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ORIGINAL ARTICLE

Expression analyses of three members of the *AtPHO1* family reveal differential interactions between signaling pathways involved in phosphate deficiency and the responses to auxin, cytokinin, and abscisic acid

Cécile Ribot · Yong Wang · Yves Poirier

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Abstract The PHO1 protein is involved in loading inorganic phosphate (Pi) to the root xylem. Ten genes homologous to *AtPHO1* are present in the *Arabidopsis thaliana* (L.) Heyn genome. From this gene family, transcript levels of only *AtPHO1*, *AtPHO1;H1* and *AtPHO1;H10* were increased by Pi-deficiency. While the up-regulation of *AtPHO1;H1* and *AtPHO1;H10* by Pi deficiency followed the same rapid kinetics and was dependent on the PHR1 transcription factor, phosphite only strongly suppressed the expression of *AtPHO1;H1* and had a minor effect on *AtPHO1;H10*. Addition of sucrose was found to increase transcript levels of both *AtPHO1* and *AtPHO1;H1* in Pi-sufficient or Pi-deficient plants, but to suppress *AtPHO1:H10* under the same conditions. Treatments of plants with auxin or cytokinin had contrasting effect depending on the gene and on the Pi status of the plants. Thus, while both hormones down-regulated expression of *AtPHO1* independently of the plant Pi status, auxin and cytokinin up-regulated *AtPHO1;H1* and *AtPHO1;H10* expression in Pi-sufficient plants and down-regulated expression in Pi-deficient plants. Treatments with abscisic acid inhibited *AtPHO1* and *AtPHO1;H1* expression in both Pi-sufficient and Pi-deficient plants, but increased *AtPHO1;H10* expression under the same conditions. The inhibition of expression by abscisic acid of *AtPHO1* and *AtPHO1;H1*, and of the Pi-starvation responsive genes *AtPHT1;1* and *AtIPS1*, was dependant on the ABI1 type 2C protein phosphatase. These results reveal that various levels of cross talk between the signal transduction pathways to Pi, sucrose and phytohormones are involved in the regulation of expression of the three *AtPHO1* homologues.

Keywords Abscisic acid · *Arabidopsis* · Auxin · Cytokinin · PHO1 · Phosphate · PHR1

Abbreviations

2,4-D	2,4-Dichlorophenoxy-acetic acid
ABA	Abscisic acid
ABI1	Abscisic acid insensitive 1
Pi	Inorganic phosphate
PHR1	Phosphate starvation response 1
PHO1	Phosphate deficient 1

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Introduction

Phosphorus is one of six essential macronutrients that plants acquire from the soil and utilize for their growth and development. Although the total amount of phosphorus in the soil may be high, inorganic phosphate (Pi), the main form of phosphorus assimilated by plants, is relatively inaccessible to plant roots because of its low solubility and high-sorption capacity in soil. This inaccessibility constitutes one of the major constraints for plant growth in natural ecosystems. To maintain phosphorus homeostasis within critical limits for optimal development, plants have evolved various systems that regulate Pi acquisition from the soil solution and its distribution to different organs and cellular compartments (Raghothama 2000; Poirier and Bucher 2002).

Until now, five protein families, implicated in phosphate transport have been identified in plants (Poirier and Bucher 2002). The PHT1 family encodes high-affinity proton-coupled Pi transporters primarily expressed in plant roots. The

characterization of loss-of-function *A. thaliana* mutants in the *AtPHT1;1* and *AtPHT1;4* genes showed a strong reduction in Pi-uptake capacity, demonstrating the major role of these transporters in phosphate absorption into roots (Misson et al. 2004; Shin et al. 2004). After penetrating in root cells, acquired Pi needs to be distributed to the various tissues of the plant as well as to the different organelles of the cells. Whereas no phosphate transporter has yet been identified in the tonoplast, specific transporters have been localized to membranes of plastids and mitochondria. These include the plastid PHT2;1 thought to function as a H^+/Pi symporter, several types of plastidic phosphate translocators acting as phosphate/sugar exchangers, and PHT3 acting as a mitochondrial Pi/H^+ symporter (Poirier and Bucher 2002).

Proper distribution of Pi among the various plant tissues requires the loading and unloading of Pi in the xylem and phloem. The *A. thaliana AtPHO1* gene has been demonstrated to have a crucial role in Pi loading to the root xylem vessel. Physiological characterization of the *pho1* mutant showed that only 3–10% of the wild-type level of Pi was translocated to shoots while Pi uptake into the root was unaffected in the mutant (Poirier et al. 1991). AtPHO1 has a large hydrophilic N-terminus and a C-terminus containing a minimum of six putative transmembrane domains (Hamburger et al. 2002). *AtPHO1* is predominantly expressed in the stellar cells of the root and of the lower part of the hypocotyl and the protein shares no sequence homology with any characterized solute transporters (Hamburger et al. 2002). Although the precise mode of action of AtPHO1 remains to be elucidated, it is clear that the protein plays an important role in phosphate homeostasis. The *A. thaliana* genome contains ten additional genes encoding proteins having significant sequence similarity and the same topology to AtPHO1, which consequently form a novel class of proteins likely involved in plant Pi transport (Wang et al. 2004). Although the promoters of a majority of *AtPHO1* family members are active in the vascular tissue, such as *AtPHO1* and *AtPHO1;H1*, some members have distinct expression pattern, such as *AtPHO1;H9*, expressed in the pollen and *AtPHO1;H10* expressed in the root epidermal/cortical cells (Wang et al. 2004). It has recently been shown that among the homologues of *AtPHO1*, only one gene, namely *AtPHO1;H1*, can complement the *pho1* mutant when expressed under the control of the *PHO1* promoter, thus revealing a limited functional redundancy for Pi loading to the root xylem among the members of the *AtPHO1* gene family (Stefanovic et al. 2007).

Over the past 5 years, several studies aimed at analyzing the pattern of gene expression using microarrays have revealed a complex network of genes that are up- and down-regulated at various points following Pi starvation either in roots or in shoots (Wang et al. 2002; Uhde-Stone et al. 2003; Wu et al. 2003; Misson et al. 2005; Morcuende et al. 2007; Müller et al. 2007). At present, relatively little is known of the components of the signal transduction cascade(s) involved in sensing Pi nutrient status and triggering a coordinated adaptive response to Pi deficiency in plants. The transcription factors PHR1, WRKY75 and BHLH32 in *A. thaliana*, and OsPTF1 in rice, as well as the small ubiquitin-related modifier E3 ligase SIZ1 in *A. thaliana*, have been identified as participating in the response of plants to Pi deficiency (Rubio et al. 2001; Miura et al. 2005; Yi et al. 2005; Chen et al. 2007; Devaiah et al. 2007). The *At4* and *IPS1* genes and the micro RNA miR399, which are all strongly induced upon Pi starvation, have been implicated in controlling the expression of PHO2, a gene encoding a ubiquitin-conjugating E2 enzyme playing an important role in phosphate distribution homeostasis under Pi deficiency (Aung et al. 2006; Bari et al. 2006; Chiou et al. 2006; Shin et al. 2006; Franco-Zorilla et al. 2007). *LTR1*, a gene encoding a multicopper oxidase, has been implicated in the reduction of primary root growth upon Pi-deficient conditions (Svistoonoff et al. 2007).

Other factors have been found to influence the responses of plants to Pi deficiency. Recent studies have indicated potential interactions between Pi-deficiency signaling and sugar-sensing pathways. Sucrose has been implicated in the transcriptional control of several genes induced upon Pi deficiency, such as the UDP glucose phosphorylase, *AtIPS1* (Martin et al. 2000), *AtACP5* (encoding an acid phosphatase) and members of the *AtPHT1* family, and phosphate deficiency typically leads to high starch and sucrose levels in shoots (Ciereszko et al. 2005; Franco-Zorrilla et al. 2005; Müller et al. 2005, 2007; Karthikeyan et al. 2006). Both hexokinase-dependent and hexokinase-independent signaling pathways are thought to be involved in the interactions between sugar sensing and Pi starvation responses (Müller et al. 2005; Karthikeyan et al. 2006). Several phytohormones have also been implicated in the response and adaptation of plants to Pi deficiency. For example, both auxin and ethylene have been implicated in modulating the developmental adaptations of roots to Pi deficiency (López-Bucio et al. 2002; Lopez-Bucio et al. 2005; Nacry et al. 2005). Microarray studies from Pi deficient plants have also revealed changes in transcripts levels of genes involved in hormone synthesis or responding to them (Wang et al. 2002; Hammond et al. 2003; Uhde-Stone et al. 2003; Wu et al. 2003; Misson et al. 2005; Morcuende et al. 2007; Müller et al. 2007). Cytokinin and its receptor CRE1 have been found to play an important role in suppressing the up-regulation of several genes following Pi deficiency (Martin et al. 2000; Franco-Zorrilla et al. 2002, 2005; Karthikeyan et al. 2002; Hou et al. 2005; Wang et al. 2006), implicating a cross talk between Pi and cytokinin signal transduction pathways.

The goal of this work was first to analyze the pattern of expression of the members of the *AtPHO1* gene family under Pi-deficiency. The influence of sugar and of the phytohormones auxin, cytokinin and abscisic acid on the expression *AtPHO1* gene family members responding to Pi-deficiency was then studied to reveal common patterns of regulation among these genes, as well as potential level of cross talk between various signal transduction pathways and the Pi-deficiency response.

Materials and methods

Plant culture and treatments

Seeds of wild-type *A. thaliana* Columbia ecotype, wild-type *A. thaliana* Landsberg *erecta* ecotype, *pho1-3*, *phr1*, *pho2*, *aba1-3* and *abi1-1* mutants were grown aseptically, under continuous light ($100\ \mu mol\ m^{-2}\ s^{-1}$) at 20°C, on agar-solidified media containing 1 mM KH_2PO_4 (pH 5.5), 5 mM KNO_3, 20 mM NH_4NO_3, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM Fe–EDTA, 50 μM H_3BO_4, 12 μM $MnSO_4$, 1 μM $ZnCl_2$, 1 μM $CuSO_4$, 0.2 μM Na_2MoO_4 and 1% sucrose. Seeds from *pho2*, *aba1-3* and *abi1-1* mutants were obtained from the Nottingham *Arabidopsis* Stock Centre (University of Nottingham, UK). For phosphate-deficient media, KH_2PO_4 was replaced by KNO_3. For phosphite treatment, a phosphite solution of 1 mM prepared from phosphorous acid (Aldrich Chemicals) and KOH (final pH of 5.5) was added to media. Stock solutions of kinetin and (±)-*cis*, *trans*-abscisic acid (ABA) were prepared in water (5 and 100 mM, respectively) with a drop of NaOH 1 N to help the dissolution, while 2,4-dichlorophenoxy-acetic acid (2,4-D) was dissolved in 100% ethanol to a concentration of 10 mM. All phytohormones were purchased from Sigma (St. Louis, MO, USA).

RNA isolation and Northern-hybridization analysis

Total RNA was extracted from plant tissues by phenol:chloroform separation and lithium chloride precipitation followed by washes with sodium acetate and ethanol as previously described (Stefanovic et al. 2007). Northern analysis was performed by separating 25 μgof total RNA on agarose gels containing formaldehyde, transferring to Nylon membranes (Hybond N+; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and hybridizing with P^{32}-radiolabelled specific probes according to standard procedures under high-stringency conditions. Specific probes corresponded to GST (Gene Specific Tags) designed in the microarray CATMA project (Crowe et al. 2003) amplified by PCR with specific set of primers or full-length cDNA.

RT-PCR analysis

Analysis of the expression profile of the *AtPHO1* family genes was done by semi-quantitative RT-PCR as described in Wang et al. (2004). Pilot experiments were performed on reverse-transcribed products of several genes to identify the number of cycles corresponding to the linear phase of amplification. Based on these results, a protocol based on 20 cycles of PCR amplifications was done for all genes. The oligonucleotides used for primers were designed to amplify a fragment of 400–600 bases at the 3′ end of the gene. The region amplified encompassed at least two exons, so that PCR fragments generated from contaminating genomic DNA could easily be distinguished from fragments amplified from cDNA based on the size of the products. Following RT and PCR amplification, as described above, the products were analyzed by Southern blot using probes derived from each member of the *AtPHO1* family using standard procedures.

Results

Distinct dynamic of changes in *AtPHO1, AtPHO1;H1* and *AtPHO1;H10* mRNA levels in response to the availability of Pi in the medium

Analysis of the expression of the *AtPHO1* and *AtPHO1;H1* genes has previously revealed their up-regulation under Pi-deficient conditions (Wang et al. 2004; Stefanovic et al. 2007). In order to examine whether other members of the *AtPHO1* gene family were up-regulated under Pi-deficient conditions, semi-quantitative RT-PCR analyses were performed to detect the gene expression pattern of all members of the *PHO1* gene family in 7-day-old seedlings grown in medium containing 1 mM Pi and then transferred either to medium containing 1 mM Pi or no phosphate for an additional 5 days (Fig. 1). Analysis revealed that in addition to *AtPHO1* and *AtPHO1;H1*, the steady state mRNA level of *AtPHO1;H10* was also elevated by phosphate deficiency. In contrast, the expression level of *AtPHO1;H2, H3, H4, H5, H7*, and *H8* genes was not influenced by 5 days of phosphate starvation, while *AtPHO1;H6* and *H9* transcripts were undetectable in seedlings at this stage of development, in agreement with previous results indicating that expression of these genes was restricted to flowers (Wang et al. 2004). Consequently, *AtPHO1*, *AtPHO1;H1* and *AtPHO1;H10* genes are the only three members of the *AtPHO1* gene family to be up-regulated by phosphate deficiency.

To better understand the dynamic of *AtPHO1*, *AtPHO1;H1* and *AtPHO1;H10* responses to phosphate starvation, an analysis of the effect of different Pi concentrations as well as a time course of response to Pi deficiency

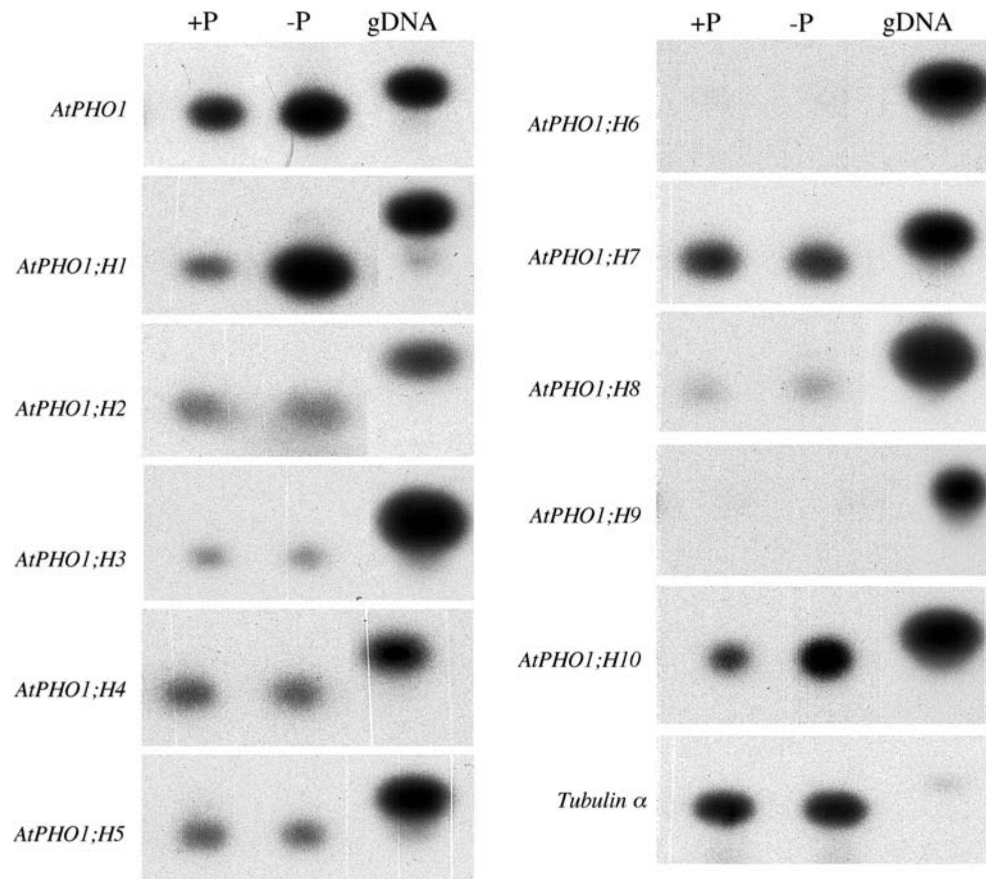

Fig. 1 Expression analysis of the members of the *AtPHO1* gene family during Pi starvation. Semi-quantitative RT-PCR were performed using RNA extracted from whole plants grown first for 7 days in media containing 1 mM Pi and then transferred to media containing 1 mM Pi (+*P*) or without Pi (−*P*) for 5 days. DNA fragment obtained by PCR performed on genomic DNA using the same oligonucleotides used for RT-PCR is shown in the *last lane* (gDNA). This control ensures that the band obtained by RT-PCR cannot be derived from the amplification of genomic DNA that could be present in the RNA preparations

was performed (Fig. 2). Seedlings were first grown for 7 days in medium containing 1 mM Pi and then transferred for 5 days to media supplemented with KH_2PO_4 at concentrations ranging from 0 to 1.25 mM. Northern blot analysis showed that the *AtPHO1;H1*, and *AtPHT1;1* transcript levels were slightly more responsive to small decrease in external Pi concentration compared to *AtPHO1* and *AtPHO1;H10* (Fig. 2a). In contrast, following the transfer of plants to medium without Pi, the increase in mRNA level was clearly more rapid for *AtPHO1;H1* and *AtPHO1;H10* compared to *AtPHO1* and *AtPHT1;1* (Fig. 2b). Similarly, the down-regulation of mRNA levels following the re-supply of Pi to plants grown in Pi-deficient media was quicker for *AtPHO1;H1* and *AtPHO1;H10*, with a return to basal expression level after 8 h of Pi re-supply, compared to *AtPHO1* and *AtPHT1;1* which returned to basal expression level only between 24 and 48 h after Pi re-supply (Fig. 2c).

Effects of phosphite and of the *phr1*, *pho1* and *pho2* mutations on *AtPHO1, AtPHO1;H1* and *AtPHO1;H10* expression

The orthophosphite anion ($H_2PO_3^-$ or HPO_3^{2-}) is a non-metabolizable analogue of phosphate that is able to suppress several typical molecular and developmental responses to Pi limitation (Ticconi et al. 2001; Varadarajan et al. 2002; Kobayashi et al. 2006). Phosphite had previously been shown to strongly suppress the up-regulation of *AtPHO1;H1* induced by Pi deficiency but to have little influence on the expression of *AtPHO1* under the same conditions (Stefanovic et al. 2007). The induction of *AtPHO1;H10* following Pi deficiency was only weakly suppressed by phosphite (Fig. 3a).

The PHR1 transcription factor was found to bind the imperfect palindromic sequence GNATATNC present in promoter regions of several genes whose expression increases during phosphate starvation stress (Rubio et al. 2001). Previous analysis had revealed the presence of the PHR1 binding sequence at position −330 bp from the transcription initiation site in the *AtPHO1;H1* gene but not in *AtPHO1* (Stefanovic et al. 2007). In agreement with the presence of these sequences, the upregulation of mRNA transcript of *AtPHO1;H1* in plants under Pi-deficient conditions was found to be under the control of PHR1, while this was not the case for the *AtPHO1* (Fig. 3b; Stefanovic et al. 2007). The *AtPHO1;H10* promoter region contains at position −996 bp upstream of the start codon a motif similar to the PHR1 element but not identical in that the first base is a T instead of a G. However, the sequence TCATATGC retains the structure of an imperfect palindromic sequence.

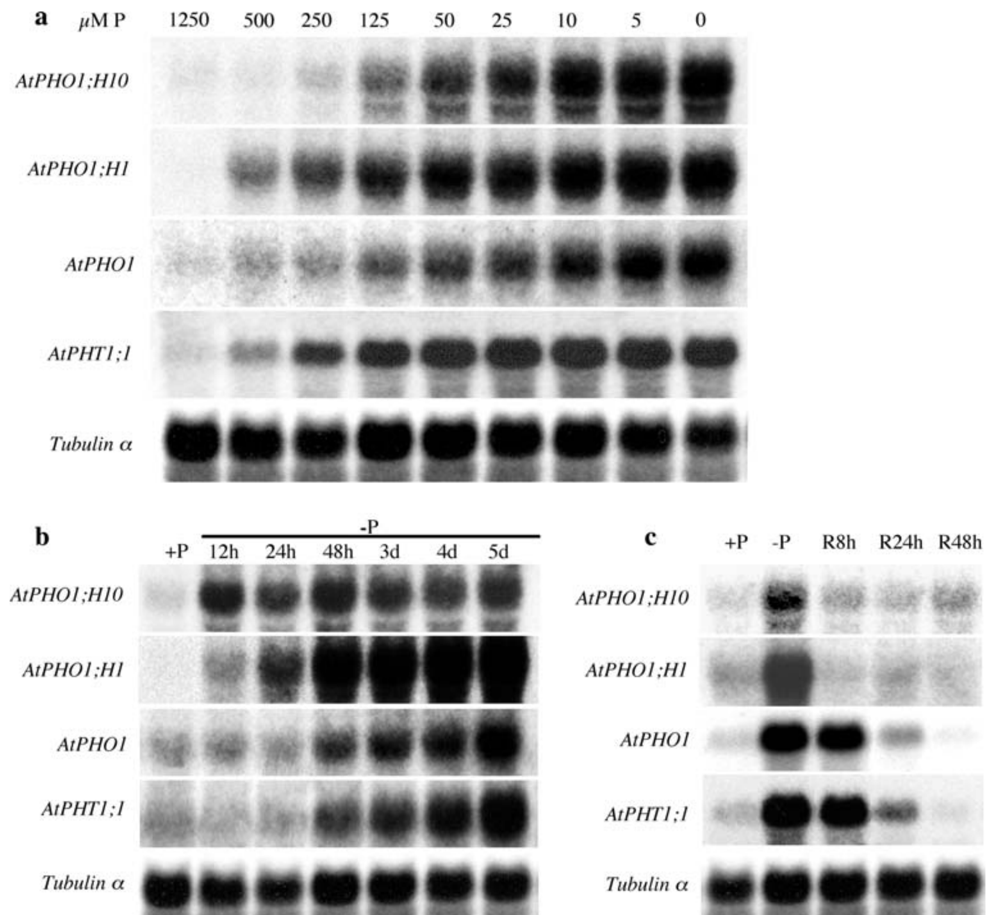

Fig. 2 Effect of Pi concentration and kinetic of response of *AtPHO1*, *AtPHO1;H1*, and *AtPHO1;H10* gene expression. **a** Plants grown for 7 days in media containing 1 mM Pi were transferred to media containing varying concentrations of Pi (0, 5, 10, 25, 50, 125, 250, 500 and 1,250 μM) for 5 days. **b** Plants grown for 7 days in media containing 1 mM Pi (+*P*) were transferred to media without Pi (−*P*) for 12, 24, 48 h, or 3, 4, and 5 days. **c** Plants grown either grown for 10 days in media containing 1 mM Pi (+*P*) or grown first for 7 days in media containing 1 mM Pi, transferred for 3 days on Pi-free media (−*P*), and then transferred to media with 1 mM Pi (*R*) for 8, 24 or 48 h. For all *panels*, total RNA was isolated from whole plants and analyzed by Northern-blot analysis

Similar to *AtPHO1;H1*, the up-regulation of *AtPHO-H10* gene expression observed in Pi-deficient wild-type plants was strongly diminished in the Pi-deficient *phr1* mutant (Fig. 3b). A similar strong reduction in gene expression under Pi deficiency was also observed for the *AtIPS1* gene, which also contains a PHR1 binding site, but not for *AtPHT1;1*, which does not have a PHR1 binding site.

The regulation of *AtPHO1, AtPHO1;H1* and *AtPHO1;H10* mRNA levels was evaluated in *pho1*, a mutant deficient in the transport of Pi to the shoot, and *pho2*, a mutant over accumulating Pi in the shoot (Poirier et al. 1991; Delhaize and Randall 1995). Both *AtPHO1;H1* and *AtPHO1;H10* transcripts were slightly over accumulated in the shoot of *pho1* mutant compared to wild-type plants grown in solid media containing 1 mM Pi (Fig. 3c). The expression profile of *AtIPS1* in *pho1* also showed a slight over accumulation in leaves, indicating a weak activation of the Pi-starvation signaling pathway in leaves of the mutant grown under high Pi conditions. However, *AtPHT1;1* mRNAs level appeared less sensitive to Pi content in *pho1* leaves as the level of expression was unchanged in *pho1* plants grown in Pi-replete media. Under Pi-deficient conditions, all genes tested were up-regulated and no differences were observed between *pho1* and wild-type plants.

For *pho2* mutant grown under Pi-sufficient conditions, a slight over-expression of *AtPHO1;H10* was observed in leaves while the level of *AtPHO1* and *AtPHO1;H1* remained unchanged in roots and shoots (Fig. 3d). In contrast, the level of *AtIPS1* transcript in *pho2* roots was increased. However, under Pi-deficient condition, no difference was found between *pho2* and wild-type for all genes studied.

Regulation of *AtPHO1, AtPHO1;H1* and *AtPHO1;H10* expression by sucrose

The regulation of *AtPHO1, AtPHO1;H1* and *AtPHO1;H10* expression by sucrose was investigated in plants grown under Pi-replete or Pi-deficient conditions (Fig. 4). While addition of 30 mM sucrose lead to a reduced expression of *AtPHO1;H10* in plants grown in media with or without Pi, sucrose addition led to an increase in mRNAs for *AtPHO1, AtPHO1;H1, AtPHT1;1* and *AtIPS1* independent of the phosphate status.

Regulation of *AtPHO1, AtPHO1;H1* and *AtPHO1;H10* expression by auxin and cytokinin

The regulation of *AtPHO1, AtPHO1;H1* and *AtPHO1;H10* expression by exogenous auxin (0.5, 10 μM 2,4-D) and

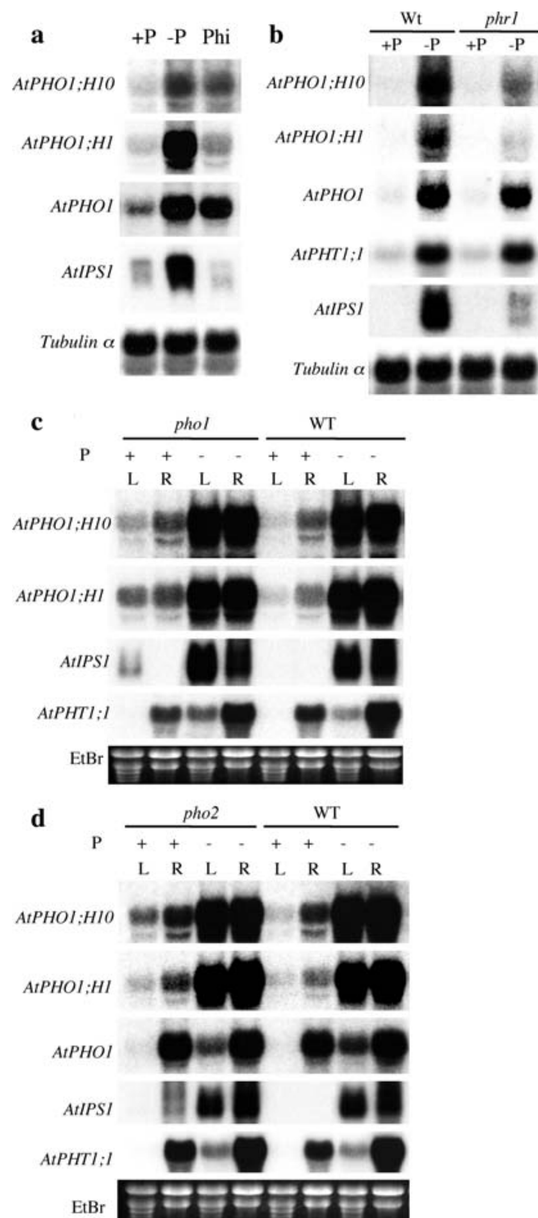

Fig. 3 Effects of phosphite and of the *phr1*, *pho1* and *pho2* mutations on *AtPHO1*, *AtPHO1;H1*, and *AtPHO1;H10* expression. **a** Plants grown for 7 days in media containing 1 mM Pi were transferred to media containing either 1 mM Pi (*+P*), no Pi (*−P*) or 1 mM phosphite (*Phi*) for 5 days. Expression profile was compared between wild-type plants and the mutants *phr1* (**b**), *pho1-3* (**c**), and *pho2* (**d**). For **b**, **c** and **d**, plants grown for 7 days in media containing 1 mM Pi were transferred to media with (+) or without (−) Pi for 5 days. Total RNA (25 µgper lane) isolated from either whole plants (**b**) or separately from leaves (*L*) of roots (*R*; **c**, **d**). For all panels, total RNA was isolated from whole plants and analyzed by Northern-blot analysis

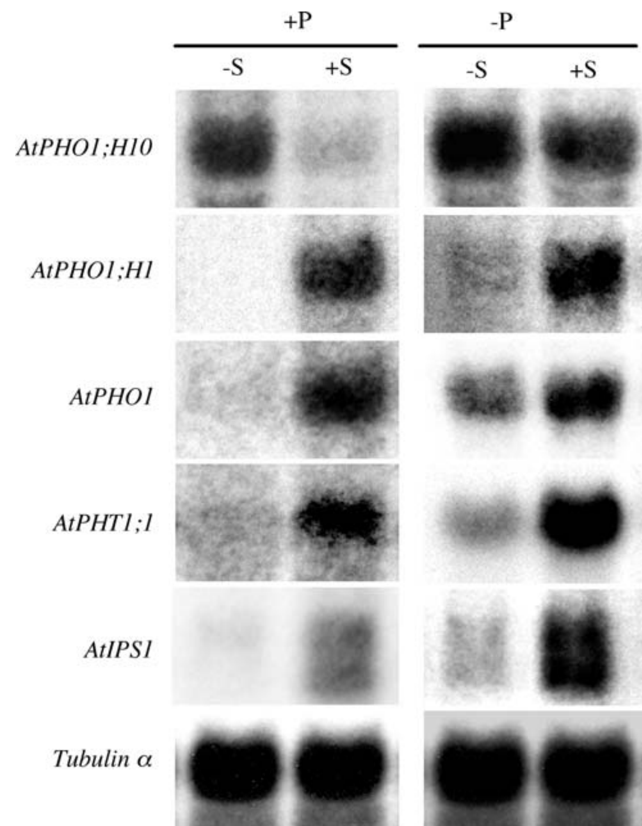

Fig. 4 Effects of sucrose supply on *AtPHO1*, *AtPHO1;H1*, and *AtPHO1;H10* expression. Plants first grown for 7 days in media without sucrose and with 1 mM Pi before being transferred and grown for an additional 5 days in media containing either 1 mM Pi (*+P*) or no Pi (*−P*) and supplemented with 30 mM sucrose (*+S*) or with no added sucrose (*−S*). Total RNA was isolated from whole plants and analyzed by Northern-blot analysis

cytokinin (10 µM kinetin) was investigated in plants grown under Pi-replete or Pi-deficient conditions. In plants grown under Pi-deficient conditions, both kinetin and 2,4-D treatments lead to a repression of *AtPHO1*, *AtPHO;H1*, *AtPHO1;H10* and *AtPHT1;1* expression (Fig. 5a, b). In contrast, for plants grown under Pi-sufficient conditions, both kinetin and 2,4-D induced the expression of *AtPHO1;H10* and *AtPHO1;H1* but repressed the induction of *AtPHO1* and *AtPHT1;1* (Fig. 5a, b).

Regulation of *AtPHO1, AtPHO1;H1* and *AtPHO1;H10* expression by abscisic acid

The basal level of expression of the *AtPHO1*, *AtPHO1;H1*, *AtPHO1;H10* and *AtPHT1;1* genes in Pi-sufficient plants was decreased following treatments with abscisic acid (ABA; Fig. 6a). In contrast, the same treatment lead to a sharp increase in *AtPHO1;H10* expression (Fig. 6a). The influence of endogenous level of ABA on the expression of *AtPHO1*, *AtPHO1;H1* and *AtPHO1;H10* was then examined using the ABA synthesis deficient mutant *aba1-3* grown under Pi-sufficient condition (Koornneef et al. 1982). While expression of *AtPHO1;H10* decreased in the *aba1-3* mutant compared to wild-type, increase of *AtPHO1*, *AtPHO1;H1* expression was observed in *aba1-3* (Fig. 6b).

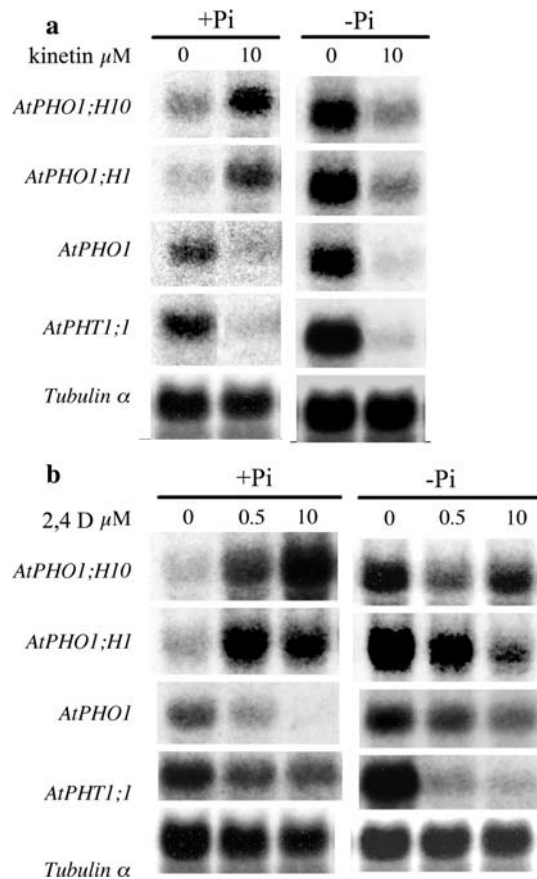

Fig. 5 Effects of cytokinin and auxin treatments on *AtPHO1*, *AtPHO1;H1*, and *AtPHO1;H10* expression. Plants grown for 7 days in media containing 1 mM Pi were transferred to media containing 1 mM Pi (+*P*) or no added Pi (−*P*) and supplemented either with the cytokinin kinetin (**a**) or the auxin 2,4-D (**b**) for 2 days. Total RNA was isolated from whole plants and analyzed by Northern-blot analysis

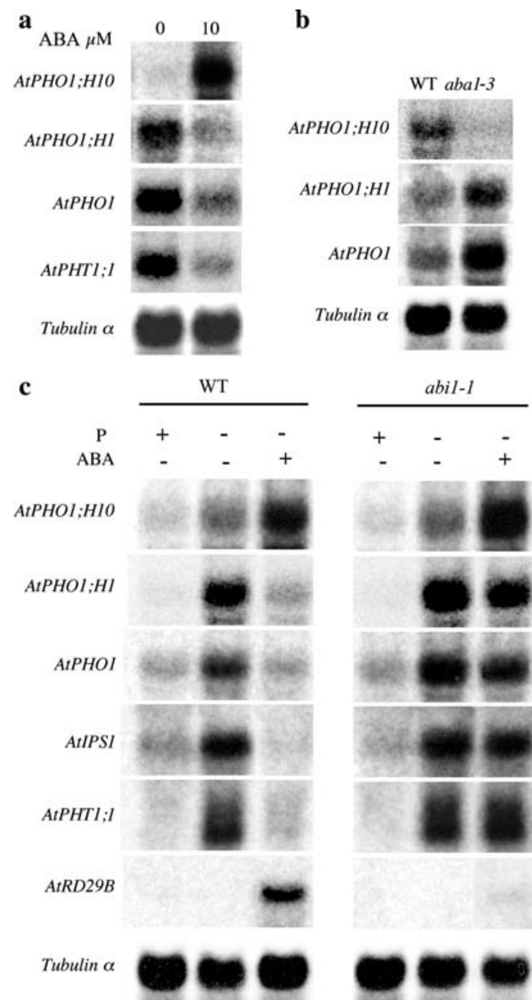

Fig. 6 Effects of ABA on the expression of the *AtPHO1*, *AtPHO1;H1* and *AtPHO1;H10* genes. **a** Wild-type plants grown for 7 days in media containing 1 mM Pi were transferred to media containing 1 mM Pi and 10 μM ABA for 2 days. **b** Wild-type plants and *aba1-3* mutant plants were grown in media with 1 mM Pi for 10 days. **c** Wild-type plants and *abi 1-1* plants were grown for 7 days in media containing 1mM Pi, and then transferred to media with (+) or without (−) Pi or ABA (10 μM) for 3 days. *AtRD29B* was used as a control for the expression of a gene induced by ABA and dependent on ABI1. For all panels, total RNA was isolated from whole plants and analyzed by Northern-blot analysis

The influence of ABA on the expression of members of the *AtPHO1* genes in plants under Pi deficiency was examined in wild-type plants and in the ABA-insensitive *abi1-1* mutant (Koornneef et al. 1982, 1984). In wild-type plants, ABA addition attenuated or abolished the increase of expression of the *AtPHO1*, *AtPHO1;H1*, and *AtPHT1;1* genes normally observed in Pi-deficient plants, while no such attenuation was observed in the *abi1-1* mutant (Fig. 6c). Similar results were also obtained for the *AtIPS1* gene (Fig. 6c). In contrast, addition of ABA to Pi-deficient wild-type plants further enhanced the expression of *AtPHO1:10* compared to untreated Pi-deficient plants, and similar results were also obtained for the *abi1-1* mutant (Fig. 6c).

Discussion

Of the three *AtPHO1* family members that are up-regulated following Pi deficiency, both *AtPHO1* and *AtPHO1;H1* have recently been shown to be involved in the transfer of Pi to the root vascular cylinder (Stefanovic et al. 2007). Analysis of the single mutants *pho1* and *pho1;h1* as well as the double mutant *pho1/pho1;h1* indicated that AtPHO1 has a primary role to play in Pi transfer to the root vascular cylinder, while the contribution of AtPHO1;H1 to the same transfer was more minor in plants grown either in medium with or without Pi (Stefanovic et al. 2007). The role of AtPHO1;H10 in phosphate homeostasis is unknown but is likely to be distinct from AtPHO1 and AtPHO1;H1. In the phylogenetic tree of AtPHO1 members, AtPHO1;H10 belongs to a clade that is distinct from AtPHO1 and AtPHO1;H1 (Wang et al. 2004). Furthermore, promoter GUS fusions revealed that the *AtPHO1;H10* promoter is

not active in root vascular tissue but rather in root epithelial and cortical cells, making it unlikely that *AtPHO1;H10* contributes to Pi transport in or out of the vascular cylinder (Wang et al. 2004).

Previous analysis revealed the presence of two pathways controlling the response of the *AtPHO1* and *AtPHO1;H1* genes to Pi deficiency, namely a pathway independent on the PHR1 transcription factor and not influenced by phosphite for *AtPHO1*, and a distinct pathway dependant on PHR1 and strongly influenced by phosphite for *AtPHO1;H1* (Stefanovic et al. 2007). The present work on the regulation of *AtPHO1*, *AtPHO;H1* and *AtPHO1;H10* provides evidence of further levels of complexity in the pathways involved in the regulation of these members of the *AtPHO1* family by Pi-deficiency.

At the level of the kinetic of transcript accumulation following shifts of plants to media with reduced or elevated Pi concentrations, *AtPHO1* and *AtPHO1;H1* behaved differently, with *AtPHO1;H1* responding quickly, within 8–12 h, to either a decrease or increase in external Pi level, while *AtPHO1* responded more slowly and gradually, typically within 24–48 h (Fig. 2). The kinetic of transcript changes for *AtPHO1;H10* was very similar to *AtPHO1;H1*. Morcuende et al. (2007) have recently applied the criteria of a rapid down-regulation of gene expression upon Pi re-supply to Pi-deficient plants in order to distinguish between genes that respond primarily to the Pi status of plants, such as *AtPHO1;H1* and *AtPHO1;H10*, as opposed to genes that may respond more to the secondary effects of Pi-deficiency on metabolism, such as the *AtPHO1* and *AtPHT1;1* genes. Several genes that are strongly up-regulated following Pi-deficiency, such as *At4*, *AtACP5*, *AtPHT2;1* and *AtRNS1* were also found to be quickly down-regulated by Pi re-supply (Müller et al. 2004). The higher responsiveness of the *AtPHO1;H1* and *AtPHO1;H10* to shift in Pi levels is also consistent with their higher expression in shoots of the *pho1* mutants, which shows reduction of Pi content in shoots even in plants grown under high external Pi concentrations (Fig. 3c; Poirier et al. 1991; Delhaize and Randall 1995).

An additional feature shared by both *AtPHO1;H1* and *AtPHO1;H10* response to Pi deficiency is their large dependence on the PHR1 transcription factor (Fig. 3b). The *phr1* mutant of *Arabidopsis* shows attenuation of several responses associated with Pi-deficiency, including reduced expression of the *AtIPS1*, *At4*, *AtRNS1* and *AtACP5* genes, reduced anthocyanin accumulation and attenuated increase in the root-to-shoot ratio (Rubio et al. 2001). PHR1 was shown to bind to the P1BS (PHR1-binding sequence) motif GNATATNC found in the At*IPS1* promoter. Microarray studies of genes induced under Pi-deficiency have revealed enrichment in genes having the P1BS motif, although numerous genes consistently induced by Pi deficiency did not contain such motif (Wang et al. 2002; Hammond et al. 2003; Uhde-Stone et al. 2003; Wu et al. 2003; Misson et al. 2005; Morcuende et al. 2007; Müller et al. 2007). Although the present study did not identify the sequences within the *AtPHO1;H10* regulatory elements to which PHR1 binds, it is likely that PHR1 binds to variants of the P1BS motif, such as potentially the imperfect palindromic sequence TCATATGC found approximately 1 kbp upstream of the start codon, indicating that a larger spectrum of genes may be under the control of PHR1 than previously expected.

One characteristic shared by both *AtPHO1* and *AtPHO1;H10* is the weak effect of phosphite on gene expression following Pi deficiency, in contrast to the strong suppression of *AtPHO1;H1* and *AtIPS1* expression following phosphite treatment (Fig. 3a). Phosphite is non-metabolized analogue of Pi that is thought to interfere specifically with early events in Pi sensing and signaling and affects a broad spectrum of Pi-starvation responses (Ticconi et al. 2001; Varadarajan et al. 2002). Altogether, these results reveal the existence of partially overlapping regulatory pathways affecting the expression of three members of the *AtPHO1* gene family in response to Pi deficiency. These include two pathways responding quickly to Pi deprivation, one involving PHR1 and strongly influenced by phosphite regulating *AtPHO1;H1*, and a second pathway involving PHR1 but weakly influenced by phosphite regulating *AtPHO1;H10*. A third pathway responding more slowly to Pi deprivation, that is independent of PHR1 and weakly influenced by phosphite regulates *AtPHO1*.

Beyond the amount of Pi available to cells, other factors have been shown to influence the response of plants to Pi deficiency, in particular sucrose and phytohormones, such as auxin and cytokinin (Torrey 1976; Franco-Zorrilla et al. 2002; Karthikeyan et al. 2002; Hou et al. 2005; Nacry et al. 2005). The present study reveals that the transcriptional regulation of *AtPHO1*, *AtPHO1;H1* and *AtPHO1;H10* in response to Pi status is also influenced by sucrose as well as phytohormones, but in manner that is gene-specific and, in the case of phytohormones, remarkably dependent of the Pi status of the plant.

Transcript level of *AtPHO1* and *AtPHO1;H1* as well as of *AtPHT1;1* and *AtIPS1* were all increased by the addition of sucrose for plants grown either under Pi-replete or Pi-deficient conditions (Fig. 4). In contrast, under the same conditions, sucrose repressed the accumulation of *AtPHO1;H10* transcript (Fig. 4). Promotion of growth of Pi-deficient roots by sucrose addition has been linked to decrease in intracellular Pi concentration and increased expression of *AtMGD3*, a gene responsive to Pi deficiency (Lai et al. 2007). Thus, the effect of sucrose on growth and on the Pi demand of cells could also play a role in the regulation of *AtPHO1* and *AtPHO1;H1*. However, besides being a metabolite, sucrose is also recognized as a signal

molecule involved in several physiological responses in plants. An influence of sucrose on Pi-responsive genes as well as an up-regulation of genes involved in carbohydrate metabolism by Pi-deficiency has been demonstrated in several studies (Nielsen et al. 1998; Cieresko et al. 2001; Lejay et al. 2003; Franco-Zorrilla et al. 2005; Müller et al. 2005; Karthikeyan et al. 2006). Recent studies have also demonstrated that several of the morphological responses linked to Pi-deficiency responses are modulated by sucrose, and that numerous genes are synergistically or antagonistically regulated by both sucrose and Pi (Karthikeyan et al. 2006; Müller et al. 2007). The present study thus reveals that *AtPHO1* and *AtPHO1;H1* genes belong to the group of Pi starvation responsive genes that are up-regulated by sucrose while *AtPHO1;H10* belongs to the group that is down-regulated by sucrose.

Addition of the cytokinin kinetin in the growth medium of phosphate-deficient plants led to the repression of the transcript level of *AtPHO1*, *AtPHO1;H1* and *AtPHO1;H10* genes (Fig. 5a). These members of the *AtPHO1* gene family are thus regulated by cytokinin under Pi-deficient conditions in the same way as *AtPHT1;1* and many other Pi-responsive genes, such as *AtIPS1* (Martin et al. 2000; Franco-Zorrilla et al. 2002; Karthikeyan et al. 2002; Hou et al. 2005; Shin et al. 2006). Pi-deficiency has been shown to lead to a decrease in the level of cytokinin that is correlated with altered root morphology (Ei-D et al. 1979; Horgan and Wareing 1980). Addition of cytokinin to Pi-starved roots has also been correlated with an increase in the intracellular Pi content and decrease in growth, thus potentially explaining the repression of Pi-starvation responsive genes by the hormone (Wang et al. 2006; Lai et al. 2007). However, in contrast to Pi-starved plants, addition of cytokinin to Pi-sufficient plants revealed different effects depending on the gene, with transcript accumulation for *AtPHO1;H10* and *AtPHO1;H1*, and transcript decrease for *AtPHO1* and *AtPHT1;1* (Fig. 5a). These data reveal an impact of cytokinin on gene expression that likely goes beyond its effect on cell growth and intracellular Pi content and that is influence by the Pi status of the plant cell.

The effects of addition of the auxin 2,4-D were similar to cytokinin, in that auxin treatment suppressed the induction of *AtPHO1;H10*, *AtPHO1;H1*, *AtPHO1* and *AtPHT1;1* in Pi-starved plants, while similar treatment induced *AtPHO1;H10* and *AtPHO1;H1* expression and repressed *AtPHO1* expression in Pi-sufficient plants (Fig. 5b). Auxin addition to roots provokes modifications of the root architecture in high Pi medium similar to the one induced by Pi deficiency, such as alteration of primary root growth and promotion of root hair and lateral root formation (Torrey 1976). Furthermore, several studies have revealed complex interactions between auxin and the root architectural modifications due to phosphate starvation (López-Bucio et al. 2002; Lopez-Bucio et al 2005; Al-Ghazi et al. 2003; Nacry et al. 2005; Jain et al. 2007). Lopez-Bucio et al. (2005) proposed the involvement of two different pathways in low phosphate stress-induced root architectural modifications where one is auxin dependent and the other auxin independent. Moreover, local changes in auxin concentrations and in auxin sensitivity in plant roots have been shown to be responsible for some specific alterations of root system architecture in adaptation to low Pi stress (Al-Ghazi et al. 2003; Lopez-Bucio et al. 2005; Nacry et al. 2005). At the gene expression level, addition of auxin has been shown to lead to variable expression pattern for Pi-starvation induced gene. Thus, while auxin addition did not influence expression of *At4*, *AtIPS1*, *AtPHT1;4*, *AtRNAse2* or *OsIPS2* under Pi-deficient conditions, similar treatment either repressed the expression of *AtPHT1;1* or induced the expression of *OsIPS1* and *AtMDG2* (Martin et al. 2000; Karthikeyan et al. 2002; Hou et al. 2005; Kobayashi et al. 2006; Shin et al. 2006). This heterogeneity in responses of gene expression may be potentially linked to the correspondence between local increase or decrease in auxin concentration or sensitivity in regions of the root or other tissues, with the distribution of the expression pattern of the various genes studied (Nacry et al. 2005). Importantly, the present study reveals that the effect of both auxin and cytokinin on the expression of Pi-starvation induced gene is highly dependent on the Pi status of the plants, and that even for two genes, such as *AtPHO1* and *AtPHO1;H1*, which have similar functions in the loading of Pi to the root xylem vessels (Stefanovic et al. 2007), the effect of these hormones can be opposite in Pi-sufficient plants.

Compared to auxin and cytokinin, the role of ABA on plant adaptation to phosphate deficiency is less well defined. ABA treatment leads to a decrease in plant weight but an increase in the root-to-shoot ratio and root hair density comparable to the one occurring during phosphate deficient conditions, suggesting that ABA could also mediate some responses of the plant to Pi starvation (Watts et al. 1981). An increase in stomatal responsiveness to applied ABA has been measured in phosphorus-stressed plants, implying that sensitivity to ABA was altered by phosphorus stress (Radin 1984). Studies performed on *Ricinus communis L.* revealed that xylem transport of ABA in Pi-deficient plants was stimulated by a factor of 6, whereas phloem transport was affected only very slightly (Jeschke et al. 1997; Jeschke and Hartung 2000). Moreover, it was reported that *abi2-1* mutants accumulate less anthocyanin in response to Pi starvation (Trull et al. 1997) and that the expression of *rab18* under Pi-starvation conditions is partially reduced in the *aba1* mutant (Ciereszko and Kleczkowski 2002), suggesting that some degree of cross-talk between ABA and Pi-starvation signaling exists in plant. However, the

comparison of growth and biochemical responses (acid phosphatase production in response to Pi deficiency) of *A. thaliana* ABA mutants *aba-1* and *abi2-1* to those of wild-type plants indicated that ABA does not have a major role in coordinating the Pi deficiency response (Trull et al. 1997). More recently, ABA treatment has been shown to repress the induction of *At4* and *OsIPS1* and *OsIPS2* in Pi-deficient plants (Hou et al. 2005; Shin et al. 2006). The present study reveals that in contrast to the effect of auxin and cytokinin, the effects of addition of exogenous ABA on gene expression were similar in Pi-sufficient and Pi-deficient plants, in that ABA repressed the expression of *AtPHO1*, *AtPHO1;H1* and *AtPHT1;1*, but activated the expression of *AtPHO1;H10* expression (Fig. 6a, c). This transcriptional regulation was further confirmed by modifying the endogenous level of ABA using the *aba1-3* mutant deficient in ABA synthesis (Fig. 6b). Importantly, the repression of *AtPHO1*, *AtPHO1;H1*, *AtIPS1*, and *AtPHT1;1* expression by addition of exogenous ABA was dependent on ABI1, a type 2C Ser/Thr protein phosphatase participating in numerous responses of plants to ABA during the germinative and post-germinative phases (Fig. 6c; Rock 2000). These results, thus, uncovered the participation of the ABA signal transduction cascade involving ABI1 in the regulation of several genes involved in Pi homeostasis, including *AtPHO1*, *AtPHO1;H1*, *AtIPS1* and *AtPHT1;1*.

In conclusion, this study shows that regulation of three homologues of the *AtPHO1* gene family by Pi deficiency involves the interaction between multiple-signaling pathways involving the plant Pi status as well as sucrose, auxin, cytokinin and ABA. The final outcome on gene expression from the integration of these parameters can sometimes be opposite, even for members of a gene family that have a similar function in Pi homeostasis, such as for *AtPHO1* and *AtPHO1;H1*, underlying the complexity of the plant's responses to Pi deficiency. Finally, ABA and the ABI1-mediated signal transduction cascade has been implicated in the regulation of several genes responding to Pi starvation, and the impact of this hormone on Pi homeostasis and the adaptation of plants to Pi deficiency deserves further attention.

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