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RESEARCH ARTICLE

Molecular characterization and expression analysis of phosphate transporter gene *TaPT2-1* in wheat (*Triticum aestivum* L.)

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Abstract **A transcript-derived fragment (TDF) showing up-regulated expression under low Pi stress and being identical to an uncharacterized phosphate transporter gene *TaPT2-1* was cloned in wheat. *TaPT2-1* was 2075 bp in length and encoded a 568-aa polypeptide. Transmembrane prediction analysis suggested that TaPT2-1 had 13 conserved transmembrane domains. *TaPT2-1* shared much higher similarities to other four homologs from *Arabidopsis thaliana*, *Solanum tuberosum*, *Capsicum frutescens*, and *Solanum melongena*. The expression of *TaPT2-1* was root specific and low Pi inducible, suggesting that it plays roles in roots and is involved in the Pi acquisition under Pi-starved condition. The promoter region of *TaPT2-1* was cloned based on genome walk analysis. Several types of *cis*-regulatory elements, such as low Pi responding and tissue specific, were identified in *TaPT2-1* promoter. The transgenic tobacco plants with the integrated *TaPT2-1* promoter *GUS* were generated, and GUS histochemical staining analysis in the roots and leaves of the transgenic plants was performed. The results of GUS staining in roots and leaves under various Pi supply conditions were in accordance with the *TaPT2-1* transcripts detected based on RT-PCR analysis. Taken together, the distinct expression of low Pi-induced and root-specific *TaPT2-1* suggested that it could be used as the potential gene resource on generation of elite crop germplasms with high Pi use efficiency in the future.**

Keywords wheat (*Triticum aestivum* L.), phosphate transporter, expression, *cis*-regulatory element

Introduction

As an essential macronutrient in plants, phosphorus (Pi) plays key roles in a range of biochemical processes associated with plant growth and development. However, most arable soils throughout the world are deficient in readily available forms of Pi, resulting in the limiting of plant growth in many crop ecosystems. It is noted that plants have evolved a range of strategies to improve the availability of soil Pi. These include both morphological and biochemical changes, such as increased root growth and branching, proliferation of root hairs, and release of root exudates, which increase plant access to Pi from the poorly available sources (Raghothama, 1999; Vance et al., 2003).

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On the other hand, the membrane-bound phosphate transporters (PTs) specifically associated with Pi uptake from soil solution and the Pi cellular transportation play critical roles for plants to adapt to the starved Pi stress. Expression of part of these transporter genes is induced in response to Pi deficiency and enables Pi to be effectively taken up against the large concentration gradient that occurs between the soil solution and internal plant tissues (Smith et al., 2000).

Pi transporters belonging to two major gene families (*Pht1* and *Pht2*) have now been identified for a range of plant species (Rausch and Bucher, 2002) and have been most extensively studied in *Arabidopsis* (Muchhal et al., 1996; Mitsukawa et al., 1997; Smith et al., 1997, 2000; Okumura et al., 1998; Karthikeyan et al., 2002; Mudge et al., 2002). The members of the gene family that are expressed in roots are typically up-regulated under Pi deficiency, but the molecular basis of the regulation has not been enough investigated. Relatively little work has been reported on the Pi

transporter family of genes in cereals, although at least eight *Pht1* genes have been identified in barley (Rae et al., 2003).

In this study, a Pi transporter gene referred to as *TaPT2-1* was cloned based on analysis of a starved Pi-induced transcript-derived fragment (TDF). The molecular properties and expression pattern of *TaPT2-1* were investigated. For further understanding of the regulation of the Pi uptake mechanism, the promoter region of *TaPT2-1* was cloned and functionally analyzed using reporter gene *GUS*. The results explored that *TaPT2-1* was expressed to be root specific and inducible under deficient Pi condition, suggesting that several *cis*-regulatory elements in the promoter region, such as PIBS, PHO-like, and ROOTMOTIFTAPOX1, may potentially be involved in regulation of the distinct expression pattern of the target gene. *TaPT2-1* also acted as a putative gene resource on generation of crop elite germplasms with high Pi use efficiency.

Materials and methods

Obtainment of a novel wheat PT gene *TaPT2-1*

A cDNA-AFLP analysis was conducted for identification of the differentially expressed genes in wheat that responded to the low Pi stress. Among the identified TDFs (Gu et al., 2009), a TDF with up-regulated expression pattern in roots under Pi stress condition was identified to share high similarity with a PT gene in *Arabidopsis* (GenBank accession number AF515591) based on BLAST search using the TDF as a query. A full-length cDNA being identical of the TDF, designated as *TaPT2-1*, was obtained in the GenBank (accession number AY293827).

Molecular characterization of *TaPT2-1*

ExPASy online tool (http://www.expasy.org/tools) was used for prediction of the polypeptide translated by *TaPT2-1*. The molecular weight and the isoelectric point (pI) of *TaPT2-1* were calculated using DNAStar software. The numbers and the positions of transmembrane domain in *TaPT2-1* were predicted online (http://www.ch.embnet.org/software/TMPRED_form.html). For generation of a phylogenetic tree of *TaPT2-1* and its homologs in plant species, the plant Pi transporter (*PT*) genes sharing high similarities with *TaPT2-1* were identified based on BLAST search. The phylogenetic tree was generated using the Megalign program provided in the DNAStar software.

Expression patterns of *TaPT2-1* under various Pi conditions

Shixin828, a wheat cultivar behaving high Pi use efficiency under Pi-deficient condition (Guo et al., 2008), was used for analysis of the *TaPT2-1* expression patterns under various Pi supply conditions. The young seedlings were hydroponically cultured in MS medium under the following conditions: a photoperiod of 12 h/12 h (day/night), temperature of 20ºC/10ºC (day/night), and a relative air humidity of 75%. At the third leaf fully expanded stage, the seedlings were low Pi treated by reducing the medium Pi from the former 2 mM to 20 μM. The tissues of root and leaf were sampled in 2, 6, 12, 24, and 48 h after low Pi treatment, respectively. The roots and leaves collected before low Pi treatment were used as the control.

Semi-quantitative RT-PCR was performed for detection of *TaPT2-1* transcripts in wheat roots and leaves. The total RNA was isolated using TRIzol reagent (Gibco-BRL, Life Technologies) according to the manufacturer's instruction. The transcripts of *TaPT2-1* in roots and leaves were detected based on one-step RT-PCR analysis (TaKaRa) by using the gene-specific primers. The primers were 5′-GATGTCTCAATCTTCTCCTTTCTT (forward) and 5′-GGTGACCTCTCAAAACAGCAACTGTTT (reverse). The program of PCR was performed as follows: 3 min at 94ºC followed by 30 cycles of 45 s at 94ºC, 30 s at 55ºC, and 2 min at 72ºC. A 7 min extension at 72ºC was added after the thermal cycling. Parallel to the detection of *TaPT2-1* transcripts, an additional RT-PCR analysis for detection of the transcripts of wheat *Tubulin*, a constitutively expressed gene, was conducted for normalization of the potential errors of *TaPT2-1* transcripts in the Pi treatments. The primers for amplification of *Tubulin* were 5′-CATGCTATCCCTCGTCTCGACCT-3′ (forward) and 5′-CATGCTATCCCTCGTCTCGACCT-3′ (reverse). Each RT-PCR analysis was performed three replicates for reproducibility.

Cloning of the *TaPT2-1* promoter

For identification of the transcriptional pattern of *TaPT2-1*, the promoter region of this gene was cloned based on genome walk analysis. For that, the genome DNA of Shixin828 was extracted using CTAB method from the leaves. Three specific primers, referred to as SP1, SP2, and SP3, with the sequence of 5′-GCCTTTGCCATCTCTGACAGCTCGG-3′, 5′-GAGAGTGTGGCGCGCGGAAGCACCA-3′, and 5′-AATGGTGGGTGGCAGCTGAGCGGCA-3′, respectively, were used for amplification of the putative *TaPT2-1* promoter. The Genome Walking kit (TaKaRa) was used for cloning of the putative promoter and the procedure was performed based on the manufacturer's descriptions.

The putative *cis*-elements in *TaPT2-1* promoter were identified based on scanning a database of plant *cis*-acting regulatory DNA elements (PLACE, http://www.dna.affrc.go.jp/PLACE/fasta.html).

Construction of a binary plasmid fused the *TaPT2-1* promoter and genetic transformation of tobacco

A 1744 bp in length flanking at the upstream of *TaPT2-1* translation start codon (ATG) was PCR amplified with the primers: 5′-TTGAATTCCCGGCAAGCTCCTCTT-3′ (forward) and 5′-TTCCATGGAGTGGCAGAAGCA-3′. After

double digestion by *Eco*RI and *Nco*I, the PCR products were integrated into binary expression vector pCAMBIA3301, which was removed of CaMV35S promoter upstream of the reporter gene *GUS*, via double digestion by *Eco*RI and *Nco*I. The generated binary plasmid was referred to as pCAMBIA3301-*TaPT2-1-GUS* and transformed tobacco leaf explants (cv. Wisconsin 35) according to the descriptions of Guo et al. (2009).

Molecular identification of the transgenic tobacco plants and assay of GUS activities

The T2 transgenic tobacco plants with the integrated *TaPT2-1* promoter were cultured in solidified MS agar medium with different Pi supply conditions (2 mM, normal, and 20 μM, Pi-starved). The generated T2 plants with the integrated empty binary vector were parallel cultured and used as the control. At the third leaf expansion stage, the genome DNA was extracted using CTAB method. The PCR was performed for identification of the positive transgenic plants by using the genome DNA as the template. The primers used in the PCR were the same as those in *TaPT2-1* promoter cloning mentioned previously. The PCR program was the same as in analysis of *TaPT2-1* expression patterns described above.

The GUS activities in the *TaPT2-1* promoter integrated plants and the control were assayed by following the descriptions of Jefferson et al. (1987).

Results

Identification of a TDF sharing high similarity with an *Arabidopsis* PT gene

A cDNA-AFLP analysis was performed to identify the differentially expressed genes responding to the low Pi stress. A TDF showing up-regulated expression in 24 and 48 h after low Pi stress was identified (Fig. 1A). The sequence of the TDF is listed in Fig. 1B. BLAST search analysis suggested that the TDF shared high similarity with an *Arabidopsis* Pi transporter gene (*PT*, GenBank accession number AF515591).

Molecular characterization of *TaPT2-1*

BLAST search analysis explored that the TDF was identical to an uncharacterized PT gene (GenBank accession number AY293827) in wheat. Based on RT-PCR by using the gene-specific primers and the 48 h low Pi-treated root cDNAs, the full-length cDNA of *TaPT2-1* was cloned. The cDNA sequence and the corresponding translated polypeptide of *TaPT2-1* are listed in Fig. 2. *TaPT2-1* was 2075 bp in length, encoding a 568-aa polypeptide with a molecular weight of 59.06 kDa and an isoelectric point (pI) of 9.51. Transmembrane prediction analysis suggested that TaPT2-1 had 13 conserved transmembrane domains. A hydrophilic loop was located at the zone between the transmembrane domains IX and X (Fig. 3). The conserved transmembrane characterization of *TaPT2-1* suggested that this wheat PT gene is possibly involved in the Pi transportation at the cellular level.

Phylogenetic tree covering the *TaPT2-1* nucleic sequence and its plant homologs was generated (Fig. 4). Of *TaPT2-1* and other 31 homologs, a total of five subgroups (Subgroups I to V) could be classified. It was shown that *TaPT2-1* shared much higher similarities to other four PT genes derived from *A. thaliana* (GenBank accession number AY293827), *S. trberosum* (GenBank accession number AY603690), *C. frutescens* (GenBank accession number EF094557), and *S. melongena* (GenBank accession number EF094558).

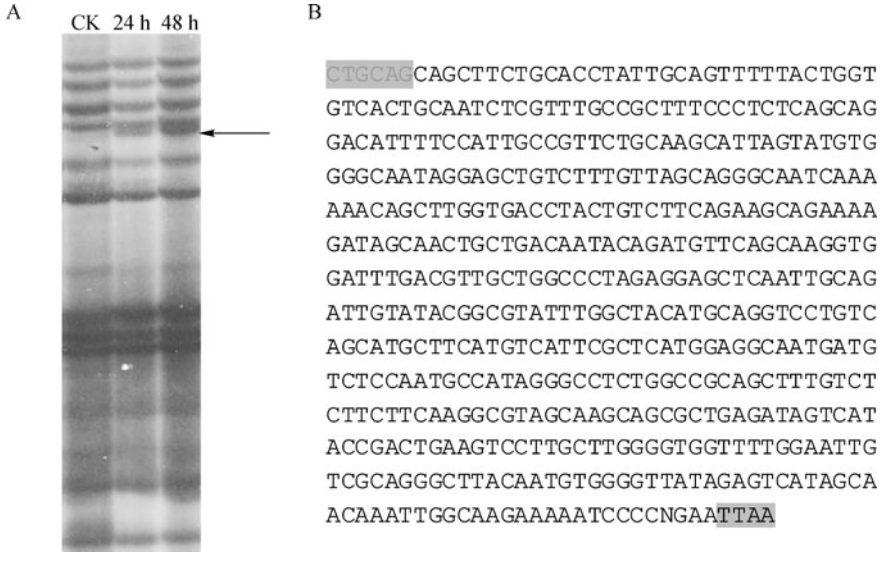

Figure 1 Identification of a TDF responding to the starved Pi stress. A: A TDF band in a polyacrylamide gel by an arrow responds to starved Pi condition of 24 and 48 h. B: The sequence of the TDF. The restriction digestion site of *Pst*I and *Mse*I, two restriction endonucleases used for digestion of the cDNAs of roots of CK, 24 h, and 48 h of starved Pi treatment, are shaded.

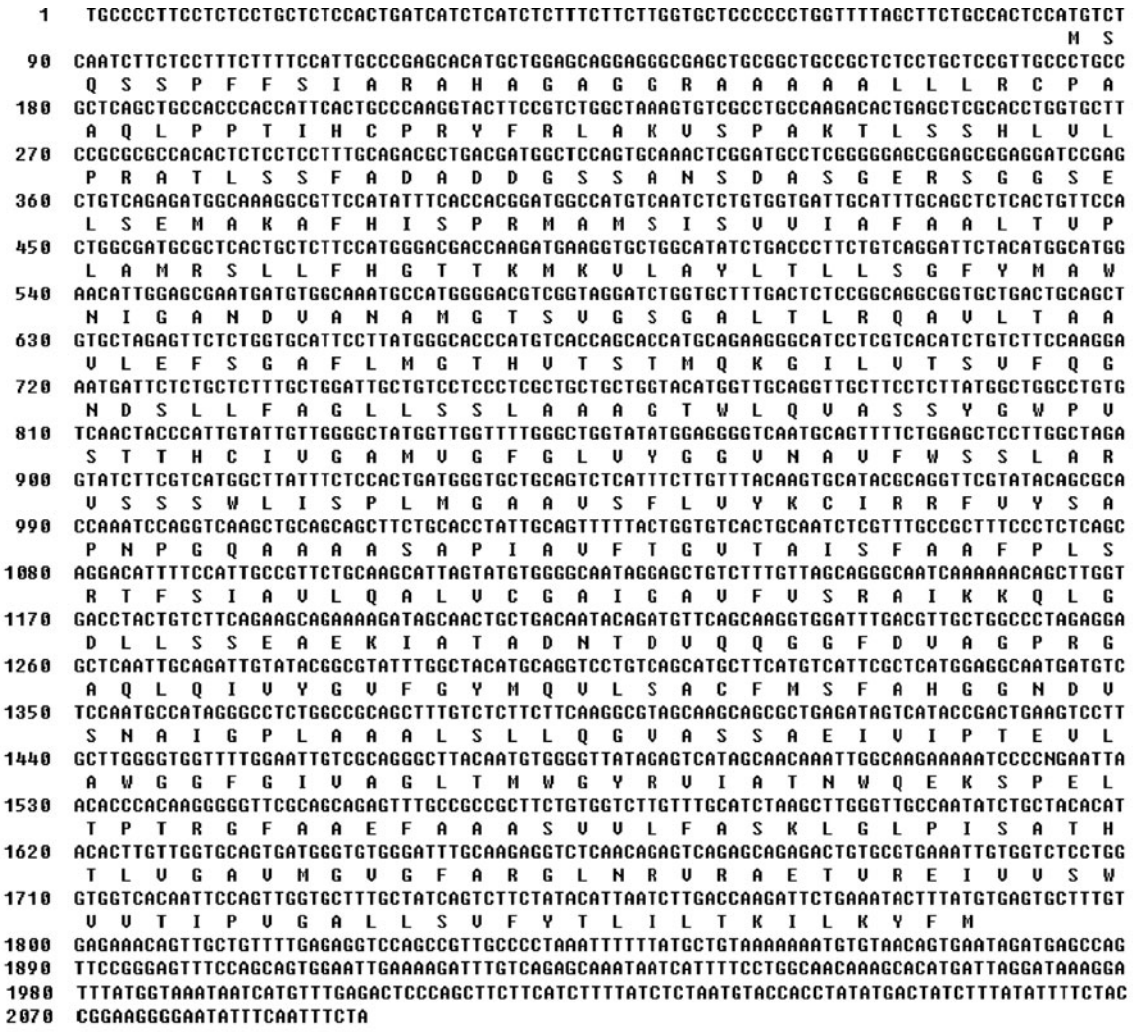

Figure 2 The cDNA sequence and the corresponding translated polypeptide of *TaPT2-1*.

Therefore, it could be speculated that *TaPT2-1* possibly originated from the same ancestor with other four homologs mentioned above.

Expression analysis showed that *TaPT2-1* was root specific; no transcripts of this gene were detected in leaves (Fig. 5). In roots, the *TaPT2-1* transcripts under normal Pi supply condition were marginally detected. Under low Pi stress, the expression level of *TaPT2-1* was elevated, showing a pattern of gradual up-regulation in a 48 h low Pi time regimen (Fig. 5). Therefore, *TaPT2-1* was suggested to play roles in roots and involved in the Pi acquisition under Pi-starved condition.

Cloning of *TaPT2-1* promoter and identification of the *cis*-regulatory elements in the promoter

A genome walk was performed for cloning of the promoter region of *TaPT2-1*. The results of three rounds of specific PCR amplification for the *TaPT2-1* promoter are listed in Fig. 6. Sequencing analysis suggested that the promoter region, flanking at the upstream of *TaPT2-1* translation start codon (ATG), was 1744 bp in length (Fig. 7). *TaPT2-1* promoter contained the conserved elements, such as TATA-BOX5 (TTATTT, positions −1051 and −1435 upstream of ATG) and CAATBOX1 (CAAT, positions −401, −686, −1035, −1127, −1223, −1327, −1347, and −1378) interacting with the RNA polymerase and regulates the transcription efficiency, respectively. Two important *cis*-regulatory motifs, PIBS (nnATATnC, positions −192, −1219, and −1385) and PHO-like (G(G/T/A)(C/T/A) GTGG, position −1121), previously identified to be involved in responding to the Pi stress, were also identified. These results suggested that the up-regulated expression of *TaPT2-1* under Pi stress was possibly associated with the existence of PIBS and PHO-like in the promoter.

Several types of *cis*-regulatory elements, such as tissue specific, defense response, auxin and salicylic acid responding, and signaling of light, sugar, and carbon metabolism,

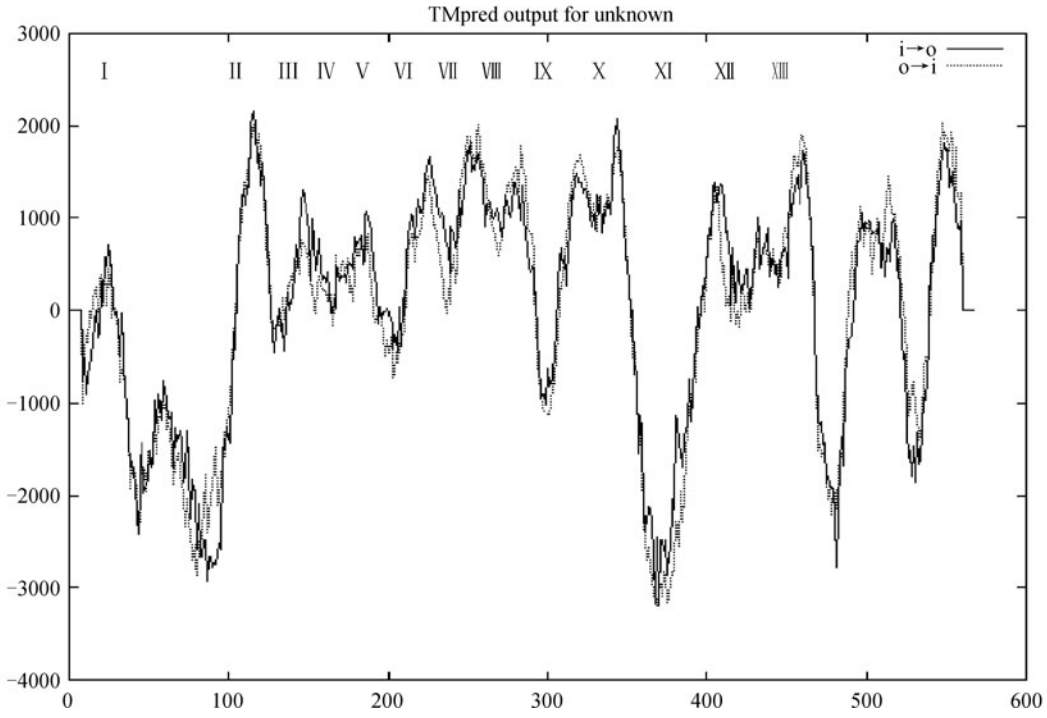

Figure 3 The diagram of transmembrane domains in TaPT2-1.

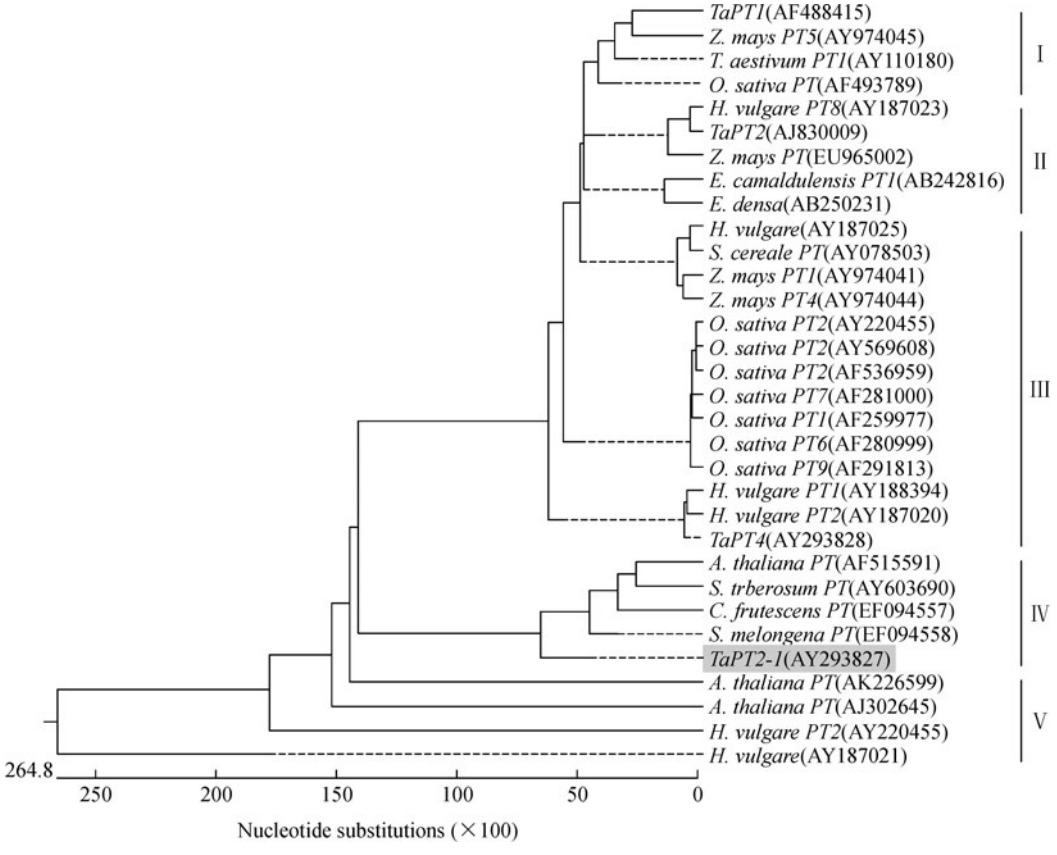

Figure 4 Phylogenetic analysis of *TaPT2-1* and its homologs in plant species.

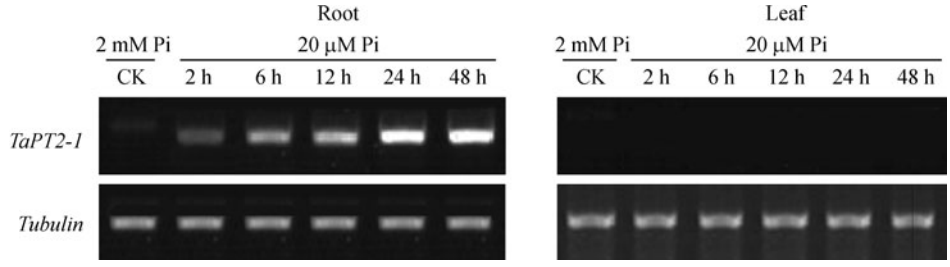

Figure 5 Expression patterns of *TaPT2-1* in roots and leaves under various Pi supply conditions.

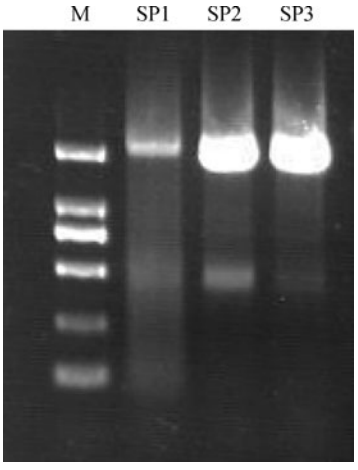

Figure 6 Cloning of *TaPT2-1* promoter region based on genome walking. SP1, SP2, and SP3 are the three rounds of specific PCR products of *TaPT2-1* promoter.

were also identified in *TaPT2-1* promoter (Table 1). Among them, the type of tissue specific included motif ROOTMOTIFTAPOX1 (position −1285), GATABOX (positions −195, −1013, −1114), and EBOXBNNAPA (positions −758, −790, −834, and −929), suggesting that they were involved in determination of *TaPT2-1* with a root-specific expression pattern. Other types of *cis*-regulatory elements in *TaPT2-1* mentioned above implied that *TaPT2-1* possibly responds to other external and internal signaling, such as abiotic stresses, part of phytohormones, and light activation.

Molecular identification of transgenic tobacco plants integrating *TaPT2-1* promoter and assays of GUS activities

The transgenic tobacco plants integrated with the *TaPT2-1-GUS* were generated via *Agrobacterium tumefaciens*-mediated approach. Four T2 independent transgenic lines (Lines 1 to 4) and the control (CK, T2 plants that transformed the empty binary vector) were subjected to molecular analysis of PCR using the specific primers of *TaPT2-1* promoter. The extracted genome DNA of CK and the transgenic plants is shown in Fig. 8A. Further PCR analysis suggested that the *TaPT2-1* promoter was all specifically amplified in the tested transgenic plants (Fig. 8B).

The roots and leaves of seedlings derived from CK and the transgenic plants at the young growth stage were sampled for GUS histochemical staining analysis. The results are shown in Fig. 9A–9C. Under normal Pi supply (2 mM Pi) condition, the GUS was strongly detected in roots and leaves of CK (Fig. 9A), with much higher levels than those in the transgenic plants (representative line, Line 2) (Fig. 9B). Under low Pi (20 μM Pi) condition, no varied GUS staining levels of roots and leaves in CK were detected (not shown). However, the GUS staining levels in the roots and leaves of the transgenic plants were intensified markedly compared to those in normal Pi supply condition (Fig. 9C). These results demonstrate that *TaPT2-1* promoter contains low Pi responding elements that are important to determine *TaPT2-1* response to the external low Pi signaling.

Discussion

The acquisition of Pi by plant roots is mediated via two phosphate transportation systems: high-affinity system and low-affinity system, which are mainly involved in transportation of Pi under Pi stress condition (μM range of Pi) and normal Pi supply condition (mM range of Pi), respectively. Pi transporters with high affinity are components of the high-affinity system and play critical roles for uptake of Pi in roots under starved Pi condition. This type of Pi transporters with low Km value generally varies between 3 and 10 μM (Raghothama, 1999). Thus far, lots of Pi transporter genes with high-affinity properties, derived from plant species, such as *M. truncatula* (*MtPT1* and *MtPT2*) (Liu et al., 2008), rice (*OsPT1*) (Seo et al., 2008), tomato (*LePT1* and *LePT2*) (Liu et al., 1998), and potato (*StPT1* and *StPT2*), are cloned and functionally characterized. On the other hand, as the components of low-affinity Pi transportation system and the Km value of 50 to 300 μM, the PTs are generally involved in the Pi uptake under rich Pi supply condition and functional in cellular Pi transportation (Raghothama, 1999). Sharing high similarities with the PTs characterized in plant species, *TaPT2-1*, is speculated to be functional on regulation of plant phosphorus nutrition in wheat.

The expression patterns of high-affinity Pi transporter genes generally induced by starved Pi stress further contributed to the activation of the Pi transportation system

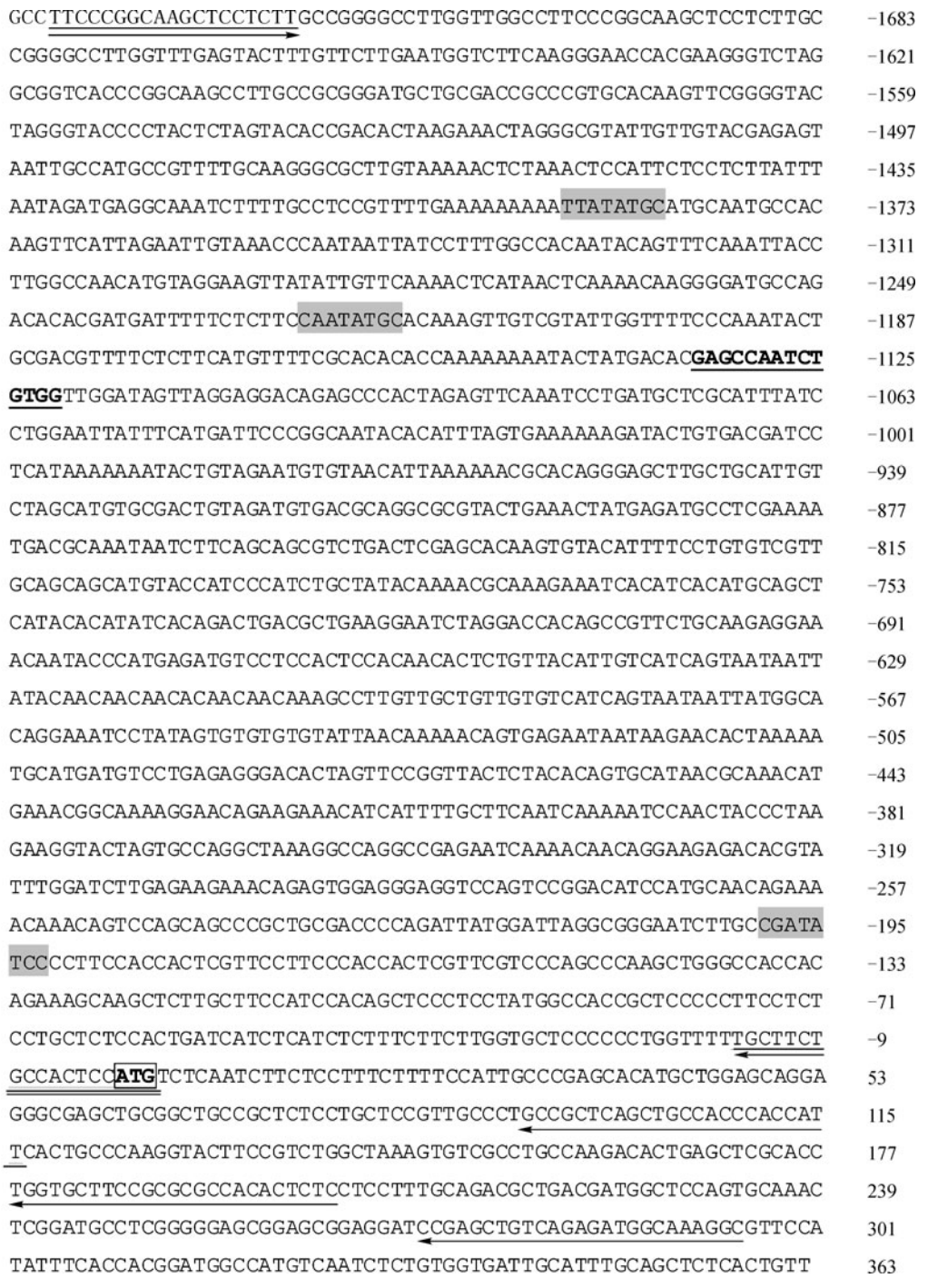

Figure 7 The *TaPT2-1* promoter sequence. The translation start codon (ATG) of *TaPT2-1* was boxed. The *cis*-regulatory elements PIBS and PHO-like putatively involved in regulation of gene expression to be low Pi responding are shaded and underlined, respectively. The primers used for the *TaPT2-1* promoter integration into binary vector pCAMBIA3301 are underlined and labeled concomitantly with forward arrow and reverse arrow, respectively. The three specific primers, SP1, SP2, and SP3, used for cloning of *TaPT2-1* promoter in genome walking, are shown by reverse arrow.

(Liu et al., 1998; Daram et al., 1999; Chiou et al., 2001). In this study, the transcripts of *TaPT2-1* were detected only in the roots, showing a pattern to be root specific. In the meantime, the expression levels of *TaPT2-1* in roots were much higher under low Pi stress than those under normal Pi supply condition, showing a pattern to be gradually elevated in a 48 h starved Pi regimen. The GUS histological staining analysis in which the reporter gene *GUS* was directed by the putative *TaPT2-1* promoter also demonstrates that the downstream gene was low Pi responding. These results suggested

Table 1 The putative important *cis*-regulatory elements identified in *TaPT2-1* promoter

Type	*cis*-regulatory element	Motif	Occurrence frequency	Position	Putative function
Transcription and regulation	TATABOX5	TTATTT	2	−1051; −1435	Interaction of RNA polymerase
	CAATBOX1	CAAT	8	−401; −686; −1035; −1127; −1223; −1327; −1347; −1378	Regulation of transcription efficiency
Low Pi responding	PIBS	nnATATnC	3	−192; −1219; −1385	Responding to low Pi
	PHO-like	G(G/T/A)(C/T/A)GTGG	1	−1121	Responding to low Pi
Tissue specific	ROOTMOTIFTAPOX1	ATATT	1	−1285	Root predominantly expression
	GATABOX	GATA	3	−195; −1013; −1114	Tissue-specific expression, light regulated
	EBOXBNNAPA	CANNTG	4	−758; −790; −834; −929	Tissue-specific activation
Defense response	ACGTATERD1	ACGT	2	−320; −1180	Early response to dehydration
	MYCCONSENSUSAT	CANNTG	4	−758; −790; −834; −929	Cold responsive
	LTRECOREATCOR15	CCGAC	1	−1531	Low temperature-responsive element
	WBOXNTERF3	TGACY	1	−846	Rapid and transit activation of transcription by wounding
	WRKY710S	TGAC	6	−731; −847; −873; −913; −1006; −1137	Defense signaling regulated via WRKY transcription factors
Auxin and salicylic acid responding	ASF1MOTIFCAMV	TGACG	3	−730; −872; −1005	Transcription activation by auxin and/or salicylic acid
Signaling of light, sugar, and carbon metabolism	SORLIP1AT	GCCAC	5	−4; −87; −136; −1330; −1373	Light regulated
	GT1CONSENSUS	GRWAAW	4	−559; −876; −1018; −1397	Light regulated
	ASF1MOTIFCAMV	TGACG	4	−730; −872; −912; −1005	Light regulated
	WBOXHVISO1	TGACT	1	−846	Sugar signaling and sugar responsive
	DOFCOREZM	AAAG	7	−127; −357; −430; −604; −773; −1016; −1213	Carbon metabolism

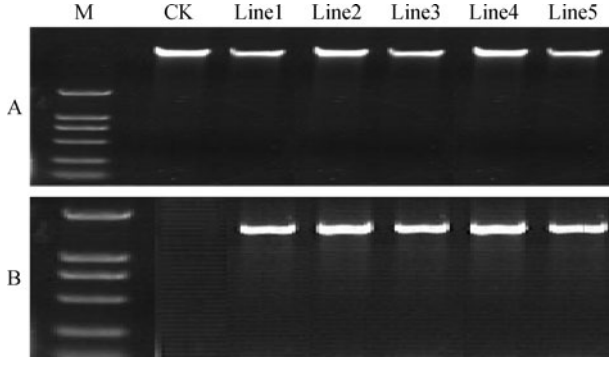

Figure 8 Molecular identification of the transgenic tobacco lines that were integrated *TaPT2-1* promoter. A: The genome DNA extracted from CK and the transgenic tobacco lines. B: PCR results in which the genome DNA extracted from the CK and the transgenic tobacco lines was used as the template.

that *TaPT2-1* was classified into high-affinity Pi transporter gene family and possibly involved in the Pi acquisition in roots under starved Pi condition.

Characterizations of the promoter are potential for identification of the transcription regulation mechanism. Previously, the promoter regions of the *Pht1* genes from barley were analyzed for identification of regulatory elements for which there is evidence of a functional role in plants (Schünmann et al., 2004). Among the promoter regions, a motif to the putative Pi-responsive P1BS element (nnATATnC, Rubio et al. 2001) was identified to be present in all *HvPht1* promoters for which sequence was available (*Pht1;1*, *Pht1;2*, *Pht1;4*, *Pht1;5*, *Pht1;6*, and *Pht1;7*; Schünmann et al., 2004). These results imply that the PIBS element, associated with the Pi starvation response (Rubio et al., 2001) and combined with another Pi-responding motif PHO-like,

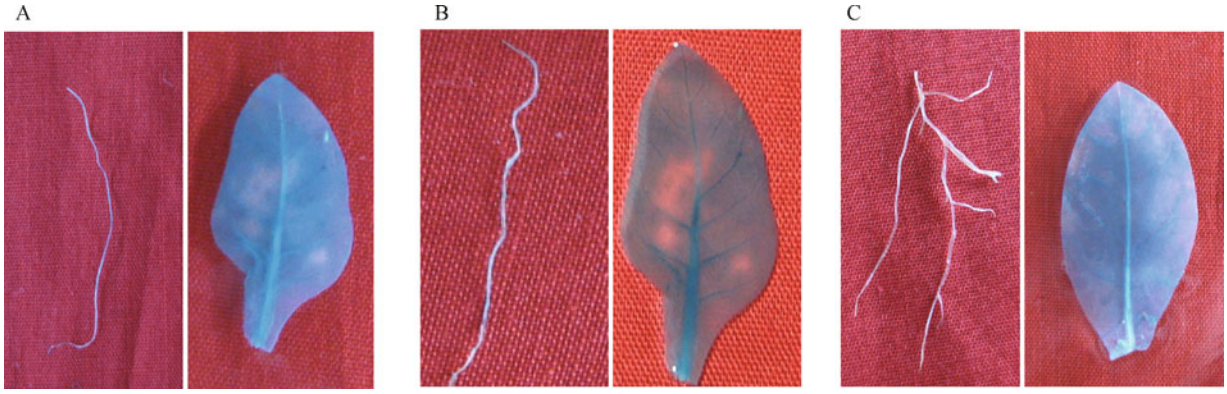

Figure 9 GUS staining analysis of the roots and leaves of control (transformed the empty binary vector) and representative transgenic plants. A: The histochemical staining of roots and leaves in which the empty binary plasmid integrated. B and C: The GUS activities of roots and leaves in which the reporter gene *GUS* was driven by *TaPT2-1* promoter under normal Pi supply condition (2 mM) and starved Pi condition (20 μM), respectively.

may serve a role in responding to low Pi signaling in the monocot plants. In this study, the PIBS motif was also identified in the *TaPT2-1* promoter and was speculated to play roles on regulation of the downstream gene with an expression pattern of low Pi responding. Previous studies have also demonstrated that the motifs of ROOTMOTIFTA-POX1, GATA, and EBOXBNNAPA are potential on determination of genes to be tissue specific (Hammond et al., 2003; Franco-Zorrilla et al., 2004). In this study, the *cis*-regulatory elements mentioned previously were all identified in the *TaPT2-1* promoter, although the occurrence frequencies of the elements varied largely. Thus, these *cis*-regulatory elements could be involved in determination of the gene with an expression pattern of root specific. Together, these results suggested that the transcription regulation of *TaPT2-1* was a comprehensive result from the actions of various putative *cis*-regulatory elements. The functions of the putative elements need to be elucidated further via analysis of the expression patterns of *GUS* or other reporter genes under the control of deleted *TaPT2-1* promoter fragments.

Previous studies clearly demonstrated that part of Pi transporter genes could improve the plant phosphorus nutrition under deficient Pi condition. The suspension tobacco cells ectopically expressing *Arabidopsis AtPT1* showed a significant increase on the fresh weight under deficient Pi supply condition (Mitsukawa et al., 1997). The rice plants in which *OsPT1*, a high-affinity Pi transporter gene was overexpressed, could improve the Pi acquisition and the plant growth when plants were grown in the limited Pi growth medium (Seo et al., 2008). With a low Pi-induced and root-specific expression pattern, *TaPT2-1* could be used as the potential gene resource on generation of elite wheat and other crop germplasms with high Pi use efficiency in the future.

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