RUNNING HEAD: Chloroplast polynucleotide phosphorylase and P starvation

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Abnormal physiological and molecular mutant phenotypes link chloroplast polynucleotide phosphorylase to the phosphorus deprivation response in Arabidopsis

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**ABSTRACT**

A prominent enzyme in organellar RNA metabolism is the exoribonuclease polynucleotide phosphorylase (PNPase), whose reversible activity is governed by the NDP/inorganic phosphate ratio. In *Chlamydomonas reinhardtii*, PNPase regulates chloroplast transcript accumulation in response to phosphorus (P) starvation, and PNPase expression is repressed by the response regulator PSR1 under these conditions. Here, we investigated the role of PNPase in the *Arabidopsis thaliana* P deprivation response, by comparing WT and *pnp* mutant plants with respect to their morphology, metabolite profiles, and transcriptomes. We found that when P-deprived, *pnp* mutants develop aborted clusters of lateral roots, which are characterized by decreased auxin responsiveness and cell division, and exhibit cell death at the root tips. Electron microscopy revealed that the collapse of root organelles is enhanced in the *pnp* mutant under P deprivation, and occurred with low frequency under P-replete conditions. Global analyses of metabolites and transcripts were carried out to understand the molecular bases of these altered P deprivation responses. We found that the *pnp* mutant expresses some elements of the deprivation response even when grown on a full nutrient medium, including altered transcript accumulation, although its total and inorganic P contents are not reduced. The *pnp* mutation also confers P status-independent responses, including but not limited to stress responses. Taken together, our data support the hypothesis that the activity of the chloroplast PNPase is involved in plant acclimation to P availability, and that it may help maintain an appropriate balance of P metabolites even under normal growth conditions.
INTRODUCTION
Organisms require phosphorus (P) continually and in relatively high amounts, and in photosynthetic systems a major use is the regeneration of ribulose-1,6-bisphosphate, the acceptor for CO₂ fixation by Rubisco. Chloroplast inorganic phosphate (Pi) pools are also affected by starch biosynthesis, since the conversion of glucose-1-phosphate to ADP-glucose, the penultimate step in the pathway, releases Pi through ATP hydrolysis. Starch is primarily synthesized during the day from excess triose phosphates and broken down at night, a step which consumes Pi through the action of starch phosphorylase and other enzymes (Zeeman et al., 2007). Thus, P is a major player in chloroplast metabolism and is intimately integrated into the carbon budget of plant cells.

Chloroplast P is also required for processes not directly related to photosynthesis, such as gene expression. In particular, the chloroplast ribonuclease polynucleotide phosphorylase (PNPase) both consumes and liberates P. PNPase in bacteria (Soreq and Littauer, 1977) and chloroplasts (Baginsky et al., 2001) is a homotrimer that degrades RNA through phosphorylytic attack, but also readily synthesizes polynucleotides using NDP or NTP precursors, a reaction which generates Pi or PPi, respectively. The purified chloroplast enzyme, like its bacterial counterpart, is readily reversible in vitro (Yehudai-Resheff et al., 2001). Several lines of work have clearly placed PNPase, in both prokaryotes and eukaryotic organelles (reviewed in Slomovic et al., 2006), as a key player in a polyadenylation-stimulated RNA degradation pathway found in plant mitochondria and chloroplasts.

While the role of PNPase in RNA metabolism is established if incompletely understood, newer evidence has linked PNPase to somewhat unrelated functions. In human cells, for example, a cytosolic fraction of mitochondrial PNPase appears to influence cell differentiation and senescence (Sarkar and Fisher, 2006) and in the mitochondrion itself, PNPase is located in the intermembrane space while mitochondrial RNA (mtRNA) is in the matrix, suggesting a metabolic rather than an RNA catalytic role for PNPase (Chen et al., 2006), through which it can nonetheless influence the accumulation of certain mtRNAs (Slomovic and Schuster, 2008). More closely related to the current study, a genetic screen carried out in Arabidopsis for resistance to fosmidomycin, which inhibits the plastid methylerythritol phosphate pathway, identified a cpPNPase null mutant which was named rif10 (Sauret-Gueto et al., 2006). This led to the suggestion that the MEP pathway might be regulated by plastid metabolic cues, which in turn could be influenced by PNPase.

The current study was stimulated by our finding that in the green alga Chlamydomonas reinhardtii, reduced expression of cpPNPase rendered cells unable to acclimate to P deprivation, whereas the same strains had WT responses to other nutrient or environmental stresses (Yehudai-Resheff et al., 2007). We also found that both the PNPase transcript and protein were repressed under P deprivation, suggesting that reduced PNPase activity is part of the overall metabolic adjustment to P limitation. Furthermore, repression of PNPase expression required the general P deprivation response regulator Psr1, a likely transcription factor (Wykoff et al., 1999), demonstrating that PNPase regulation is integrated into the global P limitation response. The orthologue of Psr1 in Arabidopsis is PHR1 (Rubio et al., 2001), among whose functions is inducing miRNAs of the miR399 family, which through long-distance signaling (Pant et al., 2008) derepress a suite of genes including some encoding P transporters, through a ubiquitination pathway involving PHO2 (Aung et al., 2006; Bari et al., 2006; Chiou et al., 2006). This pathway in turn is modulated by the noncoding RNA IPS1 (Franco-Zorrilla et al., 2007), and possibly others.
Here, we examine the role of chloroplast PNPase in Arabidopsis in the context of the P deprivation response, using null mutant alleles. The most obvious growth defect under P limitation is in the elaboration of lateral roots, where the \textit{pnp} mutant phenocopies \textit{pdr2}, a mutant thought to define a signal needed for lateral root proliferation (Ticconi et al., 2004). When gene expression was examined using array technology, we found that the \textit{pnp} mutants already induce, under normal growth conditions, genes normally only induced upon P deprivation. These and other data suggest that \textit{pnp} mutants exhibit elements of P starvation even when grown under P replete conditions. We therefore propose that chloroplast PNPase in plants, as in unicellular algae, has a key role in P metabolism.

\textbf{RESULTS}

\textbf{PNP T-DNA insertion mutants}

Chloroplast PNPase is encoded by the locus At3g03710, which specifies a 922 residue protein, consistent with the \textasciitilde 100 kDa migration of PNPase previously described from spinach and pea chloroplasts (Hayes et al., 1996; Li et al., 1998). Limited data have been obtained on Arabidopsis lines reduced or totally deficient for cpPNPase (Walter et al., 2002; Sauret-Gueto et al., 2006). To pursue the analysis of Arabidopsis cpPNPase in the context of the nutrient stress response, we selected two T-DNA insertion alleles, naming them \textit{pnp1-1} and \textit{pnp1-2} (Figure 1A). Homozygous mutants for each line were isolated following three outcrosses of the original T3 plants. UV-crosslinking was performed to confirm that the \textit{pnp1-1} mutant lacked cpPNPase,
shown in Figure 1B as a ~100 kDa RNA-binding activity. The mutants also lack complete PNP transcripts, as revealed by RT-PCR using primers flanking the respective T-DNA insertions (data not shown).

Figure 1C compares WT and pnp mutant plants after 21 days of growth on soil. The rosette leaves of pnp mutants emerged and remained pale green compared to those of WT plants, as was observed previously (Sauret-Gueto et al., 2006). In addition, the mutant plants were retarded in silique production, and both silique number and seed production were reduced (data not shown). Thus cpPNPase is required for fully normal plant development, although in its absence a full life cycle is achieved. Another feature of cpPNPase deficiency is incomplete 3' end processing of certain chloroplast transcripts including 23S rRNA and various mRNAs. These are exemplified in Figure 1D, and in the cases of 23S rRNA, rbcL and psbA, the presence of 3' extensions had been previously confirmed (Walter et al., 2002).

**Lateral root development under P limitation**

It is well established that when faced with nutrient limitation, plants seek additional sources through altered root system architecture. In the case of P limitation, both root hair density and lateral root proliferation are observed (reviewed in Lopez-Bucio et al., 2003). To see whether PNPase deficiency affected this process, we compared the root architecture of WT and mutant plants grown under +P and -P conditions. As exemplified in Figure 2, there were no substantive differences observed when several genotypes were compared under +P conditions, although the pnp mutants had a slower overall root elongation rate (not shown). We used two other mutants as controls. The first was csp41b, a nuclear mutant lacking two related chloroplast endoribonucleases (Bollenbach et al., 2009). We reasoned that if abnormal chloroplast RNA (cpRNA) metabolism had any pleiotropic effect on root architecture, that would be seen for both pnp1-1 and csp41b. A second control was pdr2, a mutant which is known to exhibit abortive lateral root initiation under -P conditions (Ticconi et al., 2004). This control was useful because as we discovered (Figure 2, lower row), the pnp1-1 mutants phenocopied pdr2. In particular, when grown under -P conditions, the pnp mutants were unable to elaborate lateral roots, instead exhibiting a proteoid root-like phenotype after four weeks of P starvation. While the same effect is observed earlier for pdr2, it should be borne in mind that pnp mutants have an overall slower growth rate (e.g. Figure 1).

![Figure 2](https://example.com)
Because the *csp41b* mutant was indistinguishable from the WT under -P conditions, we concluded that the aberrant root architecture phenotype of *pnp1-1/pnp1-2* was not related to altered cpRNA metabolism *per se*. Instead, we suspected that some function of PNPase was essential to lateral root elaboration. To investigate this phenomenon in more detail, we crossed the *pnp1-1* mutation into two backgrounds expressing GUS reporter genes, either under control of the auxin-responsive promoter elements DR5 (Ulmasov et al., 1997), or the cyclin B1 promoter (Colón-Carmona et al., 1999) as an indicator of cell division. Both reporters have previously been used in studies of root physiology under P limitation conditions (Ticconi et al., 2004; Nacry et al., 2005; Sanchez-Calderon et al., 2005).

Figures 3A and 3B show results for plants expressing DR5:GUS and CYCB1:GUS, respectively. When grown on +P medium, *pnp1-1* exhibited considerably less staining in the primary root tip than did the WT, which is consistent with its slower overall root elongation rate. When grown on -P medium, the WT exhibited reduced staining at the root tip, a result consistent with another study showing an age-dependent decrease DR5:GUS expression following P starvation as compared to seedlings grown on +P medium (Sanchez-Calderon et al., 2005). In the case of

**Figure 3.** Cell division and mortality in lateral roots developing under P deprivation.

A. Roots from plants expressing a DR5:GUS transgene were stained histochemically for GUS. Arrows point to smaller stained areas. Plants were germinated and grown for two weeks on +P medium, then transferred to +P or -P media for four weeks.

B. Roots from plants expressing a CYCB1:GUS transgene were stained histochemically, following growth as for panel A.

C. Plants were grown as for panel A. Roots were stained with Evans blue. The arrows indicate *pnp* mutant root tips which are not stained.
PNPase is a plastid protein, we examined organellar ultrastructure in roots under +P and -P conditions, as exemplified in Figure 4. Under +P conditions, we observed an increased frequency of plastoglobules in pnp1-1, as well as apparently ruptured mitochondria. Plastoglobules are associated with senescence and stress conditions, and may be a pleiotropic

**Figure 4.** Ultrastructure of WT and pnp1-1 mutant lateral root cortical cells. Plants were germinated and grown for two weeks on +P medium, then transferred to +P or -P media. Plants were observed after 1, 2, 3, and 4 weeks on -P. Here, only images of root organelles after one week on -P are shown. Cortical cells of root tip sections in the longitudinal direction were observed using transmission electron microscopy as described in Materials and Methods. Bars represent 200 nm, except for pnp1-1 on -P (plastids and mitochondria), and for the WT plastid picture on -P, where bars correspond to 500 nm. Arrows indicate features referred to in the main text.
consequence of the *pnp* mutation. The frequency of plastoglobules increased more dramatically in *pnp1-1* than in WT under -P conditions, also consistent with an enhanced stress response. In 60% of plastids in the mutant, they were arranged in a circle, as shown in Figure 4. We also noted increased mitochondrial disruption in the mutant, and occasionally in the WT, under -P conditions. Mitochondrial rupture, as measured by cytochrome c release, has been associated with cell death in male-sterile sunflower anthers (Balk and Leaver, 2001), and subsequently studied in other contexts where programmed cell death occurs in plants (Reape et al., 2008). Thus, it is possible that the mitochondrial disruption observed in *pnp1-1* under -P conditions is symptomatic of the loss of cell integrity ultimately observed (Figure 3C).

The *PNP* gene is expressed in both roots and leaves (http://mpss.udel.edu/at/ and our unpublished RT-PCR data), albeit at a much lower level in roots, raising the question of whether *pnp* mutants display an altered root architecture under -P conditions because of PNPase deficiency in roots, or in leaves, or both. To address this question, we carried out the experiments shown in Figure 5. We first excised roots from *pnp1-1* plants already exposed to -P conditions, to observe whether providing them with P would correct the root architecture deficiency. Figure 5A shows that aberrant roots did not regain normal proliferation when placed on +P medium, although some growth occurred, suggesting that the P level encountered by the roots did not alone determine their phenotype. In contrast, when root masses from *pnp1-1* plants starved for P were divided and half placed on +P medium and the other half on -P medium, normal root elongation ensued in both samples (Figure 5B). Thus, although half the *pnp* roots were in -P medium, P provided from the other roots and cycled through the

![Figure 5](http://www.plantphysiol.org)
aboveground tissues, rescued the mutant phenotype. We conclude that altered root architecture in pnp1-1 is not a cell-autonomous feature of root cells.

**Metabolite comparison of pnp1-1 and WT**

Based on the data described above, we hypothesized that PNPase deficiency either directly or indirectly affected production or transduction of a signal required for normal response to P deficiency. We first measured free or total P in WT or pnp mutant leaves and roots grown under +P and -P conditions. As shown in Figure 6, both free and total leaf P declined in both WT and mutant plants grown under -P conditions relative to +P, as was expected. For pnp1-1, we found that P levels were slightly higher in leaves, and significantly higher in roots, than those of the WT, when the plants were grown under +P conditions. On the other hand, P levels in pnp1-1 plants grown under -P conditions did not differ from those found in the WT. We conclude that the pnp1-1 mutation does not have a major effect on P content as related to fresh weight, although both leaf and root P are slightly elevated under +P conditions. We also measured P uptake in WT and mutant roots, in case differences in P uptake were masked in the P accumulation data. However, no significant differences were observed (Supplemental Figure S3).

![Figure 6](image_url)

**Figure 6.** Phosphate content in leaves and roots. P content was measured in WT and pnp1-1 mutant seedlings. Plants were germinated and grown for two weeks on +P media, then transferred to -P or +P media for an additional week. Free inorganic phosphate and total phosphate were assessed as described in Materials and Methods. Bars correspond to standard errors.

For a broader view of metabolic status, we quantified a panel of soluble, primary metabolites (Roessner et al., 2001) from WT and mutant plants grown under +P conditions, or starved between three hr and three weeks (see Supplemental Tables S1 and S2, and Supplemental Figures S1 and S2). Under +P conditions, the metabolic differences between mutant and WT were limited (Figure 7 and Supplemental Table S1). Nonetheless the sugars trehalose and isomaltose accumulated in the mutant to levels greater than four-fold higher than in the WT, whereas saccharate, gentobiose and fructose, were present at significantly lower levels in the
mutant. The mutant was additionally characterized by higher levels of O-A-Ser, H-Ser and Ala. The levels of succinate, malate and fumarate were considerably lower in the mutant than in the WT, whereas dehydroascorbate (DHA) accumulated (although it is important to note that the DHA level measured here is not absolutely representative of the in vivo amount, since the extraction conditions used here do not exactly conserve the cellular redox poise of the ascorbate pools).

When looking from a global level, the impact of P starvation was fairly similar in both genotypes. The WT displayed metabolic responses resembling those of many previous studies (Pieters et al., 2001; Uhde-Stone et al., 2003; Liu et al., 2005; Misson et al., 2005; Hernandez et al., 2007; Karthikeyan et al., 2007; Muller et al., 2007), whilst the pnp mutant generally also displayed many of these characteristic responses. Among observed differences was a far less dramatic change in the pnp mutant for the levels of fructose 6-phosphate (Fru-6P) and glucose 6-phosphate (Glc-6P). Moreover, isomaltose, mannose, saccharate, sucrose, trehalose and xylose all increased in the WT in response to P starvation, with raffinose being the only sugar which increased in both genotypes. Organic acids displayed mixed behavior in response to P deficiency: TCA intermediates downstream of the reaction catalyzed by isocitrate lyase (one of the key enzymes of nitrate assimilation; Hodges et al., 2003) decreased in both genotypes, whilst those upstream increased. Taken together, the results suggest an altered TCA cycle activity on P stress and as such are largely in keeping with those of other recent studies (for example Morcuende et al., 2007).

With the exception of GABA and proline, amino acids tend to accumulate with increasing periods of starvation in both genotypes. GABA, a stress-related metabolite (Fait et al., 2008), increased upon P starvation in the mutant, despite decreasing in the WT, whilst the increase in the plastidial aspartate family was visible following a mere 3 hr starvation in the mutant but only after one week in the WT. Indeed, the
changes in a wide range of amino acids were exacerbated in the mutant lines. To assess this statistically, two way ANOVA tests were conducted (Supplemental Table S2); these tests revealed that the majority of the metabolites that discriminated between the genotypes were amino acids, however, also included in the top 25 discriminating metabolites were the phosphorylated intermediates, Glc-6P, glycerol 1-phosphate and Fru-6P, as expected (Morcuende et al., 2007 and references therein) and a range of sugars. These results were somewhat surprising since the pattern of changes in many of these metabolites appears conserved between the genotypes, however, this clearly reflects that the altered metabolic response of pnp1-1 is quite subtle. When studied from a functional rather than chemical perspective this list also revealed a high number of stress-related metabolites such as GABA, myo-inositol, raffinose, gentiobiose, chlorogenate, DHA and salicylate which tended to respond more dramatically in the pnp mutant than in the WT.

**Transcriptome characterization of pnp1-1**

To gain further insight into how the lack of PNPase might affect plant responses to P starvation, we used microarray hybridizations to compare the transcriptomes of pnp1-1 and WT plants grown under +P or -P conditions. To be able to relate transcriptome data to the metabolite analysis, we chose two time points for which the two genotypes were most distinct, which were after 3 hr or one week of P starvation, according to principal component analysis (Supplemental Figure S2). Total rosette RNA was used with the Affymetrix ATH1 platform, to facilitate comparison with previous studies. The threshold of two-fold change was chosen with a false discovery rate (FDR) <0.05. Supplemental Tables S3 and S4 include the full datasets of the 3 hr and one week -P experiments, respectively. Because the 3 hr experiment revealed no significant regulated genes in WT or in pnp1-1 in response to P starvation, we will only discuss the one week experiment here.

We first compared the two genotypes grown on +P medium (Table I). 960 genes were found to be differentially expressed, which is perhaps not surprising given the slow growth and partial chlorosis of pnp1-1. What was noteworthy, however, was that the number of differentially expressed genes dramatically decreased when plants were grown on -P medium, to 224, indicating that WT and pnp1-1 have a more similar gene expression patterns when P is not provided.

Subsequently, the effect of P deprivation on gene expression was assessed in the WT. We found that 508 genes were regulated; 80.5% were up-regulated whereas less than 20% were repressed. We compared our dataset to other published experiments, and found general consistency, although the experimental protocols were not identical (Misson et al., 2005; Morcuende et al., 2007). A third step of the analysis was to examine the effect of P deprivation in the mutant, and we found that the number of P-regulated genes in pnp1-1 was dramatically reduced with respect to the WT, i.e. 209 vs. 508 (Table I). This is consistent with the data mentioned above, that 960 genes differentiate the two genotypes on +P but only 224 on -P. In terms of transcript regulation, we conclude that P starvation attenuates the differences between pnp1-1 and the WT.

**Classification of differentially expressed genes**

We used MapMan to determine which functional categories were most affected in various pairwise comparisons (Supplemental Table S5). When the WT and pnp1-1 were compared on +P conditions, the categories of photosynthesis, RNA regulation, cell functions, and stress responses were all identified with a p-value of $10^{-10}$ or less, and various other metabolic functions also with low p-values ($10^{-4}$ or less). Together, these categories likely include pleiotropic effects (e.g. stress responses) but may also include metabolic functions related to a
particular role of PNPase. Under -P conditions and using a p-value of 10^-4 as a cutoff, eight categories rather than 11 (as seen under +P conditions) were identified, consistent with the speculation above that P starvation attenuates differences between WT and mutant. Of these eight categories, only two were the same as for +P conditions (photosynthesis and mitochondrial metabolism), consistent with the known large-scale reprogramming of plant gene expression when facing abiotic stress. The eight categories also included three important metabolic networks: major carbohydrate metabolism, oxidative pentose phosphate pathway, and TCA cycle/organic acid transformations. Thus, under P starvation, the npn mutation affects normal gene expression as related to several major organellar and metabolic functions.

Another form of comparison was done, where we identified the 40 most regulated genes in pnp1-1 vs. WT when grown under +P conditions (Table II), 15 of which are related to defense or stress responses. Four genes related to photosynthesis are also strongly regulated. Most remarkable among them is petD, which encodes subunit IV of the cytochrome b6/f complex and is induced ≥100-fold under both +P and -P conditions, in the mutant. However, like other chloroplast-localized genes in pnp1-1, altered mRNA processing is likely to account for some of the observed increase, and furthermore, as our cDNA was primed with oligo(dT), we would amplify polyadenylated chloroplast transcripts, which it has been reported to hyperaccumulate in PNPase-deficient Arabidopsis plants (Walter et al., 2002). In contrast, two nuclear genes encoding photosynthesis proteins are strongly repressed in pnp1-1 vs. WT; these are PSBP2 (PS II oxygen evolving enhancer) and PORA (chlorophyll biosynthesis).

P-independent PNPase-regulated genes

We conducted further analysis to differentiate pleiotropic effects on gene expression related to the slower growth, chlorosis and possible general stress responses of the pnp1-1 mutant, from those which could be directly attributed to the effect of the npn mutation on chloroplast metabolism as possibly related to P deprivation responses. To do so, we compared sets of regulated genes as shown in Figure 8.

Panel A shows the overlap between pnp1-1 vs. WT on +P or -P, revealing 149 genes which are regulated in the same direction. These can be interpreted as nutrient-independent effects of the npn mutation. Representatives of these 149 genes are listed in Table III, with major categories related to photosynthesis or chloroplast functions. Fourteen genes are chloroplast-encoded and 11 of them are very strongly induced in pnp1-1, which as discussed above likely relates to the RNA maturation function of PNPase. A second cluster of genes included in the overlap in Figure 8A is conspicuous as it mainly encodes components of the PYK10 complex, which is named after an endoplasmic reticulum body-associated β-glucosidase thought to be involved in plant
defense (Nagano et al., 2005). Like the gene encoding PYK10 itself, genes encoding eight jacalin-lectin proteins, three GDSL lipase-like proteins, TSA1-like (DNA topoisomerase), and a meprin and TRAF homology domain-containing protein, are all up-regulated in the *pnp* mutant. All of these proteins except three jacalins (JAL4, 11 and 27) were found to be part of the PYK10 complex (Nagano et al., 2008), and their induction is consistent with a stress situation in the *pnp* mutant.

**Effect of the *pnp* mutation on the P starvation response**
In order to explore the apparent attenuation of P starvation-induced transcriptional responses in the *pnp1-1* mutant, we analyzed the overlap in regulated genes between *pnp1-1* -P vs. +P and WT -P vs. +P (Figure 8B). This revealed 147 genes whose expression changes in the same direction in the two genotypes, representing about 30% and 70% of WT and *pnp1-1* P-regulated genes, respectively. If one includes data below the two-fold threshold we had chosen, and allows an FDR >0.5, 92% of the non-overlapping P responsive genes in WT are also regulated by -P in the same way in *pnp1-1*. Altogether, these data indicate WT and *pnp1-1* possess largely parallel responses to P deprivation, but the average fold change of the 139 overlapping induced genes was globally lower for the *pnp1-1* plants (respectively 20.7-fold induction for *pnp1-1* and 32.4-fold for the WT), whereas the eight overlapping repressed genes had a similar average of regulation level (0.22-fold for *pnp1-1* and 0.20-fold for the WT).

**Constitutive P starvation responses in *pnp1-1***
The attenuated transcriptional response of *pnp1-1* under P starvation raised the possibility that the plants were experiencing some degree of P stress, even when grown under nominally +P conditions, and thus had constitutive P starvation responses. We therefore compared the -P transcriptional responses in WT, to the *pnp* vs. WT effects on gene expression under +P conditions (Figure 8C). The behaviors of some of the main known P-responsive genes are detailed in Table IV. The global result that emerges is that among the 508 P-responsive genes in the WT, 43% are also regulated in the same direction in *pnp1-1* relative to WT, under +P conditions. This 43%, or 221 genes, includes 166 that are induced, and 55 repressed. Among them are several major genes normally induced during P stress, such as those encoding P transporters belonging to the PHT1 family (*PHT1;1* and *PHT1;2*, detected with the same probe; *PHT1;4* and *PHT1;7*, detected with the same probe), the ribonuclease RNS1, and the transcription factor WRKY75, a positive modulator of P starvation responses and root development (Devaiah et al., 2007). Messenger RNAs encoding at least 30 other plastid-targeted proteins are also regulated in *pnp1-1* as if it were already P-starved (Supplemental Table S6). This strengthens the conclusion that plastid P metabolism is altered in *pnp1-1*.

**Verification of transcriptome results**
We performed quantitative RT-PCR to validate a portion of the expression data described above, as shown in Figure 9, selecting both P starvation-responsive genes, and genes whose expression was independently affected by the *pnp1-1* mutation. Panel A shows various protein-coding genes. We examined *PHR1*, which encodes a key P response transcription factor. As shown previously (Rubio et al., 2001), *PHR1* expression is not regulated transcriptionally by P starvation in the WT, and we found the same for the *pnp* mutant. *RNS1*, which encodes a P-starvation-induced secreted ribonuclease (Bariola et al., 1999) showed the expected induction in the WT, however it was also constitutively (under +P conditions) expressed at three-fold the WT level in *pnp1-1*. The transcripts encoding the transporters Pht1;4 and Pht1;1 are induced in the WT, as expected (Mudge et al., 2002), and in the mutant. We also verified four genes whose expression was regulated P-independently by the *pnp1-1* genotype. We confirmed that genes encoding a Jacalin-lectin and a potentially chloroplast-targeted RNase H domain-containing
protein were induced, in agreement with microarray results (Supplemental Table S7), and that two photosynthetic genes, *PORA* and *PSBP2*, were repressed.
Figure 9. Transcript levels for selected P-starvation and pnp1-1-regulated genes as determined by quantitative RT-PCR. The fold change in expression was normalized to the actin gene ACT2, and scaled to the sample with the highest expression level for each tested gene, which was defined as 1.0. Bars represent standard error. Data correspond to three biological replicates and at least two technical replicates, as detailed in Supplemental Table S7. For selected genes, relative transcript abundance is shown above the bars.

A. Validation of microarray data for selected genes. Gene symbols are used except where there is no gene name, but the product is known. Plants were germinated and grown for two weeks on +P medium, then transferred to +P or −P media for an additional week.

B. Expression level of At4 and IPS1, two P starvation markers not represented on the ATH1 microarrays, and PDR2. Plants were grown as for Panel A.

C. Plants were germinated and grown for one week on +P medium, then transferred to +P or −P media for 10 days, to replicate previously published growth conditions for the phr1 mutant (Rubio et al., 2001).
Figure 9B shows expression analysis of two P starvation-induced riboregulators, At4 and IPS1, which are not represented on ATH1 arrays. Both were strongly induced in both genotypes. Although it is not evident because of the scale of the graph, IPS1 was slightly but significantly induced in pnp1-1 under +P conditions, approximately two-fold relative to the WT. Also, we examined expression of PDR2, since mutations in that gene phenocopy pnp1-1 in terms of lateral root abortion. PDR2 expression, however, did not differ in pnp1-1. Finally, we examined the expression of the PNP gene itself, and any dependence on PHR1, because we had previously found that in Chlamydomonas (Yehudai-Resheff et al., 2007), the PNP gene is repressed by P-starvation in a Psr1-dependent manner, and PHR1 is the orthologue of Psr1. As shown in Figure 9C, PNP transcripts decreased approximately two-fold under P starvation conditions, which is comparable to the approximately three-fold decrease observed in Chlamydomonas. This decrease appeared to be PHR1-independent, however. The character of this mutant was verified by measuring IPS1 transcripts, which as expected (Nilsson et al., 2007) failed to be induced 1. In summary, our Q-RT-PCR data supported the conclusions from the microarray approach, and yielded additional information regarding the expression of riboregulators, and the regulation of the PNP gene.

DISCUSSION

Our previous report illuminated a role for the chloroplast PNPase in P starvation acclimation in Chlamydomonas (Yehudai-Resheff et al., 2007) and stimulated the studies reported here. While we conclude that chloroplast PNPase also is important for a normal P starvation response in Arabidopsis, we used a different set of analytical tools to address issues specific to the multicellular context. In one perspective, the main commonality in the two experimental systems is that cell death occurs, which in the unicellular Chlamydomonas is of course lethal, whereas in Arabidopsis it is restricted to lateral root initiates. Both results, however, point to a key role for PNPase apart from RNA metabolism.

Altered chloroplast RNA maturation in pnp mutants is associated with a pale-green leaf phenotype

Disruption of the chloroplast PNPase gene in Arabidopsis was previously noted to be associated with 3’ extensions of both mRNAs and 23S rRNA (Walter et al., 2002), which we confirmed and also observed for the atpB mRNA (Figure 1D). The pale-green phenotype is evident in emerging leaves (Figure 1C), but was not noted in Walter et al. (2002) because a cosuppressed line rather than T-DNA null mutants were used. However, the phenotype was noted where a pnp mutant allele was obtained in a genetic screen for fosmidomycin resistance (Sauret-Gueto et al., 2006). The pale-green phenotype of young tissues progressively disappears, and cotyledons are indistinguishable from those of the WT. This contrasts with deficiency in the product of the paralogous At5g14580 locus, which encodes mitochondrial PNPase. In this case, null mutants are embryo-lethal and knockdown lines hyperaccumulate antisense and intergenic transcripts, suggesting a perhaps fatal disruption in mitochondrial gene expression (Holec et al., 2006).

Chloroplast PNPase deficiency affects root adaptation to -P stress

We observed that pnp plants grown under -P conditions were unable to elaborate lateral roots (Figures 2-3), a phenomenon that phenocopies pdr2, albeit on a longer time scale. The pdr2 mutation corresponds to a point mutation in At5g23630, which encodes a P-type ATPase of group V (Ticconi, 2005). Mutations in the same gene have been associated with reduced male fertility (Jakobsen et al., 2005). We also compared the root phenotype of pnp1-1 to that of csp41b-1, the latter of which is deficient in two related chloroplast endoribonucleases, and
which has pale mature leaves (Bollenbach et al., 2009). Because csp41b-1 resembled the WT in terms of lateral roots, we conclude that neither the partial chlorosis nor the chloroplast gene expression defects of pnp1-1 are likely to be responsible for its lateral root phenotype under -P conditions.

We used two reporter genes to gain additional insight into the pnp1-1 root phenotype (Figure 3). Using the DR5 promoter to drive GUS expression, we observed reduced staining in pnp1-1, relative to the WT, whether grown under +P or -P conditions. Moreover, in most cases only one or two lateral root initiates under -P conditions stained for GUS. Since the DR5 construct essentially measures responsiveness to auxin in that tissue (Ulmasov et al., 1997), we conclude that auxin levels and/or activity are abnormally low. It has been shown that P starvation enhances auxin sensitivity in Arabidopsis roots and helps lead to higher lateral root density and inhibition of primary root elongation (Lopez-Bucio et al., 2002). Thus, our observations are consistent with the conclusion that the failure to elaborate lateral roots in pnp1-1 under low P conditions is at least in part related to a defective hormonal cue.

A second reporter gene, CYCB1:GUS, revealed no abnormalities under +P conditions, but suggested that cell division was only occurring in a single lateral root initiate within the clusters that formed in pnp1-1 under -P conditions. By staining with Evan’s Blue, we concluded that as in pdr2, mutant lateral root initiates die, followed by initiation of secondary, tertiary and quaternary lateral roots. This cell death, or at least the loss of membrane integrity, was correlated with organellar abnormalities revealed by electron microscopy (Figure 4). While we have not investigated whether lateral roots in pnp1-1 undergo programmed cell death, it is worth noting that in plants, mitochondrial abnormalities in particular but also chloroplast dysfunction or communication, have been associated with programmed cell death (e.g. Yao et al., 2004).

**P content and uptake**
To test the hypothesis that PNPase might have a role in P homeostasis, we measured total and free Pi in leaves, where PNPase is predominantly expressed as well as in roots, where a defective growth phenotype was observed. A slight increase of total P and free Pi was evident under +P conditions for pnp1-1, whereas no significant differences were noted under -P conditions WT (Figure 6). While most P-containing metabolites that we measured did not exhibit differences between genotypes, phosphorate was slightly elevated in pnp1-1 (Figure 7). Also, while P uptake did not differ between genotypes, we saw slight induction relative to WT of the gene encoding the P transporters Pht1;1 and Pht1;4, under +P conditions (Figure 9). This is consistent with a degree of P stress in pnp1-1 under +P conditions, as discussed below.

**Transcript and metabolite profiling**
Microarray data revealed an obvious reorientation of the pnp mutant transcriptome towards expression of -P responses when grown on a full nutrient medium, comprising 221 out of the 508 P starvation-regulated genes in WT. Taken together with the P transporter data discussed above, we hypothesize that pnp mutant plants adjust their metabolism as if they were already, to some extent, under phosphate stress.

A second set of genes is responding to the absence of PNPase, independent of P availability. Overlap analyses (Figure 8A) between pnp-regulated on +P and on -P compared to WT, revealed 149 genes which fall into two major metabolic classes: regulation of chloroplast activities and oxidative stress responses. Fourteen of these are encoded by the chloroplast genome, and are generally strongly upregulated. As mentioned earlier, the accumulation of these transcripts likely results from perutrbation of normal cpRNA degradation pathways. The genotype also affected 12 nucleus-encoded chloroplast proteins. On the other hand, of several
genes suggested to be responsible for the communication between chloroplast and nucleus, or retrograde signaling, none were significantly regulated. Altered photosynthesis-related gene expression in \textit{pnp1-1} is in agreement with its partial chlorosis and slow-growth phenotype.

We also examined gene expression related to the chloroplast methylenerythritol phosphate (MEP) pathway, since the \textit{pnp} mutant \textit{rif10} mutant was identified using fosmidomycin, a strong inhibitor of deoxyxylulose 5-P reductoisomerase (DXR), which catalyzes the second step (Sauret-Gueto et al., 2006). In the case of \textit{rif10}, the authors reported a similar transcript accumulation for the DXP synthase (DXS), first enzyme of the MEP pathway and for the DXR, but increased accumulation for both at the protein level. Our experiments confirmed the lack of significant regulation for two \textit{DXS} genes (\textit{DXS1} and \textit{DXS3}) and the DXR gene, but revealed 5.8-fold activation of the third \textit{DXS} gene (\textit{DXS2}; At3g21500), which is expressed at a low level under normal conditions, based on EST data (Rodriguez-Concepcion and Boronat, 2002), but in our study was also activated in the WT during P starvation. That regulation of \textit{DXS2} in \textit{pnp1-1}, rather than a post-translational effect, could explain at least in part the DXS enzyme accumulation described, although it should be noted that the activities of \textit{DXS2} and \textit{DXS3} remain to be established and indeed, it has been proposed that they do not encode functional proteins and should be renamed \textit{DXL} (for DXS-like; Phillips et al., 2008).

Overall, in \textit{pnp} mutants including \textit{rif10} and \textit{pnp1-1}, defects in the MEP pathway lead to less accumulation of chlorophyll and carotenoids. This is not only consistent with the observed chlorosis, but may make the plants subject to oxidative stress due to a decrease in photoprotective carotenoids. Finally, because this pathway is also responsible for biosynthesis of certain hormones, their levels may also be affected in \textit{pnp} mutants.

Metabolic profiling revealed limited changes between \textit{pnp1-1} and the WT in the presence of P. However \textit{pnp1-1} displayed a different response to P starvation than WT Arabidopsis (this work and the study of Morcuende et al., 2007) or common bean (Hernandez et al., 2007). For example, changes in the amino acid profile under -P conditions strongly hint of a perturbation in plastid metabolism including large changes in the aspartate family amino acids. Interestingly, these metabolites are those that cost the most ATP in their production and their rate of biosynthesis has previously been demonstrated to be highly dependent on the plastidial energy charge (Regierer et al., 2002; Carrari et al., 2005). Importantly, these changes are considerably more dramatic in the \textit{pnp} mutant than in the WT, implying a functional role for the PNP protein in this process. Although less easy to rationalize there are also large changes in cytosolic and mitochondrially-synthesized metabolites such as Gly, Ser, Ala, and GABA.

**Oxidative stress responses and organellar integrity in \textit{pnp1-1}**

At the transcriptional and metabolic levels, several lines of evidence suggest activation of oxidative stress responses in \textit{pnp1-1}. Among the metabolites, DHA is the most strongly regulated on +P, with its content increased about 15-fold. Several enzymes involved in the ascorbate cycle, and its biosynthesis, are strongly regulated in \textit{pnp1-1}. Induced genes include \textit{VTC5}, an ascorbate biosynthetic enzyme (Dowdle et al., 2007), and two ascorbate oxidases which convert L-ascorbate to monoDHA (Ishikawa et al., 2006). In contrast, the gene encoding the chloroplast thylakoid enzyme responsible for the same conversion but via H$_2$O$_2$ rather than O$_2$ scavenging, \textit{TAPX}, is repressed. Reduction of monoDHA, which is a radical, is catalyzed by monoDHA reductase, which is also strongly induced in \textit{pnp1-1}. Finally, the initiation and the activity of the ascorbate-glutathione cycle are strongly induced, probably leading to accumulation of DHA. However, the repression of the gene encoding \textit{TAPX} might lessen the ability of the \textit{pnp} mutant to detoxify the chloroplast (reviewed in Asada, 2006). It should also be noted that in a recent study on the functional role of ascorbate peroxidase in oxidative stress...
resulting from a combination of drought and heat, was associated with enhanced malic enzyme activity and a decrease in malate, leading the authors to speculate that malate metabolism plays an important role in the response of Arabidopsis to this stress combination (Koussevitzky et al., 2008). Our findings of 50% lower malate in \textit{pnp1-1} accompanying the accumulation of DHA, hint of a similar response in this mutant.

The \textit{pnp1-1} +P transcriptome revealed induction of additional genes involved in redox homeostasis including peroxidases, thioredoxins, cytochromes P450 and glutathione-S transferases. We also note the induction of one cytosolic and one chloroplast copper/zinc superoxide dismutase: \textit{CSD1} and \textit{CSD2} respectively (Kliebenstein et al., 1998). These are involved in superoxide radical detoxification, and the positive and negative regulation of genes encoding glutaredoxins. Some of this regulation was also found in the WT under P starvation.

A general consequence of reactive oxygen species accumulation can be the alteration of membrane lipids, which could lead in turn to organelle disorganization as we observed in \textit{pnp1-1} roots (Figure 4). This loss of organelle integrity might be linked to the induction of a group of genes related to the PYK10 complex, as discussed in Results. The genes encoding PYK10 complex components were found to be repressed in the \textit{nai1} mutant (Nagano et al., 2008), which lacks a bHLH transcription factor (Matsushima et al., 2004). Another target of \textit{NAI1} is the lipoxygenase \textit{LOX3}, which is induced more than 12-fold in \textit{pnp1-1} on +P; the \textit{NAI1} gene itself is induced 1.8- and 1.5-fold in \textit{pnp1-1} vs WT on +P and on -P, respectively. One study of ER bodies suggested that the PYK10 complex might form only when subcellular structures are altered or disrupted, as the complex's partners would normally be localized in different compartments (Nagano et al., 2008). The induction of PYK10 complex genes in \textit{pnp1-1} could be thus related to the loss of organelle integrity triggered by oxidative stress.

\textbf{A systemic signal may be altered in \textit{pnp1-1}}

The split-root experiment (Figure 5) raised the possibility that a systemic signal was affected in \textit{pnp1-1} plants. Such a signal has been hypothesized to arise in the root cap during P starvation (Svistoonoff et al., 2007), and may be integrated in the aerial part of the plant to stimulate adaptation in root architecture. Our results suggest that in the case of \textit{pnp1-1}, either a major metabolite and/or hormonal balance may be relevant to this issue.

One metabolite candidate would be sugars, as a tight relationship between sugar metabolism and P deprivation responses is well established, and was evident in a transcriptomic comparison between P starvation responses and sucrose-regulated metabolism (Muller et al., 2005). It was found that some P-responsive genes are more strongly induced in the presence of sucrose, and that these enhanced responses were correlated with increased lateral root density (Karthikeyan et al., 2007). It has additionally been proposed that phloem sucrose transport may integrate root responses to phosphate deprivation (reviewed in Hammond and White, 2008).

In the experiments reported here, \textit{pnp1-1} differed from the WT in particular with respect to early fairly dramatic decreases in F6P at three hours, and a delayed increase in raffinose as compared to the WT. The latter was found to accumulate in the mutant under control conditions. Additional differences in the sugar response to P starvation between the two genotypes are to be found in opposite behavior of isomaltose, and trehalose and the sugar derivative myo-inositol particularly following one week of starvation. The differences are reduced in the longest starvation period, suggesting an adjustment of sugar metabolism to P deprivation within the first hours.
Our microarray analysis also revealed gene expression changes in \textit{pnp1-1} closely related to sucrose metabolism. For example, under +P conditions several carbohydrate transporters are induced: the plastid Glc-6P translocator (GPT2), a mannitol transporter, a sugar transporter (STP4), two UDP-galactose transporters and the sucrose transporter SUC1. Also in \textit{pnp1-1}, glycolysis appears to be globally activated. While it is challenging to link particular metabolite levels to any of these changes in gene expression, it is notable that both metabolite steady-state levels and relevant genes are fluctuating in tandem.

Two other sugars, isomaltose and trehalose, increased approximately 5.5- and four-fold in \textit{pnp1-1} vs. WT, respectively, when grown on +P (Figure 7). Their accumulation patterns also differ qualitatively upon P starvation (Supplemental Figure S1). The role of isomaltose is unclear, as it is apparently not a major form of carbon exported from the chloroplast, at least in WT plants (Weise et al., 2004). Nor does isomaltose appear to be accumulating at the expense of maltose, which is the major exported form. However, we cannot exclude a deficiency of carbon export, as we have examined only total metabolites rather than their partitioning into subcellular compartments.

Trehalose is a disaccharide whose phosphorylated form, trehalose-6-phosphate (Tre-6P), appears to be an important signaling molecule related to sugar metabolism (Paul et al., 2008). Trehalose is produced in a tandem reaction commencing with the synthesis of Tre-6P from Glc-6P and uridine diphosphate (UDP)-glucose catalyzed by Tre-6P synthase (TPS); subsequently Tre-6P phosphatase (TPP) catalyzes the dephosphorylation of Tre-6P to trehalose. The accumulation of trehalose in the mutant under +P conditions might suggest an upregulation of Tre-6P dephosphorylation, a phenomenon that occurs in the WT during the short periods of P starvation and might relate to P repartitioning within the cell. On the other hand, trehalose levels could increase in response to stress condition (redox and/or P starvation). Indeed, although the role of trehalose as an osmoprotectant remains under debate, it is known to accumulate under a variety of environmental stresses including cold, osmotic imbalance, and salt. Most of its biosynthetic genes respond to plant exposure to a wide range of abiotic and perhaps also biotic stresses (Iordachescu and Imai, 2008).

Consistent with our metabolite data and published gene expression results, transcriptome analyses revealed that five genes related to trehalose biosynthesis are differentially regulated on +P in \textit{pnp1-1}. Four of them encode TPP (TPPG and TPPD are induced and TPPA and TPPH are repressed), whereas TPS1 is repressed. TPPA, TPPD and TPPG are predicted to be chloroplast targeted. Because both TPP and TPS are encoded by multigene families whose expression profiles vary widely (Paul et al., 2008), how this suite of expression changes results in increased trehalose content in \textit{pnp1-1} is difficult to pinpoint. Furthermore, since Tre-6P is an important metabolite whose concentration we have not measured directly, its relevance to the \textit{pnp} root phenotype remains an open question.

**Hormone balance in \textit{pnp1-1}**

Many publications concerning P starvation responses highlight the roles of hormones in signal transduction, particularly auxins and cytokinins (reviewed in Rubio et al., 2008; Yuan and Liu, 2008). It is not surprising, then, that many genes involved in hormone biosynthesis or degradation are regulated in the \textit{pnp} mutant on +P (Supplemental Table S4). Furthermore, expression of DR5:GUS in \textit{pnp1-1} (Figure 3A) suggested a decreased auxin activity in \textit{pnp1-1} root tips on +P as well as on -P. However, because we did not carry out detailed analyses of hormone contents, nor create genetic combinations with hormone pathway mutants, we feel it is premature to draw any direct connections between normal PNPase activity and hormone signaling.
In conclusion, the Arabidopsis and Chlamydomonas \( pnp \) mutants have few commonalities in their response to P starvation, when examined in molecular detail. However, the importance of PNPase, and more generally the chloroplast, in conferring the ability to correctly respond to P starvation is conserved. Given the considerable differences in the survival strategies of a motile, unicellular organism and a sessile, multicellular one, differences in gene regulation and the consequences of PNPase inactivation are perhaps not surprising. Examining additional evolutionarily diverse photosynthetic species in this regard, should similarly be interesting. How PNPase activity influences the ability of organisms to respond to P stress remains to be understood in detail, in particular what type of signal it generates, and how that signal is integrated into the global response pathway.

**MATERIALS AND METHODS**

**Plant material**
All the *Arabidopsis thaliana* plants used in this study are derived from the Col-0 ecotype, which was used as the wild-type. The two mutant lines, *pnp1-1* (SALK_013306) and *pnp1-2* (SALK_070705) containing a T-DNA insertion in the *PNP* gene (At3g03710), were obtained from the SIGnAL collection (Alonso et al., 2003). The precise locations of the T-DNA left borders were confirmed by DNA sequencing. PCR with the left border primer (LBB1, 5'-GCGTGGACCGCTTGTCACT-3') and gene-specific primers (*pnp1-1*, 5'-GCAAAGCTCGCTGTTTAGATG-3' and 5'-CATAGCCATGCACTTTGCGC-3'; *pnp1-2*, 5'-TACGTAGGCGAATTGTTGAGG-3' and 5'-CCACAAACAGATGCCATTAGCT-3') was used to identify T-DNA insertion alleles of *pnp1-1* and *pnp1-2*, respectively. The *pnp1-1* line was crossed with the Arabidopsis *CYCB1:GUS* (Ulmasov et al., 1997) and *DR5:GUS* (Colón-Carmona et al., 1999) lines to generate the reporter lines used in Figure 3.

**Growth conditions and phosphate starvation treatment**
Seeds were surface-sterilized and stratified at 4°C for 3-4 days. Unless noted in the Figure Legends, plants were grown as follows. Seeds were germinated in a full nutrient MS liquid medium in a controlled environmental chamber on a shaker at 25°C, under fluorescent lights (100 µmol m\(^{-2}\) s\(^{-1}\)) with a long day photoperiod (16 hr light). After 2 weeks, plantlets were transferred into Magenta boxes or onto petri plates with MS medium (1.25 mM KH\(_2\)PO\(_4\)) containing 2% (w/v) sucrose and 0.75% (w/v) phytagar (Murashige and Skoog, 1962), for the indicated periods. This medium is referred to as +P medium. For -P medium, KH\(_2\)PO\(_4\) was omitted but the potassium was compensated by K\(_2\)SO\(_4\). Plantlets were rinsed with distilled water before transfer. On soil, plants were grown on Metro Mix 360, in a growth chamber, as described above.

For the microarray and the qRT-PCR experiments, plants were germinated and grown for 2 weeks on a full nutrient MS medium (+P) containing 0.6% phytagar (w/v) and 0.5% (w/v) sucrose (to limit the sucrose effect on gene expression) at 22°C in a growth chamber under a 16 h photoperiod (with a fluorescent light intensity of 200 µmol m\(^{-2}\) s\(^{-1}\)). Then, they were transferred to fresh +P or -P MS medium. For the -P medium, KH\(_2\)PO\(_4\) was omitted but the potassium was compensated by K\(_2\)SO\(_4\). Plantlets were rinsed with distilled water before transfer. On soil, plants were grown on Metro Mix 360, in a growth chamber, as described above.

**RNA Analysis**
Total RNA was isolated using Tri Reagent according to manufacturer’s instructions (Molecular Research Center, Inc. Cincinnati, OH), separated by electrophoresis and transferred onto a GeneScreen membrane (PerkinElmer, Waltham, MA) as described previously (Bollenbach et
Gene-specific probes were labeled by random priming using 100 ng of DNA, 20 μCi of [α-32P]dCTP and the Klenow fragment (Promega, Madison, WI).

UV Crosslinking

UV crosslinking of proteins to radiolabeled RNA was performed as described previously (Lisitsky et al., 1997). The proteins (10 fmol) were mixed with [α-32P]UTP-RNA (10 fmol) in a buffer containing 10 mM HEPES-NaOH, pH 7.9, 30 mM KCl, 6 mM MgCl2, 0.05 mM EDTA, 2 mM DTT, 8% (v/v) glycerol and crosslinked immediately with 1.8 J of UV irradiation (Stratalinker 1800, Stratagene, La Jolla, CA). The RNA was then digested with 10 μg of RNase A and 30 units of RNase T1 at 37°C for 1 h. The proteins were fractionated by SDS-PAGE and analyzed by autoradiography.

Root analyses

Images of roots were recorded under a stereomicroscope (Olympus SZX12) high-performance CCD camera and imported into Photoshop Image software. Histochemical analysis of the GUS reporter enzyme activity was adapted from Jefferson (1987). Samples were stained using 2 mM X-Gluc (Sigma, St. Louis, MI, USA) dissolved in a 100 mM sodium phosphate buffer (pH 7.2) containing 0.2% Triton X-100, 2 mM K4Fe(CN)6.H2O, 2 mM K3Fe(CN)6.H2O. For cell viability analysis, roots were stained with 10% Evan's blue (w/v, Fisher, Pittsburgh, PA, USA). After washing with distilled water and mounting in 50% glycerol, root tips were viewed using the Olympus SZX12 microscope.

Transmission electronic microscopy

Roots were embedded in an epoxy resin (Spurr, 1969). The root tips, from root cap to root hair zone (approximately 2 mm in length), were cut and fixed overnight in 5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C, and post-fixed in 1% (w/v) aqueous osmium tetroxide for 3 h at room temperature and rinsed three times with distilled water. After dehydration through a graded ethanol series, the samples were rinsed in propylene oxide, infiltrated and embedded in Spurr's resin. Ultra-thin sections were cut to a 50-70 nm thickness with a diamond knife on a Leica UCT ultramicrotome (Wetzlar, Germany). The sections were stained with uranyl acetate (2.5%, w/v) and lead citrate. After staining, the sections in the middle longitudinal direction were viewed and photographed using a Philips FEI-Technai 12 microscope.

Determination of metabolite levels.

Free inorganic phosphate and total phosphate were assessed as described previously (Versaw and Harrison, 2002). For metabolic profiling, leaf samples were immediately frozen in liquid nitrogen and stored at -80°C. Extraction and quantification of metabolites was carried out using an established GC-MS based protocol (Roessner et al., 2001), with the exception that the metabolites studied also included subsequent additions to the mass spectral libraries (Schauer et al., 2005).

Principal component analysis was performed with the online tool MetaGeneAlyse (www.metagenealyse.mpimp-golm.mpg.de; Scholz et al., 2004) and TMEV software (Saeed et al., 2003). The data was normalized to the mean of the entire sample set for each metabolite and log10-transformed before the analysis. This transformation reduces the influence of rare high-measurement values, but does not change the discrimination in the dataset. Statistical analysis of the data was performed by t-test and two-way ANOVA.

Microarray hybridization and data analysis
Total RNA was isolated from seedlings, from which roots had been removed, using the RNeasy Plant Minikit (Qiagen, Valencia, CA), including DNase treatment according to the manufacturer’s instructions. RNA quality check, linear amplification, labeling, hybridization, washing and scanning were performed by the Cornell Microarray Core Facility (http://cores.lifesciences.cornell.edu/brcinfo/?f=10). Affymetrix ATH1 genome array GeneChips were used. Three biological replicates were used for each experimental condition.

Raw array data was normalized at the probe level using gcRMA (Wu, 2004). The detection calls (present, marginal, or absent) for each probe set were obtained using the mas5calls function in the Affy package (Gautier et al., 2004). Only genes with at least one present call across all the compared samples were used to identify differentially expressed genes. Significance of gene expression was determined using the LIMMA test (Smyth, 2004) and raw p-values of multiple tests were corrected using False Discovery Rate (FDR; Benjamini and Hochberg, 1995). Genes with FDR <0.05 and fold change greater than or equal to two were identified as differentially expressed. The complete dataset was deposited in NCBI Gene Expression Omnibus under accession [in process].

**Quantitative RT-PCR**

One microgram of DNase-treated RNA was reverse transcribed in a 20 µL reaction using SuperScript III (Invitrogen, Carlsbad, CA) according to the instructions, including the RNase H treatment. One µL of this cDNA was amplified using the Fast Sybr Green master mix (Applied Biosystems, Foster City, CA) and 0.66 µM of each primer in a 15 µL reaction. PCR amplification was performed using the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with the following conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The amplification specificity was checked using melting curves. The relative quantification of the samples was determined using the BioRad CFX Manager software, integrating primer efficiencies calculated from a standard curve. For each gene, the sample showing the highest intensity level was used as reference with a value of 1. The final data result from the average of three biological replicates and at least two technical repetitions. Primers are listed in Supplemental Table S8.

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Table I. The number of regulated genes in WT and/or pnp1-1 under different growth conditions.

| Experiment | data | induced$^c$ | repressed$^c$ | total |
|------------|------|-------------|---------------|-------|
| pnp1-1 +P/WT +P | 16536 | 601 | 359 | 960 |
| WT -P/WT +P | 16660 | 409 | 99 | 508 |
| pnp1-1 -P/pnp1-1 +P | 16585 | 174 | 35 | 209 |
| pnp1-1 -P/WT -P | 16751 | 154 | 70 | 224 |

$^a$ The genotype and the condition listed first correspond to the numerator of the gene expression mean ratio relative to the second, which is the denominator. For example, 601 genes are induced in the pnp1-1 mutant when compared to the WT, under +P conditions.

$^b$ Data correspond to the genes which were tagged as “present” in at least one replicate as described in Methods.

$^c$ The induced or repressed genes exhibit at least 2-fold change in expression and a false discovery rate (FDR) of < 0.05.
Table II. The 40 most up- and down-regulated genes in the *pnp1-1* mutant relative to WT when grown under +P conditions (FDR>0.05).

| Probeset ID | AGI code   | Description                                                                 | Fold change on +P | Fold change on –P<sup>b</sup> |
|-------------|------------|------------------------------------------------------------------------------|-------------------|-------------------------------|
| 244977_at   | AtCG00730  | PETD                                                                         | 102.82            | 151.68                        |
| 257673_at   | At3g20370  | Meprin and TRAF homology domain-containing protein                           | 81.11             | 15.88                         |
| 250515_at   | At5g09570  | Expressed chloroplast protein                                                | 39.15             | 1.61                          |
| 263539_at   | At2g24850  | Aminotransferase                                                           | **32.66**         | **9.07**<sup>b</sup>          |
| 267472_at   | At2g02850  | Plastocyanin-like domain-containing protein                                 | 29.93             | 3.28<sup>b</sup>              |
| 245002_at   | AtCG00270  | PSBD                                                                         | 28.93             | 34.44                         |
| 258941_at   | At3g09940  | Monodehydroascorbate reductase, putative                                    | 27.35             | ns                           |
| 248434_at   | At5g14440  | 23.5 kDa mitochondrial small HSP                                             | 26.05             | 11.35<sup>b</sup>             |
| 265051_at   | At1g52100  | Jacalin lectin family protein                                                | **26.05**         | **17.67**                     |
| 246888_at   | At5g26270  | Expressed protein                                                           | 26.02             | 18.81                        |
| 261930_at   | At1g22440  | Alcohol dehydrogenase                                                       | 24.83             | ns                           |
| 265668_at   | At2g32020  | GCN5-related N-acetyltransferase family protein                             | 22.45             | 20.99                        |
| 260522_x_at | At2g41730  | Expressed protein                                                           | 22.34             | 9.65                         |
| 266752_at   | At2g47000  | P-glycoprotein 6 (PGP6), multidrug resistant transporter                    | 21.49             | 11.89                        |
| 266246_at   | At2g27690  | Cytochrome P450                                                              | 20.82             | 5.00<sup>b</sup>              |
| 252921_at   | At4g39030  | Enhanced disease susceptibility 5 (EDS5)                                     | 20.69             | 6.70<sup>b</sup>              |
| 256159_at   | At1g30135  | JAZ8/TIFY5A (Jasmonate-ZIM-domain protein B)                                | 20.60             | ns                           |
| 254385_s_at | At4g21830  | Methionine sulfoxide reductase domain-containing protein                    | 20.56             | ns                           |
| 255110_at   | At4g08770  | Peroxidase                                                                   | 18.24             | ns                           |
| 263153_s_at | At1g54010  | Myrosinase-associated protein                                                | 18.11             | 6.18                         |
| 247718_at   | At5g59310  | Lipid transfer protein 4 (LTP4)                                              | 0.01              | 0.03                         |
| 258498_at   | At3g02480  | ABA-responsive protein-related                                              | 0.03              | ns                           |
| 262412_at   | At1g34760  | 14-3-3 protein GF14 omicron (GRF11)                                         | 0.03              | ns                           |
| 266516_at   | At2g47880  | Glutaredoxin family protein                                                 | 0.05              | ns                           |
| 266098_at   | At2g37870  | Protease inhibitor/lipid transfer protein (LTP) family protein              | 0.07              | ns                           |
| 259789_at   | At1g29395  | Stress-responsive protein                                                   | 0.08              | 0.10<sup>a</sup>              |
| 247095_at   | At5g66400  | Dehydrin (RAB18)                                                            | 0.08              | 0.04<sup>a</sup>              |
| 248197_at   | At5g54190  | NADPH-protochlorophyllide oxidoreductase A (PORA)                            | 0.1               | 0.08<sup>a</sup>              |
| 263981_at   | At2g42870  | Phytochrome Rapidly Regulated 1 (PAR1)                                      | 0.1               | ns                           |
| 247717_at   | At5g59320  | Lipid transfer protein 3 (LTP3)                                             | 0.1               | 0.13<sup>a</sup>              |
| 260831_at   | At1g06830  | Glutaredoxin family protein                                                 | 0.11              | 0.24                         |
| 245353_at   | At4g16000  | Expressed protein                                                           | 0.11              | ns                           |
| 264041_at   | At2g03710  | Agamous-like 3 (AGL3)                                                       | 0.12              | 0.1                          |
| 268873_at   | At2g44740  | CYCP4:1, cyclin family protein                                               | 0.13              | ns                           |
| 250933_at   | At5g03170  | Fascilin-like arabinogalactan-protein (FLA11)                               | 0.13              | ns                           |
| 252612_at   | At3g45160  | Expressed protein                                                           | 0.14              | 0.07                         |
| 267569_at   | At2g30790  | Photosystem II oxygen-evolving complex 23 (PSBP2)                           | 0.14              | 0.17                         |
| 261226_at   | At1g20190  | Expansin (EXP11)                                                            | 0.14              | ns                           |
| 251039_at   | At5g02020  | Expressed chloroplast protein                                                | 0.14              | 0.15                         |
| 251196_at   | At3g62950  | Glutaredoxin family protein                                                 | 0.15              | ns                           |

<sup>a</sup>The corresponding data for –P are also indicated, i.e. *pnp1-1* on –P vs. WT on -P. A full dataset is given in Table S4.

<sup>b</sup>Means the values are not significant (FDR >0.05).

“ns” indicates that the fold change is below the cutoff value of 2.

In bold, are indicated stress-related gene products.
### Table III. Selected genes regulated in pnp1-1 relative to WT independent of P status.

| Probe Set ID | AGI code | Description | Fold change on +P | Fold change on -P |
|--------------|----------|-------------|-------------------|------------------|
| 244977_at    | AtCg00730| PETD        | 102.82            | 151.68           |
| 245002_at    | AtCg00270| PSBD        | 28.93             | 34.44            |
| 245015_at    | AtCg00490| RBCL        | 16.12             | 18.88            |
| 244970_at    | AtCg00660| RPL20       | 15.03             | 26.41            |
| 245005_at    | AtCg00330| RPS14       | 10.57             | 43.80            |
| 244991_s_at  | AtCg00890| NDH.B1      | 9.54              | 18.21            |
| 244966_at    | AtCg00600| PETG        | 6.35              | 9.79             |
| 244966_at    | AtCg00600| PETG        | 5.57              | 7.03             |
| 245004_at    | AtCg00300| YCF9 (PSBZ) | 3.24              | 4.99             |
| 244930_at    | AtCg00650| RPS18       | 2.14              | 2.76             |
| 244986_at    | AtCg00840| RPL23.1     | 0.50              | 0.49             |
| 245024_at    | AtCg00120| ATPA        | 0.17              | 0.35             |
| 264856_at    | At1g24090| RNase H domain-containing protein | 7.19 | 5.56 |
| 264869_at    | At1g36180| Acetyl-CoA carboxylase 2 (ACC2) | 6.66 | 13.60 |
| 260648_at    | At1g08050| Zinc finger (C3HC4-type RING finger) family protein | 4.12 | 3.23 |
| 257667_at    | At3g20440| BE1/EMB2729 (Branching Enzyme 1), alpha-amyylase | 3.58 | 4.98 |
| 254523_at    | At4g15910| Drought-induced protein (Di21) | 3.10 | 2.75 |
| 265479_at    | At2g15760| Calmodulin-binding protein, similar to AR781 | 2.59 | 3.26 |
| 264856_at    | At1g24090| RNase H domain-containing protein | 7.19 | 5.56 |
| 265479_at    | At2g15760| Calmodulin-binding protein, similar to AR781 | 2.59 | 3.26 |
| 264991_s_at  | AtCg00890| NDH.B1      | 9.54              | 18.21            |

**Photosynthesis and chloroplast-targeted proteins**

| Probe Set ID | AGI code | Description | Fold change on +P | Fold change on -P |
|--------------|----------|-------------|-------------------|------------------|
| 244930_at    | AtCg00650| RPS18       | 2.14              | 2.76             |
| 244986_at    | AtCg00840| RPL23.1     | 0.50              | 0.49             |
| 244966_at    | AtCg00600| PETG        | 5.57              | 7.03             |
| 244966_at    | AtCg00600| PETG        | 5.57              | 7.03             |
| 245004_at    | AtCg00300| YCF9 (PSBZ) | 3.24              | 4.99             |
| 244930_at    | AtCg00650| RPS18       | 2.14              | 2.76             |
| 244986_at    | AtCg00840| RPL23.1     | 0.50              | 0.49             |
| 244966_at    | AtCg00600| PETG        | 5.57              | 7.03             |
| 244966_at    | AtCg00600| PETG        | 5.57              | 7.03             |

**PYK10 associated protein**

| Probe Set ID | AGI code | Description | Fold change on +P | Fold change on -P |
|--------------|----------|-------------|-------------------|------------------|
| 265051_at    | At1g52100| JAL11       | 26.05             | 17.67            |
| 259384_at    | At3g16450| JAL33, jacin lectin family protein | 13.28 | 4.97 |
| 266988_at    | At2g39310| JAL22       | 8.73              | 3.46             |
| 259327_at    | At3g16460| JAL34, jacin lectin family protein, similar to MBP1 (Myrosinase-Binding protein-like Protein-300B) | 7.87 | 4.32 |
| 259381_s_at  | At1g33790| JAL4, jacin lectin family protein | 5.28 | 2.11 |
| 259382_s_at  | At3g16430| JAL30, PB1 (PYK10-Binding Protein 1) | 5.60 | 3.10 |
| 263153_s_at  | At1g54000| GLL22, myrosinase-associated protein | 18.11 | 6.18 |
| 263156_at    | At1g54030| GLL25, GDSL-motif lipase | 3.57 | 2.19 |
| 259380_s_at  | At1g59260| PYK10 (phosphate starvation-response 3.1) | 10.95 | 4.27 |
| 257798_at    | At3g16590| TSA1-like (DNA topoisomerase), similar to TSK-associating protein 1 (TSA1) | 12.46 | 5.78 |
| 249817_at    | At5g23820| MD-2-related lipid recognition domain-containing protein | 3.65 | 3.68 |
| 246855_at    | At5g26280| Meprin and TRAF Homology domain-containing protein | 12.51 | 7.61 |

**RNA and DNA related proteins**

| Probe Set ID | AGI code | Description | Fold change on +P | Fold change on -P |
|--------------|----------|-------------|-------------------|------------------|
| 264640_at    | At1g10170| NF-X1 type zinc finger family protein | 4.56 | 3.03 |
| 267140_at    | At2g32250| DNA-binding protein-related, similar to transcription factor | 3.17 | 2.55 |
| 260266_at    | At1g68520| Zinc finger (B-box type) family protein | 0.40 | 0.46 |
| 262291_at    | At1g70790| C2 domain-containing protein | 0.38 | 0.35 |
| 248764_at    | At5g47640| CCAAT-box binding transcription factor subunit B (NF-YB) (HAP3 ) | 0.26 | 0.32 |
| 252504_at    | At5g46590| TRFL1 (TRF-LIKE 1) telomere repeat-binding protein | 0.26 | 0.40 |
| 263909_at    | At2g36490| HhH-GPD base excision DNA repair family protein (ROS1) | 0.16 | 0.24 |
| 264041_at    | At2g03710| SEP4 (Sepalata 4); Identical to Aegagrostis MADS-box protein AGL3 (AGL3) | 0.12 | 0.10 |

**Transport**

| Probe Set ID | AGI code | Description | Fold change on +P | Fold change on -P |
|--------------|----------|-------------|-------------------|------------------|
| 263402_at    | At2g04050| MATE efflux family protein, similar to ATDTX1, antipporter | 13.05 | 7.08 |
| 263401_at    | At2g04070| MATE efflux family protein, similar to ATDTX1, antipporter | 6.49 | 9.09 |
| 253732_at    | At4g29140| MATE efflux protein-related | 2.58 | 2.49 |
| 249188_at    | At5g42830| Transferase family protein, similar to N-hydroxycinnamoyl/benzoyltransferase 6 | 10.47 | 5.68 |
| 252504_at    | At5g46590| TRFL1 (TRF-LIKE 1) telomere repeat-binding protein | 0.26 | 0.40 |
| 263909_at    | At2g36490| HhH-GPD base excision DNA repair family protein (ROS1) | 0.16 | 0.24 |
| 264041_at    | At2g03710| SEP4 (Sepalata 4); Identical to Aegagrostis MADS-box protein AGL3 (AGL3) | 0.12 | 0.10 |
Table IV. Behavior of major genes normally involved in the phosphate deprivation response.

| Probeset ID | AGI code | Description                                                                 | pnp1-1 vs WT | WT vs WT-P | pnp1-1 vs WT | pnp1-1 vs WT-P |
|-------------|----------|------------------------------------------------------------------------------|-------------|------------|-------------|-------------|
|             |          |                                                                               | Fold Change | FDR        | Fold Change | FDR         |
|             |          |                                                                               |             |            |             |             |
| 247629_at   | At5g60410| DNA-binding family protein (SIZ1)                                            | 1.82        | 0.324      | 1.97        | 0.87        |
| 253784_at   | At4g28610| Phosphate starvation response regulator (PHR1)                              | 0.94        | 0.820      | 0.86        | 0.589       |
| 245976_at   | At5g13080| Transcription factor (WRKY75)                                               | 7.41        | 0.022      | 4.77        | 0.114       |
| 266743_at   | At2g02990| Ribonuclease 1 (RNS1)                                                       | 4.04        | 0.024      | 6.66        | 0.008       |
| 264893_at   | At1g23140| C2 domain-containing protein                                                 | 3.55        | 0.014      | 48.17       | 0.000       |
| 249152_s_at | At5g43350| Inorganic phosphate transporter (PT1) (Pht1;1/Pht1;2)                       | 7.83        | 0.001      | 40.68       | 0.000       |
| 266184_s_at | At2g39840| Phosphate transporter (PT2) (Pht1;4/Pht1;7)                                 | 9.54        | 0.003      | 36.77       | 0.000       |
| 267646_at   | At2g32830| Inorganic phosphate transporter (PHT5)                                      | 2.37        | 0.085      | 12.00       | 0.001       |
| 249151_at   | At5g43360| Inorganic phosphate transporter (PHT3)                                      | 1.03        | NA         | 1.02        | 0.000       |
| 257311_at   | At3g26570| Phosphate transporter family protein PHT2;1 (chloroplast)                   | 0.74        | 0.782      | 0.74        | 0.876       |
| 258293_at   | At3g23430| Phosphate transporter (PHO1)                                                 | 0.74        | 0.656      | 15.88       | 0.012       |
| 267456_at   | At2g33770| Ubiquitin-conjugating enzyme family protein (PHO2/UBC24)                   | 1.40        | 0.315      | 1.76        | 0.164       |
| 264204_at   | At1g22710| Sucrose transporter / sucrose-proton symporter (PHO3/PHO5)                  | 0.52        | 0.027      | 0.89        | 0.676       |
| 258158_at   | At3g17790| Acid phosphatase type 5 (ACP5)                                              | 1.78        | 0.176      | 42.25       | 0.000       |
| 260383_at   | At2g27190| Iron(III)-zinc(II) purple acid phosphatase (PAP12)                          | 1.30        | 0.074      | 5.35        | 0.000       |
| 252006_at   | At3g52820| Purple acid phosphatase (PAP22)                                             | 0.98        | 0.968      | 15.16       | 0.000       |
| 265984_at   | At2g18880| Purple acid phosphatase (PAP7)                                              | 1.03        | NA         | 10.00       | 0.307       |
| 255587_at   | At4g01480| Inorganic pyrophosphatase (ATPPA5)                                           | 2.01        | 0.013      | 5.08        | 0.000       |
| 246071_at   | At5g20150| SPX (SYG1/Pho81/XPR1) domain-containing protein (ASPX1)                    | 1.63        | 0.065      | 99.05       | 0.000       |
| 267611_at   | At2g26680| SPX (SYG1/Pho81/XPR1) domain-containing protein (ASPX1)                    | 0.78        | 0.195      | 5.17        | 0.000       |
| 266132_at   | At2g45130| SPX (SYG1/Pho81/XPR1) domain-containing protein (ASPX3)                    | 1.12        | 0.819      | 139.62      | 0.000       |
| 263599_at   | At2g01830| Histidine kinase (CRE1/AHK4/WOL)                                            | 0.40        | 0.004      | 0.55        | 0.000       |
| 258887_at   | At3g05630| Phospholipase D (PLDP2)                                                      | 0.89        | NA         | 69.58       | 0.000       |
| 258452_at   | At3g22370| Alternative oxidase 1a. mitochondrial (AOX1A)                               | 4.44        | 0.003      | 1.87        | 0.000       |
| 257642_at   | At3g26710| Basic helix-loop-helix family protein (bHLH32)                             | 0.47        | 0.134      | 0.79        | 0.000       |
| 264783_at   | At1g08650| Phosphoenolpyruvate carboxylase kinase (PPCK1)                              | 1.04        | NA         | 63.73       | 0.000       |
| 258570_at   | At3g04530| Phosphoenolpyruvate carboxylase kinase (PPCK2)                              | 1.02        | NA         | 31.95       | 0.000       |
| 249846_at   | At2g23630| ATPase E1-E2 type family protein (PDR2)                                     | 0.85        | 0.51       | 1.05        | 0.89        |

Plastid carbon transport

| Probeset ID | AGI code | Description                                                                 | pnp1-1 vs WT | WT vs WT-P | pnp1-1 vs WT | pnp1-1 vs WT-P |
|-------------|----------|------------------------------------------------------------------------------|-------------|------------|-------------|-------------|
|             |          |                                                                               | Fold Change | FDR        | Fold Change | FDR         |
|             |          |                                                                               |             |            |             |             |
| 248144_at   | At5g54800| Glucose-6-phosphate/phosphate translocator (GPT1)                            | 1.51        | 0.047      | 1.95        | 0.008       |
| 264400_at   | At1g61800| Glucose-6-phosphate/phosphate translocator (GPT2)                            | 3.49        | 0.016      | 10.56       | 0.002       |
| 252414_at   | At3g47420| Glycerol-3-phosphate transporter                                             | 1.34        | 0.796      | 33.88       | 0.016       |
| 246445_at   | At5g17630| Glucose-6-phosphate/phosphate translocator                                 | 0.89        | 0.666      | 0.77        | 0.419       |
| 248886_at   | At5g46110| Phosphate/triose-phosphate translocator (TPT)                                | 0.88        | 0.388      | 0.98        | 0.934       |
| 259185_at   | At3g01550| Triose phosphate/phosphate translocator (PTP2)                               | 0.60        | 0.831      | 0.35        | 0.856       |

Values in black are those for which the fold change is significant (fold change ≥ 2 and FDR < 0.05); for those in grey, the fold change is < 2 and/or the FDR > 0.05.