Title
Regulation and function of Pht1 family phosphate transporters in rice

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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 (Paszkowski 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 stereomicroscope 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. 2009).
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 ZEAm;Pht1;2/4, ZEAm;Pht1;3 and ZEAm;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 Ph1 PTs 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 Ph1 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 Ph1 gene family of rice. The expression of reporter genes and computational analysis of the Ph1 transporter genes in rice suggest that the involvement of Ph1 transporters not only in uptake of Pi from the soil solution but also in Pi-translocation.

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