A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation

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Phosphorus, one of the essential elements for plants, is often a limiting nutrient because of its low availability and mobility in soils. Significant changes in plant morphology and biochemical processes are associated with phosphate (Pi) deficiency. However, the molecular bases of these responses to Pi deficiency are not thoroughly elucidated. Therefore, a comprehensive survey of global gene expression in response to Pi deprivation was done by using *Arabidopsis thaliana* whole genome Affymetrix gene chip (ATH1) to quantify the spatio-temporal variations in transcript abundance of 22,810 genes. The analysis revealed a coordinated induction and suppression of 612 and 254 Pi-responsive genes, respectively. The functional classification of some of these genes indicated their involvement in various metabolic pathways, ion transport, signal transduction, transcriptional regulation, and other processes related to growth and development. This study is a detailed analysis of Pi starvation-induced changes in gene expression of the entire genome of *Arabidopsis* correlated with biochemical processes. The results not only enhance our knowledge about molecular processes associated with Pi deficiency, but also facilitate the identification of key molecular determinants for improving Pi use by crop species.

Phosphate (Pi) is an essential macronutriment required for plant growth and development. Its low availability to plants in many soils results not only from limiting amounts, but also from its association with cations and organic compounds creating insoluble complexes. Thus, Pi has become one of the major plant nutrition problems limiting growth in both acidic and calcareous soils (1). Applications of large quantities of fertilizers to correct this problem are not economically sustainable and also lead to environmental pollution. Therefore, efforts have been directed to understanding the molecular basis of plants responses to Pi deficiency and to identifying Pi-responsive genes whose expression can be manipulated to enable plant growth in low-Pi environments. Although a host of genes representing Pi transporters, phosphatases, RNases, and others (2–5) have been identified and characterized by traditional expression studies, knowledge of global changes in the expression of Pi-responsive genes is still lacking. This technical limitation has now been circumvented by recently developed microarray technology, which has been used successfully to study the effects of different abiotic stresses to large number of plant genes in parallel (6). To date, relatively small microarrays containing probes for less than one-third of all genes have been used in *Arabidopsis*, rice, and white lupin to monitor molecular responses to Pi deprivation (7–10). In *Arabidopsis*, the expression of ≈29% of 6,172 genes examined changed in response to Pi starvation for up to 3 days (10). In another study with 8,100 genes of *Arabidopsis*, differential expression of a group of 61 genes was observed after 100 h of Pi starvation (7). These studies represented the evaluation of only a part of the *Arabidopsis* genome for Pi-responsive genes. The advent of new microarray technique of Affymetrix ATH1 GeneChip, containing 22,810 *Arabidopsis* probe sets, has now made it feasible to evaluate the expression of almost all of the genes in the *Arabidopsis* genome with a sensitivity of one transcript per cell (6).

Here, we used the ATH1 GeneChip for a global evaluation of genes that are spatio-temporally regulated in response to short-, medium-, and long-term Pi deprivation. The sensitivity of this technique was corroborated by expression analysis. Identification of differentially expressed genes revealed the coordinated activation and repression of genes involved in many biochemical pathways that are closely associated with plant responses to Pi deficiency. These genes could serve as potential candidates to decipher the components of Pi-sensing mechanisms and developing strategies to improve P efficiency in crops.

Materials and Methods

Supporting Information. For further details, see Figs. 5 and 6 and Tables 1–9, which are published as supporting information on the PNAS web site.

Plant Material and Growth Conditions. *Arabidopsis thaliana* (L.) plants were raised in liquid culture and transferred in a medium with or without Pi as described (11) for evaluating the short-term (3, 6, and 12 h pooled) and medium-term (1 and 2 days pooled) effects of Pi deficiency on the gene expression. For studying long-term effects of Pi deficiency, surface-sterilized seeds were sown in square (12 × 12 cm) Petri dishes on Murashige and Skoog (MS)/10 medium, 0.5% sucrose, 0.8% agar, and supplemented with either 500 μM (P+) or 5 μM (P−) Pi; plants were grown vertically (12). Because some *Arabidopsis* genes are regulated by diurnal rhythm and circadian clocks (13), the roots and the leaves were harvested separately at the beginning and at the end of the photoperiod and pooled. Samples were rinsed with distilled water, blot-dried, and frozen in liquid nitrogen.

RNA Extraction and cRNA Preparation. Total RNA from shoot and root (long-term) and from the whole plant (short- and medium-

Abbreviations: Pi, phosphate; qRT-PCR, quantitative RT-PCR.

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term) was extracted as described (11, 12). cRNA was prepared following the manufacturer's instructions (www.affymetrix.com/support/technical/manual/expression_manual.affx).

Microarray Hybridization and Data Analysis. The Affymetrix microarrays (*Arabidopsis* ATH1 genome array) contain 22,810 probe sets representing ≈80% of the gene sequences on a single array. Labeling and hybridization on the ATH1 microarrays (one sample per chip) were performed according to the manufacturer's instructions (www.affymetrix.com/support/technical/manual/expression_manual.affx). The probe arrays were scanned and further analyzed with GENESPRING software (version 5.0; Silicon Genetics). Normalization per gene and per chip of the $\log_2$ values was performed to allow the comparison of the three independent replicates performed for each set of experiment. In addition, normalization was performed separately for each experiment and plant tissue for all measurements using the flags ("present," "marginal," or "absent") assigned by Affymetrix treatment of the arrays. However, only those transcripts that were declared present or marginal in at least three of six chips were taken into account. Such a procedure facilitated the elimination of transcripts with very low signals in both treatments (declared "absent"). This elimination was achieved by selecting the genes that were absent in at least four microarrays (four, five, or six arrays) and on this basis, complementary lists of genes were generated. Data were then analyzed for each of the experiments comprising plants deprived of Pi for different lengths of time and results were compared with corresponding P(+) plants (control). The genes that revealed significant changes in their expression in P(−) plants were selected by applying a *t* test (one-way ANOVA Welch *t* test, $P = 0.05$). Moreover, a cutoff value of 2-fold change, which is commonly used for microarray analysis (10), was adopted to discriminate expression of genes that were differentially altered in response to Pi deficiency. Annotation of the genes represented on the microarray to genomic ORFs was done with "gene description" and "gene ontology" programs of GENESPRING (based on information from the international *Arabidopsis* Genome Initiative sequencing project in collaboration with The Institute for Genome Research). To test the hybridization quality, "*Arabidopsis* control genes" coding for GAPDH, actin, tubulin, ubiquitin, and several ribosomal RNAs (25S, 5S), spotted by the manufacturer, were verified. The expression ratios [P(−)/P(+)] of the control genes were consistently in the range of 0.81–1.29.

Real-Time Quantitative RT-PCR (qRT-PCR) and Northern Analysis. A few differentially regulated Pi-responsive genes identified from the microarray analysis were selected for validation of the results by qRT-PCR and Northern analysis. cDNA was used for performing qRT-PCR (iCycler Real-Time PCR Detection System, Bio-Rad). Specific primers (T_m, 58–63°C) were designed to generate PCR products between 150 and 350 bp (Table 1). qRT-PCR of GAPDH C (At3g04120) was performed for standardization. Platinium Quantitative PCR SuperMix-UDG (Invitrogen) was used for the PCRs according to the manufacturer's protocol with a minor modification (0.33 μM of each primer in a final volume of 15 μl). Northern analysis was performed as described (11).

S6K2 Promoter Fusion. The gene *AtS6K2* (At3g08720), coding for a ribosomal protein S6 kinase is induced during Pi deficiency (Table 7). A 520-bp fragment of its promoter located upstream of the transcription start was PCR amplified and cloned into the modified binary vector pBIN-^{35}S-mgfp4 at HindIII/XbaI replacing the ^{35}S promoter (kindly provided by J. Haseloff, University of Cambridge, Cambridge, U.K.). Transgenic plants were generated by vacuum infiltration of inflorescence with *Agrobacterium* as described (14).

Quantification of Lipids, Anthocyanins, Macro- and Microelements, and Pi. Lipids, fatty acids, and anthocyanins were quantified as described (15–17). Soluble inorganic Pi was measured in plants from short- and medium-term Pi deficiency treatments (12), whereas samples from long-term treatment were mineralized in 14% HNO_3 in a microwave system (MarsX, CEM) for the determination of macro- and microelements by ICP (ICP OES Vista MPX, Varian).

^{31}P- and ^{13}C-NMR Spectroscopy. Metabolite analysis was performed by NMR as described (18) on leaf extracts from the plants grown in Petri dishes in a medium without sucrose for 20 days. The plants were enriched with ^{13}C in a growth chamber that allowed accurate regulation of the atmospheric gas composition and environmental parameters. The growth chamber conditions were: 10-h photoperiod with 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light, 22/18°C day/night temperature, and 90% humidity. CO_2 (containing 10% of $^{13}CO_2$ purchased from Euriso-Top, Saint-Aubin, France) concentration in the chamber was maintained at 350 $\mu l \cdot liter^{-1}$ during the light period by automatic injections to compensate for photosynthetic assimilation.

Results and Discussion

Phenotypic, Physiological, and Biochemical Modifications in Pi-Starved Plants. The plants were grown on low Pi (5 μM) to reduce the nonspecific effects of complete nutrient deficiency on growth. The effects of Pi deficiency treatments on some of the morphological, biochemical, and physiological traits were evaluated. A significant decline of 55% and 68% in soluble Pi content after 12 and 48 h of Pi deficiency, respectively (Fig. 1*A*) indicated a rapid effect of Pi withdrawal from the growth medium. This response to Pi deprivation could be due to small seed size of *Arabidopsis* with low Pi reserves. Generally, the amount of metabolic reserve in a seed is correlated with its size, which could be critical for seedling survival during various environmental stresses (19). However, a decline in Pi content did not result in a significant accumulation of anthocyanins (data not shown), which is one of the traits associated with Pi deficiency (1). Although many of the well characterized Pi starvation-induced genes including *Pht1;4* (10, 11), are induced during short- and medium-term Pi deficiency, the degree of Pi stress may not be sufficient enough to elicit anthocyanin accumulation. Accumulation of anthocyanins was observed in the leaves only when the plants were subjected to long-term Pi deficiency (Fig. 1*B*). In addition, there was a reduction in leaf size and a modification in the root architecture (i.e., higher densities of lateral roots and root hairs), typical responses of plants to Pi deficiency (20). Pi-deficient plants also exhibited an early arrest of the primary root growth (0.3 $cm \cdot day^{-1}$ and 2 $cm \cdot day^{-1}$ in low and high Pi, respectively), whereas secondary roots continued to grow (Fig. 1*C*). Long-term Pi deficiency-induced modifications in morphological and biochemical traits could be attributed to a decline in the total Pi content in the leaves and the roots (Fig. 1*D*). A significant decline in the concentration of K in leaves, and an appreciable increase in the concentration of S in both the roots and the leaves, was also observed (Fig. 1*D*). Likewise, higher accumulation of some micronutrients (i.e., Fe, Zn) was observed during long-term Pi deprivation (Fig. 1*E*). This observation suggests that, during Pi deficiency, the activity and/or utilization of other nutrients was altered. This finding is not surprising considering the importance of Pi in numerous energy-requiring metabolic and transport processes (21). Effects of long-term Pi deficiency on various water-soluble metabolites involved in P and C metabolism were also examined. ^{13}C-NMR spectroscopy analysis showed a reduction in the concentration of fumarate, whereas glutamine and arginine increased in Pi-deficient plants (Fig. 1*F*). The level of fumarate, a storage form of C, is known to be affected during Pi deficiency (22). Accumulation of polyaminated glutamine and arginine in Pi-deficient plants could be an adaptive response toward meeting the demand for N for protein synthesis. The ^{31}P-NMR analysis also revealed a decrease in the

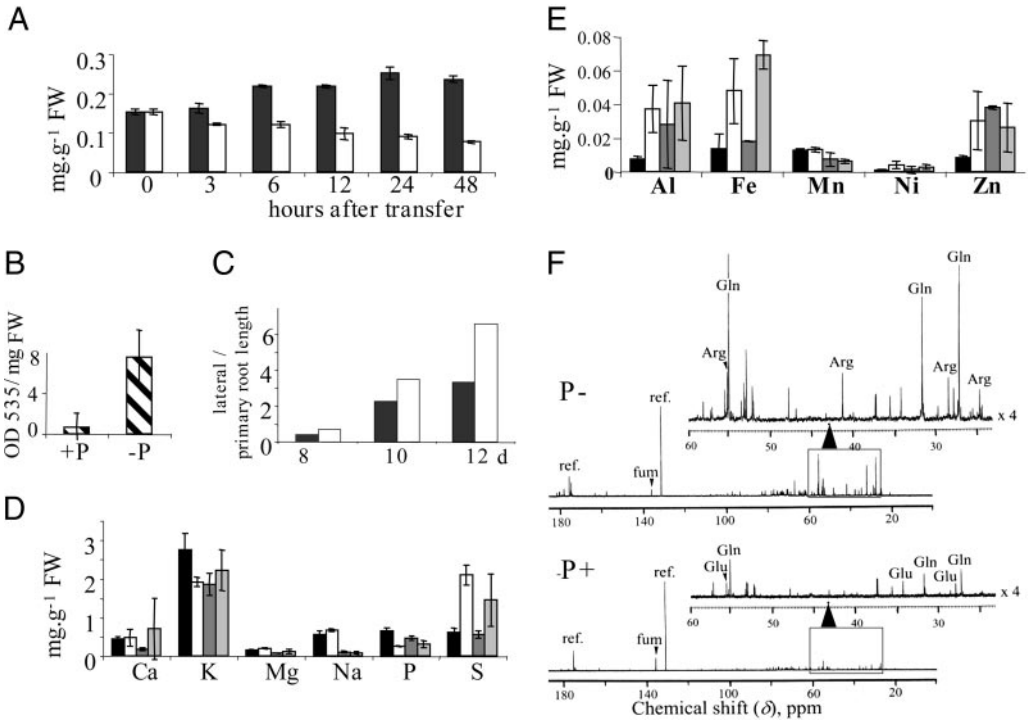

Fig. 1. Effects of Pi deficiency on some of the traits of *Arabidopsis*. (*A*) Soluble Pi in whole plant after transfer to low (open bars) or high (filled bars) Pi. (*B*) Anthocyanin accumulation in the leaves in response to Pi deficiency. (*C*) secondary/primary root length ratio in low-Pi (open bars) or high-Pi (filled bars). Macroelements (*D*) and microelements (*E*) in the high Pi (black bars) and low Pi (white bars) leaves and high Pi (dark gray bars) and low Pi (light gray bars) roots. (*F*) ^{13}C-NMR analysis. fum, fumarate; Glu, glutamate; Gln, glutamine; Arg, arginine; ref, reference (500 μmol maleate). Upper traces correspond to the enlargement of the corresponding spectrum in the rectangle in the lower trace.

concentrations of inorganic Pi and phosphorylcholine (results not shown). Reductions in both inorganic Pi and soluble phosphorylated compounds in plants grown under Pi-deficiency have been correlated with an inhibition of growth (23).

Microarray Analysis of the Spatio-Temporally Regulated Pi-Responsive Genes. ATH1 microarrays were used to evaluate the spatio-temporal regulation of genes in response to Pi deficiency. During short-term Pi deficiency (Table 2), 72 genes were induced, whereas only four genes were suppressed (Fig. 2). These numbers increased significantly (291 genes induced, 34 genes suppressed) during medium-term Pi starvation (Table 3). At these two time points, 16% of the induced genes had overlapping expression, whereas only 1 gene was suppressed (Fig. 2). Furthermore, the induction (91 genes) or suppression (22 genes) of some genes was only transient. This pattern of gene expression points to a very rapid but transient change occurring even during short periods of Pi deficiency. Modulation in the expression of the Pi-responsive genes correlated with a decline of soluble Pi content during early stages of Pi deficiency treatments (Fig. 1*A*) as reported earlier (10). Long-term Pi deprivation resulted in the differential regulation of 732 genes of which 501 were induced [228 in the roots (Table 4) and 404 in the leaves (Table 5)] and 231 were suppressed, 74 in the roots (Table 4) and 169 in the leaves (Table 5). Expression of 26.1% of the induced and 4.8% of the suppressed genes overlapped in both leaves and roots. Nevertheless, most of the genes were specific for either roots or leaves, suggesting that different plant organs respond to Pi deficiency by activating distinct sets of genes. Comparison of the microarray data from all of the three time points showed the common induction of 48 genes and suppression of only one gene (Fig. 2). These results are in agreement with results from the smaller microarrays with *Arabidopsis*, rice, and white lupin showing similar patterns of gene expression (7–10). The differential expression of Pi-responsive genes is considered an adaptive response by plants to Pi deficiency, which facilitate acquisition of sparingly available Pi and concurrent attenuation of some of the energy-requiring metabolic pathways (21). Furthermore, differential regulation of the closely related members of the purple acid phosphatase gene family was found in the roots and leaves of long-term Pi-starved plants. Likewise, the microarray discriminated among closely related members of Pht1 family (*Pht1;2* and *Pht1;3*), which share >95% sequence homology (2). This finding clearly demonstrates one advantage conferred by the use of Affymetrix ATH1 microarray (6).

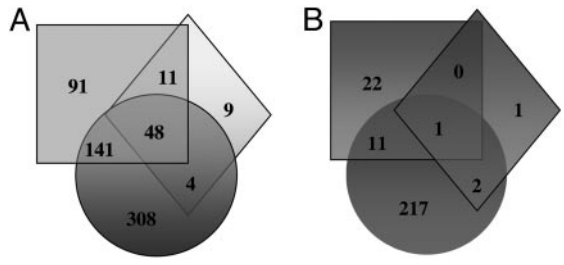

Fig. 2. Number of genes induced (*A*) or repressed (*B*) in low Pi. Comparison of short-term (diamonds), medium-term (squares), and long-term (circles) experiments. The whole plant was analyzed to monitor the regulation of Pi-responsive genes during short- and medium-term experiments. Results from leaves and roots analyzed separately were mixed as long term experiment.

Validation of the ATH1 Microarray Data. The expression levels determined from ATH1 arrays were confirmed by a combination of qRT-PCR, Northern analysis, and promoter-reporter gene fusion studies. Although qRT-PCR results were in general agreement with the microarray data, quantitative differences in the modification of expression level of some of the genes (At5g05340, At2g02990, and At5g56870) were observed (Fig. 3*A*). Extreme expression ratios of some of the genes that were also barely expressed in one of the conditions (declared absent by the Affymetrix analysis) have a poor quantitative significance (raw values of microarray results are provided in Tables 2–5). Earlier microarray analysis had also shown values derived from qPCR generally exceeded those from the array (24). Affymetrix technique was found to be more sensitive than Northern analysis (e.g., At5g56870 transcripts in the leaves, Fig. 3*B*). In addition, GFP fused to the promoter of *AtS6K2* [induced in roots of P(−) plants] revealed *in*

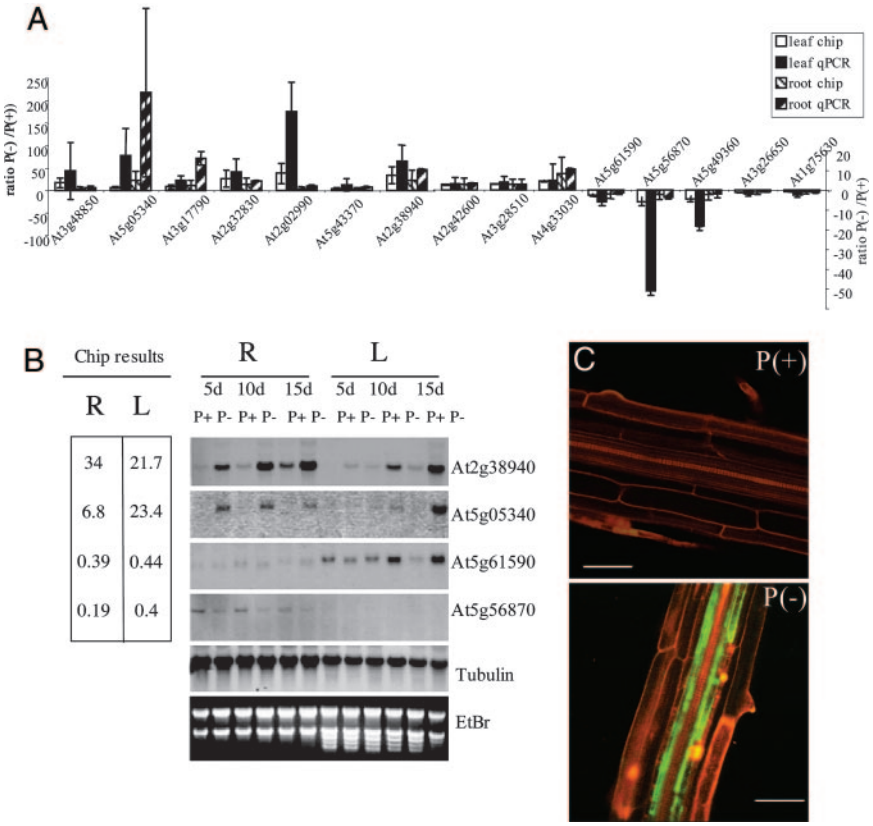

Fig. 3. Validation of ATH1 results. (*A*) Comparison of chip results and q-PCR. P(−)/P(+) ratio in leaves and roots for some selected genes are shown. Two different scales were used for the genes that were up- or down-regulated. Q-PCR and ATH1 results are means and SD of three assays performed on triplicates. (*B*) Northern blot analysis and chip results [ratio P(−)/P(+)] of Pi-responsive genes in leaves (L) and roots (R). Plants were harvested after 5, 10, and 15 days of transfer in low [P(−)] or high Pi [P(+)], and 10 μg of total RNA, isolated from the whole plant, was hybridized with ^{32}P-labeled cDNA fragments of the genes. Equivalence of RNA loading in all of the lanes is shown by ^{32}P-labeled tubulin hybridization and ethidium bromide-stained rRNA (*Lower*). (*C*) Expression of GFP fused to *AtS6K2* (At3g08720) promoter in the stele and emerging lateral roots of transgenic plants grown under high [P(+)] and low Pi [P(−)]. (Scale bar, 50 μm.)

vivo gene expression restricted only to the stele and emerging lateral roots of Pi-deprived plants (Fig. 3*C*). This result illustrates the high sensitivity of the Affymetrix technique, which allowed the evaluation of Pi-responsive genes that are either expressed at low levels or restricted to specific cell types and substantiates the robustness of transcript profiling with Affymetrix arrays (6).

Functional Classification of Pi-Responsive Genes with Altered Expression Patterns. Pi-responsive genes were categorized into different functional groups (Table 7). Approximately 84% and 75% of the induced and suppressed genes, respectively, had known functions. The first group contained genes that are related. In addition to genes harboring general metabolic functions, this study highlights the involvement of genes related to the uptake and transport of Pi and other inorganic ions and to the Pi salvage systems. Among the genes coding the Pht1 family of Pi transporters (2), *Pht1;4* was induced during short-, medium-, and long-term treatments, indicating a rapid and sustained induction of this gene in response to Pi deprivation, which is consistent with its proposed role in both acquisition and mobilization of Pi (11, 12). Some of the members of this family were induced only during medium- and/or long-term Pi-deficiency (Fig. 5*A*). Spatio-temporal regulation of the genes of Pht1 family indicates an apparent lack of functional redundancy among family members (2). Induction of *Pht3;2*, encoding a mitochondrial ATP/ADP antiporter, during medium- and long-term Pi deprivation, was shown (Fig. 5*A*). Genes involved in Pi mobilization from organic compounds such as the gene encoding glucose-6P/Pi translocator were induced only upon prolonged Pi deficiency. These "late" genes are thought to play a role in promoting the efficient use of Pi by the plant (25). It is therefore evident from the microarray results that there is Pi-deficiency-induced spatio-temporal regulation of genes involved in Pi acquisition and its mobilization; complex mechanisms necessary for plants to thrive under Pi deficiency. Late induction of *PHO1;H1*, which is involved in the loading of Pi into the xylem vessels (26), supports this view. Furthermore, there was induction of genes coding sulfate transporters (*SULTR 1;3*, *SULTR 3;4*) that may have facilitated the higher uptake of S during Pi deficiency (Fig. 1*D*), possibly to meet the demand for increased sulfolipid synthesis and for balancing the anion/cation ratio in the absence of Pi ions (27). Mechanisms involved in maintenance of Fe homeostasis (28), such as the differential and coordinated suppression of the iron transporter *IRT1* in the roots and induction of *AtFER1* (encoding a protein involved in iron storage in the chloroplast) in leaves, reflected the plant response to Fe overload induced by Pi-deficiency. These expression data correlated with the elevated concentrations of Fe, and other metals observed in Pi-deficient plants (Fig. 1*E*) may be linked to increased availability in the medium in absence of Pi. This theory was confirmed by running the chemical equilibrium model (VISUAL MINTEQ version 2.30). In addition, genes for several metal and ATP-binding cassette transporters were induced in Pi–deprived plants (Tables 6 and 7). This finding suggests that one adaptive response to Pi deficiency by the plant is to enhance its capacity to absorb and scavenge Pi-complexing metals to release sequestered Pi from the medium.

Another group of genes identified by our experiment is involved in triggering Pi-salvage via the conversion of organic phosphorus into available Pi (4, 29). Of the 29 genes that encode different purple acid phosphatases in *Arabidopsis*, 27 were present on the ATH1 microarray analysis, and 11 of them were induced by Pi deficiency (Fig. 4*B*). In addition, one of the ribonuclease genes (RNS1) was induced early in response to Pi deprivation, suggesting that ribonucleases could play a role in the remobilization of P during Pi deprivation (4). Induction of pyrophosphate-dependent phosphofructo-1-kinase and nucleotide pyrophosphatase genes during medium- and long-term Pi deficiency treatments also represent an important mechanism to reduce Pi demand in plant organs (21). The distinct induction of the expression of genes for glycerol-3-P permeases also points toward the complexity of the various adaptive modifications that occur in the plant for optimal utilization of Pi under limiting conditions.

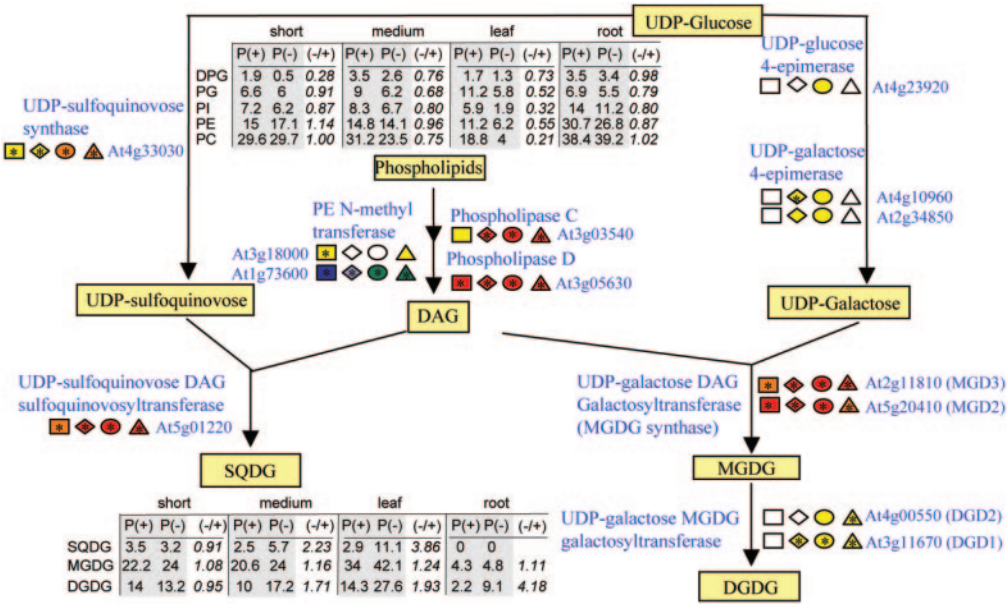

Fig. 4. Transcriptional regulation in pathway of glycosylglyceride biosynthesis in short-term (squares), medium-term (diamonds), and long-term [leaves (circles) and roots (triangles) analyzed separately] experiments. Fold change: red, >10; orange, 4–10; yellow, 2–4; white, 0.5–2; green, 0.25–0.5; pale blue, 0.1–0.25; dark blue, <0.1. *, Significant, one-way ANOVA, $P = 0.05$. (*Insets*) Metabolite quantification [% of total lipids in P(+) and P(−) and ratio P(−)/P(+)] in short-, medium- and long- (leaf, root) term treatments. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SQDG, sulfoquinovosyldiacylglycerol; MGDG, galactosyl-1,2-diacylglycerol; DGDG, digalactosyl-diacylglycerol.

Detailed analysis of Pi-responsive genes also revealed that ≈7% (44 genes) are involved in lipid biosynthetic pathways (Table 6). Of these genes, only two were suppressed. About 50% of the lipid related genes were induced within 2 days of Pi deprivation. Induced genes largely represented those coding for enzymes involved in phospholipid degradation and galacto- and sulfolipid synthesis (Fig. 4). Interestingly, only a few of the genes coding for phospholipases D (At3g05630) and C (At3g03540) were induced during Pi deficiency. These results suggest a role for these genes in the lipid metabolic pathway during Pi deficiency. Genes involved in the subsequent utilization of DAG to synthesize galactolipids (MGDG, DGDG) were strongly up-regulated at early stages of Pi deprivation, which is consistent with previous data (30). Genes coding for MGDG synthases (*MGD2* and *MGD3*) were induced 4- to 10-fold during short-term Pi deprivation; whereas expression of *DGD1* and *DGD2*, coding for DGDG synthases, was enhanced during medium- and long-term Pi deficiency, respectively. Similarly, the genes encoding UDP glucose-4-epimerase and UDP galactose-4-epimerase, which convert UDP-glucose to UDP-galactose (galactolipid precursor), were induced during medium- and long-term Pi deficiency. This induction could facilitate the production of galactose required for galactolipid synthesis. Comparatively, the genes coding for UDP-sulfoquinovose synthase and UDP-sulfoquinovosyl:DAG sulfoquinovosyltransferase exhibited early and sustained induction during Pi deficiency treatments; this was reflected by a 4-fold increase in the level of SQDG in P(−) leaves during long-term Pi deficiency. Although SQDG is not considered essential for plant development (31), under Pi deficiency it could possibly replace PG and may allow photosynthesis to continue despite a reduction in phospholipid content. *Arabidopsis* mutants defective in sulfolipid synthase show impaired growth during Pi deprivation (31). These modulations of lipid biosynthetic pathways indicate a complex mechanism to replace membrane phospholipids with nonphosphorus galacto- and sulfolipids that may have evolved to scavenge and conserve Pi in plants under Pi-limiting conditions (30). These results correlated with variations in phospholipids, sulfolipids and glycosylglycerides (Fig. 4). Alteration of the lipid content became apparent within 2 days, whereby a decrease of PG and PC was compensated by an increase of SQDG and DGDG. In leaves of plants grown in Pi-deficient medium, reduction in the levels of all phospholipids except DPG was observed. Interestingly in the P(−) roots no significant difference was detected in any of the phospholipid species including PC, but there was a substantial increase in the level of DGDG. This finding suggests that lipid composition is more sensitive to Pi deficiency in leaves than in roots, as indicated earlier (16). Despite an early induction of *MGD2* and *MGD3*, there was no significant increase in MGDG level, even during long-term Pi deficiency; this may be due to rapid conversion of MGDG into DGDG by DGD1 and DGD2, whose activity increased during long-term Pi deficiency. Furthermore, the genes *DGD1* and *DGD2* exhibited differential regulation in roots and leaves. Earlier studies have also shown the preferential biogenesis of DGD2 and DGD1 outside of plastids and in the chloroplast membrane, respectively (30, 32). The present microarray analysis revealed an early, sustained, and coordinated induction of a host of Pi-responsive genes involved in Pi acquisition and conversion of organic phosphorus into available Pi. These experiments also indicated that Pi deprivation is perceived at the molecular level as soon as Pi is withdrawn from the medium, suggesting that the plant is able to sense decrease of Pi concentration either in the medium or in cells.

Because accumulation of anthocyanin is a characteristic response of plants to long-term Pi deficiency (Fig. 1*B*), microarray data were also evaluated for the differential regulation of genes involved in its biosynthesis (Fig. 6). At each step of the anthocyanin biosynthetic pathway leading to the synthesis of cyanidin, pelargonidin, and flavonoids, at least one gene was found to be induced. Although the majority of these genes were induced only during long-term Pi deficiency treatment and more specifically in the leaves, a few of them encoding flavonol 3-*O*-glucosyltransferase were significantly up-regulated after 2 days of Pi deficiency. On average, genes were induced 3- to 4-fold, with the notable exceptions of dihydroflavonol-4-reductase, anthocyanidin synthase, and flavonone-3-hydroxylase, which showed 5- to 6-fold induction. Moreover, two genes coding for anthocyanin 5-aromatic acyltransferase and one for anthocyanin2 were induced (Table 7). Interestingly, in Pi-deficient leaf samples, there was an induction of a gene coding for chalcone synthase, which is responsible for the conversion of 4-coumarate to naringenin in flavonoid biosynthetic pathway (Fig. 6). Accumulation of naringenin has been shown to affect the transport of auxin to roots (33), which could be responsible for an altered root architecture, a hallmark of plant responses to Pi deficiency (34). In addition, the expression of some of the genes associated with phytohormone responses was also altered during Pi deficiency. For instance, genes involved in auxin response were modulated during medium-and long-term Pi-deprivation (Table 7).

A gene for an auxin response element, which was induced in Pi-deficient roots, is known to be involved in transcriptional regulation (35). Likewise, ethylene-response genes also showed differential regulation in response to Pi deprivation. The role of auxin and ethylene in the Pi-starvation response is well established and thus it is not surprising to see the changes in the expression of genes involved in the response pathways for these hormones.

Pi deficiency is known to cause significant stress to the plant and this was reflected in modulation of an array of stress-related genes (Tables 6 and 7). A large number of them are related to disease or pathogen resistance (e.g., chitinases, PR-proteins) and toxin catabolism (GSTs). General features of oxidative stress [superoxide dismutase (SOD), peroxidase, GST, cytochrome P450] are also strongly induced in Pi-deficient samples mainly in long-term experiments (Table 7). This finding suggests interactions among several stress-related pathways that may have functional implications in plant survival under Pi deficiency. Pi starvation also resulted in spatio-temporal expression of the genes (10 induced and two suppressed) involved in controlling the level of reactive oxygen species (ROS) (Table 7). They encode different ROS-scavenging enzymes such as SOD, monodehydroascorbate reductase (MDAR), glutathione peroxidase (GPX). One of the genes coding for NADPH oxidase-like enzyme that is responsible for generating ROS showed a transient suppression during medium-term Pi deprivation. Recent microarray analysis of *Arabidopsis*, subjected to different abiotic stresses, had also demonstrated differential regulation of 152 genes coding for different enzymes involved in scavenging and generation of ROS (36).

In Pi-limiting conditions, genes coding for enzymes involved in protein degradation (six genes) and protein biosynthesis (21 genes) are induced and suppressed, respectively (Table 7), suggesting that initiation of Pi recycling processes. Furthermore, genes coding for protein phosphatases and kinases were up-regulated during Pi deficiency. Some of the protein kinase genes were also found to be down-regulated in leaves and roots of Pi-deficient plants (Table 7).

During medium-and long-term Pi deficiency, modulation in the expression of the genes encoding various enzymes involved in cell wall metabolism was observed, and this finding is in conformity with earlier microarray analysis (9). Majority of these induced genes (2- to 10-fold) encoded enzymes like xyloglucan endo-1,4-β-D-glucanase, polygalacturonase inhibiting protein-1, putative pectinesterase, (1,4)-β-mannan endohydrolase precursor, and polygalacturonase inhibiting protein. A few genes for xylosidase or cellulose synthase-like protein were found to be down-regulated. Aside from the production of galactose to support increased synthesis of MGDG and DGDG, increased galactose may also have a critical role in cell wall biosynthesis of the modified root architecture, as shown by transcript analysis of lateral root induction (37). Analysis of long-term Pi-deprived plants revealed that genes for these enzymes were mainly modulated in roots.

Because Pi deficiency responses are known to be regulated at the transcription level (1), microarray data were further analyzed for the Pi-deficiency-induced genes encoding transcription regulatory elements. The expression of a total of 80 genes, presumed to be associated with transcriptional regulation of gene expression, was altered during Pi deficiency (Table 6). A few of them were up-regulated during short-term (five genes) and medium-term (10 genes) Pi deficiency. However, their induction was more pronounced (47 genes) during long-term Pi deprivation. Interestingly, the induction of only a small number of transcription factor genes overlapped during different stages of Pi deficiency. This finding suggests that specific sets of transcription factors are involved in regulating early and late responses of plants to Pi deficiency. To gain further insight into the mode of regulation of Pi-responsive genes, the conserved sequences located upstream (-1 to $-2{,}000$ bp) of the ATG start codon were analyzed (Tables 8 and 9). Promoters of the Pi-responsive genes coding for Pi transporters, phosphatases, and those involved in protein synthesis were found to be significantly enriched with the PHR1 binding sequence, which is recognized by a MYB-domain-containing transcription factor (5). Furthermore, deduced protein sequence of some of the Pi-responsive genes harbored an SPX domain, which has been identified in proteins involved in either transport or sensing of Pi (26).

Induction of phosphatases and kinases further suggests the involvement of numerous posttranslational modifications, which remain to be identified. This study thus presents a global analysis of plant transcriptomic responses to Pi deficiency and physiological and biochemical correlations with observed phenotypes. The list of putative targets established will significantly add to our knowledge about the complex molecular processes associated with Pi nutrition.

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