A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae

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Plants have evolved a number of adaptive responses to cope with growth in conditions of limited phosphate (Pi) supply involving biochemical, metabolic, and developmental changes. We prepared an EMS-mutagenized M_2 population of an *Arabidopsis thaliana* transgenic line harboring a reporter gene specifically responsive to Pi starvation (*AtIPS1*::*GUS*), and screened for mutants altered in Pi starvation regulation. One of the mutants, *phr1* (*phosphate starvation response 1*), displayed reduced response of *AtIPS1*::*GUS* to Pi starvation, and also had a broad range of Pi starvation responses impaired, including the responsiveness of various other Pi starvation-induced genes and metabolic responses, such as the increase in anthocyanin accumulation. *PHR1* was positionally cloned and shown be related to the *PHOSPHORUS STARVATION RESPONSE 1* (*PSR1*) gene from *Chlamydomonas reinhardtii*. A GFP::PHR1 protein fusion was localized in the nucleus independently of Pi status, as is the case for PSR1. *PHR1* is expressed in Pi sufficient conditions and, in contrast to *PSR1*, is only weakly responsive to Pi starvation. PHR1, PSR1, and other members of the protein family share a MYB domain and a predicted coiled–coil (CC) domain, defining a subtype within the MYB superfamily, the MYB–CC family. Therefore, PHR1 was found to bind as a dimer to an imperfect palindromic sequence. PHR1-binding sequences are present in the promoter of Pi starvation-responsive structural genes, indicating that this protein acts downstream in the Pi starvation signaling pathway.

[*Key Words*: *Arabidopsis thaliana*; *Chlamydomonas reinhardtii*; coiled–coil domain; MYB domain; Pi starvation; transcription factor]

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Phosphorus is an essential macronutrient for growth and development of living organisms. It is a constituent of key molecules such as ATP, nucleic acids, or phospholipids, and as phosphate, pyrophosphate, ATP, ADP, or AMP, plays a crucial role in energy transfer, metabolic regulation, and protein activation (Marschner 1995). Phosphorus is one of the most limiting nutrients for plants because the form that is preferentially assimilable, phosphate (Pi), is unevenly distributed in soils and >80% is immobile and not readily available to roots (Holford 1997).

Plants have evolved adaptive responses to cope with growth under conditions of limited phosphate availability (for review, see Raghothama 1999). Biochemical and metabolic adaptations involve changes that increase the availability of endogenous and exogenous inorganic phosphate (Pi), including increased secretion of organic acids from roots, the induction of high affinity phosphate transporters, RNases, and phosphatases (Clarkson 1985; Lipton et al. 1987; Goldstein et al. 1988; Krannitz et al. 1991; Theodorou and Plaxton 1993; Bariola et al. 1994; Duff et al. 1994; Green 1994; Muchhal et al. 1996; Smith et al. 1997; C.M. Liu et al. 1998; H. Liu et al. 1998) and changes in thylakoid lipid composition, whereby a decrease in phosphatidylglycerol is accompanied by an increase in sulpholipids (Essigmann et al. 1998). Additional adaptations involve alterations in the rate of photosynthesis and photosynthate partitioning, the accumulation of the light protecting, anthocyanin pigments, and the utilization of alternative glycolytic or respiration pathways (Duff et al. 1989). These alternative glycolytic and respiratory pathways circumvent steps requiring phosphate or adenylate, contributing to the plant survival during prolonged periods of phosphate deprivation (Duff et al. 1989).

Developmental responses involve changes in root growth and architecture that enhance the exploitation of

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soil phosphate resources and include increases in root/shoot ratio, root hair proliferation and length, and lateral root number (Bates and Lynch 1996). Some plants further modify the soil scavenging potential of their roots by forming lateral proliferations (proteoids) or establishing symbiotic associations with mycorrhizal fungi (for review, see Harrison 1999; Watt and Evans 1999).

Several genes responsive to Pi starvation have been isolated recently from vascular plants (for review, see Raghothama 1999), and encode high-affinity Pi transporters, acid phosphatases, and RNases, among other proteins (Bariola et al. 1994; Muchhal et al. 1996; C.M. Liu et al. 1998; H. Liu et al. 1998; del Pozo et al. 1999; Haran et al. 2000). Members of the *Mt4/TPSI1* gene family are also characterized by their highly specific responsiveness to Pi starvation. These genes encode RNAs with short nonconserved reading frames (Burleigh and Harrison 1997, 1999; Liu et al. 1997; Martín et al. 2000). The existence of various genes responding to Pi starvation suggests that plants are also endowed with a phosphate starvation regulon, as is the case for yeast and *Escherichia coli* (for reviews, see Torriani 1990; Lenburg and O'Shea 1996). In contrast to the situation with these microorganisms, very little is known about the mechanisms governing responses to phosphate starvation in vascular plants. Mutants of *Arabidopsis thaliana* have been isolated that are affected in phosphate accumulation, such as *pho1* or *pho2*, or an acid phosphatase activity (Poirier et al. 1991; Delhaize and Randall 1995; Trull and Deikman 1998), but the structural or regulatory roles of the genes are not known. In addition, several Pi starvation response mutants have been identified recently by use of an elegant conditional genetic screen (Chen et al. 2000), but the corresponding genes have not yet been identified. One regulatory gene of the Pi starvation response has been identified and cloned from the unicellular algae *Chlamydomonas reinhardtii*, and shown to encode a member of the MYB transcription factor superfamily (Wykoff et al. 1999).

We have taken advantage of the availability of transgenic *A. thaliana* plants harboring a reporter gene specifically induced by Pi starvation (*AtIPS1::GUS*; Martín et al. 2000) to initiate the molecular genetic dissection of Pi starvation signaling. We report on the identification and characterization of a phosphate starvation response mutant, *phr1*, which is impaired in various aspects of the response, such as the induction of Pi starvation-responsive genes and anthocyanin synthesis. We show that *PHR1* encodes a transcription factor related to the PHOSPHORUS STARVATION RESPONSE 1 (PSR1) protein from *C. reinhardtii*, suggesting that the increase in complexity of the Pi starvation response during the evolution of multicellular vascular plants was achieved, at least in part, via recruitment of new functions under the control of a MYB-based regulatory system pre-existing in unicellular photosynthetic ancestors. We also show that PHR1 binds as a dimer to sequences present in the promoter of Pi starvation-responsive genes, in line with the presence in this protein of a coiled–coil domain shared with PSR1 and other members of the MYB–CC family.

Results

Isolation of the phosphate starvation response mutant phr1

The *AtIPS1* gene, like other members of the *Mt4/TPSI1* family, is specifically responsive to Pi starvation. A translational fusion between *AtIPS1* and the coding region of the *GUS* gene also displays a specific response to Pi starvation in transgenic *A. thaliana* plants (Martín et al. 2000); transgenic plants harboring this reporter gene are therefore suitable for identifying mutants with altered Pi starvation responses. M_2 seedlings of an EMS-mutagenized population were screened by use of a nondestructive GUS staining assay (Martin et al. 1997). Putative mutants were identified as follows: nine-day-old seedlings (~25,000) grown in medium lacking Pi were stained with GUS for 6 h, during which time plates were examined every hour. Seedlings showing reduced GUS staining were selected as candidates for further analysis; 17 mutant candidates were selected, and M_3 progeny was obtained from 15 of them. After preliminary phenotypic analysis (not shown), *phr1-1*, which was affected in the expression of several Pi starvation-inducible genes, and which did not accumulate anthocyanin during Pi starvation stress (see following section and Figs. 1 and 2, below), was selected for further analysis.

The fact that *phr1-1* did not accumulate anthocyanin in response to Pi starvation suggested a simple screen for *phr1* alleles. After pre-screening 100,000 seedlings grown under Pi starvation conditions for colorless cotyledons, followed by the analysis of GUS activity, we identified a single additional mutant, *phr1-2,* with reduced GUS staining. Results of crossing experiments (not shown) indicated that both mutants were recessive and allelic. Prior to the phenotypic analysis (detailed in the next section), the *phr1-1* allele was backcrossed four times with the wild-type transgenic reporter line.

phr1 *mutant alleles are impaired in different Pi starvation responses*

In addition to the study of the expression of the *AtIPS1::GUS* reporter gene, several metabolic and developmental traits influenced by Pi starvation, as well as the expression of six Pi starvation-responsive genes, were examined in the *phr1-1* and *phr1-2* mutant alleles (Figs. 1 and 2, below). The *phr1* mutations resulted in reduced GUS activity driven by *AtIPS1::GUS* in all parts of Pi starved plants (Fig. 1A). In addition, Pi starvation-induced increases in anthocyanin accumulation and, to a lesser, although in a statistically significant extent ($P < 0.02$), in the root-to-shoot growth ratio, were impaired in the plants homozygous for either of the *phr1* alleles (Fig. 1B,C). The effect of *phr1* mutations on these two traits was specific for Pi starvation stress, as no significant difference was observed between mutant alleles and wild type in anthocyanin accumulation or on the root/shoot growth ratio under nitrogen starvation conditions (Fig. 1B,C). Moreover, the mutants showed increased anthocyanin synthesis in response to the stress-

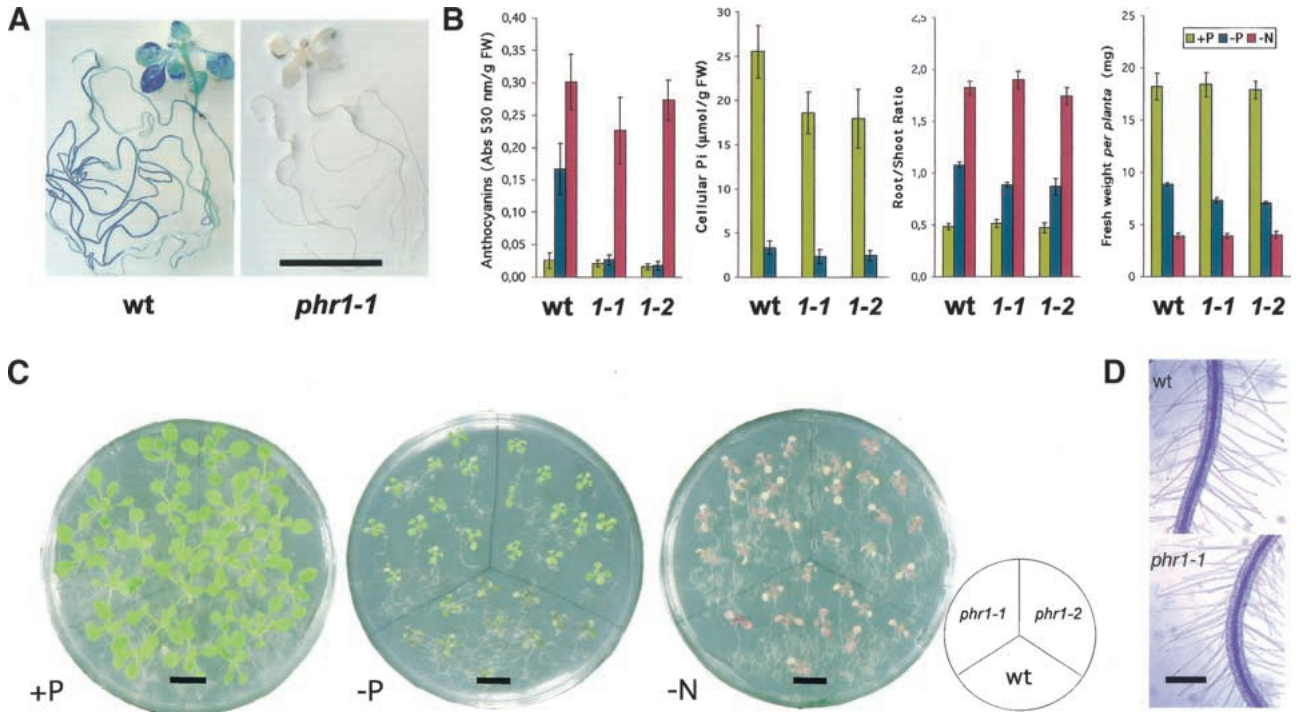


Figure 1. Characterization of the *phr1* mutant alleles. (*A*) Histochemical analysis of GUS activity driven by the *AtIPS1*::*GUS* reporter gene in response to phosphate starvation in wild type (wt; *left*) and in the *phr1-1* mutant (*right*). Scale bar, 1 cm. (*B*) Histograms of metabolic (anthocyanin and Pi content) and developmental (root/shoot growth ratio and total weight) parameters of the wild-type (wt) and *phr1-1* (*1-1*) and *phr1-2* (*1-2*) mutant alleles, grown under different nutrient regimes; complete medium (+P), Pi starvation (–P), or nitrogen starvation (–N) regimes. (*C*) Plates containing the wild-type (*bottom*) and the *phr1-1* (*left*) and *phr1-2* (*right*) mutant alleles grown on different nutrient regimes. Scale bars, 1 cm. (*D*) Detail showing root hairs of wild type and *phr1-1* grown under Pi starvation conditions. Scale bar, 0.5 mm. The analyses were conducted on plants grown in complete medium for 5 d, then transferred to complete medium or to medium lacking Pi or N for 7 d, except in the cases of the Pi and N starvation shown in *C*, in which the starvation lasted for 12 d. Data represent means of at least six independent measurements. Standard deviations are indicated by bars. Statistically significant differences using the Student's *t* test between wild-type and *phr1* alleles were observed for anthocyanin accumulation, root-to-shoot growth ratio, and total weight for plants grown under Pi starvation conditions ($P < 0.01$), as well as for total Pi content for plants grown under Pi sufficient conditions ($P < 0.02$).

related hormones abscisic acid and jasmonic acid similar to wild-type plants (not shown). Both mutations resulted in a statistically significant decrease in the Pi content of the plant ($P < 0.01$) when plants were grown under Pi sufficient conditions, and in a decrease in plant growth under Pi starvation conditions ($P < 0.01$; Fig. 1B). In contrast, no effect of the mutations of *PHR1* was observed for the Pi starvation-induced increases in root hair length and number (Fig. 1D).

The effect of the *phr1* mutations on the expression of Pi starvation-induced genes was examined by use of Northern analysis. Six Pi starvation-induced genes were analyzed, including *AtIPS1* and *At4*, members of the *Mt4/TPSI1* family (Burleigh and Harrison 1997; Martín et al. 2000); *AtPT1*, *AtACP5*, and *RNS1*, encoding a high-affinity Pi transporter, an acid phosphatase, and a RNase, respectively (Bariola et al. 1994; Muchhal et al. 1996; del Pozo et al. 1999); and *AtIPS3*, encoding a protein of unknown function (J.C. del Pozo, J. Iglesias, V. Rubio, A. Leyva, and J. Paz-Ares, unpubl.). The Pi starvation inducibility of all six genes examined was reduced in the plants homozygous for either *phr1* allele, the effect being most pronounced on *AtIPS1*, *At4*, and *RNS1* (Fig. 2).

PHR1 *encodes a member of the MYB superfamily conserved between* A. thaliana *and* C. reinhardtii

The *PHR1* gene was cloned by a map-based chromosome walking procedure on the basis of a cross between the *phr1-1* mutant (Columbia ecotype) and the Landsberg wild-type ecotype. By use of 2100 F_2 seedlings showing the *phr1* phenotype, the *PHR1* gene was mapped to chromosome 4 (between markers RPS2 and prha) by a series of simple sequence length polymorphism markers (SSLP; Bell and Ecker 1994) and cleaved, amplified, polymorphic sequences (CAPS; Konieczny and Ausubel 1993) available in databases. The mapping was further refined by use of new SSLP markers generated from sequence information from the *A. thaliana* genome sequencing project (see Materials and Methods). As a result, the *PHR1* locus was defined to a region of ~120 kb between BACs F20O9 and F16A16 (Fig. 3A). Within this region, candidate genes were considered that showed homology to yeast Pi starvation signaling genes (*PHO80*, *PHO81*, *PHO85*, *PHO2*, and *PHO4*) (Bajwa et al. 1984; Legrain et al. 1986; Sengstag and Hinnen 1987; Madden et al. 1988; Gilliquet et al. 1990; Creasy et al. 1993) or to the recently

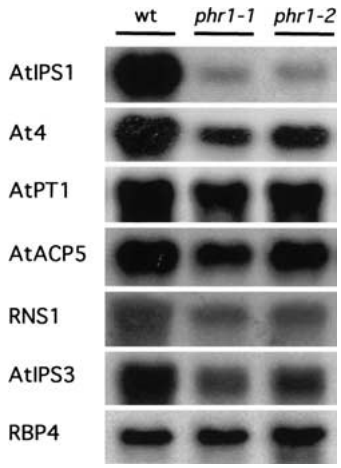


Figure 2. Northern analysis of the effect of *phr1* mutations on the expression of Pi starvation-responsive genes. Wild-type and mutant *phr1-1* and *phr1-2* alleles were grown for 5 d in complete medium, transferred to medium lacking Pi, and collected at 7 d. Total RNA was isolated and RNA gel blots containing 10 µg of these samples were hybridized to the *AtIPS1* probe and subsequently rehybridized to probes corresponding to the related gene as follows: *At4* (Burleigh and Harrison 1999); Pi transporter *AtPT1* (Muchhal et al. 1996); *RNS1* gene (Bariola et al. 1994); type 5 acid phosphatase *AtACP5* (del Pozo et al. 1999); *AtIPS3* gene, encoding a protein of unknown function (J.C. del Pozo, J. Iglesias, V. Rubio, A. Leyva, and J. Paz-Ares, unpubl.); *RBP4* gene (encoding the ribosome binding protein 4; C. Konnz, unpubl.) used as loading control.

defined *PSR1* gene from the unicellular algae *C. reinhardtii* (Wykoff et al. 1999). Only a single gene (*AT4g28610;* protein CAB81449.1) in this region showed homology to any of these genes (specifically, to the *C. reinhardtii PSR1* gene). Transformation of the *phr1-1* mutant with a 5-kb genomic region, spanning the coding region plus sequences 2 kb upstream of the translation initiation codon and 1.3-kb sequences downstream of the termination codon, cloned in the binary vector pBIB (Becker 1990), rescued the wild-type phenotype in 12 of 19 transformants (Fig. 3B).

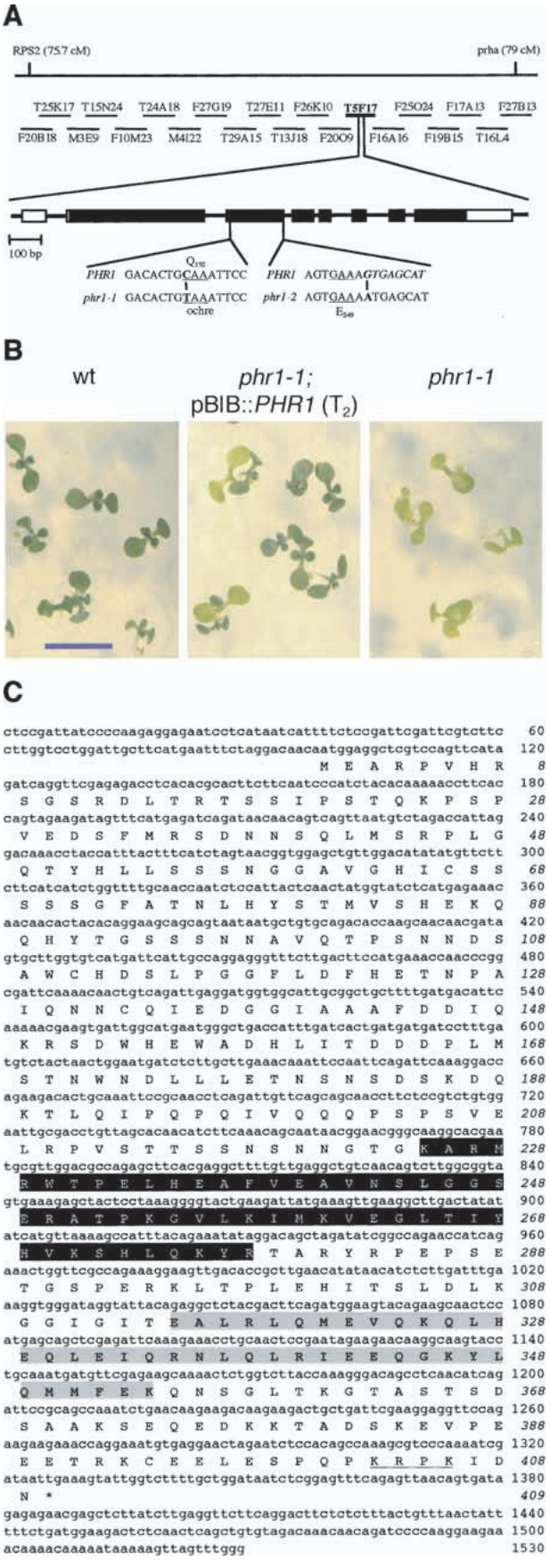
To define experimentally the structure of the *PHR1* gene, overlapping cDNA fragments were isolated following the Marathon RACE protocol (Clontech) by use of oligonucleotides derived from the gene sequence (gene *AT4g28610,* EMBL accession no. AL161573; see Materials and Methods). The cDNA sequence (Fig. 3C) showed that the predicted intron/exon structure in gene *AT4g28610* is correct except for the sixth exon, which uses an AG acceptor site 39 nucleotides downstream of that predicted (position 1195 instead of 1156, in which the A of the start codon is defined as nucleotide 1). In addition, the first 91 nucleotides of the cDNA sequence shown in Figure 3C correspond to a 5′ untranslated exon. Sequencing of the transcribed region of the two alleles revealed that each contained a point mutation. In the case of the *phr1-1* allele, the mutation was a C-to-T transition at nucleotide 663 of the genomic sequence (in which the A of the predicted start codon is defined as nucleotide 1), which results in the replacement of Gln 192 with an ochre stop codon. The *phr1-2* mutation is a G-to-A transition at nucleotide 838, which results in the loss of a splice donor site in the third intron, and probably generates a truncated protein.

The presence of a single repeat MYB domain classifies PHR1, as well as PSR1, as members of the MYB superfamily of DNA-binding proteins. A singular characteristic of the PHR1 and PSR1 proteins is that they share a second motif predicted to adopt a coiled–coil conformation (using `COILS` at www.ch.embnet.org/software/COILS_form.html), which is a potential dimerization motif. Searches in the SPTREMBL or EMBL databanks allowed us to identify 18 proteins (15 in *A. thaliana*) containing both domains (Fig. 4A). A phylogram of all of these proteins was constructed using the neighbor-joining method (Saitou and Nei 1987) of the `CLUSTAL` program (Higgins et al. 1996). Two subgroups can be distinguished, with PHR1 and the *C. reinhardtii* PSR1 protein belonging to the same subgroup (I) (Fig. 4B).

Sequence-specific binding of PHR1 in the promoter of phosphate starvation-responsive genes

To test the sequence-specific DNA-binding properties of PHR1, we performed electrophoretic mobility shift assays (EMSA) with in vitro-translated PHR1 protein and DNA fragments from the 5′ region (upstream of the first ATG) of *AtIPS1*, a gene whose Pi starvation induction is severely impaired in *phr1-1* and *phr1-2* (Fig. 2). Five overlapping fragments encompassing 707 bp upstream of the first ATG were amplified and radiolabeled by PCR and incubated with the in vitro-translated PHR1 protein. A slower migrating band was observed when either of two overlapping fragments (a and b) were incubated with PHR1 (Fig. 5A). To confirm that the shifted band contained PHR1 and to examine which part of the PHR1 protein was responsible for the binding activity observed, several carboxy- or amino-terminally truncated derivatives were generated and subjected to binding and EMSA. As a probe, the 45-bp fragment representing the overlap between fragments a and b (extending from –612 to –568) was used. In all cases in which the retarded band was observed, the mobility shift correlated with the size of the truncated protein, confirming the presence of PHR1 in the protein–DNA complex (Fig 5B). At least 207 amino-terminal amino acids (Δ207Nt) close to the start of the MYB domain could be deleted without compromising DNA binding. In contrast, deletion of 78 amino acids in the carboxy-terminal region abolished DNA binding of the truncated protein. This deletion does not affect the MYB domain, but the second domain conserved between PHR1 and PSR1 from *C. reinhardtii*, in agreement with the idea that the coiled–coil domain of these proteins is also necessary for correct DNA binding.

To further delimit the PHR1-binding site, DNA-binding assays and EMSAs were performed by use of three overlapping deletion derivatives of the 45-bp fragment (spanning positions; –612 to –593, –599 to –575, and –586 to –568), As a result, only one (–599 to –575) was



bound by PHR1 (not shown). Scanning mutagenesis, consisting of consecutive 4-bp substitutions throughout this 25-bp fragment, was performed, and the substitution derivatives were subjected to binding and EMSA with PHR1 (not shown). As a result, a 10-bp sequence (Fig. 5C) was found to be sufficient for PHR1 binding. This sequence included an imperfectly palindromic sequence GCATATTC. Analysis of the effect of mutations on the 10-bp sequence further highlighted the relevance of the imperfect-palindromic sequence for binding by PHR1. Mutations in the sequence of the imperfect palindrome did not greatly affect binding by PHR1. The same was true for mutations in positions 2 and 7 (C and T, respectively), the positions that do not conform to the rules of a canonical palindromic sequence.

Given that mutations at *PHR1* affected the activity of all Pi starvation-induced genes tested, we asked whether they contained the PHR1-binding sequence (GNATATNC, P1BS) in their promoter region. All Pi starvation-induced genes examined contained a P1BS-related sequence (Table 1). The P1BS present in the *AtIPS3* gene is bound by PHR1 (Fig. 5D).

The imperfect-palindromic nature of the PHR1 binding sequence (P1BS) raised the possibility that PHR1 would bind its target as a dimer. To address this question, the strategy of Hope and Struhl (1987) was followed. The full-length and the ΔNt207 derivative were translated in vitro, alone or in combination, and the resulting products were tested in DNA binding and EMSA with P1BS. In addition to the band corresponding to the full-length or the deletion derivative, a band of intermediate mobility appeared when the cotranslation products

Figure 3. Positional cloning and structure of the *PHR1* gene. (*A*) *PHR1* was first mapped between CAPS markers RPS2 and phra, and finally narrowed the physical localization between BACs F20O9 and F16A16. Within this region, a homolog to the *PSR1* gene from *C. reinhardtii* (Wykoff et al. 1999) was identified (*At4g28610*). Sequencing of the region corresponding to the *PSR1* homolog in the two alleles, *phr1-1* and *phr1-2* revealed that each had a mutation in this gene. The mutation in *phr1-1* was a C-to-T transition, causing the introduction of a premature stop codon. The mutation in *phr1-2* was also a G-to-A substitution, which impaired a GT splicing donor site. Nucleotides in the intron are shown in italics. The exon structure derived from comparison of the genomic and cDNA sequences is highlighted with boxes (empty, noncoding exons, or parts; full, coding exons, or parts). (*B*) Complementation of the *phr1-1* mutant with plasmid *pBIB::PHR1*, harboring the *PHR1*-coding region plus 2 kb upstream and 1.3 kb downstream sequences. T_2 progeny of a transgenic plant harboring a copy of the *pBIB::PHR1* T-DNA (*middle*), displays a 3:1 segregation of the colored phenotype when germinated directly in Pi starvation medium. Control progeny from wild-type and *phr1-1* homozygous plants are shown at *left* and *right*, respectively. Scale bar, 0.5 cm. (*C*) Nucleotide and deduced amino acid sequence from the *PHR1* cDNA. The two regions conserved between PHR1 and the *C. reinhardtii* PSR1 protein, corresponding to the MYB domain and to a predicted coiled–coil domain, are highlighted in reverse contrast and gray, respectively. A putative nuclear localization signal is shown (underlined).

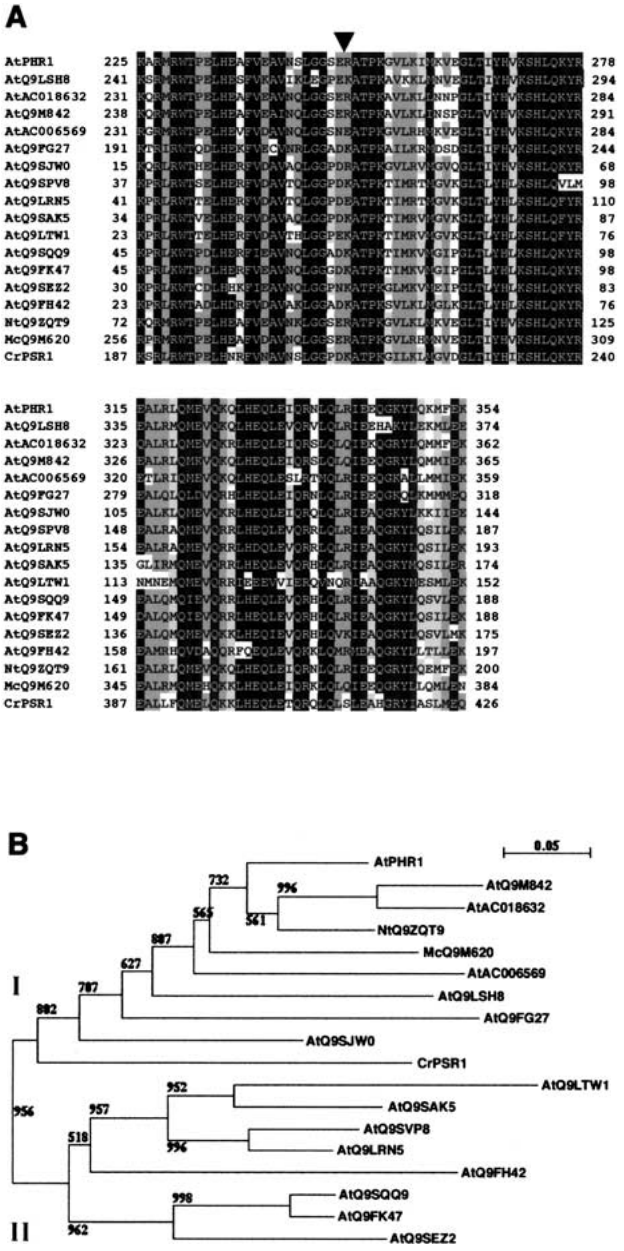


Figure 4. Sequence comparison among PHR1 and related proteins in databanks. (*A*) Alignment of the MYB (*top*) and predicted coiled–coil (*bottom*) conserved domains constructed by use of the CLUSTAL program (Higgins et al. 1996). The protein accession number given in the SPTREMBL or EMBL databanks is preceded by a species identifier as follows: *A. thaliana* (At), *Nicotiana tabacum* (Nt), *Mesembryanthemum crystallinum* (Mc), and *C. reinhardtii* (Cr). Arrowhead indicates the position of an insertion of 8 and 16 amino acid residues in the Q9SVP8 and Q9LRN5 sequences, respectively. Alignment was colored according to the average BLOSUM62 score (0.5–1.49, light gray; 1.5–2.9, gray; ≥3.0, black). (*B*) Phylogram of proteins described in *A* sharing the MYB and predicted coiled–coil conserved domains, constructed by use of the CLUSTAL (Higgins et al. 1996) program and the neighbor-joining method (Saitou and Nei 1987). The bootstrap (Felsenstein 1992) value of each node is indicated (of 1000 samples). Scale bar, 0.05 substitutions/site. To construct the alignment and the tree, only the two conserved regions were considered.

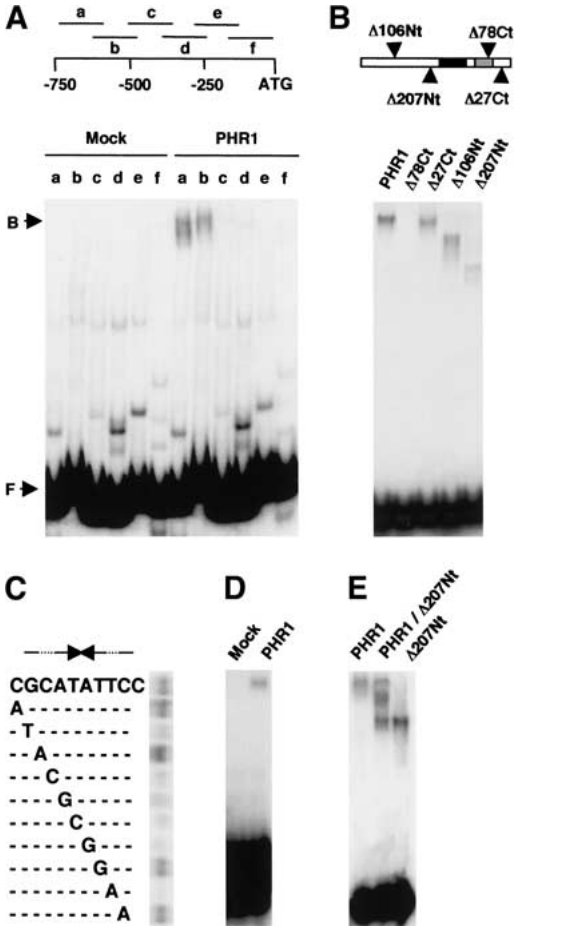


Figure 5. Sequence-specific DNA-binding properties of PHR1. (*A*) EMSA of in vitro translated PHR1 protein binding to overlapping DNA fragments from the 5′ region of *AtIPS1*. Mock-translated reticulocyte lysate was used in the indicated lanes. The DNA fragments used in the experiment are represented diagrammatically at *top*, and span 707 bp upstream of the first ATG of *AtIPS1* (a, from –726 to –568; b, from –612 to –440; c, from –484 to –343; d, from –384 to –245; e, from –285 to –130; f, from –152 to –19). Arrows show PHR1-bound or free DNA (B or F, respectively). Other bands detected in the autoradiograph are shared for each fragment between the lanes corresponding to the mock-translated and to the in vitro-translated PHR1 protein, and thus represent interactions between the fragment and proteins of the reticulocyte lysate. (*B*) EMSA of amino-terminal and carboxy-terminal deletion derivatives binding to the overlapping region between fragments a and b (from –612 to –568). A diagrammatic representation of the PHR1 protein is shown at *top*. The black and gray boxes represent the MYB and the predicted coiled–coil domains, respectively. Arrows show the amino terminus and the carboxyl terminus of the amino- and carboxy-terminally truncated derivatives, respectively. (*C*) Scan mutagenesis of the 10 bp sequence containing the PHR1-binding site (P1BS). Wild-type P1BS is shown with the imperfect palindromic repeats indicated by arrows. Base changes in the mutants tested are indicated in the lines below. Broken lines indicate positions with the same base as in the wild-type P1BS. (*D*) EMSA of PHR1 to the P1BS-related sequence present in the *AtIPS3* promoter (see Table 1). (*E*) PHR1 homodimerization. EMSA was conducted with the full-size PHR1 protein and with the Δ207Nt deletion derivative binding to the sequence used in *B*. Proteins were translated in vitro, alone, or in combination.

were used (Fig. 5E). This intermediate mobility band corresponds to the mobility expected for a heterodimer, indicating that PHR1 recognizes its target as a dimer.

PHR1 *transcription and nuclear localization of a GFP::PHR1 fusion protein is found independent of Pi status*

To evaluate whether control of *PHR1* activity occurs pretranslationally, we examined transcript levels in plants grown under different conditions. As shown in Figure 6, *PHR1* RNA is detected independently of the Pi status of the plant, but in contrast to *PSR1* in *C. reinhardtii* counterpart, is only moderately responsive to Pi starvation (twofold induction for *PHR1* vs. 13-fold induction for *PSR1*; Wykoff et al. 1999).

A common mechanism to regulate nutrient starvation responses is to control the subcellular localization of a transcription factor (O'Neill et al. 1996; Beck and Hall 1999). To test whether this could be the case for PHR1, we prepared a *35S::GFP::PHR1* chimeric gene in which the 35S promoter drives the expression of the coding region of the green fluorescence protein 5 (Siemering et al. 1996) fused to the full-size *PHR1* ORF. The vectors containing the *35S::GFP::PHR1* fusion gene or the *35S::GFP* gene alone were used to transform wild-type and *phr1* mutant plants. Analysis of the *phr1* plants transformed with the fusion protein showed that it could phenotypically complement the Pi starvation response defect, indicating that the GFP::PHR1 fusion protein is functional (data not shown). Microscopic analysis showed that, whereas in the plants harboring the control *GFP* construct, fluorescence was distributed throughout the cell, in the plants harboring the *GFP::PHR1* construct, fluorescence was found in the nucleus, both in the wild-type and in the mutant *phr1* grown under any Pi regimen. This is shown in Figure 7 for the case of wild-type transformed plants, and indicates that the subcellular localization to the nucleus is independent of the Pi status. A similar Pi status-independent nuclear localization was found for the *C. reinhardtii* PSR1 protein (Wykoff et al. 1999).

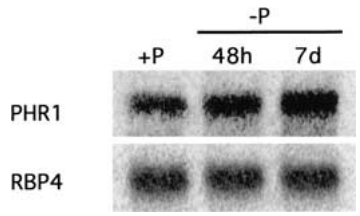


Figure 6. Northern analysis of *PHR1* gene expression. *A. thaliana* plants were grown in complete medium for 5 d, then transferred to medium containing Pi (+P) for 7 d or lacking Pi (–P) for 2 or 7 d. Poly A^+-enriched RNA was isolated from these samples and RNA gel blots containing 0.5 µg of these samples were hybridized to the *PHR1* probe and subsequently rehybridized to a probe corresponding to the *RBP4* gene used as loading control.

Discussion

The mechanisms underlying Pi starvation signaling are well understood in bacteria and yeast (for reviews, see Torriani 1990; Lenburg and O'Shea 1996). However, little is known about this process in vascular plants. In this study, we have isolated and characterized a Pi starvation response mutant and cloned the corresponding gene *PHR1*, the first gene involved in the control of Pi responses in vascular plants to be characterized at the molecular level.

The *phr1* mutations isolated in this work affect a broad spectrum of Pi starvation responses, including some responses shared with other stress responses. For example, they affect anthocyanin accumulation, which is also increased in response to nitrogen starvation and to a large number of environmental stresses, including cold and UV radiation (Dixon and Paiva 1995; Mol et al. 1996). This defect in anthocyanin accumulation in the *phr1* mutants is specific for phosphate starvation, however, as no alteration in its accumulation is observed in these mutants in response to nitrogen starvation (Fig. 1B,D) or to the stress-related hormones tested, abscisic and jasmonic acids (data not shown). These observations indicate that responses common to several different stresses may be controlled by signaling pathways spe-

Table 1. *Sequences related to the PHR1-binding site found at the upstream region of phosphate starvation-responsive genes from several plant species*

Gene	Species	Sequence	Position	Reference
AtlPS1	*Arabidopsis thaliana*	GCATATTC	–598	Martin et al. 2000)
AtlPS3	*Arabidopsis thaliana*	GAATATGC	–570	(J.C. del Pozo, J. Iglesia, V. Rubio,
		GAATATGC	–745	A. Leyva, and J. Paz-Ares, unpubl.)
AtACP5	*Arabidopsis thaliana*	GAATATCC	–290	(del Pozo et al. 1999)
AtPT1	*Arabidopsis thaliana*	GTATATCC	–200	(Muchhal et al. 1996)
At4	*Arabidopsis thaliana*	GCATATTC	–245	(Burleigh and Harrison 1999)
		GTATATGC	–782	
RNS1	*Arabidopsis thaliana*	GTATATAC	–188	(Bariola et al. 1994)
PAP1	*Arabidopsis thaliana*	GGATATAC	–149	(Haran et al. 2000)
TPSI1	*Lycopersicum esculentum*	GCATATCC	–551	(Liu et al. 1997)
Mt4	*Medicago truncatula*	GCATATCC	–230	(Burleigh and Harrison 1997)

The position is given for the most 5′-upstream nucleotide with respect to the first ATG in the transcribed region.

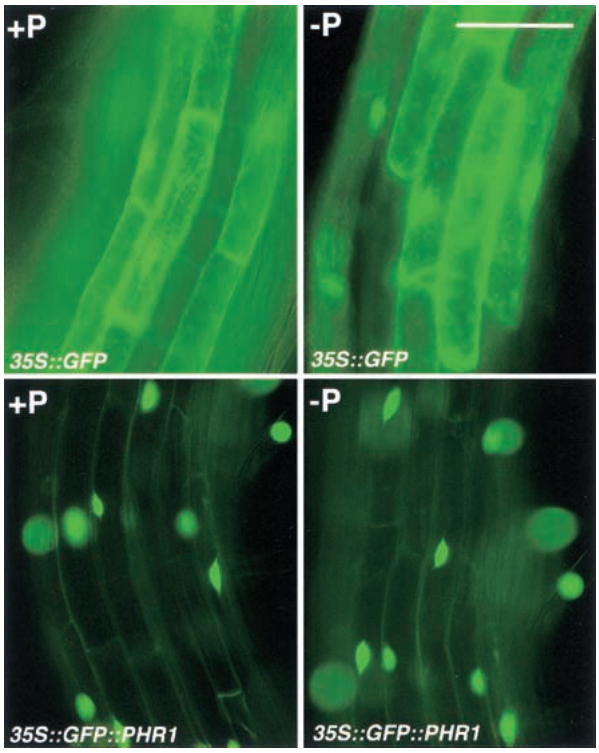


Figure 7. Subcellular localization of a GFP::PHR1 fusion protein in wild-type plants grown under Pi sufficient and Pi starvation conditions. Microscopic images of root cells from transgenic *A. thaliana* Col-0 plants harboring a control gene *35S::GFP* (*top*) or a *35S::GFP::PHR1* fusion gene (*bottom*). Plants were grown in complete medium for 5 d, then transferred to medium containing Pi (+P, *left*) or lacking Pi (–P, *right*) for 7 d before analysis. Scale bar, 20 μm.

cific to each stress type. In addition, they suggest that anthocyanin accumulation is a primary response to Pi starvation, rather than a secondary nonspecific side effect controlled by a common mechanism as suspected previously (Trull et al. 1997), as the *phr1* mutant is stressed with respect to Pi when grown under Pi starvation conditions (Fig. 1B).

PHR1 was cloned by chromosome walking assisted by the choice of gene candidates and was shown to be related to the *PSR1* gene from *C. reinhardtii*. This finding further underlines the importance of *C. reinhardtii* as a model system for photosynthetic eukaryotes. In addition, it shows that evolution of the Pi starvation rescue system in vascular plants involved the recruitment of new responses, such as anthocyanin accumulation, to a conserved regulatory system preexisting in the unicellular ancestors. The PHR1 protein is 409 amino acids in size and contains two domains shared with PSR1, a MYB-related domain, characteristic of DNA-binding proteins, and a predicted coiled–coil domain, potentially involved in protein–protein interactions. In line with the presence of these two domains, DNA-binding assays showed that PHR1 binds to DNA as a dimer in a sequence-specific manner. Thus, the MYB domain is likely to be involved in sequence-specific recognition, and the coiled–coil domain may be the dimerization domain. In agreement with this interpretation, a deletion derivative of PHR1 lacking part of the coiled–coil domain showed impaired high-affinity sequence-specific DNA binding.

The combination of a characteristic MYB and a coiled–coil domain is shared by several other plant proteins (15 in *A. thaliana*) defining a family that we term the MYB–CC family. Phylogenetic analysis within this family reveals two subgroups, I and II. PHR1 and PSR1 from *C. reinhardtii* belong to subgroup I, which contains several other members from *A. thaliana* more closely related to PHR1 than to PSR1. This raises the possibility of functional heterodimeric interactions between MYB–CC proteins through their coiled–coil domains, as well as the possibility of partial redundancy between members of this family. In line with this hypothesis, the two *phr1* mutants studied, which probably carry loss-of-function mutations, only showed partial impairment of most of the molecular/physiological responses studied, in contrast to the case of the *psr1* mutations in *C. reinhardtii*.

Scanning mutagenesis of the DNA target site for PHR1 allowed us to determine the sequence requirements for interaction. PHR1 binds to an imperfect palindrome of 8 bp. Interestingly, this sequence is present in all Pi starvation-induced genes examined except PHR1 itself, including those acting in the scavenging, mobilization, and uptake of Pi. We conclude that PHR1 (or its highly related counterparts) acts downstream in the Pi starvation signal-transduction pathway, and does not appear to be self regulated transcriptionally.

It remains to be shown whether other members of the MYB–CC family control other aspects of the Pi starvation response. It is well known that there are two types of response to Pi deprivation in plants, one which is systemically controlled by whole-plant Pi status and the other governed by local Pi status (Drew 1975; Drew and Saker 1984; Burleigh and Harrison 1999). Notably, of the various responses studied, the only ones that were unaffected in the *phr1* alleles were root hair length and number, which are the only ones known to be controlled by local Pi status (Bates and Lynch 1996).

Expression of *PHR1*, like that of *PSR1*, is detected independent of the Pi status. In both cases, there is increased RNA accumulation in response to Pi starvation, although to a lesser extent in the case of *PHR1*. Similarly, both PHR1 and PSR1 proteins are localized in the nucleus under any Pi regime. The presumed presence of PHR1 in the nucleus of plants grown in Pi sufficient conditions raises the questions as to its role under these conditions and to its regulation. In this regard, it is noticeable that the Pi content of the mutant is lower than that of the wild type when grown under a Pi sufficient regime, suggesting that *PHR1* participates in the control of the plant Pi status under any Pi regime. On the other hand, PHR1 is either post-translationally modified or the protein is a constitutively active, specific component of Pi starvation signaling and a second component of the signaling cascade responds to the signal.

The relevance of the Pi starvation rescue system in the context of phosphorus nutrition efficiency is indicated

by the fact that the *phr1* mutants display reduced growth under Pi starvation conditions (Fig. 1B). The identification and cloning of *PHR1* provides a potentially useful tool toward engineering plants that require less phosphate fertilizer.

Materials and methods

Strains and growth conditions

All *A. thaliana* plants used in this study, including mutants and transgenic plants, were on the Columbia (Col-0) or the Landsberg *erecta* background. Plants were grown in complete medium (+P) as described by Bates and Lynch (1996), using one-strength nutrient salts (Johnson et al. 1957). In the Pi deficient medium (–P), KH_2PO_4 was replaced by equimolar amounts of KCl_2. In nitrogen deficient medium (–N), $Ca(NO_3)_2$ and KNO_3 were replaced by equimolar amounts of $CaCl_2$ and K_2SO_4, respectively. Growth chamber conditions were 22°C, 60% humidity, and a 16-h light/8-h dark photoperiod with 100 μE/m^2 per sec of white light.

Isolation of mutants

A. thaliana seeds (50,000) of a homozygous line harboring the phosphate starvation responsive *AtIPS1::GUS* reporter gene (Martín et al. 2000) were mutagenized with ethyl EMS by treating hydrated seeds (soaked overnight in distilled water) with 0.3% EMS for 13 h, then sowed directly onto soil in 56 pots. Seeds were harvested separately as M_1 families. M_2 seeds (450) from each M_1 family were plated directly on low-phosphorus medium and, after 9 d, screened for GUS expression using a nondestructive assay (Martin et al. 1997). Putative mutants showing impaired phosphate starvation response were recovered on fresh complete medium and transferred to soil. M_3 seeds were retested for inheritance of the observed phenotype.

A search for *phr1-1* alleles was performed by taking advantage of the colorless phenotype of *phr1* plants grown under Pi starvation conditions. M_2 seedlings (100,000) were grown under Pi starvation conditions for 12 d, and plants showing colorless cotyledons were subsequently subjected to analysis of GUS activity, and the one showing reduced GUS activity was selected. Prior to the phenotypic analysis, the *phr1-1* allele was backcrossed four times with the wild-type transgenic reporter line.

Physiological measurements

Anthocyanin was extracted from rosettes of plants grown on complete medium (+P) for 5 d, then transferred to +P, –P, or –N medium for 7 d. Anthocyanin content was measured as described previously (Swain and Hillis 1959). The method of Ames (1966) was used to determine the cellular phosphorus content of seedlings grown on complete medium (+P) for 5 d, and then transferred to +P or –P medium for 7 d. Mean values were compared by use of a Student's *t* test.

Molecular procedures

Routine molecular work was performed as described previously, except where indicated (Sambrook et al. 1989; del Pozo et al. 1999). Genomic DNA was isolated as described by Doyle and Doyle (1990). A full-length cDNA of *PHR1* was isolated by use of the Marathon RACE Kit as described by the manufacturer (Clontech), Expand High Fidelity polymerase (Boehringer), the AP1 primer (from the Marathon RACE Kit) and the *PHR1* primers 5′-GATTCGTCTTCCTTGGTACCGGATTGCTTC-3′ and 5′-GGGATTGAAGAAGTGCGTGTGAGG-3′. These PCR fragments were cloned into *Sma*I-digested pBluescript SK plasmids (Stratagene) and sequenced.

Genetic analysis and positional cloning of PHR1

phr1-1 mutant plants were backcrossed four times to wild-type plants (Col-0 ecotype) to test the linkage of the different phenotypes observed to a single recessive mutation. For mapping purposes, *phr1-1* plants (Col-0 ecotype) were crossed to wild-type plants of the Landsberg *erecta* ecotype (L*er*). Twelve-day-old F_2 seedlings that displayed the anthocyaninless phenotype under phosphorus starvation were isolated. DNA from these plants was prepared and used to analyze linkage of the *phr1-1* mutation to previously described SSLP (Bell and Ecker 1994) and CAPS (Konieczny and Ausubel 1993). *PHR1* was mapped to chromosome 4 between RPS2 and prha CAPS markers. To identify the mutant gene, we generated molecular markers (Table 2).

Plant transformation

Two types of constructs were used to transform *A. thaliana* plants by use of the vacuum infiltration method (Bechtold et al. 1993). One corresponded to the genomic DNA containing the *PHR1* gene, which was used to complement the mutant *phr1-1*. The genomic fragment was obtained by PCR amplification from wild-type plants using as primers: 5′-CAACGAAGATTAC GAAGCTCGAAAGTACG-3′ (positions –2063 to –2035 relative to the start of translation) and 5′-CATCGAAGGCT GAGATACTGCTGGAGGTCGG-3′ (positions 2580 to 2550). The PCR fragment was digested with *Hin*dIII and cloned in the binary vector pBIB plasmid (Becker 1990), which confers hygromycin resistance in planta.

The second construct corresponded to a *GFP::PHR1* translational fusion used to examine the subcellular localization of PHR1. To do this, a *PHR1* fragment of 1327 bp was amplified by PCR using the *PHR1* primers 5′-AAAAAAAGATCTATTC GTCTTCCTTGGTCCTGGATTGC-3′ and 5′- GAAGAACCT CAAGATAAGAGCTCG-3′. This fragment was digested with *Bgl*II and fused translationally to the 3′ end of the *GFP* ORF contained in the pAVA393 plasmid, which encodes GFP version *5* (Siemering et al. 1996). This construct and the pAVA393 (as a positive control) were used to transform both the wild type and the *phr1* mutant.

Visualization of GFP and derivatives

GFP fluorescence was visualized by use of a Leica DM-R microscope (Leica Microsystem, Wetzlar GmBH, Germany). GFP

Table 2. *New markers generated for positional cloning of* PHR1

Marker[a]	Size Col (bp)/Ler	Primers
T15N24	Col (177) > Ler	5′-GAAGTTTCCACAGACGAGG-3′ 5′-CAGCTAAGGGTTCTGCATCC-3′
F27G19	Col (179) > Ler	5′-CCCACTTCAACACAATTCTTCC-3′ 5′-CAATTGCAGCTACAGATCGC-3′
F20O9	Col (135) < Ler	5′-CTCATAATGCATTGAACCC-3′ 5′-GTTACAATACCATGCACG-3′
F16A16	Col (167) > Ler	5′-GCATATCAAACTCAGAGG-3′ 5′-CACATATCTAAATACAGACC-3′
F19B15	Col (181) > Ler	5′-GTATGTTTTTCAATGTATTTGG-3′ 5′-GACAACTACTTTTGTGGATG-3′

[a]All were named for the BAC from which they are derived.

excitation was performed with standard FITC filters. Images of roots were taken through FITC filters with an Apogee KX85 CCD camera (Apogee Instruments), splitting the emission signal into two channels, one for GFP emission (green channel) and one for autofluorescence (red channel, not shown). To visualize nuclei, roots were submerged in a DAPI solution (1 µg/mL DAPI in 100 mM phosphate buffer, 0.5% Triton X-100) for 1 h, and the nuclear-specific dye DAPI was visualized with light microscopy to certify the identity of nuclei (data not shown).

Protein synthesis, DNA-binding reactions, and EMSA

Full-length PHR1 and the PHR1 deletion derivatives were generated by in vitro translation (or cotranslation in the dimerization experiments) using the flexi-rabbit reticulocyte system (Promega) as described (Solano et al. 1997). PCR and labeling of promoter fragments and oligonucleotides, DNA-binding reactions, and EMSA were performed as described (Solano et al. 1995). The 45-bp promoter fragment of the *AtIPS1* gene was obtained by PCR amplification using the following primers: 5′-CAATTTTGGTAACGCGCATATTCC-3′ and 5′-GAGAATT TTGGATCACCGATG-3′.

The deletion derivatives of the 45-bp promoter fragment were obtained by annealing and end filling by use of the Klenow fragment of DNA polymerase I and the following sets of two overlapping primers: AtIPS1AF, 5′-CAATTTTGGTAACGCG CATA-3′; *AtIPS1*AR, 5′-TATGCGCGTTACCAAAATTG-3′; AtIPS1BF, 5′-GCGCATATTCCATCGGATGA-3′; *AtIPS1*BR, 5′-TTGGATCATCCGATGGA-3′; AtIPS1CF, 5′-CGGATGAT CCAAAATTCTC-3′; *AtIPS1*CR, 5′-GAGAATTTTGGATCAT CCG-3′.

Mutant versions of the phosphate starvation response box were obtained by consecutive 4-bp substitutions or by point mutation of the *AtIPS1*B primers.

The promoter fragment of the *AtIPS3* gene was obtained by end filling the following overlapping primers: *AtIPS3*F, 5′-CCAAATATGGGCTAAGACCAACG-3′; *AtIPS3*R, 5′-CACA TTTCATAAGAATGAATATGC-3′.

Computer programs for protein and nucleic acid analysis

Databank searches (Swissprot, EMBL, and NCBI) were performed by use of the FASTA and BLAST programs (Pearson and Lipman 1988; Altschul et al. 1990). Alignment, tree construction by the neighbor-joining method, and its bootstrapping (1000 samples) were performed as described previously (Saitou and Nei 1987; Felsenstein 1992; Higgins et al. 1996).

Accession number of PHR1 *cDNA sequence*

The *PHR1* cDNA sequence has been deposited in the EMBL databank under accession number AJ310799.

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