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RESEARCH PAPER

Acclimation responses of *Arabidopsis thaliana* to sustained phosphite treatments

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Abstract

Phosphite ($H_2PO_3^-$) induces a range of physiological and developmental responses in plants by disturbing the homeostasis of the macronutrient phosphate. Because of its close structural resemblance to phosphate, phosphite impairs the sensing, membrane transport, and subcellular compartmentation of phosphate. In addition, phosphite induces plant defence responses by an as yet unknown mode of action. In this study, the acclimation of *Arabidopsis thaliana* plants to a sustained phosphite supply in the growth medium was investigated and compared with plants growing under varying phosphate supplies. Unlike phosphate, phosphite did not suppress the formation of lateral roots in several *Arabidopsis* accessions. In addition, the expression of well-documented phosphate-starvation-induced genes, such as *miRNA399d* and *At4*, was not repressed by phosphite accumulation, whilst the induction of *PHT1;1* and *PAP1* was accentuated. Thus, a mimicking of phosphate by phosphite was not observed for these classical phosphate-starvation responses. Metabolomic analysis of phosphite-treated plants showed changes in several metabolite pools, most prominently those of aspartate, asparagine, glutamate, and serine. These alterations in amino acid pools provide novel insights for the understanding of phosphite-induced pathogen resistance.

Key words: *Arabidopsis*, phosphate, phosphate-starvation response, phosphite.

Introduction

Phosphorus is an essential macronutrient for plants and is taken up and assimilated in the form of inorganic phosphate ($H_2PO_4^-$, Pi). The Pi concentrations in most soils are very low, and Pi availability is further decreased by immobilization, such as by the formation of insoluble complexes of Fe and Al oxides (Gerke, 1992). Plants have developed mechanisms to cope with Pi limitation. The inducible developmental and metabolic adaptations that enhance uptake and utilization are together referred to as the phosphate-starvation response (PSR) (Raghothama, 1999; Chiou and Lin, 2011). Uptake of Pi from the soil can be enhanced by secretion from the roots of organic acids, such as citrate or malate, and phosphatases to mobilize Pi from mineralized and organic forms (Duff *et al*., 1994; Ryan *et al*., 2001). Morphological alterations of root architecture, such as decreased primary root growth and increased lateral root and root-hair production, enhance the capacity to forage Pi from the richer topsoil layers (Lynch and Brown, 2001; Peret *et al*., 2011). Some plant species are able to generate specialized root structures composed of dense clusters of lateral roots or form associations

Abbreviations: GC-MS, gas chromatography–mass spectrometry; LRD, lateral root density; PFK, phosphofructokinase; Phi, phosphite; inorganic phosphate; PSR, phosphate-starvation response; SE, standard error.

with mycorrhizal fungi to form a symbiotic partnership to enhance Pi acquisition (Shane and Lambers, 2005; Bucher, 2007). The developmental and physiological processes controlled by Pi availability are under complex control through local and systemic signalling as well as external and internal Pi concentrations (Thibaud *et al.*, 2010).

The PSR includes the transcriptional induction of genes such as high-affinity Pi transporters or phosphatases to increase Pi acquisition, remobilization from storage compounds such as phytate and phospholipids, and redistribution between tissues and/or subcellular compartments. At the post-transcriptional level, the miRNA399/PHO2/PHR1 regulon has been identified as an important mechanism to control Pi homeostasis. Under Pi deficiency, *miRNA399* transcription is highly upregulated by the transcription factor PHR1 to direct the degradation of the mRNA for the E2 ubiquitin conjugase PHO2 (Bari *et al.*, 2006; Chiou *et al.*, 2006). The regulated degradation of PHO2 transcripts by miRNA399 is in turn attenuated by the regulatory RNAs IPS1 and At4 (Franco-Zorrilla *et al.*, 2007). PHO2 controls Pi concentrations by regulating protein turnover through ubiquitination of a set of target proteins that include PHO1 and probably also Pi transporters (Liu *et al.*, 2012). As a consequence, a *pho2* mutant, as well as *miRNA399* overexpressing *Arabidopsis* lines, hyperaccumulate Pi (Aung *et al.*, 2006; Chiou *et al.*, 2006). Furthermore, the phloem mobility of miRNA399 has been interpreted to indicate that miRNA399 is a signal in shoot-to-root communication (Pant *et al.*, 2008). Transcriptional regulation has also been found for genes that are part of the acclimation to limited Pi supply, for example for *SQD2* and *NMT3* involved in the remodelling of membrane lipid composition (Essigmann *et al.*, 1998; Hartel *et al.*, 2000).

It is not clear how Pi levels are perceived in plants. In yeast, a phosphate transporter (PHO84) has been identified that signals Pi status through a conformational change induced by Pi binding that is independent of the transport of Pi across the membrane (Popova *et al.*, 2010). A similar transceptor function has also been identified for the nitrate transporter NRT1.1/CHL1 in *Arabidopsis*, indicating that similar sensing mechanisms could exist for Pi in plants (Ho *et al.*, 2009).

Post-translational regulation of the PSR involves metabolic adjustments to cope with the changed availability of Pi for enzymatic reactions and their regulation (Plaxton and Tran, 2011). Pi is a structural component of many organic components, such as DNA, RNA, phospholipids, and phosphorylated sugars. Therefore, Pi deficiency has profound effects on plant metabolism, such as leading to the accumulation of starch in the shoot (Ghosh and Preiss, 1966; Fliege *et al.*, 1978). Another example is the remodelling of membrane lipid composition by replacing phospholipids with sulfo- and galactolipids to release Pi from this membrane component and conserve Pi (Essigmann *et al.*, 1998; Hartel *et al.*, 2000; Lambers *et al.*, 2012). With Pi being a substrate in many more important pathways of primary metabolism, the metabolic pools of Pi in the various subcellular compartments need to be regulated, with the vacuole being the major storage compartment releasing Pi under a low external supply (Lee and Ratcliffe, 1993; Mimura *et al.*, 1996; Danova-Alt *et al.*, 2008; Pratt *et al.*, 2009). Recent results from ^{31}P-NMR studies gave estimates of subcellular Pi pools with concentrations in the range of 60–80 µM in the cytosol and 7 mM in organellar mitochondrial and plastidal pools combined (Pratt *et al.*, 2009). This same study provided evidence for considerable fluctuation of cytosolic but not of organellar concentrations under a Pi supply, which could be used to sense changes in cellular levels and trigger the signalling cascade of the PSR.

Phosphite ($H_2PO_3^-$, Phi) is an analogue of phosphate in which a hydroxyl group is replaced by a hydrogen atom. It enters the cell via Pi transporters, therefore competing with Pi for uptake, and is mobile within the plant (Ouimette and Coffey, 1989; Carswell *et al.*, 1996; Danova-Alt *et al.*, 2008). On the other hand, Phi is used in the control of plant pathogens, especially oomycetes such as *Phytophthora* or *Pythium* species; it has been used for decades as the active agent in biostats such as Fosetyl-Al (Cohen and Coffey, 1986; Guest and Grant, 1991; Hardy *et al.*, 2001). High concentrations of Phi directly inhibit growth of oomycete mycelia, and this has been attributed to interference of Phi with key reactions by competing with Pi for the enzyme catalytic sites, leading to altered abundances of metabolites such as ATP, NAD, polyphosphates, and pyrophosphate (Griffith *et al.*, 1990; Barchietto *et al.*, 1992; Niere *et al.*, 1994). In addition to this direct effect of Phi on pathogens, it also activates plant defence responses (Molina *et al.*, 1998; Friedrich *et al.*, 2001; Eshraghi *et al.*, 2011). This Phi-induced resistance depends on the signalling component NPR1 (non-expressor of PR1) and the accumulation of salicylic acid (Molina *et al.*, 1998; Friedrich *et al.*, 2001). Recently, it has been shown that Phi pre-treatment primes plants for a stronger defence response when subsequently challenged with *Hyaloperonospora arabidopsidis* and this priming involves the mitogen-activated protein kinase MPK4 (Massoud *et al.*, 2012).

The chemical resemblance of Phi to Pi has made it an attractive tool to investigate Pi sensing. Phi is metabolically inert in plants, does not enter the organic P pool and thus accumulates in the tissues (Carswell *et al.*, 1996, 1997; Danova-Alt *et al.*, 2008; Berkowitz *et al.*, 2011). The treatment of severely Pi-starved plants with Phi suppresses the activation of many PSR genes and it is therefore assumed that Phi is recognized by the same sensing mechanism as Pi. The Phi-dependent downregulation of the PSR then leads to an accentuation of Pi deficiency, for example by decreasing Pi uptake capacity. This interference with the Pi regulatory network has been suggested to cause the plant growth-inhibiting effect of Phi (Carswell *et al.*, 1996, 1997; Ticconi *et al.*, 2001; Varadarajan *et al.*, 2002). ^{31}P-NMR studies using tobacco BY2 cells have shown that Phi accumulates in the cytoplasm of P-deprived cells and that resupply of Pi leads to efflux of Phi from the cell. In Pi-sufficient cells, however, Phi accumulates in the vacuole, illustrating the complex interactions of Pi and Phi with respect to cell compartmentation, sensing, and physiology (Danova-Alt *et al.*, 2008).

To understand the specific effects of Phi on plant growth and metabolism, it is necessary to compare directly the effects of Phi-treated plants with plants under Pi limitation. In this study, *Arabidopsis* plants grown on medium containing Phi

and Pi in varying ratios but at a constant combined concentration were investigated. Growth parameters, root development, and gene expression patterns of Phi-treated plant were compared with those of plants grown with various Pi supplies. In addition, Phi-induced metabolic adjustments with potential relevance for plant defence mechanisms were identified.

Materials and methods

Plant material

Seeds of accessions of *Arabidopsis thaliana* (L.) Heynh. used in this study were obtained from the European *Arabidopsis* stock centre (Col-0, N1093) and the Versailles Resource Centre (Col-4, AV187; Jea, AV25; Nd-1, AV220; Sha, AV236; Tsu-1, AV239). *Arabidopsis* plants were grown on a modified MS medium (Murashige and Skoog, 1962) in a vertical plate system for 10 d. The medium contained 2 mM NH_4NO_3, 1.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, 3 mM KNO_3, 3 mM MES (pH 5.8), 0.5% (w/v) sucrose and 0.8% (w/v) agar. The agar contributed ~10 µM Pi. Phosphate was added as KH_2PO_4 (pH 5.8). KCl was substituted for KH_2PO_4 to maintain a constant osmotic potential. A freshly prepared 0.1 M potassium phosphite stock solution was prepared from phosphorous acid by adjusting to pH 5.8 with KOH and was added to the medium as required. Micronutrients were used according to Murashige & Skoog (1962) and obtained from Austratec (Bayswater, Australia). After sterilization [2 min in 70% (v/v) ethanol, 5 min in 5% (v/v) sodium hypochlorite], the seeds were resuspended in 0.1% (w/v) agarose and stratified for 2 d at 4 °C. The seeds were transferred to 10 × 10 cm square plates, which were then sealed with microporous tape, and the plants were grown in a controlled-environment growth chamber with a 10 h photoperiod at ~100 µmol m^{-2} s^{-1}. For metabolomic analyses, plants were grown for 3 weeks in horizontal 90 mm Petri dishes sealed with microporous tape under the same growth conditions as described above. For analysis of growth responses, all experiments were repeated in five independent replicates consisting of eight seedlings each.

Phosphate and phosphite quantification

Phosphate and phosphite were extracted from ~10 mg of shoot or root tissue with 1 % (v/v) acetic acid at 1:10 (w/v) using a tissue lyser (Qiagen, Doncaster, Australia) for 1 min at 25 Hz. After clearing the lysate by centrifugation at 14 000*g* and 4 °C for 15 min, the supernatant was assayed for phosphate and phosphite as described previously (Ames, 1966; Berkowitz *et al*., 2011).

Root growth measurements

For measurements of root growth, the position of the root tip was marked every 24 h for the 10-day growth period. Plates were then scanned for analysis of root length, growth rate, and lateral root number using ImageJ software with the NeuronJ plug-in (Meijering *et al*., 2004).

Gene expression analyses

For each treatment, RNA was extracted from five independent biological replicate samples and reverse transcribed using Plant RNA Mini and Tetro cDNA synthesis kits according to the manufacturer's instructions (Bioline, Alexandria, Australia). Quantitative real-time PCR on a Rotor Gene 6000 platform (Qiagen) was performed with 2 ng of cDNA and a SensiFast SYBR No-ROX kit (Bioline) as described previously (Jost *et al*., 2007). Relative expression levels were calculated using the comparative $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) and normalized to the internal reference genes *PDF2*, *UBC9* and *UPL7* (Czechowski *et al*., 2005). For evaluation of reference genes and amplification efficiencies, the geNORM and LinRegPCR algorithms, respectively, were used (Vandesompele *et al*., 2002; Ramakers *et al*., 2003). Primers were designed using Primer Express software (Applied Biosystems, Mulgrave, Australia) and the QuantPrime tool (Arvidsson *et al*., 2008). Primer sequences and Arabidopsis Genome Initiative (AGI) gene identifiers are given in the supplementary data (Table S1 at *JXB* online).

Metabolomic analysis by gas chromatography–mass spectrometry (GC-MS)

Polar metabolites were extracted from shoot tissue harvested from eight biologically independent experiments 3 h after the onset of light and immediately frozen in liquid nitrogen. After homogenization in a tissue lyser (Tissue Lyser II; Qiagen), 30 mg of frozen tissue powder was extracted with 500 µl extraction solution (20:3 methanol:water including 10 µg ml^{-1} of ribitol as an internal standard) for 20 min at 75 °C with shaking at 1200 rpm in a thermomixer (Thermo mixer compact; Eppendorf, North Ryde, Australia). After centrifugation at 16 100*g* for 3 min at room temperature, 60 µl of the supernatant was dried under vacuum and the polar metabolites redissolved by incubation in 20 µl of methoxyamine hydrochloride (20 mg ml^{-1}) in pyridine at 37 °C with shaking at 750 rpm for 90 min. For derivatization, 30 µl of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide was added and the mixture was incubated at 37 °C with shaking at 750 rpm for 30 min. A retention time standard (10 µl) containing 140 nl ml^{-1} each of *n*-dodecane, *n*-pentadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotriacontane and *n*-hexatriacontane in pyridine was added to each sample. GC-MS analysis was performed by injecting 1 µl of derivatized sample using the splitless mode on an Agilent 7890GC/5975MSD system (Agilent, Mulgrave, Australia) equipped with a Varian Factor 4 column and operated in scan mode (range: 50–650 *m*/*z*). Metabolites were identified by comparing retention times and ionization patterns with standards and the National Institute of Standards and Technology mass spectral library. The integrated signal of the total ion current for each peak was compared between samples after normalization. Metabolites were normalized first using the internal standard and then the sample weight.

Statistical analyses

For statistical analyses, the SPSS Statistics Base software package (version 17; IBM, New York, USA) was used. Analysis of variance with a Tukey HSD test was performed for multiple comparisons.

Results

Growth responses to sustained Phi supply

Because detailed reports on the acclimation of plants under sustained Phi supply that also take into account internal Pi and Phi concentrations are lacking, this study analysed the responses of *Arabidopsis* Col-0 seedlings to varying Pi and Phi concentrations in the growth medium at a constant combined total concentration of 0.5 mM. Seeds were germinated directly on medium containing differing Phi:Pi ratios ranging from 1:1 to 50:1. Growth was compared with that of control seedlings grown on medium containing Pi only at concentrations over the same range (Fig. 1, Table 1). For the Phi-treated seedlings, the Pi and Phi concentrations of the shoots and roots varied according to the external supply. Interestingly, the total concentration of Pi+Phi in both tissues was remarkably constant, despite the substantial variation in the concentrations of the two anions in the growth medium (Fig. 1).

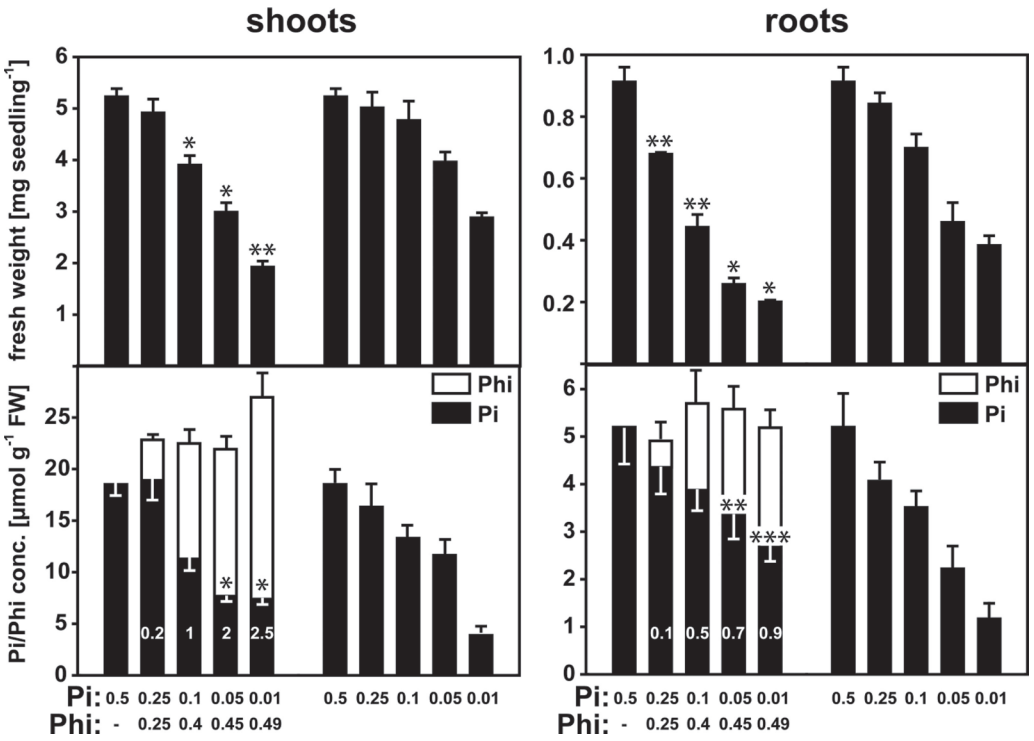

Fig. 1. Fresh weights and Pi and Phi concentrations of *Arabidopsis* (Col-0) shoots and roots under varying Pi and Phi supplies. Plants were grown in a vertical plate system for 10 d before harvesting the shoot and root tissues. Numbers in bars represent the corresponding ratios of Phi and Pi concentrations in the tissue. For each sample, eight plants grown on one plate were pooled, and for each treatment five independent biological replicates were performed (n=5 pools of eight per treatment). Results are given as means ±standard error (SE). Significant differences between Phi treatments and their corresponding controls grown on the same Pi concentration are indicated (ANOVA Tukey: *P <0.05; ** P < 0.01; *** P < 0.001).

Shoot fresh weights for seedlings grown on 0.25 mM Pi+0.25 mM Phi did not differ significantly from seedlings grown on 0.25 mM Pi alone. Quantification of Pi and Phi tissue concentrations revealed that the seedlings supplied with Phi had the same shoot Pi concentration as plants supplied with Pi but not Phi. However, despite the equimolar supply of Pi and Phi, the Phi accumulation was only 20% of that of Pi (Fig. 1). Higher Phi supplies led to a decrease in shoot biomass that was accentuated compared with that of control seedlings grown at the same external Pi. Interestingly, the seedlings treated with 0.1 mM Pi+0.4 mM Phi and 0.05 mM Pi had equal shoot Pi concentrations and similar shoot fresh weights. Thus, for this Phi treatment, the reduced biomass was explained mainly by a reduction in the Pi concentrations in the shoot tissue, and the accumulation of Phi seemed to have little direct effect on shoot biomass. However, the seedlings that contained the highest concentration of Phi in the shoots had an accentuated reduction in shoot biomass compared with seedlings grown on the lowest Pi supply. Surprisingly, in the two treatments containing 0.01 mM Pi, the seedlings that were supplied with Phi showed a higher Pi concentration in the shoots.

Phosphite accumulation had a more pronounced effect on decreasing the fresh weight of the roots than on that of

Table 1. Comparison of fresh weights and Pi/Phi tissue concentrations of Phi-treated and non-treated plants of *Arabidopsis thaliana* (see also Fig. 1)Fresh weights (FW) and Pi tissue concentrations of Phi-treated plants are expressed relative to Pi-sufficient plants grown on 0.5 mM Pi or control plants with the same Pi concentration in the medium but lacking Phi. Results are given as means of five independent biological replicates with eight seedlings each.

	Leaves				Roots			
	% of 0.5 mM Pi		% of Pi control		% of 0.5 mM Pi		% of Pi control	
P_w/Phi treatment	FW	[Pi]	FW	[Pi]	FW	[Pi]	FW	[Pi]
0.25 mM/0.25 mM	93	88	98	115	74	78	80	107
0.1 mM/0.4 mM	74	71	81	85	48	67	63	110
0.05 mM/0.45 mM	57	62	75	66	28	42	56	151
0.01 mM/0.49 mM	36	20	66	190	21	22	52	229

the shoots (Fig. 1, Table 1), which resulted in an increase in shoot:root ratio (Fig. S1 at *JXB* online). Across all treatments, seedlings grown on Phi-containing medium had a lower root biomass than seedlings that had a similar Pi tissue concentration in the absence of Phi. For example, the roots of seedlings exposed to the 0.05 mM Pi+0.45 mM Phi and the 0.1 mM Pi treatments showed similar Pi concentrations, but the Phi-treated seedlings had a much lower root biomass. The Phi concentration in the roots was only comparable to the Pi concentration when Phi was in a ten- to 50-fold external excess (Fig. 1), and was only equimolar in the 0.01 mM Pi+0.49 mM Phi treatment. At this highest Phi:Pi ratio, the root fresh weight was reduced by about 80% compared with seedlings in the 0.5 mM Pi treatment, whilst the shoot fresh weight was only reduced by ~25% (Table 1).

Across all Phi treatments tested, the Phi:Pi tissue ratio was consistently about twofold higher in the shoots than in the roots, indicating preferential transport and accumulation of Phi in the shoot. However, this did not lead to an accentuated impact on shoot biomass, as the relative decrease in biomass with increasing external Phi was higher in the roots. This discrepancy might indicate a higher Phi tolerance of the shoot tissue. A surprising observation was the consistently higher Pi concentration in roots of Phi-treated seedlings compared with seedlings grown at the same Pi supply. A similar effect was observed for shoot tissue but only at the highest external Phi:Pi ratios.

Phosphite does not suppress increases in lateral root density (LRD) under Pi limitation

Next, the effect of Phi on root development under our growth conditions was examined. Plants grown at lower Pi supplies showed the characteristic reduction in primary root length (Fig. 2A), as reported previously (Peret *et al.*, 2011). Phi only had an effect on primary root length at the highest concentration tested, with a 68% reduction in primary root length compared with seedlings grown at the same external Pi supply (Fig. 2A). The primary root growth rate in the 0.25 mM Phi treatment was reduced by ~1 mm d^{-1} from 4 d after germination compared with the control (Fig. 2B, C). Interestingly, primary roots exposed to either 0.1 mM Pi+0.4 mM Phi or 0.05 mM Pi+0.45 mM Phi initially had decreasing growth rates, which recovered from 4 d after germination and increased steadily for the rest of the experiment, leading to root lengths comparable to roots grown on the same Pi but without Phi in the medium. Only primary roots of seedlings growing at the lowest Pi supplies had a constant deceleration in growth that ultimately caused the shortest primary roots across treatments. This effect was stronger in the presence of Phi.

LRD (the number of lateral roots per cm of primary root) for seedlings grown in the absence of Phi increased with decreasing Pi supply, as expected (Fig. 3). Intriguingly, Phi treatment had no effect on LRD at the lowest Phi:Pi ratio tested, whilst plants growing on 0.1 mM Phi+0.4 mM Pi had a higher LRD than plants on 0.1 mM Pi alone (Fig. 3). This was surprising, as the combined total concentration of Pi+Phi in

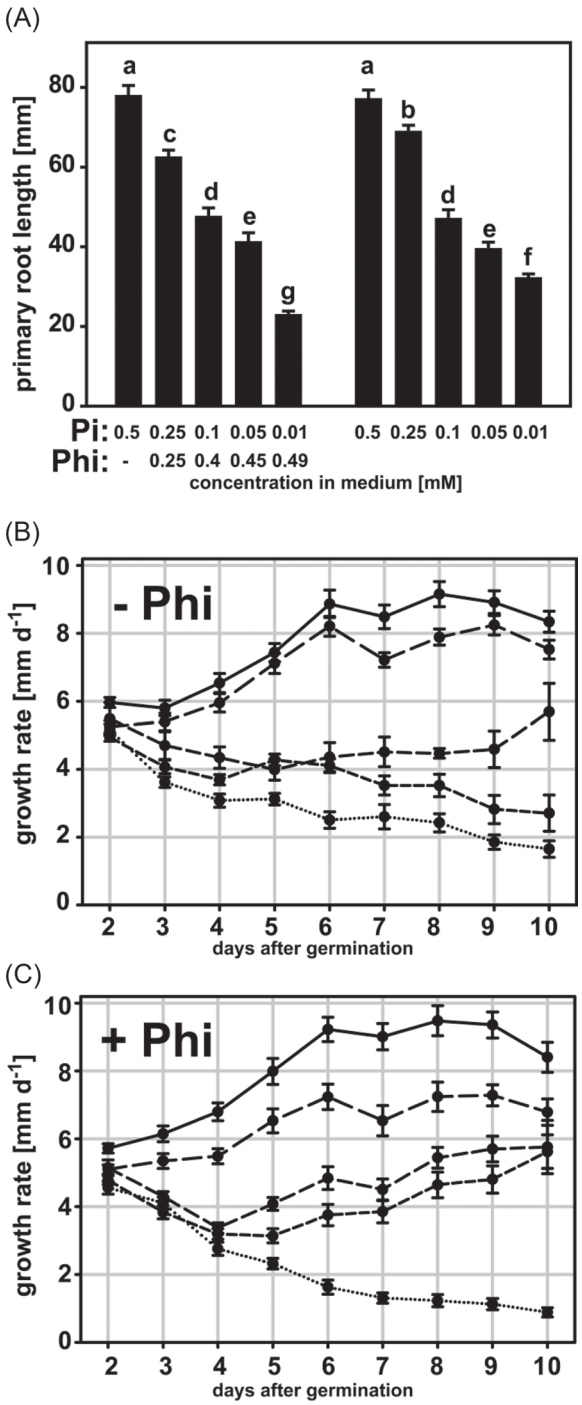

Fig. 2. Lengths and growth rates of *Arabidopsis* (Col-0) primary roots in response to external Phi and Pi supply. (A) Primary root lengths of plants grown in a vertical plate system for 10 d with varying Phi and Pi concentrations in the growth medium. Means with different letters are significantly different (ANOVA Tukey, P <0.05). (B, C) Root growth rates for plants grown with decreasing Pi (B) and variation of Phi:Pi ratios (C) in the medium. Data points are means ±SE of five biological replicates with eight seedlings each. Medium concentrations in (B) and (C): solid line, 0.5 mM Pi; long-dashed line, 0.25 mM Pi (+0.25 mM Phi); medium-dashed line, 0.1 mM Pi (+0.4 mM Phi); short-dashed line, 0.05 mM Pi (+0.45 mM Phi); dotted line, 0.01 mM Pi (+0.49 mM Phi).

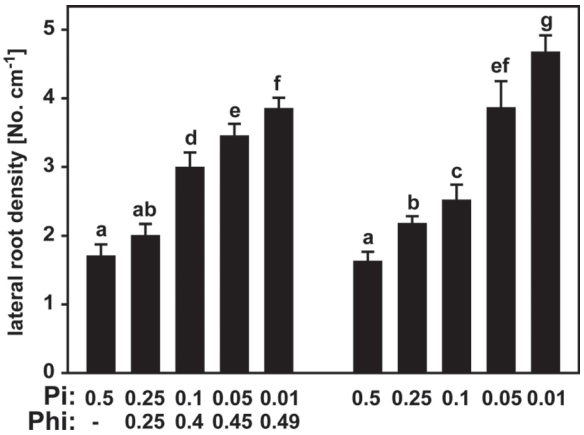

Fig. 3. LRD of *Arabidopsis* (Col-0) plants grown on medium with different Phi and Pi concentrations (mM). Primary root lengths and the numbers of lateral roots (>100 μm) were determined from five biological replicates with eight seedlings each. Results are given as means ±SE. Means with different letters are significantly different (ANOVA Tukey, $P < 0.05$).

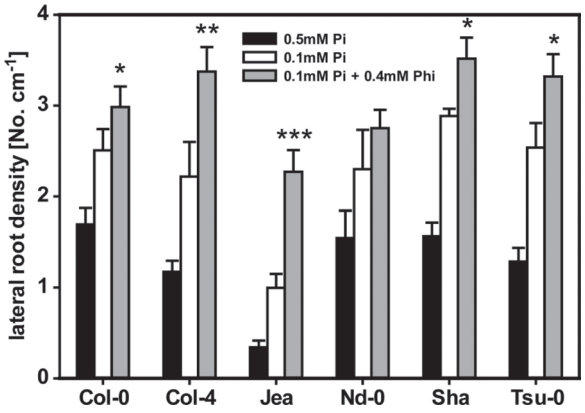

Fig. 4. Comparison of LRD for six *Arabidopsis* accessions grown on growth medium containing 0.5 mM Pi, 0.1 mM Pi, or 0.1 mM Pi+0.4 mM Phi. For each accession, at least 16 seedlings per treatment were analysed (four independent replicates with four seedlings each). Results are given as means ±SE. Statistically significant differences between the 0.1 mM Pi and the 0.1 mM Pi+0.4 mM Phi treatments are indicated (ANOVA Tukey, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

the root and shoot tissues was unchanged, and also across all treatments the combined concentration of Pi and Phi in the growth medium was constant at 0.5 mM (Fig. 1). Thus, Phi did not seem to have a suppressing effect on LRD similar to that of Pi. Only for the two highest Phi concentrations was the increase in LRD reduced compared with the corresponding control seedlings, but these Phi-treated seedlings also had a higher Pi concentration than the control seedlings grown on the same Pi concentration in the absence of Phi (Fig. 1). Therefore, even at these higher concentrations, Phi had very little impact on suppressing lateral root formation, and instead had rather an indirect effect leading to elevated Pi in the root tissue, which subsequently resulted in a decreased LRD when compared with the corresponding control plants.

As a first step to analyse the natural genetic variation of *Arabidopsis* accessions in the response to Phi, the LRD of another five accessions in addition to the Col-0 accession used in our other experiments was analysed. All of these accessions showed an increase in LRD on 0.1 mM Pi when compared with growth on 0.5 mM Pi (Fig. 4). Interestingly, we observed an even higher LRD for plants grown on 0.1 mM Pi+0.4 mM Phi, especially for the accessions Col-4, Jea, Sha and Tsu-0 with a 1.5- to twofold increase in LRD, and a less pronounced increase for Col-0 and Nd-0. Thus, Phi did not suppress lateral roots in a similar fashion to Pi but instead led to an accentuated increase in LRD.

Gene expression analyses

As Phi was not able to suppress an increase in LRD under Pi limitation, a known PSR, the effects of Phi treatment on the expression of genes involved in the PSR and others associated with Pi metabolism were examined next. For these experiments, shoots from seedlings exposed to 0.1 mM Pi+0.4 mM Phi and 0.05 mM Pi were compared. These two shoot sets had comparable Pi concentrations, which were lower than those of seedlings supplied with 0.5 mM Pi (Fig. 1). The shoots of these Phi-treated seedlings also accumulated similar concentrations of both Phi and Pi. The transcript levels for a set of genes involved in the primary Pi regulatory network (*At4*, *miRNA399d*, *PHR1*, *PHO1*, and *PHO2*) and downstream genes involved in the adaptation to low-Pi levels (*PHT1;1*, *PHT1;4*, *PAP1*, *NMT3*, *SQD2*, and *ASK11*) were quantified. As expected, transcripts for the well-documented Pi-starvation-induced genes *At4* and *miRNA399d* showed strong induction (~60-fold) under Pi limitation (Fig. 5). Transcripts of *PHT1;1*, *PHT1;4*, *SQD2*, *PAP1* and *PHO1* showed a milder induction (between 2.5- and eightfold), whilst *NMT3* expression was repressed (approx. twofold). These results confirmed that these seedlings were sensing and responding to the reduced availability and tissue accumulation of Pi (Bari *et al.*, 2006; Chiou *et al.*, 2006). Surprisingly, *At4*, *miRNA399d*, *PHO1* and *PHT1;4* transcripts had a very similar response in the Phi-treated seedlings (Fig. 5). The expression of these genes was not affected by the substantial accumulation of Phi in the leaf tissue. Thus, under these growth conditions, seedlings were still able to sense and respond to the depletion of Pi and this was not suppressed by Phi. However, Phi suppressed the induction and repression of genes important for the remodelling of membrane lipid composition, *SQD2* and *NMT3*, respectively. By contrast, the observed induction in gene expression of *PHT1;1*, *PAP1* and *ASK11* under Pi limitation was accentuated in the presence of Phi. These results demonstrated a multifaceted impact of Phi on gene regulation beyond the direct mimicking of Pi in sensing mechanisms.

Metabolic changes induced by phosphite treatment

Current evidence suggests that plants do not assimilate Phi into organic compounds and that oxidation of Phi to Pi as occurs in microbes is unlikely (Carswell *et al.*, 1997;

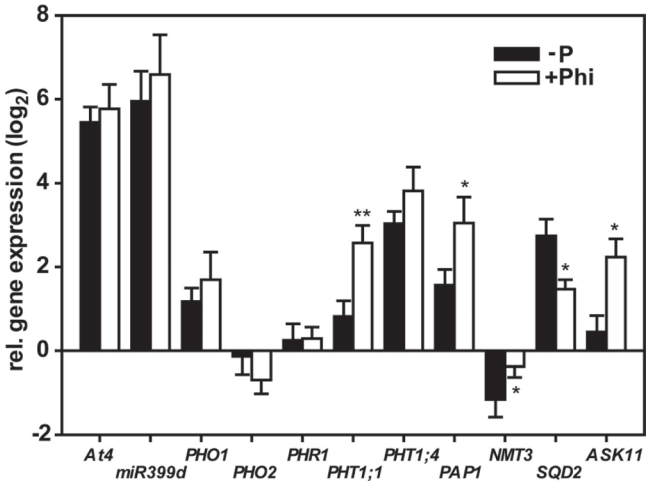

Fig. 5. Gene expression analysis for leaf tissue of 10-d-old *Arabidopsis* (Col-0) seedlings. Shown are quantitative RT-PCR results for Pi-deficient plants grown on 0.05 mM Pi (–P) and 0.1 mM Pi+0.4 mM Phi (+Phi). For both treatments, expression levels are given relative to control plants with a sufficient Pi supply (0.5 mM Pi) and normalized to the reference genes *PDF2*, *UBC9* and *UPL7*. Results are given as means ±SE (n=5). Statistically significant differences between the –P and +Phi treatments are indicated (ANOVA Tukey, $^{*}P < 0.05$; $^{**}P < 0.01$).

Danova-Alt *et al*., 2008; Berkowitz *et al*., 2011). However, this does not rule out the possibility that Phi affects metabolism by mimicking Pi, for example by competing for catalytic sites or in the allosteric regulation of enzymatic reactions.

An untargeted approach to measure metabolite levels in Phi-treated and untreated control plants by GC-MS was carried out to probe for Phi-induced metabolomic changes. To differentiate between Pi-dependent and Phi-specific differences in metabolite pools, control plants were supplied with either high (0.5 mM) or low (0.05 mM) Pi in the growth medium. Accordingly, these growth conditions resulted in high and low Pi concentrations, respectively, in the harvested shoot tissues after 3 weeks of growth (Fig. 6A). In parallel, and on the basis of earlier experiments, the Phi treatment was exposure of plants to 0.5 mM Pi+2.5 mM Phi in the growth medium. This treatment yielded plants that had accumulated shoot Pi to concentrations that were intermediate compared with those obtained in the shoots of the control plants exposed to either high or low Pi supply. The Phi concentrations at the time of harvest were below the threshold where Phi indirectly affects shoot growth. The fresh weight of the Phi-treated plants was reduced by only 13% compared with the high-Pi-treated plants, whereas the fresh weight of plants supplied with low Pi was reduced by 36% (Fig. 6B). The plants exposed to low Pi, however, had not accumulated anthocyanins (data not shown) and were therefore not severely P limited. This conclusion was supported by the still substantial Pi concentration in the shoots. Therefore, after being exposed to either Phi or low Pi, these plants were not severely P limited, and any differences in metabolite abundance were expected to represent metabolic adjustments rather than a stress response brought on by severe Pi deficiency.

In total, we were able to robustly quantify 112 polar metabolites by GC-MS. Of these, 84 metabolites could be identified through peak comparison with mass spectra libraries. For quantitative comparison of the metabolites present in the shoots of plants exposed to the low-Pi and Phi treatments, we normalized the data to the metabolite levels obtained from shoots of plants supplied with high (0.5 mM) Pi. A total of 28 metabolites showed a significant difference (ANOVA Tukey, $P < 0.05$) in abundance of more than 20% in at least one comparison with the control (Fig. 7).

The most prominent group of primary metabolites for which differences across treatments were observed were the amino acids asparagine, glutamic acid, aspartic acid, and serine, which showed contrasting changes in the two treatments. The abundance of each of these amino acids was 40–60% lower in the shoots of the Phi-treated plants, but slightly higher (10–20%) in the shoots of plants exposed to low Pi when compared with the control plants.

The largest differences in metabolite abundance in the shoots of plants exposed to low Pi were found for the organic acids citrate and threonate, which were about twofold more abundant than in control tissues. For the Phi treatments, both

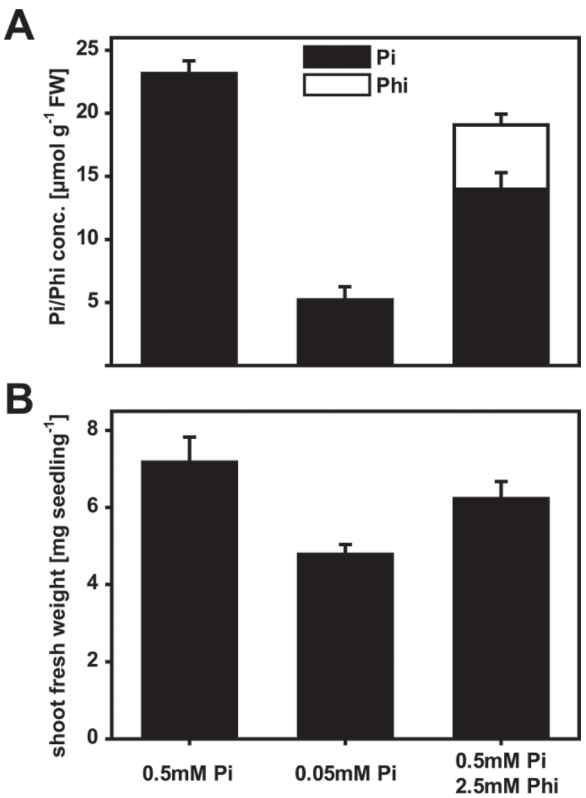

Fig. 6. Metabolomic profiling. *Arabidopsis* plants (Col-0) were grown for 3 weeks on three different growth media to allow comparison of Phi-treated (0.5 mM Pi+2.5 mM Phi) and Pi-limited (0.05 mM Pi) plants with Pi-sufficient (0.5 mM Pi) control plants. (A) Shoot Pi and Phi concentrations. (B) Shoot fresh weights. Results are given as means ±SE from eight independent biological replicates.

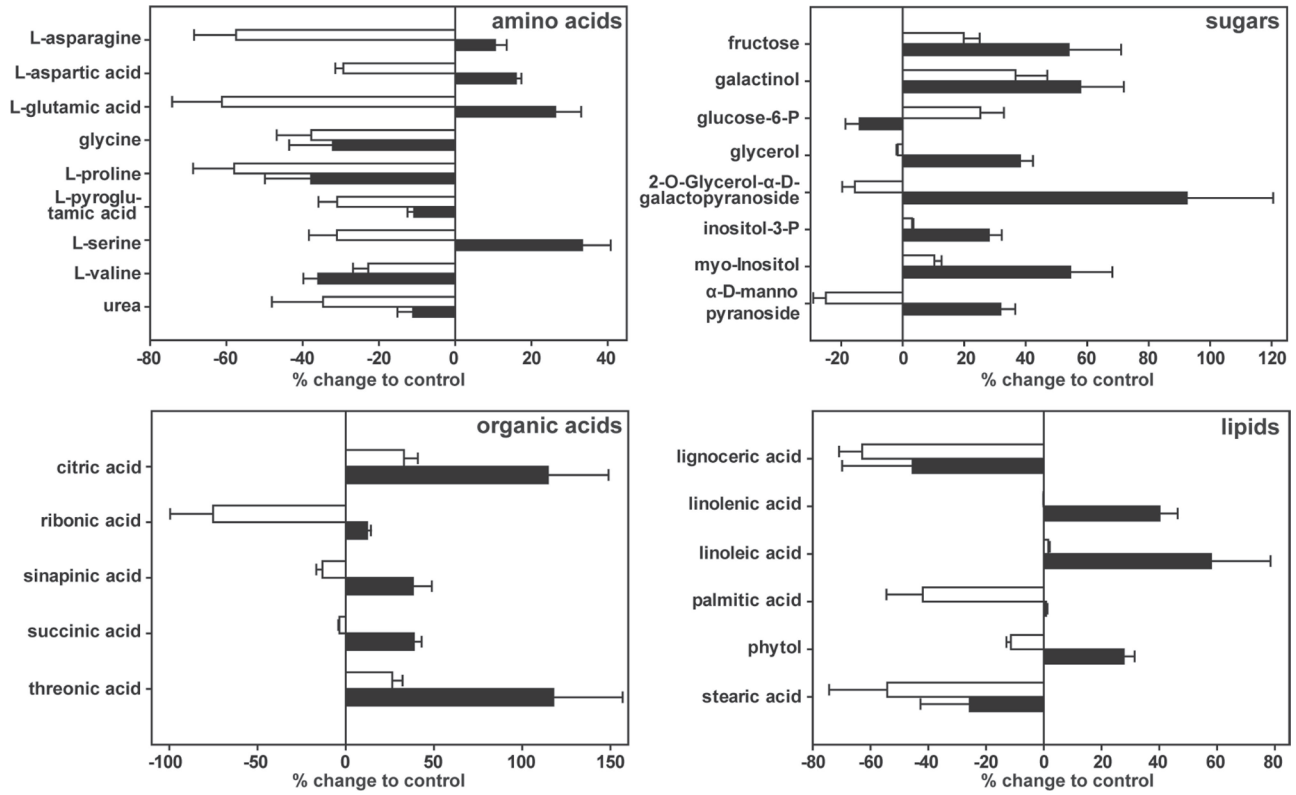

Fig. 7. Effect of Phi accumulation and Pi limitation on levels of polar metabolites in 3-week-old *Arabidopsis* (Col-0) shoots. Changes in metabolite pools of Phi-treated (0.5 mM Pi+2.5 mM Phi, open bars) and Pi-limited (0.05 mM Pi, filled bars) plants are given in as percentages of the results for the Pi-sufficient (0.5 mM Pi) control plants. Shown are only metabolites identified by GC-MS analysis as having a greater than 20% change for at least one comparison are shown (ANOVA Tukey, P <0.05). Results are given as means ±SE of eight independent biological replicates per treatment.

metabolites were about 25% more abundant. Organic acids such as citrate have frequently been found to be elevated under P deficiency, probably to increase pools to allow for excretion and improve Pi uptake from the soil (Koyama *et al.*, 2000; Narang *et al.*, 2000). An increase in *myo*-inositol and inositol-3-phosphate in the low-Pi samples and to a lesser degree for Phi treatments might indicate the mobilization of Pi from, or a reduced flux into, storage pools. Both these metabolites are intermediates in the biosynthesis and degradation of the phosphate-storage compound phytate (inositol hexakisphosphate) (Raboy, 2003). Similarly, replacement of phospholipids by galacto- and sulfolipids is an important means of releasing Pi from membrane lipids and remodelling plant lipid composition by providing precursors for lipid biosynthesis (Essigmann *et al.*, 1998; Hartel *et al.*, 2000; Li *et al.*, 2006). Correspondingly, we were able to observe changes for the major fatty acids linolenic and linoleic acid. For both, levels decreased in the low-Pi but not in the Phi-treated samples. Palmitic acid on the other hand shows a decreased abundance in the Phi-treated shoots only, whilst stearic acid levels dropped in both treatments. A slight decrease in glucose-6-phosphate in the Pi-limited plants and an increase in the Phi treatments was also detected. Several metabolomic studies have reported decreases in levels of phosphorylated sugars under P deficiency (Rychter and Randall, 1994; Morcuende *et al.*, 2007; Huang *et al.*, 2008).

Overall, 11 and one metabolite(s) were specifically more abundant in the low-Pi and Phi treatments, respectively, whilst four metabolites increased in abundance in both treatments (Fig. S2A at *JXB* online). For the Phi-treated plants, nine metabolites, most notably the central amino acids asparagine, aspartic acid, serine, and glutamic acid and its degradation product pyroglutamic acid were less abundant and six metabolites differed in their abundance between both treatments (Fig. S2A).

Discussion

Impact of phosphite accumulation on plant growth

Understanding the consequences of Phi accumulation in plants is complicated by the different molecular processes that Phi may impact on and their possible interactions. Several studies have reported an inhibitory effect of Phi on the growth of plants (Carswell *et al.*, 1996, 1997; Ticconi *et al.*, 2001; Varadarajan *et al.*, 2002; Thao *et al.*, 2008*a*, *b*). However, knowledge on the cause of the inhibitory effect of Phi on plant growth is limited, and detailed studies on the interaction of Pi and Phi on root architecture are not available. Phi competes with Pi for uptake by the same membrane transport system and should therefore, at least at high external concentrations, reduce the amount of Pi entering the cells

and lead to Pi depletion of the plant (Carswell *et al*., 1996; Danova-Alt *et al*., 2008). In addition, Phi influences subcellular compartmentation of Pi (Danova-Alt *et al*., 2008; Pratt *et al*., 2009) and both processes will impinge on Pi homeostasis with downstream effects on Pi-dependent metabolism. At the same time, the pseudo-pyramidal Phi closely resembles tetrahedral Pi in its geometry, which suggests a potential for Phi to mimic Pi and impair Pi-sensing mechanisms or Pi-dependent enzyme reactions and their allosteric regulation. In this study, a constant combined tissue concentration of Phi+Pi in Phi-treated plants across a wide range of external concentration ratios was observed, suggesting a mechanism integrating Pi and Phi concentrations and regulating the homeostasis in both shoot and root tissues. Intriguingly, a higher Pi concentration in roots of seedlings grown in the presence of Phi was detected when compared with those grown on the same medium lacking Phi. A correspondingly higher shoot Pi concentration with Phi treatment was apparent only for the highest Phi supply. As plants are unable to oxidize Phi to Pi and the shoot accumulated Phi to a higher extent than the roots, this increased root Pi concentration is probably caused by an increased uptake of Pi from the medium and/or retention in the root. As the expression of *miRNA399d* was not suppressed by Phi, the uptake of Pi by high-affinity transporters might not be downregulated under sustained Phi treatments. Inhibition of Pi assimilation into the organic P pool as a consequence of Phi accumulation could also lead to increased Pi concentrations in the roots. This could occur either through forcing more Pi into the vacuolar storage pool, thereby decreasing Pi availability in the cytosol and organelles, or by directly inhibiting metabolic processes. These results highlight the necessity to determine Phi and Pi concentrations in the tissues to be able to differentiate between direct effects of Phi, for example on the PSR or metabolism, and indirect effects on Pi homeostasis.

Suppression of the PSR by phosphite

The perception of Phi by the plant has the potential to suppress the PSR. In support of this hypothesis, several studies have shown the capacity of Phi to suppress the PSR in severely Pi-starved plants when transferred to medium containing high concentrations of Phi, for example suppression of Pi-transporter genes and the inhibition of root-hair growth or anthocyanin accumulation (Carswell *et al*., 1996; Ticconi *et al*., 2001; Varadarajan *et al*., 2002). Another characteristic developmental pattern influenced by Pi availability is the alteration of root architecture as part of the PSR. Plants respond to reduced Pi supply by slowing primary root growth and increasing LRD, which is regulated locally by the external Pi concentration (Thibaud *et al*., 2010). Surprisingly, under acclimation to Phi, seedlings showed an increase in LRD comparable to Pi-deficient plants in this study. This was the case even though the combined concentrations of both anions in the external medium were constant and overall tissue concentrations were also the same, i.e. there were no changes in external or internal [Pi+Phi] across all treatments. These results suggest that plants are able to differentiate between the two anions at least to some extent and sense the depletion of Pi even in the presence of Phi. This was not only the case for the accession Col-0 but also for several other *Arabidopsis* accessions we tested for changes in LRD, which responded even more strongly. Similar studies have also shown a varying sensitivity of *Arabidopsis* accessions to Pi deficiency (Chevalier *et al*., 2003; Reymond *et al*., 2006).

In addition, our gene expression studies also indicated that plants accumulating Phi can still sense Pi deficiency. Pi-starvation-induced (PSI) genes of the primary regulatory network (*At4*, *miRNA399d*, *PHO1*, and *PHT1;4*) remained upregulated, although Phi accumulation resulted in a combined Pi+Phi tissue concentration that was comparable to plants on a high Pi supply. By contrast, several other studies have reported repression of several PSI genes (e.g. *At4*, *PAP1*, *PHT1;1* and *IPS1*) by Phi treatment in severely Pi-starved plants (Ticconi *et al*., 2001; Varadarajan *et al*., 2002; Ribot *et al*., 2008), but Ribot *et al*. (2008) also found no suppression of *PHO1* by Phi. A possible explanation for these differences in response might be a distinct subcellular localization of Phi and/or Pi under different experimental conditions. In Pi-sufficient plants, Phi preferentially accumulates in the vacuole, whereas in Pi-starved plants, Phi accumulates in the cytoplasm (Danova-Alt *et al*., 2008). Such differential compartmentation might explain differences in the ability of Phi to interfere with Pi-sensing mechanisms and subsequently the regulation of gene expression. In our experiments, the induction and repression of *SQD2* and *NMT3*, respectively, was attenuated by Phi, whilst the induction of *PHT1;1*, *PAP1* and *ASK11* was accentuated. At the same time, the induction of *miRNA399d*, *At4* and *PHT1;4* was not affected by Phi. These results indicate alternative sensing mechanisms of the Pi (and Phi) status within the plant. A possible mechanism allowing the plant to differentiate between Pi and Phi could be the additional sensing of a P-containing or a derived component as a measure of flux of free Pi into the organic P pool. Members of the *NMT* gene family have already been implicated in a stress signalling pathway involving the phospholipid-derived second messenger phosphatidic acid (Jost *et al*., 2009). Such regulation would be reminiscent of other two major macronutrients nitrate and sulfate, for which downstream assimilation products, i.e. glutamate/glutamine and glutathione, respectively, are part of the nutrient-sensing network (Lappartient *et al*., 1999; Gutierrez *et al*., 2008).

Metabolomic response to phosphite accumulation

Several enzymes, such as glyceraldyde-3-phosphate dehydrogenase and phosphorylases such as starch phosphorylase, use Pi as a substrate and many enzymes of primary metabolism are regulated by Pi, such as phosphofructokinase (PFK), sucrose phosphate synthase, ADP-glucose pyrophosphorylase, and glutathione synthetase (Sowokinos and Preiss, 1982; Doehlert and Huber, 1984; Hausler *et al*., 1989; Jez and Cahoon, 2004). Because of its steric similarity to Pi, Phi probably mimics and competes with Pi for binding in catalytic or regulatory sites of enzymes, causing competitive or allosteric inhibition. Indeed, for ATP-dependent PFK, but not PPi-dependent

PFK, of *Brassica nigra*, Phi inhibits *in vitro* enzyme activity (Carswell *et al.*, 1996). Similarly, several glycolytic enzymes from *Phytophthora palmivora* and yeast are also inhibited by Phi (Stehmann and Grant, 2000), indicating that the regulation of enzyme activity by Phi may be of wider significance.

Metabolomic analyses identified alterations in a number of metabolite pools. These results showed the ability of Phi to induce specific shifts, which for some metabolites contrasted with those of Pi-limited plants. We found that Phi specifically decreased levels of the central amino acids asparagine, aspartate, glutamate, and serine (Figs 7 and S2). Alterations in amino acid levels have also been observed for severely P-limited *Arabidopsis* and barley plants (Morcuende *et al.*, 2007; Huang *et al.*, 2008).

Although these results do not allow the underlying specific targets of Phi causing these changes to be identified, they reveal that even moderate accumulation of Phi influences plant metabolic processes. This may provide clues to increase our understanding of the mechanisms of Phi-induced resistance of plants to oomycete pathogens such as *Phytophthora* and *Pythium* species (Guest and Grant, 1991). The molecular mode of action of Phi in inducing resistance has not yet been identified, but studies in *Arabidopsis* have implicated salicylic acid and its major signalling component NPR1, as well as a MAP kinase regulatory network (Molina *et al.*, 1998; Friedrich *et al.*, 2001; Eshraghi *et al.*, 2011; Massoud *et al.*, 2012). Several recent studies have identified a role of primary metabolites, especially amino acids, in the establishment of resistance against plant pathogens (Song *et al.*, 2004; Chanda *et al.*, 2008; van Damme *et al.*, 2009; Liu *et al.*, 2010; Park *et al.*, 2010; Brauc *et al.*, 2011, 2012; Chanda *et al.*, 2011; Hwang *et al.*, 2011; Stuttmann *et al.*, 2011; Voll *et al.*, 2012). These studies show that the alteration of specific metabolite levels by feeding or genetic manipulation of plant metabolic pathways can lead to resistance against pathogens. Interestingly, alterations in the abundance of amino acids derived from the aspartate pathway have been identified to be important for resistance against the oomycete pathogen *H. arabidopsidis* and the bacterial pathogen *Pseudomonas syringae* (van Damme *et al.*, 2009; Stuttmann *et al.*, 2011; Navarova *et al.*, 2012). As we found Phi-induced changes in levels of aspartate and the related amino acids asparagine and glutamate (Fig. S2B), we surmise that these are similarly important for Phi-induced resistance against oomycete pathogens. Currently, it is not clear how these metabolic changes induce resistance. It has been suggested that, due to the evolutionary relationship between plants and oomycetes, metabolic pathways are conserved and metabolic perturbations in the host decrease the colonization ability of the pathogen (Stuttmann *et al.*, 2011). These results indicate the possibility for a mode of Phi action by inducing alterations in primary metabolite pools leading to pathogen resistance.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Shoot:root ratios of plant under +Phi and –Pi treatments as in shown in Fig. 1.

Supplementary Fig. S2. (A) Representation of metabolites with overlapping and contrasting changes in Phi-treated and Pi-limited *Arabidopsis* plants. (B) Simplified metabolic pathways showing interconnections of metabolites with altered abundance (in bold) under Phi treatment or Pi limitation.

Supplementary Table S1. Sequences of the primers used in gene expression experiments.

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