Running head: Role of the rice \textit{PHO1} genes in phosphate homeostasis

Yves Poirier  
Département de Biologie Moléculaire Végétale  
Biophore  
Université de Lausanne  
CH-1015 Lausanne, Switzerland  
tel : 41 21 692 4222  
fax, 41 21 692 4195  
email, yves.poirier@unil.ch

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Characterization of the rice *PHO1* gene family reveals a key role for *OsPHO1;2* in phosphate homeostasis and the evolution of a distinct clade in dicotyledons

David Secco, Arnaud Baumann and Yves Poirier*

Département de Biologie Moléculaire Végétale, Biophore, Université de Lausanne, CH-1015 Lausanne, Switzerland
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* To whom correspondence should be addressed. Fax: 41 21 692 4195; email: yves.poirier@unil.ch
Abstract

Phosphate homeostasis was studied in a monocotyledonous model plant through the characterization of the PHO1 gene family in rice (*Oryza sativa*). Bioinformatics and phylogenetic analysis showed that the rice genome has three PHO1 homologues, which cluster with the Arabidopsis *AtPHO1* and *AtPHO1;H1*, the only two genes known to be involved in root-to-shoot transfer of phosphate. In contrast to the Arabidopsis PHO1 gene family, all three rice PHO1 genes have a cis-natural antisense transcript located at the 5’ end of the genes. Strand-specific Q-PCR analyses revealed distinct patterns of expression for sense and antisense transcripts for all three genes, both at the level of tissue expression and in response to nutrient stress. The most abundantly expressed gene was *OsPHO1;2* in the roots, for both sense and antisense transcripts. However, while *OsPHO1;2* sense transcript was relatively stable under various nutrient deficiencies, the antisense transcript was highly induced by Pi deficiency. Characterization of *Ospho1;1* and *Ospho1;2* insertion mutants revealed that only *Ospho1;2* mutants had defects in Pi homeostasis, namely strong reduction in Pi transfer from root to shoot, which was accompanied by low shoot and high root Pi. Our data identify *OsPHO1;2* as playing a key role in the transfer of Pi from roots to shoots in rice, and indicate that this gene could be regulated by its cis-NAT. Furthermore, phylogenetic analysis of PHO1 homologues in monocotyledons and dicotyledons revealed the emergence of a distinct clade of PHO1 genes in dicotyledons, which include members having roles other than long-distance Pi transport.
Introduction

Inorganic phosphate (Pi) is essential for plant growth and plays a pivotal role in metabolism. Although high amounts of phosphorus can be found in soil, readily available Pi levels are often hindered by its high sorption capacity and its low solubility in soil. The concentration of Pi in most soils is usually lower than 10 µM and may even drop to submicromolar levels at the root/soil interface. Pi deficiency limits plant growth and development, and significantly decreases production yield of crops. In order to maintain Pi concentration within critical limits for optimal development, plants have evolved a wide variety of responses at the morphological, physiological, biochemical and gene expression level aimed at regulating and optimizing Pi acquisition from the soil solution and its distribution to different organs and sub-cellular compartments (Raghothama, 2000; Abel et al., 2002; Poirier and Bucher, 2002).

Although the first genes involved in phosphate transport were characterized in Arabidopsis thaliana (Muchhal et al., 1996), the study of Pi transport and homeostasis at the molecular level has been expanded to other plants. This is particularly true for genes encoding the high affinity Pi transporters belonging to the Phl1 family and involved in Pi uptake into cells, as well as some aspect of the Pi-deficiency signaling cascades, which have been studied in barrel medic, potato, tomato, rice, barley, and maize (Leggewie et al., 1997; Liu et al., 1998a; Liu et al., 1998b; Chiou et al., 2001; Paszkowski et al., 2002; Hou et al., 2005; Nagy et al., 2005; Yi et al., 2005; Nagy et al., 2006; Tesfaye et al., 2007; Zhou et al., 2008). However, other aspects of Pi homeostasis have been less well studied at the molecular level in plants other than Arabidopsis, such as the role of the low affinity Pi transporters Phl2 (Rausch et al., 2004), as well as members of the PHO1 family (Hamburger et al., 2002).

PHO1 has first been identified in A. thaliana as a gene playing an important role in the transfer of Pi from roots to shoots (Poirier et al., 1991). The Atphol mutant is deficient in loading Pi acquired by the roots to the xylem vessel, resulting in strong Pi deficiency in all the aboveground tissues (Poirier et al., 1991). The AtPHO1 gene was cloned by a map-based approach and shown to encode a protein with no homology to characterized solute transporters, including the family of Phl1 H⁺-Pi co-transporters (Hamburger et al., 2002). AtPHO1 contains two domains, named SPX and EXS, which have been identified in Saccharomyces cerevisiae proteins playing a role in either Pi transport or sensing, as well as sorting proteins to endomembranes. AtPHO1 is mainly expressed in the stellar cells of the
root and the lower part of the hypocotyl, consistent with its role in Pi transfer to the xylem (Hamburger et al., 2002). The *A. thaliana* PHO1 family comprises 11 members (Wang et al., 2004). Complementation studies revealed that, among the *AtPHO1* family, only *AtPHO1* and *AtPHO1;H1* could rescue the defects of the *Atpho1* mutation, indicating that only these two genes are involved in long distance Pi transport from roots to shoots (Stefanovic et al., 2007). The role of the other nine *Arabidopsis* PHO1 genes is largely unknown, except for the *PHO1;H4* homologue, which plays a role in the response of hypocotyls to blue light (Kang and Ni, 2006), as well as seed size (Zhou et al., 2009) and flowering (Zhou and Ni, 2009). The moss *Physcomitrella patens* also contains 7 homologues to *AtPHO1*, even though moss does not have a vascular system and is not known to necessitate long-distance transport of Pi (Wang et al., 2008). Together, these results indicate that members of the PHO1 family have roles that go beyond Pi transport and homeostasis.

Rice is one of the most important cereal crops, feeding about half of the world population. Understanding the mechanisms regulating nutrient homeostasis, and in particular Pi, is of main importance to increase yield while minimizing the negative impact of soil fertilization on the environment. Herein, we characterize the PHO1 gene family in rice. We identified three homologues of PHO1 in rice, which all cluster with the Arabidopsis *AtPHO1* and *AtPHO1;H1*. Interestingly, the three rice genes have *cis*-natural antisense transcripts (*cis*-NATs). To investigate the potential role of the rice PHO1 genes family in Pi homeostasis and their regulation by *cis*-NATs, we analyzed the expression profiles of the sense and antisense transcripts, in the shoots and in the roots, in response to different nutrient stresses, and in different tissues. Furthermore, we isolated and characterized knock-out mutants for *OsPHO1;1* and *OsPHO1;2*, revealing that *OsPHO1;2* plays a major role in Pi distribution in rice. Phylogenetic analysis of PHO1 genes in monocotyledons and dicotyledons revealed the evolution of a distinct clade of PHO1 genes only in dicotyledons, which include members having roles other than long-distance Pi transport.
Results

Phylogenetic analysis of the PHO1 homologues in rice

Analysis of the rice genome databases revealed three genes with homology to the *A. thaliana* *AtPHO1*, referred to as *OsPHO1;1* (Os01g02000), *OsPHO1;2* (Os02g56510) and *OsPHO1;3* (Os06g29790). Phylogenetic analysis revealed that all three rice PHO1 proteins clustered with the *A. thaliana* *AtPHO1* and *AtPHO1;H1*, known to be involved in Pi transfer from roots to shoot (Stefanovic et al., 2007) (Fig. 1A). *OsPHO1;2* showed the highest level of homology to Arabidopsis PHO1, with 52% of amino acid identity and 70% of similarity (Fig. 1B). Among the rice PHO1 family, sequence conservation ranging from 43% amino acid identity and 64% similarity between *OsPHO1;1* and *OsPHO1;2*, to 62% and 75% of amino acid identity and similarity for *OsPHO1;1* and *OsPHO1;3*. These levels of homology are similar to those observed for the Arabidopsis and the moss *Physcomitrella patens* PHO1 family members (Wang et al., 2004; Wang et al., 2008). The two identified domains of the PHO1 proteins, namely the tripartite SPX N-terminal domains and the C-terminal EXS domain, were both highly conserved in the rice PHO1 proteins (Supplementary Figure S1).

To assess the diversity of the PHO1 gene family beyond rice and Arabidopsis, a phylogenetic tree was constructed which further included the PHO1 homologues from the monocotyledonous plants maize (*Zea mays*, 3 genes), sorghum (*Sorghum bicolor*, 3 genes), and *Brachypodium distachyon* (2 genes), as well as from the dicotyledonous plants papaya (*Carica papaya*, 5 genes), grapevine (*Vitis vinifera*, 8 genes), poplar (*Populus trichocarpa*, 9 genes), and barrel medic (*Medicago truncatula*, 3 genes) (Fig. 1A). These 47 genes were forming 2 distinct clades, with clade I regrouping genes homologous to the Arabidopsis *AtPHO1* and *AtPHO1;H1*, the only two genes known to be involved in long-distance Pi transport (Stefanovic et al., 2007), and clade II regrouping genes homologous to the Arabidopsis *AtPHO1;H2* until *AtPHO1;H10*, which have functions other than long-distance Pi transport (Fig. 1A). Remarkably, monocotyledons only had PHO1 members belonging to clade I, while dicotyledons had members belonging to both clades I and II.
The rice PHO1 genes are associated with cis-natural antisense transcripts

Analysis of the rice genome databases revealed the presence of antisense transcripts associated with all three rice PHO1 genes (Fig. 2). These cis-NATs were overlapping with the 5’ coding exons of the genes and, in addition for OSPHO1;1 and OsPHO1;2, the 5’untranslated region. The presence of these cis-NATs was further confirmed by RT-PCR using oligonucleotides allowing the specific amplification of either the sense or antisense transcripts, as well as the sequencing of the corresponding cDNAs (data not shown; Supplementary Table 1). The cis-NATs for OsPHO1;1 and OsPHO1;3 contained two introns while the cis-NATs for OsPHO1;2 was intronless. Analysis of the Arabidopsis genome failed to reveal any cis-NATs associated with the members of the PHO1 gene family in Arabidopsis. While the open reading frame of the OsPHO1;1 cis-NAT was short, with the potential of coding only a 56 amino acid peptide, the open reading reading frames of cis-NATs for OsPHO1;2 and OsPHO1;3 were significantly longer and could potentially encode proteins of 160 and 172 amino acids, respectively (Supplementary Figure S2). No significant sequence conservation was found between the polypeptides potentially encoded by the three cis-NATs, and no significant homology could be found with proteins in the NCBI database (data not shown).

Spatial expression of PHO1 genes in rice

To gain insight into the spatio-temporal pattern of expression of the PHO1 members in rice, strand specific real time RT-Q-PCR were performed on different organs and at different stages of development for plants grown in soil (Fig. 3). OsPHO1;2 sense transcript was most strongly expressed in roots while it was expressed at a relative low and constant level in all the other tissues tested (Fig. 3B). Interestingly, OsPHO1;2 antisense transcript was particularly strongly expressed in flowers during pollination, and to a lesser extent in roots. For OsPHO1;1 the predominant expression levels of the sense transcript were observed in flowers before and during pollination, as well as in roots (Fig. 3A). The antisense transcript of OsPHO1;1 was weakly expressed in all tissues tested with a slightly higher expression level in the roots. The expression of OsPHO1;3 sense transcript was the lowest of all three genes and was slightly more abundant in flowers as well as in leave blades (Fig. 3C). The antisense transcript associated with OsPHO1;3 was weakly expressed in all tissues. Comparison of the sense transcript expression in the roots for the three OsPHO1 genes revealed that OsPHO1;2 is expressed at levels 30- to 350-fold above that of OsPHO1;1 and OsPHO1;3, respectively.
Responses of OsPHO1 genes to N, P, K, S and Fe nutrient starvation

To examine the potential induction of the rice PHO1 genes by nutrient deficiency, transcript abundance was assessed strand-specific RT-Q-PCR in plants grown for two weeks in media deficient in either nitrate, phosphate, potassium, sulfate or iron, which was sufficient for the first symptoms of nutrient deficiency to appear. For these analysis, a value of 2-fold induction was used as a threshold. Analyses showed that OsPHO1;1 sense transcript was only weakly up-regulated in shoots by potassium deficiency (Fig. 4A). No significant induction of the OsPHO1;1 antisense transcript was detected for either shoots for roots (Fig. 4B). Phosphate deficiency up-regulated the OsPHO1;2 sense transcript weakly in shoots (2.1-fold) but induced the more strongly the OsPHO1;2 antisense in roots (4.5-fold) (Fig. 4C-D). For OsPHO1;3, the sense transcript showed up-regulation upon Pi starvation in the shoots and in the roots (3.5- and 2.5-fold, respectively) (Fig. 4E). The OsPHO1;3 antisense transcript was up-regulated by nitrate starvation (4-fold) in roots (Fig. 4F).

Transcriptional regulation of the OsPHO1 transcripts in response to Pi starvation

To further characterize the response of the rice PHO1 genes specifically to Pi deprivation, pre-germinated seedlings were grown for 7 days on Pi-sufficient medium and then transferred to Pi-deficient medium for 3 to 30 days, before being re-supplemented with Pi for up to 4 days. Assessment of the inorganic phosphate content in the shoots and in the roots (Fig. 5G, H), as well as the expression of the OsIPS1 transcript (Supplemental Figure S3), which has been previously shown to be strongly up-regulated by low Pi status (Hou et al., 2005), confirmed that the plants were effectively experiencing Pi deficiency.

Upon Pi starvation, the sense transcripts for both OsPHO1;1 and OsPHO1;2 remained relatively stable in both shoots and roots, with the exception for a transient increase in OsPHO1;1 expression in roots at 5- and 8-days of Pi deficiency (Fig. 5A-D). In contrast, the antisense transcripts generally increased upon deficiency, with a sharper increase observed for shoots compared to roots. The increase in OsPHO1;1 and OsPHO1;2 antisense upon Pi-deficiency leads to a low ratio of antisense/sense transcripts under Pi-sufficient conditions and high ratio upon extended Pi-deficient conditions (Fig. 5A-D). This shift in the transcript ratios was quickly reversed upon Pi re-supply. For the OsPHO1;3 gene, there was a general trend of increased sense transcript abundance upon Pi deficiency in both roots and shoots, with a quick
decrease in transcript abundance upon Pi re-supply (Fig 5E, F). In contrast, the level of *OsPHO1;3* antisense transcript remained stable upon all treatments. It is of interest to note that in most cases, the increase in sense or antisense transcript level observed for the three *OsPHO1* genes occurred relatively late, typically after 11 to 14 days upon Pi withdrawal, while the increase in *OsIPS1* occurred more quickly, between 3 to 5 days upon Pi withdrawal (Supplementary Figure S3).

**Mutation in OsPHO1;2 sense transcript causes defective root-to-shoot Pi transfer**

To investigate the functions of the rice *PHO1* gene family, we searched different rice genomic resources for publicly available mutants. Two independents null mutants were found for both the *OsPHO1;1* and *OsPHO1;2* genes, while no mutant was available for *OsPHO1;3*. For all four mutants, the insertion site of the T-DNA or transposon was checked by PCR and insertions were found to be within exons. Analysis by RT-PCR failed to detect transcripts of the mutated gene in all cases (Fig. 6A, B; Fig 7A, B).

Growth and Pi content of root and shoot was assessed for plants grown under hydroponic conditions with 1 mM Pi for 5-7 weeks. No significant difference was observed between wild type and the two independent mutants *Ospho1;1-1* and *Ospho1;1-2* for the biomass and Pi content in both roots and shoots (Fig. 6C-E). In contrast, under similar conditions, the two independent mutants *Ospho1;2-1* and *Ospho1;2-2* showed a net decrease in shoot and root biomass (Fig. 7C, D). Furthermore, the mutant plants had approximately 2-fold less Pi in the shoots and 4-fold more Pi in the roots compared to wild type plants (Fig. 7E, right histograms).

The Pi content in both roots and roots was further examined for plants initially germinated in water for 7 days and grown for an additional 7 days in medium with 1 mM Pi (time 0) before transferring to medium without Pi for 10, 20 and 30 days (Fig 7E). At time 0, the amount of root Pi in the *pho1;2* mutants was 6- to 7-fold higher compared to wild type, while only a slight decrease in shoot Pi content was observed for the mutants. Upon transfer in nutrient solution without Pi for 10 days, the mutants displayed 3- to 5-fold higher Pi content than wild type in both shoots and roots, while uniformly low internal Pi content was measured in both shoots and roots for both wild type and mutants when grown for 20 and 30 days in medium without Pi. Strikingly, as soon as 2 days after re-supply of Pi to plants grown for 30 days in...
Pi-deficient medium, the mutants had 2- to 4-fold higher Pi content in the roots than the wild type, and 2- to 3-fold less Pi in the shoots (Fig. 7E, penultimate histograms). Assessment of the level of Pi transfer from roots to shoots in plants grown in Pi-deficient medium revealed a 25-fold reduction in the Ospho1;2 mutants compared to wild type (Fig. 7F). In contrast, no difference was observed between wild type and the Ospho1;2 mutants in the extent of transfer of Pi from shoots to roots in plants grown under Pi-deficient conditions (Supplemental Figure S4).

No consistent changes in the expression of the sense and antisense transcripts for OsPHO1;1 were observed in the shoots or roots of the two Ospho1;2 mutants (Fig. 8A, B). While a small 1.5-fold reduction in the expression of the OsPHO1;2 antisense transcript was observed in the shoots for both Ospho1;2 mutants, no consistent change was found in roots (Fig. 8C, D). The expression of the sense OsPHO1;3 transcript in mutants was increased 4-fold in shoots and 2- to 5-5 fold in roots, while no changes were observed for either tissues for the antisense OsPHO1;3 transcript (Fig. 8E, F). However, the transcript level of the sense OsPHO1;3 transcript in the roots of the mutants remains at least 50-fold lower then the OsPHO1;2 sense transcript in wild type plants.
Discussion

Identifying the molecular players controlling shoot Pi content in plants is of main importance to improve crop growth and yield in Pi depleted soils. In this work, we studied the involvement of members of the rice PHO1 family in Pi homeostasis. Analysis of mutants in the rice OsPHO1;2 reveals a major role for this gene in the transfer of phosphate from the root to the shoot. This was most directly revealed by the large reduction in the fraction of Pi acquired by the root that is transferred to the shoot in two independent Ospho1;2 mutants compared to wild type, while the transfer of Pi from shoots to roots remained unaffected. This large reduction in root-to-shoot transfer of Pi was further reflected by the over-accumulation of Pi in the roots and the reduction of leaf Pi content in the Ospho1;2 mutants grown under Pi-sufficient conditions. The disruption in Pi transfer to the shoot has an important impact on plant growth, as reflected by a decrease in both shoot and root growth. Although a minor phenotypic effect of the small reduction in the expression of the OsPHO1;2 antisense transcript in the two T-DNA mutants cannot be excluded, the absence of changes in expression of the same antisense transcript in roots of the mutants strongly suggest that the deficiency in the transfer of Pi to the shoot is primarily caused by the lack of the sense OsPHO1;2 transcript in the mutants.

The rice OsPHO1;2 appears to be the functional homologue of the Arabidopsis AtPHO1 gene, since the Atpho1 mutant of Arabidopsis also showed sharp reduction in root-to-shoot transfer of Pi and strong negative effects on plant growth (Poirier et al., 1991). Recent characterization of knock-down mutants in the rice Pht1;2 and Pht1;6 genes, encoding a low- and high-affinity H⁺-Pi co-transporters, respectively, as well as of the knockout rice mutant in the proton pump OsA8 also revealed a role for these genes in the translocation of Pi to the shoot (Ai et al., 2009; Chang et al., 2009). However, the changes in shoot and root Pi concentrations observed in these mutants were significantly smaller than for the Ospho1;2 mutant. Furthermore, for plants with knock-down of Pht1;2 and Pht1;6 expression, reduction in shoot Pi was detected only for plants grown under low external Pi, consistent with the induction of mRNA accumulation and strength of promoter activity of the OsPht1;2 and OsPht1;6 gene under Pi deprivation (Ai et al., 2009). In contrast, mutation of the OsPHO1;2 gene had a stronger effect on growth and Pi levels in plants grown under Pi-sufficient conditions.
Mutations in the OsPHO1;1 gene appear to have no effects on plant growth or Pi content in roots or shoots. It is possible that this lack of phenotype may be due simply to the relatively weak expression of OsPHO1;1 in the root relative to OsPHO1;2. Alternatively, it remains possible that OsPHO1;1 may have a primary role in Pi homeostasis in tissues other than roots. The observed increase in OsPHO1;3 expression in roots of the Ospho1;2 mutants suggests a compensatory mechanism and a potential redundancy between OsPHO1;2 and OsPHO1;3. A similar redundancy and compensatory effects has been noted for the Arabidopsis PHO1 and PHO1;H1 genes (Stefanovic et al., 2007). However, at the amino acid level, OsPHO1;1 is more closely related to OsPHO1;3 than to OsPHO1;2. Furthermore, OsPHO1;1 is significantly expressed in flowers before and during pollination and its pattern of expression, as detected by Q-RT-PCR, largely overlaps with the OsPHO1;3 gene. Together, these results indicate also a possible redundancy in the role of OsPHO1;1 and OsPHO1;3. Deciphering the potential role of OsPHO1;1 and OsPHO1;3 will necessitate the analysis of double and perhaps triple mutants in the OsPHO1 genes.

Phylogenetic analysis of the PHO1 family in four monocotyledons (rice, maize, sorghum and Brachypodium distachyon) and five dicotyledons (Arabidopsis, papaya, barrel medic, poplar and grapevine) revealed the presence of two distinct clades. Of the 11 PHO1 genes present in Arabidopsis, only AtPHO1 and AtPHO1;H1 belonged to clade I while all other Arabidopsis PHO1 homologues belong to clade II. AtPHO1 and its closest homologue AtPHO1;H1 have previously been shown to be the only members of the AtPHO1 gene family to be able to complement the Arabidopsis pho1 mutant, and thus participate in Pi transfer from roots to shoots (Stefanovic et al., 2007). Furthermore, while the promoters of the AtPHO1 and AtPHO1;H1 genes are primarily active in the vascular cylinder, other members of the gene family show expression in a wide variety of tissues, including hydathodes, trichomes and pollen grains (Wang et al., 2004), or respond to various stresses and signaling molecules other than Pi, such as abscisic acid or the jasmonic acid precursor 12-oxo-phytodienoic acid (Ribot et al., 2008a; Ribot et al., 2008b). Together, these data indicate that members of the Arabidopsis PHO1 gene family belonging to clade II have roles that are quite distinct from long-distance Pi transport. This conclusion is further supported by the finding that the Arabidopsis PHO1 homologue AtPHO1;H4 (also known as SHB1) is involved in controlling hypocotyl growth under blue light (Kang and Ni, 2006), as well as seed size (Zhou et al., 2009) and flowering (Zhou and Ni, 2009). In this context, it is surprising that all members of the PHO1 family in the monocotyledons studied belong to clade I, while dicotyledons have
PHO1 members in both clade I and clade II. These results suggest the emergence of a divergent group of PHO1 genes only in dicotyledons, which may coincide with the acquisition of a novel role for this gene. Since the role of PHO1 members belonging to clade II is only currently known for the AtPHO1;H4 (SHB1) gene, it is unclear what common functionality, if any, can be assigned to the PHO1 genes of clades II, and why this functionality appear to be restricted to monocotyledons.

A feature distinguishing the rice PHO1 genes from their Arabidopsis homologues is the presence of cis-NATs in all three rice PHO1 genes whereas none are found for the Arabidopsis PHO1 genes. Cis-NATs are defined as RNA transcript generated from the same genomic loci as their sense transcript, but on the opposite DNA strand, thus generating a transcript containing a region with perfect complementarity to the sense transcript. Cis-NATs are often not encoding proteins, and the lack of homology among the potential open reading frames of the three OsPHO1 cis-NATs as well as against the NCBI proteome database suggest that the OsPHO1 cis-NATs are also non-coding. It has been estimated that rice and Arabidopsis have respectively 7 and 9% of genes having a cis-NAT (Osato et al., 2003; Jen et al., 2005; Wang et al., 2005; Lapidot and Pilpel, 2006b). Despite being widespread, their functional significance is unclear. Recent studies suggest that cis-NATs could be involved in the control of expression of the primary sense transcript through a variety of mechanisms, including transcriptional or translational interference, RNA masking, double-stranded RNA-dependent mechanisms and chromatin remodeling via methylation (Lapidot and Pilpel, 2006a; Werner et al., 2009). In plants, expression of cis-NAT was shown to influence the expression of the flowering gene FLC, the salt-stress genes P5CDH and SRO5, the cellulose synthase CesA6 and the cytokinin biosynthetic gene Sho, via the generation of small RNAs (Borsani et al., 2005; Swiezewski et al., 2007; Zubko and Meyer, 2007; Held et al., 2008). However, it is estimated that the majority of cis-NATs present in Arabidopsis do not generate small RNAs (Henz et al., 2007).

It is striking that while the cis-NAT associated with the OsPHO1;3 gene does not appear to be regulated either developmentally or in response to Pi deficiency, the cis-NATs of OsPHO1;1 and OsPHO1;2 are strongly up-regulated by Pi deficiency, while the corresponding sense transcript remains relatively stable. Such a pattern of expression suggests a potential role for these cis-NATs in controlling the expression of the OsPHO1;1 and OsPHO1;2 genes. It has previously been observed that cis-NATs pairs tended to have more anti-correlated expression
patterns than non-overlapping neighboring transcripts, and that this transcription pattern could sometimes be associated with the generation of small RNAs (Henz et al., 2007; Werner et al., 2009). In addition, intronless cis-NAT, such as found for OsPHO1;2, has been proposed to be a very rapid way of regulating the sense transcript by avoiding splicing mechanisms (Lapidot and Pilpel, 2006a). However, the complex and wide range of action of cis-NATs, coupled with the few well described examples of their roles and mode of action, makes the search of the putative underlying regulatory mechanisms challenging. Furthermore, caution must be exercised in interpreting the potential effects of regulated expression of the sense and antisense transcripts because the analysis was performed at the whole organ level and it is not known if the expression of the sense and antisense OsPHO1 transcripts occur in the same cells and tissues in all cases.

The relatively slow response of OsPHO1;1 sense and OsPHO1;2 antisense transcripts to Pi deficiency suggests that these transcripts are more likely to respond to changes in the concentration of internal Pi or Pi-containing metabolites instead of sensing the external Pi concentration. Indeed, increased levels of expressions of these transcripts is linked with to low internal Pi status. In Arabidopsis, Pi levels in roots drops by 70% after only 4 days of Pi starvation, while in rice it took 8 days for roots to reach a similar decrease. Similar results were reported in the moss Physcomitrella patens (Wang et al., 2008), suggesting that Arabidopsis has very little phosphate storage capacity, compared to rice and moss.
Materials and Methods

Database searches, sequence alignment and phylogenetic analysis. The identification of PHO1 homologs in monocotyledons and dicotyledons was performed using the tBLASTn program and the Arabidopsis PHO1 amino acid sequence (NP 188985) as a query, in the PLAZA1.0 software (http://bioinformatics.psb.ugent.be/plaza/) and the Phytozome 4.0 software (http://www.phytozome.net/). The unrooted phylogenetic tree of the PHO1 family mono- and dicotyledons was made using the neighbor-joining method and displayed using the MEGA4 program (http://www.megasoftware.net/index.html). The rice sense and antisense transcripts were identified from the Rice Functional Genomic Express Database (http://signal.salk.edu/cgi-bin/RiceGE). Information on transcripts for OsPHO1;1 sense (AK242852), OsPHO1;1 antisense (AK241522), OsPHO1;2 sense (AK100323), OsPHO1;2 antisense (AK071338), OsPHO1;3 sense (AK067110) and OsPHO1;3 antisense (AK1092049) was obtained from KOME (http://cdna01.dna.affrc.go.jp/cDNA/) (Satoh et al.; Kikuchi et al., 2003).

Plant materials and stress treatments.

For hydroponic culture of O. sativa, the plants were grown for 3 to 6 weeks under non-sterile conditions. Seeds were placed on a floating polystyrene raft, filled with wholes and closed at the bottom by a plastic net. These systems were disposed on top of 5L tanks filled with tap water for the first week, and then with nutrient solution for the additional weeks. For studying a time-course of gene expression under Pi deficiency, rice (Oryza sativa L. cv. Nipponbare) seeds were first pre-germinated in water for 7 days and then placed for 7 days in hydroponic culture under controlled conditions in a phytotron (13 h light, 28°C/11 h dark, 22°C cycle) with full Hoagland media (1 mM Pi), and then subjected to Pi deficiency (0 mM Pi) or normal condition (1 mM Pi) for various amounts of time before being harvested for RNA extraction and Pi content measurements. Hydroponic solution was changed every 5 days. For studying gene expression under different nutrient starvation conditions, seeds were first pre-germinated in water for 7 days, and then grown for 2 weeks in normal Hoagland medium (control) or nutrient deficient media before collecting tissues for RNA extraction. For Pi-deficient medium, Pi was removed from solution. For nitrate-deficient medium, equimolar amounts of K2SO4 and CaCl2 were used instead of KNO3 and Ca(NO3)2. For potassium-deficient medium, equimolar amounts of Ca(NO3)2, CaCl2 and NH4H2PO4 were used instead of KNO3, KCl and KH2PO4. For sulfate-deficient medium, equimolar amounts of MgCl2 was
used instead of MgSO₄, and finally for iron-deficient medium, iron citrate was removed. Care was taken to adjust the media to pH 5.5 using 2.4 mM MES. Plants have also been grown in a greenhouse (12 h light, 30°C/12 h dark, 22°C cycle) in fertilized soil.

**RNA extraction and expression analysis.**

Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen) following manufacturer’s instructions. cDNA was synthesized from 1 µg of DNaseI-treated total RNA (Fermentas) using oligo(dT) and Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer’s instructions. Strand specific real-time PCR was performed in a 25 µl volume containing 5 µl of cDNA, 12.5 µl of 2x SYBR green mix (Axon lab), and 0.25 µM of each primer. Analysis was performed in an MX3000P real-time PCR system (Stratagene) with cycle conditions of an initial denaturing at 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 60°C for 1 min, and 72 °C for 1 min. The transcript specific primers used are listed in Supplementary Table 1. All data were normalized against the expression level of the rice ubiquitin gene (Os06g46770).

**Identification of the Ospho1 mutants.**

The Tos17 insertion mutants Ospho1;1-2 (NG7033), Ospho1;2-1 (NC0015), and Ospho1;2-2 (NE2044) were obtained from the Rice Genome Resource Center, Japan. The homozygous mutants were identified using primers provided on the Rice Genome Resource Center website (http://tos.nias.affrc.go.jp/). The Ospho1;1-1 (1A-24127) T-DNA insertion mutant was obtained from Postech RISD (http://www.postech.ac.kr/life/pfg/risd/)

**Pi content determination and long distance transfer of Pi.**

Determination of Pi in tissues was measured by releasing the cellular content of cells into water by repeated freeze-thaw cycle, or by incubation for 1 hour at 85°C, and quantifying Pi by the molybdate assay according to the procedure of Ames (Ames, 1966). Transfer of Pi from roots to shoots was assessed by growing plants under hydroponic conditions for 3 weeks in a Pi-deficient medium and then immersing roots in a medium containing 33P radiolabelled phosphate (2 µCi/ml; 10 µM Pi) for 2 hours. Following extensive washing of the roots with 1mM cold phosphate, the radioactivity present in roots and shoots was measured separately by scintillation counting. Pi transfer from root to shoot was obtained by dividing shoot radioactivity by whole plant radioactivity. To measure the extend of Pi transfer from shoots to roots, around 2 cm of the tip of leaves of plants grown for 7 days in water and 7 days in Pi-
sufficient medium (1mM Pi) were dipped into a solution containing 3 µCi/mL of $^{33}$P radiolabelled phosphate (10 µM) and 0.01% TritonX-100 for 24 h, followed by washing with a non-radioactive 1mM phosphate solution. Plants were then transferred for 10 additional days in a Pi-deficient Hoagland medium. The portion of the plant which had been dipped into the radiolabelled solution was then discarded, and the radioactivity present in roots and shoots was measured separately by scintillation counting. The extent of Pi transfer from shoots to roots was obtained by dividing root radioactivity by whole plant radioactivity.
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Figure legends

Figure 1. Phylogenetic analysis of the PHO1 family in monocotyledons and dicotyledons. (A) Unrooted phylogenetic tree of the PHO1 family from Oryza sativa (Os), Sorghum bicolor (Sb) Zea mays (Zm), Brachypodium distachyon (Bd), Arabidopsis thaliana (At), Carica papaya (Cp), Populus trichocarpa (Pt), Vitis vinifera (Vv), and Medicago truncatula (Mt). The tree was made using the neighbor-joining method. The two major clades are encircled, the Arabidopsis genes are underlined and the rice genes are in bold. (B) Identity/similarity matrix for rice and Arabidopsis PHO1 proteins. Amino acid identity and similarity are indicated by first and second number, respectively.

Figure 2. Structure of the OsPHO1 genes with their respective cis-NAT. Exons are indicated by black boxes. Numbers indicate the length of the mRNA, in base pair, and the large arrow heads indicates the orientation of transcription. Small arrows indicate the location of the primers used for strand specific Q-RT-PCR.

Figure 3. Spatial expression of the OsPHO1 transcripts. Sense and antisense transcript levels for OsPHO1;1 (A), OsPHO1;2 (B) and OsPHO1;3 (C) in seven-day-old seedlings, flowers before, during and after pollination, leaf sheath, leaf blade and roots are represented. Apart for seedlings, all other tissues were harvested from plants grown in soil for 5-6 month in a greenhouse. Plants were fertilized weekly with a nutrient solution containing 1 mM Pi. All data are means of three replicates with error bars indicating SD, and represented as transcript level per 10^6 copies of ubiquitin.

Figure 4. Expression pattern of the OsPHO1 transcripts in response to different nutrient deficiencies. After a week of pre-germination in water, plants were grown for 2 weeks in complete medium (Ctrl) or in medium deficient either in sulfur (-S), phosphate (-Pi), nitrogen (-N), potassium (-K) or iron (-Fe), before being harvested. Expression levels of both sense (A, C, E) and antisense (B, D, F) transcripts were assessed using strand specific Q-RT-PCR for OsPHO1;1 (A, B), OsPHO1;2 (C, D), and OsPHO1;3 (E, F). All data are means of three replicates with error bars indicating SD, and represented as transcript level per 10^6 copies of ubiquitin.
Figure 5. Expression pattern of the OsPHO1 transcripts in response to Pi starvation. Strand specific Q-RT-PCR were performed to detect the levels of expression of sense (white) and antisense (black) transcripts of OsPHO1;1 (A, B), OsPHO1;2 (C, D) and OsPHO1;3 (E, F) transcripts in shoots (A, C, E) and roots (B, D, F). After a week of pre-germination in water, the plants were grown for a week in presence of 1 mM Pi before being transferred to Pi deficient media (T=0 days) for the desired period of time (from 0 to 30 days). After a 30 days of Pi starvation, plants were transferred to Pi sufficient conditions for one to four days. Control plants (+Pi) were grown for 34 days in presence of 1 mM Pi. All data are means of three replicates with error bars indicating SD, and represented as transcript level per 10^6 copies of ubiquitin. Inorganic phosphate content in the aerial part (G) and in the roots (H) is represented. Error bars are SD, n=6.

Figure 6. Functional characterization of Ospho1;1 mutants. (A) Positions of Tos17 and T-DNA insertions in OsPHO1;1 gene are indicated by arrow heads. Black boxes represent exons and black lines represent introns. Small arrows represent the gene specific primer (FL1 and RL1) used for RT-PCR. (B) RT-PCR using RNA of roots from mutants and wild type (WT) plants. (C) Morphological appearance of Ospho1;1 null mutants plants compared to WT. Plants were pre-germinated in water for 7 days and then grown hydroponically for six weeks in presence of full Hoagland medium (1 mM Pi), before being harvested. Black bar represent 10 cm. Fresh weight (D) and Pi content (E) were assessed for shoots and roots of ospho1;1 mutants and WT after 6 weeks of growth in medium with 1 mM Pi. All data are means of six replicates with error bars indicating SD.

Figure 7. Functional characterization of rice pho1;2 mutants. (A) Positions of Tos17 insertions in OsPHO1;2 gene are indicated by arrow heads. Black boxes represent exons and black lines represent introns. Small arrows represent the gene specific primer (FL2 and RL2) used for RT-PCR. (B) RT-PCR using RNA of roots from mutants and wild type (WT) plants. (C) Morphological appearance of Ospho1;2 null mutants plants compared to WT. Plants were pre-germinated in water for 7 days and then grown hydroponically for four weeks in presence of full Hoagland medium (1 mM Pi), before being harvested. Black bar represent 10 cm. (D) Fresh weight was assessed for shoots and roots of Ospho1;2 mutants and WT after 4 weeks of growth in medium with 1 mM Pi. (E) Pi content was assessed in roots and shoots for Ospho1;2 mutants and WT. Plants initially germinated in water for 7 days and grown for an
additional 7 days in medium with 1 mM Pi (time 0) before transferring them to medium without Pi for 10, 20 and 30 days. After 30 days in Pi-deficient medium, plants were transferred back in medium with 1 mM Pi for 2 days (30d + 2d). Control plants (right histograms +Pi) were grown for 34 days in medium with 1 mM Pi. (F) Translocation rate of Pi from roots to shoots of 3-week-old plants grown hydroponically in Pi-deficient medium. All data are means of six replicates with error bars indicating SD.

**Figure 8. Expression of the OsPHO1 gene family in the Ospho1;2 mutants.** Strand specific Q-RT-PCR were performed to detect the levels of expression of sense (white) and/or antisense (black) transcripts of *OsPHO1;1* (A, B), *OsPHO1;2* (C, D) and *OsPHO1;3* (E, F) transcripts in shoots (A, C, E) and roots (B, D, F), in WT and in the two *Ospho1;2* mutants. After a week of pre-germination in water, the plants were grown for 5 weeks in medium containing 1 mM Pi, before harvesting shoots and roots separately. All data are means of three replicates with error bars indicating SD, and represented as transcript level per 10^6 copies of ubiquitin.
A

B

| OsPHO1;1 | OsPHO1;2 | OsPHO1;3 |
|---------|---------|---------|
| 48/69   | 52/70   | 46/67   |
| 43/64   | 62/75   | 44/64   |
A

OsPHO1;1 transcript levels

B

OsPHO1;2 transcript levels

C

OsPHO1;3 transcript levels

Sense
Antisense

Seedling
Flowers before pollination
Flowers during pollination
Flowers after pollination
Leaf sheath
Leaf blade
Root
Normalized transcript level

A

Sense transcripts

OsPHO1;1 sense

B

Antisense transcripts

OsPHO1;1 antisense

C

OsPHO1;2 sense

D

OsPHO1;2 antisense

E

OsPHO1;3 sense

F

OsPHO1;3 antisense

Ctrl -S -Pi -N -K -Fe

Shoot Root

Ctrl -S -Pi -N -K -Fe

Shoot Root
