Differentiating phosphate-dependent and phosphate-independent systemic phosphate-starvation response networks in Arabidopsis thaliana through the application of phosphite

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Abstract

Phosphite is a less oxidized form of phosphorus than phosphate. Phosphite is considered to be taken up by the plant through phosphate transporters. It can mimic phosphate to some extent, but it is not metabolized into organophosphates. Phosphite could therefore interfere with phosphate signalling networks. Typical physiological and transcriptional responses to low phosphate availability were investigated and the short-term kinetics of their reversion by phosphite, compared with phosphate, were determined in both roots and shoots of Arabidopsis thaliana. Phosphite treatment resulted in a strong growth arrest. It mimicked phosphate in causing a reduction in leaf anthocyanins and in the expression of a subset of the phosphate-starvation-responsive genes. However, the kinetics of the response were slower than for phosphate, which may be due to discrimination against phosphite by phosphate transporters PHT1;8 and PHT1;9 causing delayed shoot accumulation of phosphite. Transcripts encoding PHT1;7, lipid-remodelling enzymes such as SQD2, and phosphocholine-producing NMT3 were highly responsive to phosphite, suggesting their regulation by a direct phosphate-sensing network. Genes encoding components associated with the ‘PHO regulon’ in plants, such as At4, IPS1, and PHO1;H1, generally responded more slowly to phosphite than to phosphate, except for SPX1 in roots and MIR399d in shoots. Two uncharacterized phosphate-responsive E3 ligase genes, PUB35 and C3HC4, were also highly phosphite responsive. These results show that phosphite is a valuable tool to identify network components directly responsive to phosphate.

Key words: Arabidopsis thaliana, phosphate-starvation response, phosphate transport, phosphite, phosphonate, phosphorous acid, phosphorus signalling networks, PSR genes, transcriptional regulation.

Introduction

Phosphite (H₂PO₃⁻, Phi) is a less oxidized form of phosphorus (P) than phosphate (H₂PO₄⁻, Pᵢ). Phi is highly water soluble and less prone than Pᵢ to adsorb to soil particles, which makes it more accessible to plants (Ruthbaum and Baille, 1964). Phi competes with the essential macronutrient Pᵢ for uptake by plants, most probably through both high- and

Abbreviations: P, phosphorus; Phi, phosphite; Pᵢ, inorganic phosphorus/phosphate/H₂PO₄⁻, PSR, phosphate-starvation-responsive.

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low-affinity transport systems (d’arcy-Lameta and Bompeix, 1991; Danova-Alt et al., 2008). Phi uptake is strongly and competitively inhibited in the presence of P, (Pratt et al., 2009). Within the plant, Phi can be translocated, and it preferentially accumulates in sink tissues (Nartvanranant et al., 2004).

Phosphite was once abundant in the oceans, but it has been oxidized over time (Pasek et al., 2013). Many microbes have retained the ability to oxidize Phi to P, and even use it as a reducing agent, namely for sulphate reduction (Poehlein et al., 2013). Plants, however, are not able to metabolize Phi (McDonald et al., 2001). Instead, P-limited plants are highly sensitive to Phi and display toxicity symptoms such as leaf chlorosis and stunted growth (McDonald et al., 2001; Ratjen and Gerendas, 2009; Thao and Yamakawa, 2009). Other detrimental effects caused by Phi are the arrest of primary root growth, yellowing of the leaf lamina of young leaves, and a patchy accumulation of anthocyanins in older leaves (Varadarajan et al., 2002). Pratt et al. (2009) also showed that respiration rates declined upon Phi treatment of P-limited sycamore cells. It was recently found that the accumulation of Phi impacts on metabolism in Arabidopsis thaliana, leading to changes in the levels of several central metabolites (Berkowitz et al., 2013).

Phi also triggers broad-spectrum resistance against pathogens with a (hemibiotrophic) lifestyle, such as oomycetes, fungi, and nematodes (Smillie et al., 1989; Hofgaard et al., 2010; Dias-Arieira et al., 2013; Percival and Banks, 2014). Phi has been suggested to act as a priming agent of plant defence responses in a number of plant–pathogen interactions (Machinandiarena et al., 2012; Massoud et al., 2012; Dalio et al., 2014). However, it is unclear how the primary recognition of Phi takes place, and which molecular pathways are altered within the plant subsequently to induce this primed state of heightened defence. Given that Phi is transported by P transporters, these primary molecular interactions could trigger changes in signal perception (Schothorst et al., 2013).

Phi accumulates in both the cytosol and organelles, while exposed to Phi treatment of P-limited A. thaliana accession Col-0 and three PHT1 trans- porter mutants was therefore determined. Root growth and a patchy accumulation of anthocyanins in older leaves (et al., 2003). Interestingly, the combined concentrations of Phi plus P, within roots and shoots of A. thaliana were remarkably constant, regardless of their ratio in the growth medium, demonstrating that plants sense both P, and Phi and adjust their uptake and allocation accordingly (Berkowitz et al., 2013).

Due to its physical similarity to P, and non-metabolizable nature, Phi has been used as a tool to understand P-dependent signalling networks in plants. In several studies, Phi in fact seemed to mimic P, effectively. Brassica nigra seedlings germinated on low-P, media in the presence of high (1–10 mM) Phi concentrations had reduced activation of P-starvation-induced phosphoenolpyruvate phosphatase and pyrophosphate-dependent phosphofructokinase compared with P-limited control plants (Carswell et al., 1996). While Phi did not affect the total adenylate pool in P-limited Brassica napus suspension cells in the same way as P, it did cause changes in the in vivo phosphorylation status of a number of proteins (Carswell et al., 1997). In A. thaliana, Ticconi et al. (2001) observed that Phi prevented the induction of transcripts from the P-starvation-responsive (PSR) genes ACP5, At4, and PT2 upon 14 d exposure of P-sufficient seedlings to a medium lacking P, but containing high concentrations of Phi. The same plants showed reduced in vitro activities of PSR ribonucleases RNS1 and RNS2 and of an acid phosphatase. Within 1 d of transfer of P-sufficient A. thaliana seedlings to a medium lacking P, Phi suppressed the typical root hair formation and transcript accumulation of purple acid phosphatase PAPI and P transporters PT1 and PT2 that occur upon P withdrawal (Varadarajan et al., 2002). Exposure of A. thaliana to Phi prevented not only PSR MGD2 and MGD3 expression, but also changes in glycerolipid profiles that accompany P-limited growth (Kobayashi et al., 2006). In P-limited tomato seedlings, Phi mimicked P, in promoting proteolytic turnover of purple acid phosphatases (Bozzo et al., 2004). In rice, long-term exposure (5–7 d) to Phi suppressed the P-starvation-induced expression of OsIPS1 and OsIPS2 (Hou et al., 2005). In tobacco BY-2 cells, Phi caused the reversion of autophagic protein turnover triggered by P, deprivation (Tasaki et al., 2014).

The first evidence suggesting that Phi and P, have discrete effects on P signalling networks came from work by Stefanovic et al. (2007), who showed that transcripts of PHO1 and its close paralogue PHO1;H1 differentially accumulated in plants treated with P, or Phi. The PHR1-dependent induction of PHO1;H1 under P-limiting conditions was attenuated by Phi, while the PHR1-independent induction of PHO1 was not. This effect does not directly depend on the MYB transcription factor PHR1, because, unlike for PHO1;H1, the induction of another PHR1-regulated paralogue, PHO1;H10, was not affected by Phi (Ribot et al., 2008). Interestingly, both PHO1 and PHO1;H1 transcripts were less abundant in the P-limited pho2 mutant and more strongly induced in the P-limited pdr2 mutant compared with those in the wild type (Stefanovic et al., 2007). Disruption of the gene encoding endoplasmic reticulum (ER)-resident P-type ATPase PDR2 affected local P-sensing networks and heightened the sensitivity and amplitude of metabolic responses to P limitation (Ticconi et al., 2004). The conditional pdr2 short-root phenotype was reversible by Phi. These observations strongly suggest that Phi mimics P, in local signalling networks, irrespective of the plant’s P status.

Studies have so far addressed the question of whether Phi can prevent the long-term accumulation of PSR gene transcripts. In this study, the question of whether the shorter term kinetics of Phi suppression were similar to those of P, was addressed (Müller et al., 2004; Morcuende et al., 2007). Organ-level accumulation of both P, and Phi in P-limited seedlings in A. thaliana accession Col-0 and three PHT1 transporter mutants was therefore determined. Root growth and anthocyanin accumulation as well as gene expression profiles
in response to Phi treatment or P, resupply were monitored in P-limited Col-0 seedlings over a time-course from 1 d to 7 d.

Materials and methods

Plant material and growth conditions

Seeds of *A. thaliana* (L.) Heynh. Col-0 and homozygous T-DNA insertion lines for *phit1;1–2* (SALK 088568C) (Shin et al., 2004), *phit1;8* (SALK 056529, Lapis-Gaza et al., 2014), and *phit1;9–1* (SALK 050730) (Remy et al., 2012) were surface-sterilized for 2 min in 70% (v/v) ethanol and 5 min in 5% (v/v) NaOCl, before being rinsed five times in sterile water. Seeds were resuspended in sterile 0.1% (w/v) agar and stratified in the dark for 24–48 h at 4 °C. Seedlings (12 per plate) were grown vertically on 10×10 cm plates containing 50 ml of nutrient solution [1 mM Ca(NO₃)₂, 2 mM KNO₃, 0.5 mM MgSO₄, 0.25 mM KH₂PO₄, 40 μM Fe-EDTA, 25 μM H₃BO₃, 2 μM MnCl₂, 2 μM ZnSO₄, 0.5 μM CuSO₄, 0.075 μM (NH₄)₆MoO₄.2H₂O, 0.15 μM CoCl₂, 50 μM KCl, pH 5.8] with 0.5% (w/v) 2-(N-morpholino)ethanesulphonic acid and 1% (w/v) sucrose, and solidified with 0.7% (w/v) agar (Plant TC Agar, cat.#A111, PhytoTechnology Laboratories, Shawnee Mission, KS, USA). Plates were sealed with 3M™ Micropore medical tape (Intouch Direct, Springwood, Laboratories, Shawnee Mission, KS, USA). Seedlings were grown in a 10/14 h day/night cycle with 200 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR) at 21 °C (day), 19 °C (night), and 65% relative humidity. The plant-available P, present in the agar added another 5 μM to the medium. This amount is within the range of P concentrations across gelling agents (Jain et al., 2009). Preliminary experiments showed that concentrations of P ranging from 250 μM to 1 mM do not limit seedling growth in this system (data not shown). For the experiment, seedlings grown on a medium with 250 μM P for 5 d were grown for 4 d on plates without P, supplementation (containing 250 μM KCl instead) before being transferred to plates containing minimal P (5 μM residual P in agar), or equimolar concentrations (250 μM) of either P, or Phi. The Phi solution was prepared from a fresh batch of phosphorous acid (99%, Sigma Aldrich, Castle Hill, Australia) as a filter-sterilized 250 mM stock. The pH was adjusted to pH 5.8 with KOH. There was <0.1% oxidation of Phi to P, in this solution during 1 month storage at 4 °C.

At harvest, the 12 seedlings on each plate were pooled into one sample. Roots were rinsed in MilliQ water for 5 min. Roots and shoots were blotted dry and shock-frozen in liquid nitrogen. Harvesting started 3 h after the beginning of the light period in synchrony with the experimental time-course to ensure that plants were at a comparable physiological state.

Root growth analysis and microscopy

After emergence of the radicle or transfer to a new plate, the position of the primary root tip was marked at 24 h intervals. Prior to transfer or harvest, the seedlings were scanned at 600 dpi resolution to determine root and root hair length, growth rate, and lateral root number (LSM Image Browser v4.2; Carl Zeiss Microscopy GmbH, Jena, Germany).

For microscopy (Axioplan Universal microscope; Carl Zeiss Microscopy GmbH), roots were mounted onto slides in water under glass cover slips. Images were electronically processed (AxioVision4; Carl Zeiss Microscopy GmbH).

Metabolite quantification

Fifteen volumes of 1% (v/v) acetic acid were added to frozen plant powder (30–50 mg) and homogenized for three cycles of 45 s at 5000 rpm in the presence of two ceramic beads (ø 2 mm, Precellys 24 Tissue Disruptor; Bertin Technologies, Montigny-le-Bretonneux, France). After incubation for 15 min on ice, the homogenization process was repeated once. Cleared supernatants were used to determine organ P concentrations via the reduction of a phosphomolybdate complex by ascorbic acid (Ames, 1966). Phi concentrations were determined using the same extracts in a high-throughput enzymatic fluorescence assay (Berkowitz et al., 2011).

Anthocyanins in leaf samples were determined using a pH-differential method (Wrolstad et al., 2005). Concentrations were calculated using the molar absorptivity of cyanidin-3-glucoside (ε=26 900 M⁻¹cm⁻¹), the predominant anthocyanin in *A. thaliana* leaves (Tohge et al., 2005).

Relative quantification of transcript abundance

mRNA was captured from tissue homogenates using oligo(dT)₃₄-coated magnetic beads (Dynabeads, Life Technologies Australia Pty Ltd, Mulgrave, Australia) and converted to cDNA as previously described (Jost et al., 2007). Aliquots of 0.5 ng of cDNA were amplified in a 10 μl reaction volume containing 0.5 μM of each primer and PCR master mix (Power SYBR® Green, Applied Biosystems, Scoresby, Australia). Quantitative PCR and threshold cycle (Ct) determination were performed using a fluorescence baseline setting of 0.3 (7500 FAST Real-Time PCR System, Applied Biosystems, Scoresby, Australia). Data were normalized against *PP2AA3* (formerly *PDF2*) and *UBC9* reference genes (Czechowski et al., 2005). PCR efficiencies for each primer pair were determined using the LinReg algorithm (Ruijter et al., 2009) (Supplementary Table S1 available at JXB online). Data were expressed either relative to normalized Ct values in control samples (ΔΔCt) or as 40–ΔCt values that correlate with the relative transcript expression of the gene of interest (Bari et al., 2006). The detection limit of the assay was calculated to be a 40–ΔCt value of 25.7±0.1. Statistical analysis

Statistically significant differences between treatments were determined using analysis of variance (ANOVA) and defined as P≤0.05 (SigmaStat v. 12.3, Systat Software Inc., San Jose, CA, USA). Two-way ANOVA followed by Tukey’s post-hoc test was used to separate means. Hierarchical clustering was performed using squared Euclidean distance and complete linkage (J-Express 2012, Norwegian Bioinformatics Platform and Norwegian Microarray Consortium (http://www.molmine.com)) http://jexpress.bioinfo.no/site/ (last accessed 27 January 2015) (Dysvik and Jonassen, 2001).

Results

Phosphite strongly reduced plant biomass production

A vertical growth system was used for *A. thaliana* Col-0 seedlings that allowed a direct comparison of the effects of Phi versus P, on the repression of P-starvation responses without the confounding effects of competition between P, and Phi. Using this system, P-limited seedlings were subjected to continued P, deprivation, P, resupply, or Phi treatment. Plant biomass did not differ significantly among the treatments within the first 2 d of transfer (Fig. 1). After 3 d of treatment, both the P-limited seedlings and those resupplied with P, had greater root and shoot biomass than seedlings at days 1 and 2, while the biomass of the Phi-treated seedlings was unchanged. Over the next 4 d, seedlings resupplied with P, recovered from P limitation with a proportional increase in both root and shoot biomass that maintained the root-to-shoot ratio at 0.30±0.01. P-limited seedlings preferentially allocated resources to roots over shoots, leading to a final root-to-shoot ratio of 0.47±0.03. Despite the greater partitioning of biomass to roots, the root biomass after 7 d of further P
Phosphite strongly inhibited primary root elongation

Seeds germinated on high-P$_i$ media showed steady primary root growth from 2 d after sowing (Fig. 2A). Root growth was initially maintained when the seedlings were transferred to a P$_i$-deficient medium 5 d after sowing. Imposing P$_i$ resupply or Phi treatments after 4 d of P$_i$ withdrawal did not affect root elongation during the first day (Fig. 2A). Two days after the transfer to the final medium (day 11), roots of both P-limited and P$_i$-resupplied seedlings grew at similar rates (Fig. 2B). In contrast, primary roots of Phi-treated seedlings showed much lower growth rates during this period, and elongation ceased completely within the next 48 h. Root growth in P$_i$-resupplied seedlings accelerated exponentially during this same time period (Fig. 2B), with roots reaching the bottom of the 10-cm plate by 6 d after imposing the treatment. Root growth in P-limited seedlings decelerated by 2%, resulting in a final total root length that was almost 30% shorter than in P$_i$-resupplied seedlings. These results show that, unlike P$_i$ resupply, Phi treatment accentuated the reduction in root growth caused by P$_i$ depletion.

Phosphite altered seedling root architecture

At the end of the time-course experiment, high-resolution scans of primary root segments initiated on day 3 after the final transfer were used to analyse the effects of the three treatments on root development (Fig. 2C, D). The chosen root segment was proximal to the root apex, at the beginning of the root branching zone (Dubrovsky and Forde, 2012). The short-root phenotype caused by Phi resulted in an almost 2-fold greater lateral root density than in P-limited seedlings in this newly formed section of the root (Fig. 2C). Remarkably, the number of lateral roots per segment in Phi-treated seedlings (2.3±0.2) was 2-fold lower than that in P$_i$-limited (4.7±0.3) and P$_i$-resupplied (5.0±0.5) segments. Primary root growth in P$_i$-resupplied seedlings decreased lateral root density by nearly 2-fold compared with P-limited seedlings. While lateral roots elongated similarly under both P$_i$ limitation and P$_i$ resupply, emergence of lateral roots was inhibited in the presence of Phi. This phenomenon was also observed in a hydroponics growth system, where transfer to different nutrient solutions is less damaging to roots (Supplementary Fig. S1 at JXB online; note that in order to compensate for slower uptake of Phi over P$_i$, 1 mM Phi was used in this experiment).

Root hairs of Phi-treated seedlings were 57% shorter than in P-limited seedlings (Fig. 2D). This shortening was similar to the 52% reduction observed for seedlings resupplied with P$_i$. A concomitant reduction in root hair density by 38% for Phi-treated seedlings (14±1 mm$^{-1}$) compared with P-limited seedlings (22±1 mm$^{-1}$) was also very similar to the 44% reduction observed in P$_i$-resupplied seedlings (13±1 mm$^{-1}$). Hence this local response to P$_i$ resulting in fewer and shorter root hairs appears to be mimicked by Phi.

Anthocyanin accumulation in P-limited seedlings was repressed by both P$_i$ and Phi

Anthocyanins accumulated to significant levels in leaves of seedlings after a total of 11 d of growth on minimal P$_i$ media (4 d P$_i$ withdrawal+7 d treatment; Fig. 3). This slow accumulation indicates that the seedlings were not highly stressed by the P$_i$ deprivation imposed during the early stage of the experiment, and were probably accessing and gradually depleting P reserves that accumulated during the initial 5-d growth on P$_i$-containing medium. Seedlings resupplied with P$_i$ after a starvation period of 4 d had lower levels of anthocyanins within 2 d of treatment. Phi-treated seedlings also had reduced leaf anthocyanin levels within the first 2 d of treatment, but not as low as in P$_i$-resupplied seedlings. In Phi-treated seedlings, the leaf anthocyanin concentration was higher at day 7 than in P$_i$-resupplied seedlings, but was 72% lower than in P-limited seedlings. Therefore, Phi attenuated anthocyanin accumulation in P-limited plants that was completely suppressed by P$_i$ resupply.

Root-to-shoot transport favoured P$_i$ over Phi

To appreciate fully the differences in the physiological and molecular responses to Phi compared with P$_i$, the accumulation of both anions in roots and shoots was determined over time. While roots accumulated both P$_i$ and Phi equally within...
Changes in root architecture in response to phosphate (P) resupply and phosphite (Phi) treatment. (A) Primary root growth over the course of the experiment. Seedlings were germinated on media containing 250 μM P (filled circles). After 5 d, they were transferred to media containing minimal P (5 μM, open circles) before being transferred to plates with minimal P (5 μM, open circles), high P (250 μM, filled triangles), or Phi (250 μM, open triangles). Arrows indicate transfer to new plates. (B) Root growth rates in response to treatments. Symbols are the same as in (A). Shown in (A, B) are means ±SE, n=16 (four seedling roots each were measured individually from four separate plates). (C) Lateral root density in seedlings harvested 7 d after transfer to minimal P (white bars), high P (black bars), or 250 μM Phi (grey bars). Emerging lateral roots were counted in root segments that were formed 3 d after transfer. Shown are means ±SE, n=10 (five seedlings each from two plates). (D) Root hair length of seedlings harvested 7 d after transfer to minimal P (white bars), high P (black bars), or 250 μM Phi (grey bars). Shown are means ±SE, n=30 (3 root hairs ×5 seedlings ×2 plates). Statistically significant differences across time points in (A, B) were determined by Tukey’s HSD for treatments relative to P-limited seedlings at P<0.001. In (C) and (D), pairwise multiple comparisons between treatments identified statistically significant differences at P<0.005.

1 d of exposure, there was a delay in the accumulation of Phi relative to that of P in the shoot (Fig. 4). Shoot P concentrations reached ~13 μmol g⁻¹ fresh weight (FW) within 1 d of resupply (Fig. 4A) which was greater than the level of free P in seedlings continuously grown on a sufficient P supply (~5 μmol g⁻¹ FW). The shoot P concentration nearly doubled over the next 6 d to a final concentration of ~21 μmol g⁻¹ FW. In roots, P levels increased to 9 μmol g⁻¹ FW within 1 d of resupply, matching the P concentration found in roots of seedlings continuously receiving P (~10 μmol g⁻¹ FW). P concentrations remained at this level for several days, before dropping to 6 μmol g⁻¹ FW by day 7 (Fig. 4B). The drop in P was probably due to a combination of depletion from the medium, continued export to the shoot, conversion to organic P compounds, and internal dilution by root growth. In roots of Phi-treated seedlings, Phi accumulated to similar levels as P in the experiment, with a final concentration of 12 μmol g⁻¹ FW. In shoots of Phi-treated seedlings, Phi concentrations were lower than the P concentrations in P-resupplied seedlings at the two earliest time points (3 μmol g⁻¹ FW; Fig. 4A). After 3 d, the shoot Phi concentration of 10 μmol g⁻¹ FW caught up with the shoot P concentration found after only 1 d of P resupply. At the final harvest, the shoot Phi concentration of 27 μmol g⁻¹ FW in Phi-treated seedlings was higher than that of the free P concentration in resupplied seedlings, probably due to metabolic conversion of P but not Phi into organic compounds. In shoots of P-limited seedlings, the P concentration declined over the course of the experiment to a final concentration of 1.5 μmol g⁻¹ FW. The P concentration in roots of P-limited plants (2 μmol g⁻¹ FW) was constant over the time-course. In roots and shoots of Phi-treated seedlings, the P concentration (3 μmol and 4 μmol P g⁻¹ FW, respectively) was constant over time, and P concentrations at
the final harvest were higher than those in P-limited organs, most probably due to Phi-induced P\textsubscript{i} retention in the vacuole (Pratt et al., 2009).

Phosphite tissue accumulation was differentially affected among a set of pht1 mutants

To gather direct evidence that Phi is transported by P\textsubscript{i} transporters of the PHT1 family, the Phi accumulation in roots and shoots of homozygous T-DNA insertion lines was analysed in the Col-0 background lacking either PHT1;1, one of the major P\textsubscript{i} transporters at the root–soil interface (Shin et al., 2004), PHT1;8, or PHT1;9. The latter two PHT1 transporters are involved in translocation of P\textsubscript{i} to the shoot (Lapis-Gaza et al., 2014). Seedlings were grown on vertical plates and depleted of P\textsubscript{i} as described above, before supplying them with either 250 \(\mu\)M P\textsubscript{i} or 250 \(\mu\)M Phi for 24 h prior to harvest. P\textsubscript{i} starvation led to similar residual organ P\textsubscript{i} concentrations across genotypes (Fig. 5). Compared with the corresponding wild-type Col-0, the pht1;1–2 mutant accumulated 58% less P\textsubscript{i} in roots and 22% less P\textsubscript{i} in shoots of P\textsubscript{i}-resupplied seedlings over the 24-h period (Fig. 5). The effect of this mutation on Phi uptake by P-limited seedlings was significantly more pronounced, leading to 71% less Phi in roots and 84% less Phi in shoots of the mutant than in the wild type. Knocking out PHT1;8 or PHT1;9 had no effect on either root or shoot P\textsubscript{i} accumulation. In contrast to pht1;1–2, Phi concentrations in roots of both pht1;8 and pht1;9–1 were similar to those in the wild type, but Phi accumulation in shoots was reduced by 76% for pht1;8 and by 60% for pht1;9–1 compared with Col-0, the same extent as seen in pht1;1–2. The basal organ P\textsubscript{i} concentrations in Phi-treated seedlings were similar across mutants. The same trends in organ P\textsubscript{i} and Phi concentrations were observed after 2 d of treatment, although differences between Col-0 and the three mutants were diminished by day 7 (Supplementary Fig. S2 at JXB online). Throughout the time-course, root and shoot biomass accumulation was

![Fig. 4. Kinetics of phosphate (P\textsubscript{i}) and phosphite (Phi) accumulation in seedling organs. Five-day-old seedlings were depleted of P\textsubscript{i} for 4 d before being transferred to plates for the different treatments as indicated. (A) Shoot and (B) root accumulation of P\textsubscript{i} in phosphorus-limited seedlings (white bars), upon P\textsubscript{i} resupply (black bars), or with Phi treatment (grey bars). Phi accumulation in Phi-treated seedlings is shown as red bars. Shown are means ±SE, n=3 or n=4 replicates with 12 seedlings each. Statistically significant differences between time and treatments are indicated by different letters according to Tukey’s HSD at P<0.001.](image1)

![Fig. 5. Phosphite (P\textsubscript{i}) and phosphite (Phi) accumulation in P-limited Col-0 and pht1 mutant organs after 1 d of P\textsubscript{i} resupply or Phi treatment. Five-day-old seedlings were depleted of P\textsubscript{i} for 4 d before being treated as indicated. (A) Shoot and (B) root accumulation of P\textsubscript{i} in P-limited (white bars), P\textsubscript{i}-resupplied (black bars), and Phi-treated (grey bars) seedlings and accumulation of Phi (red bars). Shown are means ±SE, n=3 replicates with 12 seedlings each. Genotypes and treatments with a letter in common are not significantly different according to Tukey’s HSD at P<0.05.](image2)
largely unaffected by the lack of individual PHT1 proteins (Supplementary Fig. S3).

**Phosphite altered transcript accumulation for a subset of P$_i$-responsive genes**

The short-term effect of Phi on PSR gene expression was assessed by quantitative reverse-transcriptase PCR (qRT-PCR) for a set of well-documented PSR genes representing various metabolic and regulatory steps within plant P signalling networks (Hammond et al., 2003; Wu et al., 2003; Misson et al., 2005; Morcuende et al., 2007; Woo et al., 2012). If Phi was a true P$_i$ analogue and sensed in the same way as P$_i$ by as yet unidentified cellular signalling components, one would expect the effect of the two chemicals on transcript profiles to be similar; that is, lower transcript levels for P$_i$-starvation-induced genes and higher transcript abundance for genes involved in organophosphate biosynthesis or encoding negative regulators such as the E2 ubiquitin conjugase PHO2 (Aung et al., 2006; Bari et al., 2006) or the F-box protein FBX2 and transcription factor BHLH32 (Chen et al., 2008).

The selected PSR genes showed the previously documented expression changes within 1 d of P$_i$ resupply (Fig. 6). Surprisingly, 33% of the target genes showed no significant change in transcript abundance in response to Phi in shoots of P-limited plants over the 7-d treatment period (Fig. 6A, grey transcript names). Within this non-responsive group were the P$_i$ transporter gene PHT1;4, as well as genes involved in P$_i$ metabolism (ACP5, G3PP1, NMT3, PAPI, PLD $\xi$, and RNS1). In shoots, the majority of PSR genes tested showed an attenuated response to Phi treatment with a 1 d or 2 d delay compared with P$_i$ resupply. This set included genes encoding regulatory components such as At4, IPS1, PHO1;H1, and SPX1, as well as genes encoding protein kinase PPK2 and sulpholipid synthase SQD2 (Fig. 6A, red clusters). In contrast, other genes responded strongly to Phi, as they did to P$_i$, resupply. These responses included an 8-fold suppression within 24 h of Phi treatment for PHT1;7 transcript amounts, with a further 16-fold drop within 2 d of treatment. Similarly, transcripts encoding U-box-containing E3 ligase PUB35 were less abundant in shoots within 24 h of Phi treatment. A milder suppression compared with P$_i$ was observed for the primary transcript of regulatory microRNA miR399d. Transcripts encoding transcription factor BHLH32, E3 ubiquitin ligase C3HC4, and transport facilitator PHF1 responded more slowly but similarly to both P$_i$ resupply and Phi treatment, with a >4-fold lower abundance than in shoots of P-limited seedlings at the end of the experiment. **PHO2** transcripts showed an unexpected profile in shoots, with 2- to 4-fold lower levels in P$_i$-resupplied over P-limited seedlings. Phi treatment triggered a similar 2-fold decline in **PHO2** transcripts within 3 d of treatment.

Despite the fact that Phi accumulated as quickly as P$_i$ in roots, 43% of the tested P-responsive transcripts did not respond to Phi in this organ (Fig. 6B, grey transcript names). Transcripts from ACP5, G3PP1, PLD2, and PHT1;4 were among those that were also identified as being non-responsive to Phi in shoots. In roots, Phi-non-responsive transcripts included those from At4, PHO1;H1, PHF1, and PPCK2, all of which responded to Phi to some extent in shoots. On the other hand, transcripts encoding phosphatase PAP1 and

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**Fig. 6.** Effect of phosphate (P$_i$) and phosphite (Phi) on transcript abundance in P-limited seedlings. Hierarchical cluster analysis of a time-course on relative transcript abundance in P-limited Arabidopsis thaliana (A) shoots and (B) roots in response to P$_i$ resupply or Phi treatment. Mean log$_2$ expression ratios (–ΔΔCt) relative to the normalized expression change abundance in response to both Pi and Phi treatment, while those in grey (*) are unresponsive to Phi treatment (*P*≤0.05). Raw data were normalized against the transcript abundance of PP2AA3 and UBC9 reference genes. Clusters that contain Phi-responsive transcripts are highlighted by red lines in the tree. Transcripts in black change abundance in response to both Pi and Phi treatment, while those in grey(*) are unresponsive to Phi treatment (**P**≤0.05). PHO1 and PHO2 transcripts are highlighted in red. Transcripts in blue show no significant change in abundance across treatments. Details on individual transcript expression patterns and statistical analysis can be found in Supplementary Table S2 at JXB online.
ribonuclease RNS1 were more responsive to Phi in roots compared with shoots. As in shoots, NMT3 transcript abundance in roots increased 2-fold in response to Phi within 24 h of treatment, but transcript levels did not continue to increase and were 11-fold lower compared with roots of P̶ ̶ resupplied plants on day 7 (Supplementary Table S2 at JXB online). PHT1;7, PUB35, SPXI, and SQD2 were highly Phi responsive in roots as well as in shoots. However, the response was relatively delayed in roots for PHT1;7 and PUB35, while SPXI and SQD2 transcripts were more quickly suppressed in roots than in shoots. C3HC4, IPS1, and pmiR399d transcript abundance showed a weaker response to Phi in roots compared with shoots. In contrast to shoots, PHO1 transcript abundance did not respond to P̶ resupply in roots. Curiously, within 48 h of Phi treatment, PHO1 transcript abundance was ~2-fold greater than that in roots of P̶ limited plants and continued to increase throughout the time-course. PHO2 transcript abundance in roots did not respond to either P̶ or Phi treatment.

It has to be noted that seedlings were not severely P̶ starved at the beginning of the experiment. Evidence for this was the small changes in transcript abundance in organs of P̶ limited control plants at day 1 of the experiment compared with transcript levels in plants continuously supplied with P̶. As a consequence, transcript levels of the target P̶ starvation-induced genes continued to increase over the time-course in P̶ limited control plants. This was also the case for those transcripts that did not show a response to Phi in Phi-treated seedlings.

In contrast to the gradual response to P̶ deprivation, P̶ resupply led to the suppression of P̶ starvation-induced genes within 24 h (Supplementary Table S2 at JXB online). Thereafter, transcript abundance remained at the newly established lower levels for the rest of the time-course. Exceptions to this expression profile were those of microRNA antagonists IPS1 and At4, which showed a more gradual response to P̶ resupply in both roots and shoots. In shoots, PHT1;4 transcripts also showed this gradual decrease in abundance in response to P̶. Unlike all other target genes, transcripts from both IPS1 and PHT1;4 decreased in abundance to below the level observed in shoots of seedlings that were continuously supplied with P̶. In roots, PHO2 transcript levels tended to increase transiently within 24 h of P̶ resupply, rather than showing a sustained increase over P̶ limited plants. PHO2 transcripts did not respond to P̶ resupply in shoots.

**Discussion**

Phi has been demonstrated to suppress the induction of P̶ starvation responses. This conclusion was drawn from a series of experiments where P̶-starved plants were transferred to P̶-containing or P̶-free media supplemented with increasing Phi concentrations, or where seeds were germinated on these media (Carswell et al., 1996; Ticconi et al., 2001; Varadarajan et al., 2002; Berkowitz et al., 2013; Eshraghi et al., 2014). Thus, these studies focused on the ability of Phi to interfere with the induction of PSR genes in response to P̶ removal or the lack of P̶ supply. The experimental set-up used in this study allowed direct comparison of Phi and P̶ effects on the suppression of P̶ starvation responses through monitoring plant growth, P̶ anion and anthocyanin accumulation, as well as PSR gene expression. The experimental set-up has several advantages. (i) Withdrawal of P̶ from the medium prior to Phi treatment avoids competition between the two anions for uptake. (ii) A direct comparison of Phi and P̶ effects on the suppression of PSR genes can be conducted. (iii) Phi accumulation in the cytosol and organelles should be favoured over the vacuole under these conditions, so that more direct effects on metabolism and gene regulatory networks can be observed. (iv) The kinetic dependences of these effects on the accumulation of both P̶ anions in roots and shoots can be determined.

**Discrimination between P̶ and Phi by PHT1 transporters**

The differential movement of Phi and P̶ into the shoots of plants suggests different affinities for these molecules within their transport routes. Measurements of transport kinetics in different systems have concluded that P̶ transporters are able to transport Phi, albeit with a lower affinity than for P̶ (d’arcy-Lameta and Botmeix, 1991; Pratt et al., 2004; Danova-Alt et al., 2008; Basheer et al., 2011). This means that Phi can bind to P̶ transporter proteins without inducing the same conformational changes necessary for efficient transport (Basheer et al., 2011). It is unknown if all plant PHT transporters interact with Phi with the same affinity or whether some discriminate more strongly against Phi. In this study, the more pronounced delay in root-to-shoot transport of Phi in the pht1;8 and pht1;9-1 mutants than in wild-type seedlings, without a delay in Phi uptake, suggests that the encoded transporters discriminate more strongly against Phi than PHT1;1. The fact that discrimination is stronger in the absence of either PHT1;8 or PHT1;9 could mean that the two only partially complement each other (Lapis-Gaza et al., 2014) which would slow down transport even further. Alternatively, a third transport process, perhaps involving the Phi exporter PHO1 (Arpat et al., 2012), could be implicated in the stronger discrimination between P̶ and Phi in both mutants. The alleviation of the Phi discrimination phenotype over time is most probably due to remobilization processes between sink and source organs involving other PHT transporters, such as PHT1;5 (Nagarajan et al., 2011).

Differential recognition of Phi by different PHT proteins may modulate not only transport activity, but also signalling events associated with this activity (Schlothorst et al., 2013). It is unclear whether such a ‘transceptor’ function applies to the plant PHT family, but complex post-translational regulation has already been shown. Bayle et al. (2011) showed that some high-affinity PHT1 proteins undergo complex post-translational modifications, including protein phosphorylation. PHT1 protein abundance is also controlled by ubiquitin-mediated protein degradation (Lin et al., 2013; Park et al., 2014). Both PHT1;8 and PHT1;9 proteins can be distinguished from other family members by the presence of a PEST [proline, glutamic acid (E), serine, threonine] domain.
that mediates phosphorylation-dependent protein degradation in many systems (Rechsteiner and Rogers, 1996), for example the high- and low-affinity P$_i$ transporters in yeast (Lagerstedt et al., 2004; Estrella et al., 2008).

Differential expression of ‘PHO regulon’ genes in response to local P$_i$ signalling in roots and shoots

There is mounting evidence that the local and systemic control of PSR gene expression is governed by different signalling circuits in roots and shoots, and that different circuits within each organ respond either to the direct perception of P$_i$ or to a more indirect process involving downstream metabolites or other as yet unidentified signals (Müller et al., 2004; Bari et al., 2006; Thibaud et al., 2010; Woo et al., 2012; Rojas-Triana et al., 2013). The discrimination between P$_i$ and Phi by PHT1;8 and PHT1;9 shown in this study leads to a delayed accumulation of Phi in shoots. This delayed accumulation of Phi may hence be an elegant tool for dissecting direct sensing of P$_i$ from other potential signals of P status in the shoot. PHT1;7 and pri-MIR399d transcripts in the present study were suppressed earlier in shoots than in roots and responded before Phi accumulated to significant levels. This would place them into an early-response circuit more directly connected to a P$_i$-specific sensor in the root-to-shoot transport route. PHT1;7 and pri-MIR399d expression was deregulated in the phi1;9-1 (Lapis-Gaza et al., 2014) and the phr1 mutant, but not the pho2 mutant (Bari et al., 2006). Slower shoot accumulation of Phi correlated with an attenuated down-regulation of a select subset of PSR genes closely associated with the ‘PHO regulon’, such as At4, IPS1, SPX1, PHF1, and PHO1;H1. This would support their response to local P$_i$ or, in this case, Phi availability in the shoot. Interestingly, these genes were also deregulated in both P-limited phr1 and P$_i$-resupplied pho2 mutants (Bari et al., 2006). These findings may indicate that early P$_i$- and Phi-responsive genes are more directly connected to PHR1, possibly through a SIZ1 domain protein (Puga et al., 2012). Transcripts encoding another SPX domain protein, SPX1, responded to Phi in both roots and shoots. SPX1 is a competitive inhibitor of PHR1 binding to the P1BS element in PSR gene promoters (Puga et al., 2014). Its interaction with PHR1 is also highly dependent on the presence of either P$_i$ or Phi. In contrast to most PSR genes in the present study, it responded much more quickly to Phi in roots. Both PHO1;H1 and SPX1 are regulated in a PHR1- and PHO2-dependent manner (Bari et al., 2006), but SPX1 is also controlled by SIZ1 (Duan et al., 2008). The latter may explain its more direct response to local Phi concentrations in the root (Miura et al., 2011). This would put SIZ1 into a position close to the local P$_i$- and Phi-sensing module in roots (Fig. 7). Surprisingly, SPX1 is also systemically regulated in P-limited roots in a split-root system (Thibaud et al., 2010).

PHO2 transcripts encoding an E2 ubiquitin conjugase (Aung et al., 2006; Bari et al., 2006) accumulated transiently in P$_i$-resupplied roots, but were largely unresponsive to Phi treatment. This suggests that PHO2 is connected to a signalling circuit that responds very sensitively to changes in overall P status, perhaps through monitoring concentrations of a downstream P metabolite (Klecker et al., 2014; Pant et al., 2015). In support of this interpretation, P-sufficient pht1;9 mutants showed a stronger accumulation of At4 and pri-MIR399d transcripts, and lower transcript accumulation of PHO2 in shoots which did not correlate with P$_i$, concentrations in pht1;9 roots or shoots (Lapis-Gaza et al., 2014). The decline in PHO2 transcripts over the treatment period could therefore be an early response to the P$_i$ depletion of the media resulting in lower levels of a downstream P metabolite. This P$_i$ depletion after 7 d of treatment would also explain the observed lower P$_i$ concentration and the higher transcript abundance for PHO1 and SPX1 in roots as well as increasing transcript levels for PHT1;7 and pri-MIR399d in shoots of P$_i$-resupplied seedlings. In shoots of P$_i$-resupplied seedlings, PHO2 expression was even lower than that in P-limited seedlings. Since At4 and IPS1 transcript levels were significantly lower in shoots in response to either P$_i$ or Phi treatment, the late increase in pri-MIR399d transcript abundance, which underlies PHO2 repression, might explain the further drop in PHO2 transcript amounts in the shoot. In contrast to roots, this response was mimicked by Phi to some extent, again highlighting the differences in P$_i$ perception between the two organs.
All the genes mentioned in this section respond very quickly to changes in P status. However, there is a clear distinction in the regulation of \( \text{SPX1} \) that responds very early in roots, \( \text{PHO1} \), which seems to respond to signals associated with growth, \( \text{PHO2} \) which responds to unknown downstream P signals, and all other components of the ‘PHO regulon’ that do show strong responses to both P and Phi, especially in shoots. It is possible that the first perception of P takes place during root-to-shoot transport or within the shoot itself. Conversely, PSR gene expression in the root largely responds to secondary, shoot-derived signals as previously demonstrated (Bari et al., 2006; Lin et al., 2008; Thibaud et al., 2010).

**Phosphite-dependent expression changes in roots affect transcripts for local lipid-remodelling pathways**

In roots, transcripts encoding sulpholipid synthase SQD2 that catalyses the last step in sulpholipid biosynthesis and phosphoethanolamine \( N \)-methyltransferase NMT3 that synthesizes the head group of the phospholipid phosphatidylcholine responded very quickly to both P and Phi, while their response was slower in shoots. By contrast, \( \text{PLD}_\zeta \) transcripts encoding a phospholipase D isoform showed a response to P, but not to Phi. NMT3 is one of the few genes that respond to P independently of PHR1 and PHO2 in \( \text{A. thaliana} \) seedlings (Bari et al., 2006). In the study of Woo et al. (2012), many lipid-remodelling genes such as \( \text{PLD}_\zeta \) and SQD2 were among the group of genes that specifically responded to P in both roots and shoots. Their response to P was PHR1-dependent, but undisturbed in \( \text{pho2} \) seedlings (Bari et al., 2006). They were also systemically regulated in a split-root system, but their induction was attenuated compared with that in P-limited control roots (Thibaud et al., 2010). These genes were also highly responsive to Phi in an earlier acclimation study (Berkowitz et al., 2013). Kobayashi et al. (2006) demonstrated that the promoter of another lipid-remodelling gene, \( \text{MGD}_2 \), responds very strongly to Phi in roots and shoots, and that Phi is able to cause the modification of shoot lipid profiles in

**Fig. 7.** A model for the sequence of changes in phosphate-starvation-responsive (PSR) gene expression observed in roots and shoots of phosphorus-limited \( \text{A. thaliana} \) seedlings in response to phosphite (Phi) treatment. Blue pathway: the discrimination of Phi by PHT1;9 and subsequently by PHT1;8 during xylem loading (1) may indicate the recognition by a receptor that signals the availability of phosphate (P) and Phi to the shoot, possibly involving SiZ1 (2). This sequence of events may primarily affect PHT1;7 expression in the shoot (3), followed by the consecutive suppression of other PSR genes within 24 h (4-5) or later after 3 d when Phi finally started to accumulate in the shoot (7-8). Green pathway: in roots, early local recognition of Phi is possibly restricted to the suppression of SQD2 within 24 h (3) and of the less responsive SPX1 and PPCK2 as well as to the induction of NMT3 (5). Compared with shoots, PHT1;7, RNS1 and a couple of transcripts in group 8 (in grey) responded more slowly in roots, most probably indicating that their expression in roots is regulated by PHO2 and relies on systemic signalling, perhaps through reduced levels of mir399d in the phloem (blue dotted arrow). Curiously, \( \text{PHO1} \) expression in roots increased within 3 d of Phi treatment, which may indicate its connection to independent regulatory networks (purple 8) that directly respond to the overall P status of the plant or the growth inhibition triggered by Phi. Note that the number of genes responding equally well to either P, or Phi (red first letter in gene name) was greater in shoots than in roots. Gene names in black indicate a 2-fold expression change in response to Phi over P-limited controls (Fig. 6). An orange border indicates a 4-fold expression change. A bold red border indicates an 8-fold change. Grey names indicate non-significant changes. Red arrows following gene names indicate suppression (↓) or induction (↑) within 24 h of Phi exposure, while black arrows indicate a response within 3 d of treatment. An asterisk indicates a Phi-specific expression change that was not observed in P-resupplied seedlings.
Phosphate effects on root architecture may be caused by altered accumulation of transcripts encoding proteins involved in protein turnover and vesicle trafficking

In this study, a U-box/ARM-repeat E3 ligase gene, PUB35, was responsive to the plant’s P status and was among a small group of PSR genes that were highly responsive to Phi, especially in roots. U-box and RING-finger E3 ligases, such as PUB35 and C3HC4, that responded to both P, and Phi in the present study, are highly responsive to the plant’s P status, with many of them showing PHR1/PHL1-dependent regulation (Rojas-Triana et al., 2013). The U-box E3 ligase, PUB9, has recently been implicated in linking auxin-dependent and P-regulated lateral root emergence with vesicle trafficking. PUB9 interacts with the S-domain receptor kinase ARK2 that is implicated in P-derived signal recognition (Deb et al., 2014). The ark2-1/pub9-1 double mutant features shorter primary roots under low P supply, thus mimicking the Phi-induced phenotype in this study. While it has been demonstrated that several PUB E3 ligases can interact with ARK2 in vitro (Samuel et al., 2008), this has yet to be demonstrated for PUB35.

What makes this potential link between P signalling and Phi recognition particularly intriguing is the fact that U-box proteins have also been implicated in triggering plant immunity (Gonzalez-Lamothe et al., 2006; Trujillo et al., 2008). Many of the 64 predicted U-box-containing proteins in A. thaliana are associated with mono-ubiquitination and proteasomal degradation of signalling components during stress responses that trigger cell death (Yee and Goring, 2009).

Indirect effects of Phi treatment on plant growth

In P-limited cell suspension cultures, Phi exacerbates P starvation by inhibiting vacuolar efflux of P (Pratt et al., 2009). This could explain the arrest in primary and lateral root growth observed upon Phi treatment in this study and upon longer term Phi exposure (Berkowitz et al., 2013; Eshraghi et al., 2014). In both instances, the growth arrest was much more severe than the slowing of primary root elongation observed upon P withdrawal alone. However, plants in the present study were not experiencing severe P starvation, given that anthocyanin levels in leaves of P-limited controls only started to increase towards the end of the experiment. Also, a significant reduction of both root and shoot biomass in the presence of Phi compared with that of both continued P-limited growth and P resupply was only observed on the third day of treatment, at the time when Phi first significantly accumulated in shoots. The inhibitory effect that Phi has on organ growth might thus be directly triggered by the accumulation of Phi within the cytosol and organelles of the shoot (Danova-Alt et al., 2008; Pratt et al., 2009). As would be expected from a mildly cytotoxic agent, Phi then seems to affect both root and shoot growth in a similar fashion. This is very different from the opposing hormonal effects on root and leaf development (King et al., 1995; Werner et al., 2003).

These observations would imply that whilst Phi is a great tool to tease apart direct, P-triggered effects on P signalling networks from those further downstream, care has to be taken to interpret longer term effects due to its immediate toxicity on many P-dependent metabolic pathways.

Conclusion

The present results indicate that Phi is perceived as P and suggest that this perception is stronger in shoots than in roots. The perception of Phi most probably affects distinct regulatory circuits in both organs, and is more closely associated with factors that interact with PHR1-associated networks, such as SIZ1 (Miura et al., 2011). The strong root architectural changes induced by Phi in P-limited plants are most probably invoked by its interference with local signalling components that affect lipid remodelling (PLD(c2), SQD2, NMT3) and protein turnover (PUB35, C3HC4). In the longer term, Phi severely affects plant growth, most probably by inhibiting vital P-dependent metabolic pathways. Several of these pathways have the potential to trigger the priming of plant defences. Used with caution, Phi can be a useful tool in further disentangling these complex interactions.

Supplementary data

Supplementary data are available at JXB online.
Figure S1. Root phenotypic responses in phosphorus-limited plants to phosphate resupply or phosphate treatment.
Figure S2. Phosphate and phosphite accumulation in roots and shoots of Col-0 and pht1 mutants over time.
Figure S3. Biomass accumulation in roots and shoots of Col-0 and pht1 mutants over time.
Table S1. Information on target genes and primers used in qRT-PCR analyses.
Table S2. Time-course of relative transcript abundance of known phosphate-responsive genes in phosphorus-limited A. thaliana seedlings in response to either phosphate resupply or phosphite treatment.

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