Introduction

Phosphorus (P) is taken up by plant roots from soil as inorganic phosphate (Pi). However, most of the Pi in native soils is present as Pi-esters or metal ion salts, which are not readily available to plants. This stability and insolubility results in lower concentrations (~10 μM) of Pi in soil solutions (Holford, 1997), while the concentration in the cytoplasm of plant cells is generally greater than 10 mM (Mimura, 1999). Plants have therefore evolved a range of strategies to increase the availability of soil P and its uptake against large concentration gradient via high-affinity Pi-transporters (PTs) (Rausch and Bucher, 2002; Smith *et al.* 2000).

PTs belonging to two major gene families (*Pht1* and *Pht2*) have now been identified in several plant species (Raghothama, 1999; Rausch and Bucher, 2002; Bucher, 2007; Chen et al. 2007; Javot *et al.* 2007). There are multiple members of PTs belonging to the Pht1 family in many plant genomes. The genomes of *Arabidopsis thaliana* and rice (*Oryza sativa*) contain nine and 13 members of PTs in the Pht1 family, respectively (Mudge *et al.* 2002; Paszkowski *et al.* 2002). At least five homologous genes of Pht1 PTs have been isolated in *Zea mays* (maize) (Nagy *et al.* 2006), solanaceous species (Chen *et al.* 2007), *Medicago truncatula* (Liu *et al.* 2008), and eight members in the barley genome (Schünmann *et al.* 2004a). In *Arabidopsis*, the expression patterns of reporter genes driven by their native promoters of PT genes showed that four out of nine PTs in the Pht1 family were expressed in the root epidermis, and were induced under Pi-deprivation (Mudge *et al.* 2002). In maize, transcripts of five identified PTs were quite abundant in Pi-starved roots and leaves (Nagy *et al.* 2006). Some plant PTs belonging to the Pht1 family are predominantly expressed in above ground parts including stems, leaves, cotyledons, tubers, flowers , grains and seeds (Karthikeyan *et al.* 2002, Mudge *et al.* 2002; Rae *et al.* 2003; Nagy *et al.* 2006; Ai *et al.* 2008). It was speculated that these PTs are involved in the translocation of Pi within the plant (Mudge *et al.* 2002; Raghothama and Karthikeyan, 2005). In addition to the Pi-regulated PTs, many mycorrhiza-induced or enhanced PTs in the Pht1 family have been characterized in the roots of solanaceous species (Rausch *et al.* 2001; Nagy *et al.* 2005; Chen *et al.* 2007), legume species (Harrison *et al.* 2002; Maeda *et al.* 2006; Javot *et al.* 2007), and cereal crops (Paszkwoski *et al.* 2002; 2005; Nagy *et al.* 2006; Glassop *et al.* 2005; 2007).

In contrast to thorough characterization of the Pht1 genes in *Arabidopsis*, much less work has been done with the Pht1 genes in rice. Real time quantitative RT-PCR (qRT-PCR) analyses of expression of all 13 members of Pht1 genes in rice have resulted in identification of *OsPT11* as a PT specifically activated during mycorrhizal symbiosis (Paszkowski *et al.* 2002) and *OsPT13* as a mycorrhiza-enhanced gene (Güimil *et al.* 2005; Glassop *et al.* 2007). We have investigated expression patterns of the Pht1 genes in the model cultivar of rice (*Oryza sativa* ssp. *Japonica* cv. Nipponbare) grown in nutrient solutions with different Pi levels. We have shown that *OsPht1;2* and *OsPht1;6* have different functions and kinetic properties in uptake and translocation of Pi in rice (Ai *et al.* 2009). Here we report the spatial and temporal expression patterns of the other Pht1 genes in rice and discuss the relationship between three *cis*-regulatory elements, W-box, PHO1 and P1BS, and the Pi-regulated expression of Pht1 genes in rice plants. These results provide a better understanding of the roles of Pht1 genes in rice that play in Pi-acquisition and translocation.

Materials and methods

Plant growth conditions and RT-PCR analysis

The treatments of seeds and seedlings and growth conditions were as described previously (Ai *et al.* 2009). After 10 d of growth, the seedlings were transferred to nutrient solution either with 0.3 mM Pi (Pi sufficient) or without the inclusion of Pi (Pi deficient). The solution pH was adjusted to 5.5 and solution was replaced every 3 d.

For detecting histochemical localization of the reporter gene in stamen and caryopses, the transgenic rice plants selected by hygromycin were grown in normal soil until the materials were harvested at different reproductive stages. Extraction of total plant RNA and RT-PCR analysis methods were same as described by Ai et al. (2009). These experiments were done independently three times.

Preparation of OsPTs promoters and generation of expression vectors

For the isolation of the *OsPTs* (GeneBank accession numbers *OsPT1* (AF536961); *OsPT3* (AF536963); *OsPT4* (AF536964); *OsPT5* (AF536965); *OsPT7* (AF536967); *OsPT8* (AF536968); *OsPT9* (AF536969); *OsPT10* (AF536970) promoter, 5' upstream regions were PCR amplified with *Oryza sativa* L ssp. Japonica, Nipponbare genomic DNA using specific primers (Supplementary Table S2). Restriction enzyme sites were incorporated in the primers to facilitate cloning into the expression vectors. The amplified fragment was cloned into pMD19-T vector (TaKaRa) and the PCR products were confirmed by restriction enzyme digestion and DNA sequencing. The promoter fragments were digested from pMD19-T vector and cloned to the β-glucuronidase (GUS) reporter genes in the binary vectors pS1aG-3 (kindly provided by Dr. Delhaize, CSIRO Plant Industry, Australia). The expression vectors were transferred to *Agrobacterium tumefaciens* strain EHA105 by electroporation and used for rice transformation as described by Upadhyaya *et al.* (2000).

Reporter gene assays

Histochemical analysis of GUS activity was done as described previously (Jefferson *et al.* 1987). The samples were submerged in GUS reaction mix (0.05 mM sodium phosphate buffer pH 7.0, 1 mM X-gluc, and 0.1% (v/v) Triton X -100), and incubated at 37˚C overnight. Green tissues were transferred to ethanol to remove chlorophyll prior to observation. The stained tissues were photographed using an Olympus MVX10 steromicroscope with color CCD camera (Olympus Instrument, Japan).

Sequence extraction and alignments

Multiple sequence alignments of protein sequences of the *OsPTs*, *HORvu;PTs*, *ZEAmaPTs* and *AtPht1s* were done using the Clustal X 1.81 program with default multiple alignment parameters. The phylogenetic analysis was carried out by the neighbor-joining method. The phylogenetic tree was constructed using MEGA 3.1 program with a bootstrap analysis of 1000 re-sampling replications. The protein sequences for alignment were obtained from NCBI.

Results

Expression pattern of the Pht1 transporters under Pi-sufficient and Pi-deficient conditions

In accordance with the recommendations of the Commission for Plant Gene Nomenclature of the International Society for Plant Molecular Biology (Bucher *et al.*

2001), 13 members of the rice PT genes in the Pht1 family were named as *ORYsa;Pht1;1* through *ORYsa;Pht1;13*. For simplicity, here we call them *OsPT1* through *OsPT13* (Paszkowski, *et al.* 2002). According to the published sequences of 13 Pht1 genes in Nipponbare, the cultivar used for sequencing the genome of Japonica species of rice (Goff *et al.* 2002), we designed gene-specific primers (Table S1). The specificities and regulation of transcriptional expression of the Pht1 transporter genes were examined in roots and leaves of rice grown in Pi-deficient and Pi-sufficient nutrient solutions.

We observed that nine out of all 13 Pht1 transporter genes were expressed in both Pi-deprived roots and leaves. The transcript levels of *OsPT2, OsPT3, OsPT6* and *OsPT7* were higher and significantly enhanced by Pi deficiency in roots. Expression of both *OsPT4* and *OsPT8* was abundant and constitutive at both Pi levels; however, *OsPT4* was expressed exclusively in the roots.

Tissue specificity and Pi-responsiveness of the Pht1 promoter expression in rice

Using transgenic rice plants expressing the GUS reporter gene driven by the promoters, we previously detected the tissue specificity and Pi-responsiveness of expression of *OsPT2* and *OsPT6* in rice (Ai *et al.* 2009). Here, the rest of 13 Pht1 transporter genes in rice have been further characterized. The promoter-GUS sequences were introduced into Nipponbare cultivar, and progenies of the transgenic lines were prepared for the detection of the spatial distribution.

The GUS detection in this article and our previous study (Ai *et al.* 2009) showed that *OsPT1*, *OsPT2*, *OsPT3*, *OsPT4*, *OsPT5*, *OsPT6*, *OsPT7* and *OsPT8* were expressed in the roots, expression of *OsPT5* and *OsPT7* was rather weak, and that most of them were induced to a varying degree by Pi-deprivation, supporting the idea that most plant Pht1 transporters studied to date are expressed in roots and induced by Pi-starvation (Karthikeyan *et al.* 2002; Mudge *et al.* 2002). GUS was constitutively expressed in both roots and leaves of *OsPT8* promoter carrying plants. There was no expression of the reporter gene in the root cap in any of the transgenic lines.

In contrast to the expression in the roots, the *OsPT1*, *OsPT2*, *OsPT6*, *OsPT7* and *OsPT8* promoters also expressed GUS in the root-shoot junctions and leaves at relatively low levels, and induction of *OsPT1*, *OsPT2*, *OsPT6* and *OsPT7* was observed under Pi-deprivation. *OsPT1*, *OsPT2*, *OsPT3*, *OsPT4*, *OsPT6*, *OsPT7* and *OsPT8* promoters also directed expression of GUS in some mature organs under Pi supply. *OsPT1* and *OsPT8* promoters directed expression in stamens, caryopses and the growing points of germinated seeds, while for *OsPT3* and *OsPT4* promoter enhanced expression in the actively growing points of germinated seeds.

Identification of motifs related to the Pi starvation response in the Pht1 promoters in rice

In order to explore the Pi-regulatory molecular mechanism of these PT genes in rice, we first used the motif-building program MEME to identify conserved candidate regulatory motifs shared by the Pi-regulated PTs from plants and fungi, including P1BS motif (GNATATNC), PHO-like element (C(G/T/A) (C/T/A)GTGG) and W boxes (TTGACC/T). The available sequences, including the 5` un-translated regions of the encoded genes, immediately upstream of the translation start, were compared for 13 of the Pht1 genes (Table S2).

All OsPht1 promoters investigated, except *OsPT1, OsPT4* and *OsPT10*, have P1BS motifs with multiple copies being present for the *OsPT3, OsPT7, OsPT8, OsPT9* and *OsPT11* promoters. The PHO-like elements are present in 8 of all the OsPht1

promoters investigated (*OsPT1, OsPT6, OsPT7, OsPT8, OsPT9, OsPT11, OsPT12* and *OsPT13*). W boxes were identified in most of the OsPht1 promoters except for *OsPT8, OsPT9, OsPT10* and *OsPT13*, with multiple copies being present for *OsPT1, OsPT2, OsPT3, OsPT4, OsPT6, OsPT7, OsPT11* and *OsPT12* promoters.

Discussion

Pht1 promoter-GUS and RT-PCR showed here and in our previous study (Ai, *et al.* 2009) that at least 8 members (*OsPT1*, *OsPT2*, *OsPT3*, *OsPT4*, *OsPT5*, *OsPT6*, *OsPT7* and *OsPT8*) of 13 Pht1 genes are expressed in rice roots, and that most of them are induced by Pi-deprivation. These rice genes are likely to play roles similar to that of previously characterized genes in *Arabidopsis*, barley, tomato and maize (Mudge *et al.* 2002; Schünmann *et al.* 2004a; Nagy *et al.* 2005), being involved in Pi uptake from the soil or remobilization of Pi (Smith *et al.* 2003). At least three members of the Pht1 family of Pi-transporters in rice, *OsPT1*, *OsPT6* and *OsPT8* were found to direct reporter gene expression in the root epidermis, very similar to the expressions of *ARAth;Pht1;1, ARAth;Pht1;2, ARAth;Pht1;3* and *ARAth;Pht1;4* in *Arabidopsis* (Mudge *et al.* 2002), indicating that they are likely to be involved in Pi uptake from soil solution. Among the Pht1 Pi-transporters in rice, *OsPT3* and *OsPT4* were specifically expressed in the roots**.** The promoters of these genes could be useful in developing the transgenic rice that are able to respond to Pi deficiency.

To sustain plant growth and development, Pi acquired from soil is transported from the root to the shoot via the xylem. It is released from phloem cells in sink organs and subsequently transferred to surrounding organs, tissues and cells. High expression levels of *OsPT1*, *OsPT2*, *OsPT6*, *OsPT7* and *OsPT8* were detectable in root-shoot junctions and leaves under Pi-starvation, indicating that they may be involved in translocation of Pi from root to shoot in rice and they are also likely to play a role in redistribution of Pi to young organs during leaf senescence (Poirier *et al.* 2002; Rausch *et al.* 2004). These results are in accordance with previous results in maize, where there was an increase in *ZEAma;Pht1;2/4*, *ZEAma;Pht1;3* and *ZEAma;Pht1;6* transcript levels in leaves under Pi-deprived conditions (Nagy *et al.* 2006).

Phytate (inositol hexakisphosphate, IP6) is the major storage form of P in grains and seeds (Marschner *et al.* 1995), typically comprising >1% of the dry weight, and is responsible for approximately two-thirds of total seed P. During seed germination, Pi is released, redistributed to growing parts of the plant for growth and development (Nagy *et al.* 2005). We found higher activities of *OsPT1*, *OsPT3*, *OsPT4* and *OsPT8* promoters in the growing points of germinated seeds, suggesting that these genes may be involved in P release and remobilization. *Pht1;5* in *Arabidopsis* is likely to perform a similar role during this process (Mudge *et al.* 2002).

Both the physiological and molecular evidence that is emerging highlights the fact that plant growth and development requires the coordinated activation of many different Pi transport processes (Smith, *et al.* 2003). It is clear that transporters are involved in Pi-transport in tissues other than roots and leaves. Examples includes remobilization of stored Pi from leaves via the phloem (Rae *et al.* 2003), and Pi uptake in elongating pollen tubes (Mudge *et al.* 2002; Nagy *et al.* 2006). Here, both GUS detection of the promoter-reporter gene fusions and sensitive RT-PCR analysis showed that four of the reporter genes of the Pht1 family (*OsPT9*, *OsPT10*, *OsPT11* and *OsPT13*) could not be detected in roots, leaves or mature tissues under both high and low Pi-supply. The transcripts of *OsPT5* were very low in abundance in the

Pi-deficient roots. Because *OsPT11* and *OsPT13* are mycorrhiza-regulated PTs in rice (Paszkowski *et al.* 2002; Güimil *et al.* 2005; Glassop *et al.* 2007), the expression would not be expected under the conditions used here.

The overall nutritional status of the plant may also influence the regulation of the transcription of Pi transporters. There could be accumulation of very high level of Pi (Welch *et al.* 1982; Smith, *et al.* 2003) and up-regulation of some Pht1 Pi-transporters in zinc-deficient roots (Huang et al., 2000). Furthermore, P, K and Fe deficiency changed the expressions of *LePT1*, a Pi-transporter, and *LeNRT2.1*, a nitrate transporter (Wang *et al.* 2002; Wang *et al.* 2001). Nitrogen deficiency could up-regulate a number of Pi-transporters (Wang *et al.* 2001). We speculate that some nutrients may have a specific role in the signal transduction pathway regulating the expression of these genes which were not expressed under both high- and lo-Pi supply environments in rice. This is an interesting area of study that needs further attention with rice.

The expression regulation of Pi-deficiency-induced genes, including Pi-transporters, is in part due to the interaction of nuclear-localized transcription factors with the specific protein-binding *cis*-elements of the genes (Mukatira *et al.* 2001). Previously, several transcription factors involved in the regulation of Pi stress responses have been discovered and characterized (Rubio *et al.* 2001; Yi *et al.* 2005; Devaiah *et al.* 2007a; Devaiah *et al.* 2007b). Sequences related to the PHR1-binding site, P1BS-like elements, and specific binding-element, the conserved W boxes (TTGAC/T), which are involved in the regulation of gene expression in P-starved plants, have been checked at the upstream regions of a range of Pi-starvation-responsive genes in dicot plants (Schünmann *et al.* 2004; Rubio *et al.* 2001; Hammond *et al.* 2003; Devaiah *et al.* 2007a). We here identified some of these motifs in all of the Pht1 promoters in rice. The presence of the P1BS motif in *OsPT2*, *OsPT3*, *OsPT6* and *OsPT7*, which were significantly induced by Pi-deprivation, suggesting that P1BS-like element is likely to be involved in modulating Pi stress responses in rice plants. The differential level of increase under Pi-starvation appeared to be correlated with a combination of the number and type of the P-regulated *cis*-elements predicted in the promoter regions of the transporter genes. The PTs which are abundantly expressed and induced by Pi-deprivation in both roots and leaves, have multiple predicted P1BS-like, W boxes and PHO elements on their promoters, suggesting that these *cis*-elements have a stronger role in responding to Pi-starvation in rice. In addition, there is no PHO element on the promoter regions of *OsPT2* and *OsPT3* which were abundantly expressed and strongly induced by Pi-starvation, demonstrating that PHO element is not essential for Pi-deprivation induced gene expression in plants.

In summary, this investigation and our previous work have provided a survey and characterization of all members of Pht1 gene family of rice. The expression of reporter genes and computational analysis of the Pht1 transporter genes in rice suggest that the involvement of Pht1 transporters not only in uptake of Pi from the soil solution but also in Pi-translocation.

Acknowledgements

This work was supported by China 973 Program (2005CB120903), China National Natural Science Foundation (30871582).

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