an 83 bp region from the 5' UTR. This region is only 70% identical to the same region isolated in this work (data not shown), and has significant differences in the PIBS-like motif. This suggests that the remaining upstream regulatory region may also be different. It will be very interesting to identify and compare the *TaPT2* regulatory sequences from varieties such as Xiaoyan54 that differ from the Dalcahue variety in terms of *TaPT2* transcript levels, and/or tolerance to low Pi levels. This type of analysis may provide insight

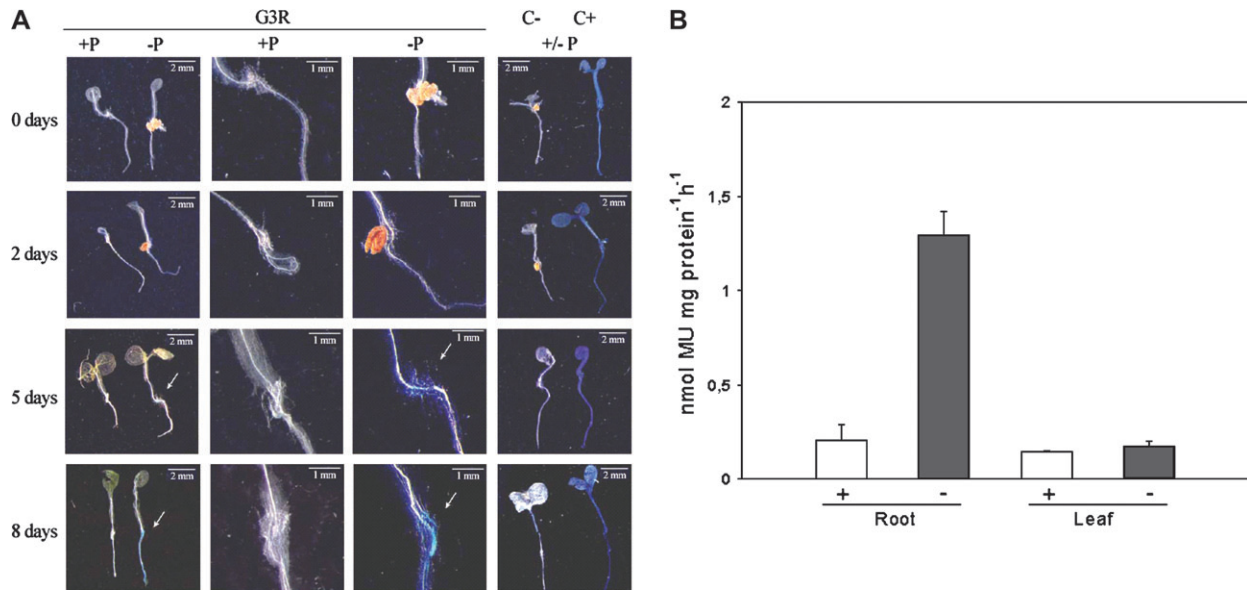


Fig. 5. GUS activity in transgenic *Arabidopsis thaliana* that contains the *TaPT2* promoter-*uidA* fusion. (A) Histochemical localization of GUS activity. Activity was detected in roots after 5 d and 8 d of Pi starvation (arrows). Negative control (C-), transgenic plants containing the promoterless construct, pBI101.1, and positive control (C+), 35S promoter-*uidA*, are shown. (B) Fluorometric determination of GUS activity. Plants were grown for 5 d in the presence of 0.1 mM Pi and then transferred to a medium without Pi for 5 d. Significant levels of GUS activity were detected in roots of Pi-deprived plants. Bars represent standard deviation, $n=3$.

into identifying the *cis*-elements responsible for this type of transcriptional regulation.

High-affinity Pi transporters have been identified in both dicots and monocots. Some of these transporters have been shown to be expressed exclusively in the aerial portion of the plant, others exclusively in specific mycorrhiza associations, and yet others are expressed only in roots of Pi-starved plants. The high-affinity Pi transporters whose expression is altered depending upon Pi conditions include the *TaPT2* from wheat, its putative barley orthologues, *HvPT1* and *HvPT2* (Smith *et al.*, 1999; Schünmann *et al.*, 2004a), the rice orthologue *OsPT2* (Paszowski *et al.*, 2002), and several members of the Pht1 *Arabidopsis* family (Mudge *et al.*, 2002). The high sequence identity existing within the coding regions of *TaPT2*, *HvPt1*, and *HvPT2* extends into the promoter regions, suggesting that the transcriptional regulation of these genes may be evolutionarily conserved. Schünmann *et al.* (2004b) have shown that a minimum regulatory region (the P1BS motif) within the *HvPT1* promoter is necessary to regulate expression in a tissue- and Pi-specific manner. Although a complete P1BS motif has not been identified in the *TaPT2* promoter, one of the four motifs that have been identified (motif 4: ATATRCA) contains five of the base pairs found in the P1BS motif. The high conservation of this candidate motif in the promoters of others Pi transporters in rice, barley, and *Arabidopsis* suggests that this motif is functionally conserved and may play a role in inducing expression under Pi-deficient conditions in the root. This motif was not found in the sequence of transporter promoters that are

expressed in flowers (*AtPT6*), shoots (*HvPT6*), or specifically with mycorrhiza (*OsPT11* and *HvPT8*).

Some studies have shown that there is high conservation in the regulatory mechanisms that define the phosphate-signalling pathway, among them the transcription factor *PHR1* which has been studied in *Arabidopsis thaliana*, *Oryza sativa*, and the unicellular alga *Chlamydomonas reinhardtii* (Rubio *et al.*, 2001; Bari *et al.*, 2006). However, until now, there was no evidence that this pathway exists in wheat. Analyses of the *TaPT2* promoter fragment in wheat and *Arabidopsis* provide indirect evidence that suggests that this pathway is present and functionally conserved in wheat.

There are very few examples in the literature of monocot gene promoters that are functional in a dicot plant (Liu *et al.*, 2003; Iwamoto *et al.*, 2004). Koyama *et al.* (2005) have shown that the promoter of the *Arabidopsis* high-affinity Pi transporter, *AtPT1*, is able to drive GUS expression in a root-specific and Pi-dependent manner in both *Arabidopsis* and rice. Karandashov *et al.* (2004) tested the activity of mycorrhiza-specific Pi transporter promoters of two dicot species (potato and *Medicago truncatula*) and a monocot (rice) in several dicot host plants. They observed that only the dicot promoters were functionally regulated in dicots, whereas the promoter from rice was not. The results from Karandashov *et al.* (2004), therefore suggests that not all promoters will behave the same in dicots and monocots.

In this paper, it has been demonstrated that an isolated fragment of the *TaPT2* promoter is sufficient to drive GUS expression in a Pi-dependent manner in the roots of

both monocots (wheat) and dicots (*Arabidopsis*). This demonstrates that the transcriptional regulation of these high-affinity Pi transporters is conserved between monocots and dicots. Additionally, this suggests that the Pi mycorrhiza-specific transporters are not regulated in a similar manner.

The analyses of the transgenic wheat used in the present study and *Arabidopsis* lines show that the *TaPT2* promoter activity is low. This observation agrees with the low activity observed by Schünmann *et al.* (2004a) in the *HvPT1* and *HvPT2* promoters. This low level of activity may be due to the absence of regulatory regions further upstream of the 579 bp fragment or regulatory regions in an intron. It has been observed in monocots that an intron between the promoter and the coding region increases the expression level considerably without changing the expression pattern (Schünmann *et al.*, 2004a).

It is important to mention that in both dicots and monocots (*Arabidopsis* and wheat, respectively) maximal GUS expression was detected after 7 d of Pi deprivation. This result is similar to that observed in *Arabidopsis* microarray analyses performed by Misson *et al.* (2005).

The conservation of the *TaPT2* promoter activity in both wheat and *Arabidopsis* suggests that the model plant *Arabidopsis thaliana* may be used to study the transcriptional regulation of this and/or other wheat transporters, the *trans*-acting factors that may regulate this promoter, and the functionality of the new motifs identified in this work.

Supplementary data

A phylogenetic tree analysis was performed among TaPT2 and other plant and fungal Pi transporters.

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