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Essential role of MYB transcription factor: PvPHR1 and microRNA: PvmiR399 in phosphorus-deficiency signalling in common bean roots

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ABSTRACT

Phosphorus (P), an essential element for plants, is one of the most limiting nutrients for plant growth. A few transcription factor (TF) genes involved in P-starvation signalling have been characterized for *Arabidopsis thaliana* and rice. Crop production of common bean (*Phaseolus vulgaris* L.), the most important legume for human consumption, is often limited by low P in the soil. Despite its agronomic importance, nothing is known about transcriptional regulation in P-deficient bean plants. We functionally characterized the P-deficiency-induced MYB TF TC3604 (Dana Farber Cancer Institute, Common Bean Gene Index v.2.0), ortholog to *AtPHR1* (*PvPHR1*). For its study, we applied RNAi technology in bean composite plants. PvPHR1 is a positive regulator of genes implicated in P transport, remobilization and homeostasis. Although there are no reports on the regulatory roles of microRNAs (miRNA) in bean, we demonstrated that PvmiR399 is an essential component of the PvPHR1 signalling pathway. The analysis of *DICER-like1* (*PvDCL1*) silenced bean composite plants suppressed for accumulation of PvmiR399 and other miRNAs suggested that miR399 is a negative regulator of the ubiquitin E2 conjugase: *PvPHO2* expression. Our results set the basis for understanding the signalling for P-starvation responses in common bean and may contribute to crop improvement.

Key-words: abiotic stress; microRNA regulation; transcription factors.

INTRODUCTION

Phosphorus (P) is an essential element for plant growth and development. One of the most important constraints for plant growth is low P availability because of its association with cations (Fe and Al) or organic compounds that create insoluble inaccessible complexes. Plants have evolved several morphological, physiological and biochemical adaptations to cope with P-deficiency (–P) that include mycorrhizal symbiosis, decreased growth rate, modification of root architecture for increased surface area, remobilization of internal inorganic phosphate, modification of carbon metabolism bypassing P requiring steps, synthesis and secretion of acid phosphatases (AP), exudation of organic acids, and enhanced expression of phosphate transporters (for reviews, see Raghothama 1999; Smith 2001; López-Bucio, Cruz-Ramírez & Herrera-Estrella 2003; Vance, Udhe-Stone & Allan 2003; Shulaev *et al.* 2008).

Several P-deficiency adaptation responses are regulated at the transcriptional level (Raghothama 1999; Franco-Zorrilla *et al.* 2004). Microarray/gene chip analyses in *Arabidopsis thaliana* have allowed the identification of differentially expressed genes involved in the plant adaptation to –P (Hammond *et al.* 2003; Wu *et al.* 2003; Misson *et al.* 2005; Morcuende *et al.* 2007; Müller *et al.* 2007). Likewise, phenotypic analysis of different mutants has demonstrated four P-starvation signalling pathways that are controlled by the *AtPHR1*, *AtWRKY75*, *AtZAT6* and *AtBHLH32* transcription factors (TFs) (Rubio *et al.* 2001; Chen *et al.* 2007; Devaiah, Karthikeyan & Raghothama 2007a; Devaiah, Nagarajna & Raghothama 2007b; Nilsson, Müller & Nielsen 2007). The AtPHR1 signalling pathway regulates the expression of genes implicated in P remobilization, transport, homeostasis and anthocyanin biosynthesis (Rubio *et al.* 2001; Nilsson *et al.* 2007). The AtWRKY75, AtZAT6 and AtBHLH32 signalling pathways, in addition to regulating gene expression of some of these processes including carbon metabolism modification, are implicated in regulating changes in root architecture (Chen *et al.* 2007; Devaiah *et al.* 2007a,b).

Besides the transcriptional regulation of adaptation to –P, P homeostasis in *Arabidopsis* is post-transcriptionally regulated by the microRNA 399 (miR399), an essential component of the PHR1 signalling pathway (Fujii *et al.* 2005; Aung *et al.* 2006; Bari *et al.* 2006; Chiou *et al.* 2006). This microRNA (miRNA) is positively regulated by AtPHR1 (Aung *et al.* 2006; Bari *et al.* 2006) and is negatively regulated by

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IPS1/At4 by means of the target mimicry mechanism (Franco-Zorrilla *et al.* 2007). The relevant role of miR399 during P starvation is exerted by promoting the degradation of the *PHO2* (ubiquitin E2 conjugase) mRNA, because PHO2 is a negative regulator of the high-affinity phosphate transporter PHT1 (Fujii *et al.* 2005; Chiou *et al.* 2006).

Common bean (*Phaseolus vulgaris* L.) is the world's most important grain legume for direct human consumption. Environmental factors such as low soil nitrogen and P levels, and acid soil conditions are important constraints for bean production in most areas of Latin America and Africa where the crop is grown (Brougthon *et al.* 2003; Graham *et al.* 2003). Despite the agronomic importance of bean, there is little information on its regulation of P-starvation responses. In an attempt to understand these responses, we have performed a macroarray analysis of –P bean roots from a Mesoamerican bean genotype and identified 124 differentially expressed genes (Hernández *et al.* 2007). Tian *et al.* (2007) identified 240 differentially expressed genes from P-deficient roots of an Andean bean genotype by screening a suppression subtractive hybridization library. Furthermore, using real-time RT-PCR profiling, we identified three MYB TF induced in P-deficient bean roots (Hernández *et al.* 2007). We hypothesized that these induced TF may be involved in the transcriptional regulation of P-deficiency-induced genes.

The aim of this work was to investigate if the common bean MYB TF [TC3604, tentative consensus assigned by the Computational Biology and Functional Genomics Laboratory, Dana Farber Cancer Institute (DFCI), Common Bean Gene Index, v.2.0], orthologous to *AtPHR1*, hereby denominated as *PvPHR1*, is a regulator of the P-deficiency response. We used a novel bean transformation method (Estrada-Navarrete *et al.* 2007) in conjunction with the RNAi gene-silencing technology, utilized for the first time for this species. In order to investigate if miR399 participates in the PvPHR1 signalling pathway, we analysed the expression of P-deficiency response genes in *PvDCL1*-RNAi silenced bean composite plants.

MATERIALS AND METHODS

Plant material and growth conditions

The common bean (*P. vulgaris*) Mesoamerican 'Negro Jamapa 81' cultivar was used in this study. Seeds were surface sterilized and germinated in sterile conditions for 2 d and then planted in pots with vermiculite. Plants were grown in a greenhouse with a controlled environment (26–28 °C, 16 h photoperiod) and were watered with nutrient solution (Summerfield, Huxley & Minchin 1977) with 1 mM (P sufficiency, +P) or 5 μM PO_4 (P-deficiency, –P). Transcript expression analyses were performed in roots and leaves of plants grown for 21 d. Bean composite plants with transgenic roots were generated as reported (Estrada-Navarrete *et al.* 2007, see further discussion). Hairy roots (3–6 cm long) emerging from the *Agrobacterium rhizogenes* infection site were observed during the second week post-infection. After confirming the presence of reporter gene (see further discussion), their normal (untransformed) root system was cut, and composite plants were replanted in pots with vermiculite. These were grown for 21 d under controlled environmental conditions watered with +P or –P nutrient solution, as described previously. After this period, transgenic roots and non-transformed aerial tissues were collected in liquid nitrogen and were stored (–80 °C) until used for phenotypic analyses. Free P contents were determined from different organs of 21-day-old composite plant as reported (Hernández *et al.* 2007). Data from Table 1 represent the average ± standard error of nine replicates from three independent experiments using composite bean plants with high silencing level (80–95%).

Cloning of full-length PvPHR1 cDNA and of mature PvmiR399

PvPHR1 full-length cDNA sequence was cloned based on ESTs partial sequences obtained from nodule and roots cDNA libraries (Ramírez *et al.* 2005), assigned as TC3604 (DFCI/Common Bean Gene Index, v. 2.0). Two primers were designed for *PvPHR1* PCR gene amplification by 5′ rapid amplification of cDNA ends (RACE) (GSP5: CTGCCAC CACAATCTATGTGCTGACCATGA) and 3′ RACE (GSP3: CCGGATTCCTCATCTGATGAAGGGAAAA AGG). The SMART-RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) was used. PCR-amplified cDNA fragments were cloned into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and were sequenced. For overlaps of new sequences SeqManII program in the DNASTAR software package was used (DNASTAR, Inc., Madison, WI, USA). The amino acid sequence alignment between *AtPHR1* and *PvPHR1* was performed using MultAlign software developed by Corpet (1988). The identification of putative sumoylation sites was carried out using the online tools SUMOplot provided by Abgent primary antibody Company (Flanders Court, San

Table 1. Free phosphate concentrations in different organs of *PvPHR1*-RNAi bean composite plants with high silencing level (80–95%) and control composite plants grown under P deficiency

	Control (empty vector) (μmol g^{-1} FW)	*PvPHR1-RNAi* silenced plants (μmol g^{-1} FW)	*P* value
Leaves	2.48 ± 0.131	0.96 ± 0.125	6.44E-06
Stems	3.74 ± 0.516	0.34 ± 0.03	3.33E-05
Roots	1.01 ± 0.029	0.78 ± 0.105	3.87E-02

Data represent the average ± SE of nine replicates from three independent experiments.

Diego, CA, USA) and for predictions of coiled-coil regions, COILS at http://www.ch.embnet.org/software/COILS/form.html was used. The mature miR399 was cloned from a small RNA library of bean seedlings prepared as reported (Elbashir, Lendeckel & Tuschl 2001). This library was used as template for PCR amplification of the AtmiR399 using consensus sequence-specific primers (5′ADmiR399: ACG GAA TTC CTC ACT TGC CAA AGG AGA; 3′ADmiR399: CTG GAA TTC GCG GTT CAG GGC AA) and adaptor sequence primers. The PCR product was cloned in pCR 2.1-TOPO vector (Invitrogen) and was sequenced.

Plasmid construction and plant transformation

In order to obtain the RNAi constructs, we first constructed the pTDT-DC-RNAi vector. This was derived from pBA-DC-RNAi (Jang *et al.* 2007) by replacing the BASTA gene (cut with StuI and BglII) with the tdTomato gene (cut with EcoR1, blunted, and BamHI) from the pRSET-BtdTomato vector (Shaner *et al.* 2004).

The design of primers for gene amplification was based on the reported EST sequences from bean cDNA libraries (Ramírez *et al.* 2005). To generate RNAi constructs, fragments unique to *PvPHR1* (290 bp) or *PvDCL1* (228 bp) coding sequences were amplified using gene-specific forward primers (*PvPHR1*: 5′ACCTGAAAAAGATAAT TGAAGAA; *PvDCL1*: GGATGATGAAAACGGAAAA AGAA) and caccT7 reverse primer (CACCTAATAC GACTCACTARAGGG). The amplified fragments were cloned in the pENTR/SD/D-TOPO vector and were sequenced (Invitrogen). The resulting pENTR-*PHR1* or pENTR-*DCL1* plasmids were recombined into the pTDT-DC-RNAi binary vector. The correct orientation was confirmed by PCR using the WRKY-5-Rev (GCAGA GGAGGAGAAGCTTCTAG) or WRKY-3-Fwd primer (CTTCTCCAACCACAGGAATTCATC) and caccT7 primer (Supporting Information Fig. S1). The resulting pTDT-*PHR1*-RNAi and pTDT-*DCL1*-RNAi plasmids were introduced by electroporation into *A. rhizogenes* K599 and were then used for plant transformation. Bean composite plants were generated as reported (Estrada-Navarrete *et al.* 2007). Putative transgenic hairy roots were confirmed by checking for the presence of red flourescence, resulting from the expression of the tdTomato reporter gene by confocal microscopy. The original root system and all the transgenic roots, except one, were removed to avoid root chimeras, and the composite plants were grown in the greenhouse for 21 d in +P or –P conditions, as described.

RNA extraction and analysis

Total RNA was isolated from 1 g frozen roots or leaves of bean plants and transgenic roots of bean composite plants, grown under similar +P or –P conditions in independent experiments, as reported (Ramírez *et al.* 2005). Isolated RNA preparations were used for semiquantitative RT-PCR (sRT-PCR) and low-molecular-weight RNA-blot analyses. Quantification of transcript levels from the bean genes was performed by two-step RT-PCR using polythymine deoxynucleotide primer following the manufacturer's instructions (Clontech). Bean gene annotation, primer oligonucleotide sequences and sRT-PCR conditions used are shown in Supporting Information Table S1. ^{32}P-radiolabelled probes for miRNAs were synthesized using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) and the primers shown in Supporting Information Table S1. miRNAs were detected in roots of bean plants and in transgenic roots of bean composite plants grown in +P or –P by low-molecular-weight RNA-gel hybridization. Gels, hybridization and washing conditions were performed as reported (Reyes & Chua 2007). The intensity of bands from sRT-PCR amplification or RNA-blot hybridization was quantified by densitometry using the ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA, USA), and the –P/+P expression ratios were obtained.

RESULTS

Identification of putative PHR1 signalling pathway bean genes

We have identified 10 P-starvation-induced common bean genes encoding for putative TF from different gene families (Graham *et al.* 2006; Hernández *et al.* 2007). In this work, we further characterized the TC3604 gene (DFCI/Common Bean Gene Index, v.2.0, http://compbio.dfci.harvard.edu/tgi/) classified in the MYB superfamily and induced twofold in –P roots (Hernández *et al.* 2007). TC3604 is proposed as the bean ortholog of the *AtPHR1* (AJ310799) gene, TF implicated in signalling of the response to P starvation in *Arabidopsis* and rice (Rubio *et al.* 2001; Zhou *et al.* 2008), hence designated *PvPHR1*. The full-length *PvPHR1* cDNA clone (1.38 kb) (accession number EU500763) contained a 984 bp open-reading frame (ORF) with 63% identity to *AtPHR1*. It encodes for a deduced 328 amino acid protein (Mr = 35.63 kDa). The characteristic MYB domain for DNA binding, coiled-coil domain for protein–protein interaction, and three putative sumoylation sites could be identified from the deduced amino acid sequence (Fig. 1a). *PvPHR1* was induced in leaves (twofold) and roots (1.6-fold) of P-deficient bean plants (Fig. 1b).

In *Arabidopsis*, miR399 is part of the AtPHR1 signalling pathway and plays an important role in P homeostasis (Bari *et al.* 2006; Chiou *et al.* 2006). In an attempt to identify miRNAs possibly involved in bean stress responses, we prepared a miRNA library from bean seedlings and identified sequences that were homologous to *Arabidopsis* miRNAs (Arenas-Huertero *et al.*, unpublished data). From this library, we cloned a miRNA identical in sequence to AtmiR399*b* and *c*, hereby designated as PvmiR399 (Supporting Information Fig. S2a). Moreover, an *in silico* analysis of the National Center for Biotechnology Information (NCBI) dbEST led us to identify clone EG594372 as the reverse complement sequence containing the mature PvmiR399. RNA folding of this

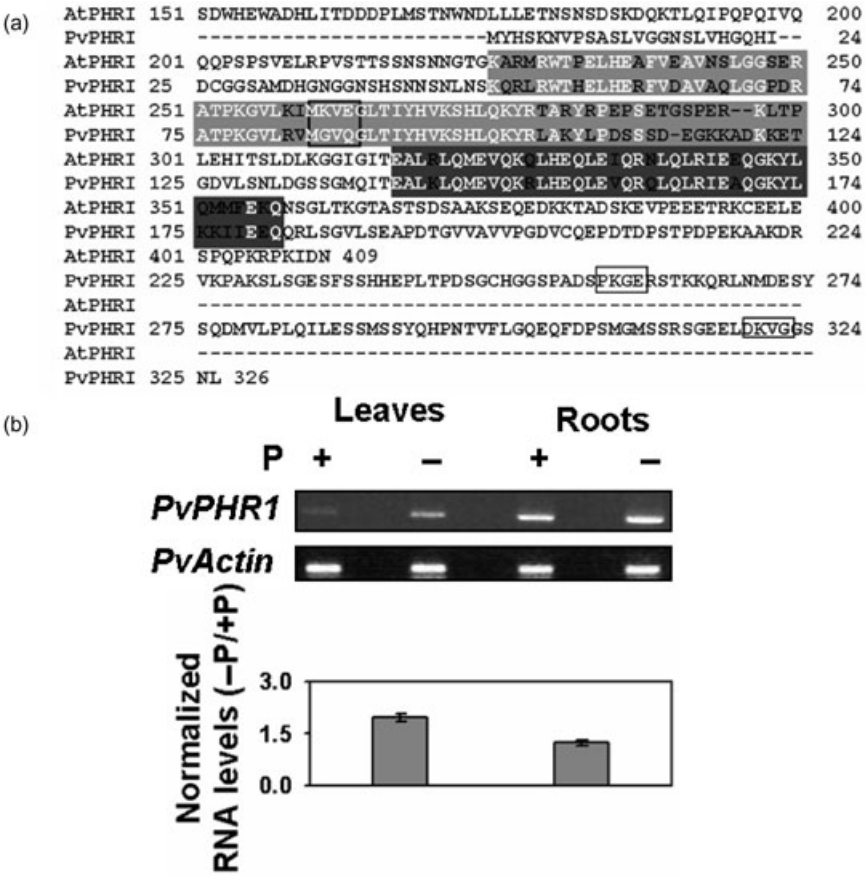

Figure 1. *PvPHR1* cDNA clone. (a) Comparison between *Arabidopsis* PHR1 and PvPHR1 deduced amino acid sequences. Conserved regions corresponding to the MYB domain (light grey), coiled-coil domain (dark grey) and putative sumoylation sites (inside a rectangle) are highlighted. White letters indicate conserved amino acids within each domain. (b) Transcript levels of *PvPHR1* in leaves and roots of P-sufficient (+P) and P-deficient (–P) bean plants were detected by semiquantitative RT-PCR (sRT-PCR) using the actin gene as control for uniform sRT-PCR conditions. The intensity of the bands from amplified products was quantified densitometrically, and the –P/+P normalized expression ratios were obtained. Values are mean ± SE of three biological replicates.

sequence using the mfold program developed by Zuker (2003) is consistent with it being the PvmiR399 precursor (data not shown). PvmiR399 accumulation was highly induced (80-fold) in roots of P-deficient bean plants when compared with control samples under P sufficiency (+P) (Fig. 2a).

In order to investigate the regulatory role of PvmiR399, we searched for bean orthologs of *Dicer-like* (*DCL*) genes implicated in miRNAs biogenesis. Four *DCL* genes involved in the generation of small RNAs have been identified in plants. Specifically, the DCL1 protein is the major player involved in miRNA biogenesis; however, in its absence, it can be partially substituted by DCL2 (Deleris *et al.* 2006; Jones-Rhoades, Bartel & Bartel 2006). Searching the Common Bean Gene Index (DFCI), we identified an EST, CV544176, which has 80% identity to *AtDCL1*, hereby designated as *PvDCL1* (Supporting Information Fig. S2b). Importantly, this gene did not respond to –P treatment (data not shown).

In addition, we identified orthologs of two genes also implicated in P homeostasis: *At4* (TC7206, *Pv4*) and *PHO2* (TC8137, *PvPHO2*) (Aung *et al.* 2006; Shin *et al.* 2006) (Supporting Information Fig. S2c,d). *At4* and its ortholog from the legume *Medicago truncatula* (*Mt4*) belong to the *IPS1* (induced by phosphate starvation) gene family and lack a long ORF, containing instead a series of short overlapping ORFs. These genes play an important role in the translocation of P from roots to shoots (Shin *et al.* 2006). We found a clear induction of *Pv4* in leaves (18-fold) and roots (29-fold) of P-deficient bean plants when compared with +P plants (Fig. 2b). The ubiquitin E2 conjugase *AtPHO2* is a negative regulator of the AtPHR1 signalling pathway (Fujii *et al.* 2005; Chiou *et al.* 2006). In agreement, *PvPHO2* was repressed in leaves (twofold) and roots (1.8-fold) of P-deficient bean plants when compared with +P plants (Fig. 2c).

PvPHR1 participates in P-deficiency signalling in beans

In order to demonstrate the possible role of *PvPHR1* in the regulation of gene expression in P-starved bean roots, we undertook the RNAi gene-silencing approach in composite plants. The protocol for the generation of composite bean plants was recently reported as an alternative for stable transformation, especially for species recalcitrant to transformation such as common bean (Estrada-Navarrete *et al.* 2007).

The pTDT-DC-RNAi, with the Gateway cassette driven by the CaMV 35S promoter and the tdTomato (red fluorescent protein) reporter gene (Shaner *et al.* 2004) for identification of the transgenic roots by confocal microscopy, was constructed. This vector was used for the *PvPHR1*-RNAi gene construct consisting of inverted repeats of a *PvPHR1*

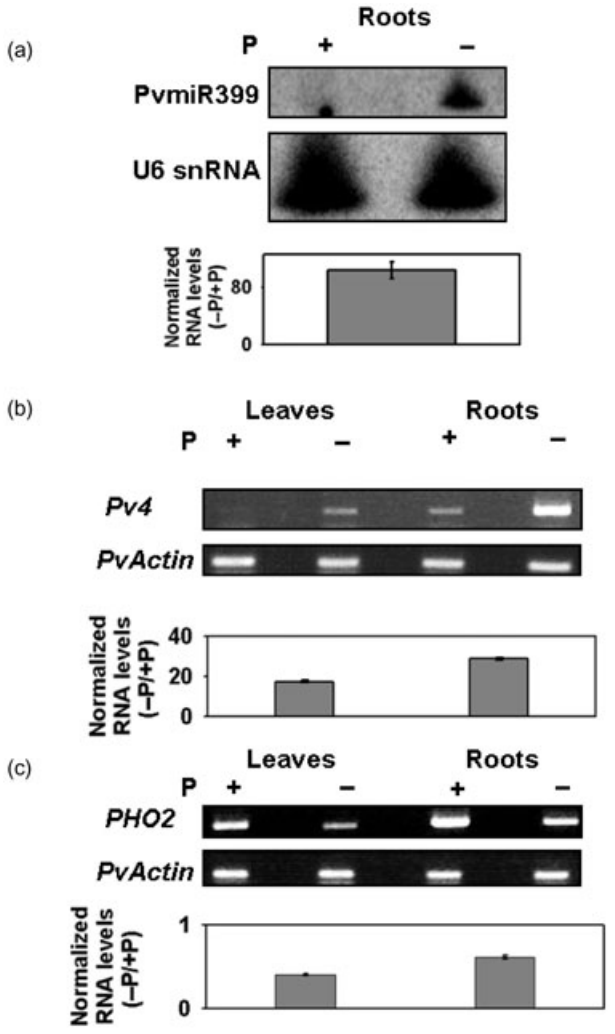

Figure 2. Expression of PvmiR399, *Pv4* and *PvPHO2* in leaves and roots of P-deficient and P-sufficient bean plants. (a) PvmiR399 levels in roots were detected by RNA-blot analysis using U6snRNA as loading control. Transcript levels of *Pv4* (b) and *PvPHO2* (c) in leaves and roots were detected by semiquantitative RT-PCR (sRT-PCR) using the actin gene as control for uniform sRT-PCR conditions. The intensity of the bands was quantified densitometrically, and the –P/+P normalized expression ratios were obtained. Values are mean ± SE of three biological replicates.

gene fragment (Supporting Information Fig. S1a). The expression of *PvPHR1*-RNAi was verified in putative transgenic roots from composite bean plants (Supporting Information Fig. S1b).

In order to confirm *PvPHR1*gene silencing, sRT-PCR analysis was performed in composite bean plants expressing the *PvPHR1*-RNAi construct, as compared with control composite plants transformed with an empty vector. Each transgenic root analysed showed a specific level of *PvPHR1* transcript, indicating different degrees of gene silencing, resulting from a different transformation event in each root. The comparison of *PvPHR1* transcript levels of RNAi versus the control plants grown in –P revealed a reduction ranging from 15 to 95% among the analysed composite plants (Fig. 3a). The level of *PvPHR1* gene silencing showed an inverse correlation with the transcript level of two P-responsive target genes *PvPHT1* (phosphate transporter) and *PvAP5* (Fig. 3a).

To identify the PvPHR1 target genes, we evaluated the transcript level of selected P-deficiency-induced genes (Hernández *et al.* 2007) (Supporting Information Table S1) in the *PvPHR1*-RNAi plant with the highest degree of gene silencing (95%, Fig. 3a) as compared with control (empty vector) composite plants under +P and –P (Fig. 4a). While the –P control plant showed an evident induction of P-responsive genes, the silenced plant showed a reduction of target gene transcript levels (Fig. 4a). Transcript levels of three genes involved in P remobilization, namely *PvAP*, *PvAPC5* and *PvRNS* (RNase), showed a

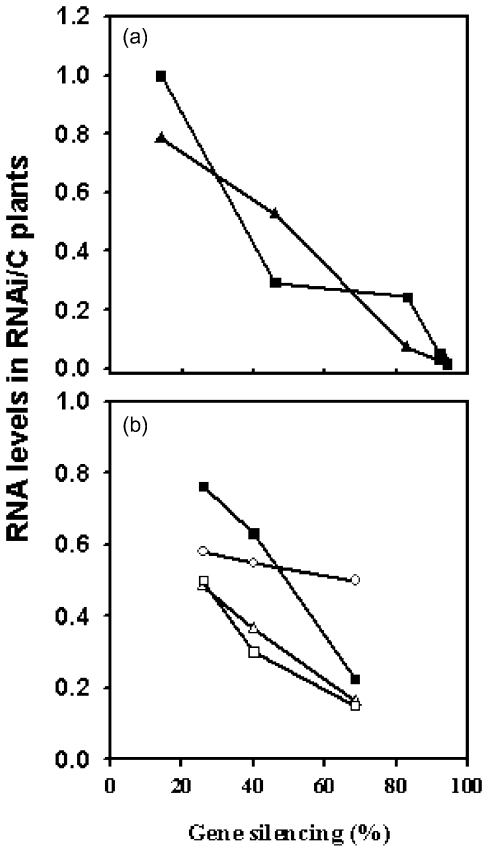

Figure 3. Effect of *PvPHR1* or *PvDCL1* RNAi gene silencing on the expression of P-responsive genes and miRNAs. Data from five *PvPHR1*-RNAi plants (a) and three *PvDCL1*-RNAi plants (b) with increasing percentage of gene silencing each, grown in –P. Transcript levels of *PvPHT1* (■) and *PvAPC5* (▲) were evaluated by semiquantitative RT-PCR (sRT-PCR) in transgenic roots of each composite plant. Transcript levels from PvmiR399 (△) and from orthologs of AtmiR159 (□) and AtmiR160 (○) were evaluated by RNA-blot analysis. The intensity of the bands from amplified products and from hybridization was quantified densitometrically, normalizing that from RNAi silenced plants to that from control (empty vector) plants.

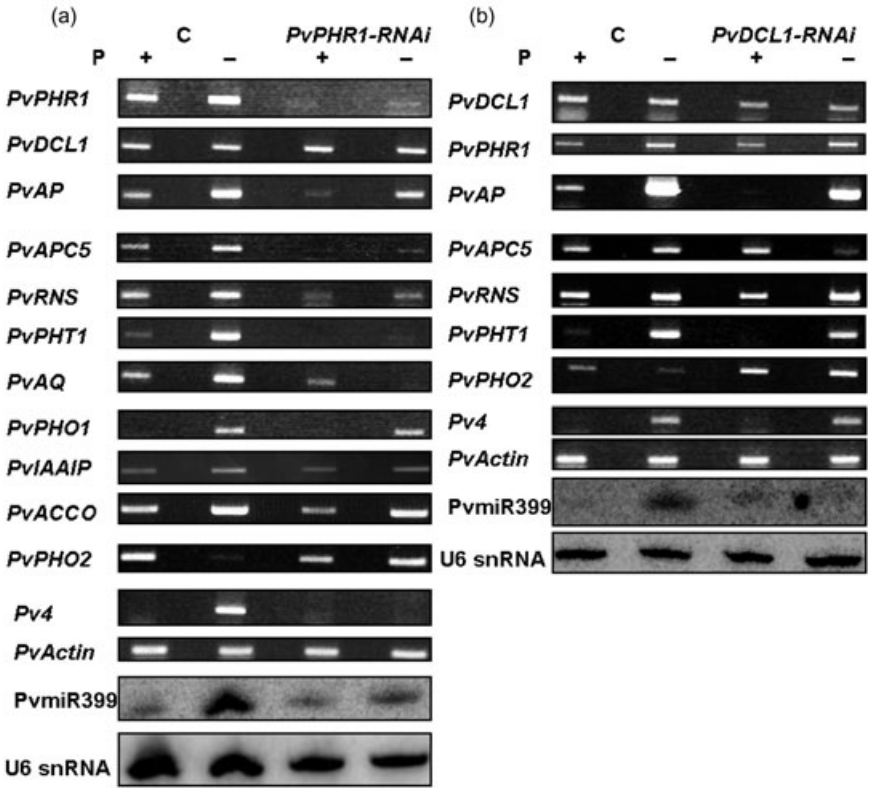

Figure 4. Evaluation of target gene transcript levels in roots of composite bean plants with the highest degree of *PvPHR1* (a) or *PvDCL1* (b) gene silencing. Control (empty vector, C) and RNAi silenced plants grown in +P or –P were analysed. Transcript levels for the indicated *Phaseolus vulgaris* genes were determined by semiquantitative RT-PCR (sRT-PCR), and PvmiR399 levels by RNA-blot analysis using the actin and U6 snRNA as controls, respectively.

reduced response to P deficiency in the *PvPHR1* silenced plant as compared with the control plant. In addition, the –P induction of *PvPHT1* and aquaporin (*PvAQ*), both involved in transport, decreased significantly. The expression of the *PvPHO1* gene, a P translocator involved in loading root P into the xylem vessel, was not affected in *PvPHR1*-RNAi plants. A highly increased expression of *PvPHO2*, involved in P homeostasis, was observed in the *PvPHR1* silenced plant under P deficiency. In addition, the expression of PvmiR399 in –P was significantly reduced despite the unaffected expression of *PvDCL1*. The *Pv4* transcript level was not detected in +P conditions, while in –P, the silenced plant showed a complete inhibition as opposed to the control plant. We determined the transcript levels of 1-aminocyclopropane-1-carboxylate oxidase (*PvACCO*) for ethylene biosynthesis and an indole acetic acid-induced protein gene (*PvIAAIP*). Because both phytohormone-related genes are induced in P-deficient bean roots (Hernández *et al.* 2007), they could be implicated in the root architecture modification response under P starvation (Bonser, Lynch & Snapp 1996; Lynch & Brown 2001; López-Bucio *et al.* 2003). As shown in Fig. 4a, the –P response of these genes was not affected in the *PvPHR1*-RNAi composite bean plants.

The P-deficiency phenotype of bean composite plants was also assessed in relation to the concentration of free P in different plant organs. *PvPHR1*-RNAi composite bean plants showed a decrease of free P content in their leaves (2.5-fold), stems (11-fold) and roots (1.3-fold) as compared with control composite plants (Table 1).

PvmiR399 and PvPHO2 are components of the PvPHR1 signalling pathway in beans

With the rational that miRNA levels would be suppressed in plants with reduced levels of DCL1, we analysed *PvDCL1* silenced composite plants as a tool to investigate the participation of PvmiR399 in the PvPHR1 signalling pathway through the regulation of *PvPHO2*. Composite plants bearing the *PvDCL1*-RNAi gene construct driven by the CaMV 35S promoter (Supporting Information Fig. S1c,d) showed a 20–70% reduction of *PvDCL1* transcript levels as compared with the control plants (Fig. 3b). *PvDCL1*-RNAi composite plants showed an inverse correlation between *PvDCL1* silencing levels and the abundance of three bean miRNAs, homologous to *Arabidopsis* miR399, miR159 and miR160, thus indicating that reduced amounts of *PvDCL1* transcript affected miRNA biogenesis and accumulation (Fig. 3b). An inverse correlation between *PvDCL1* transcript and the P-responsive gene *PvPHT1* was also observed in *PvDCL1*-RNAi plants grown in –P, suggesting the participation of miR399 in signalling for P-deficient response (Fig. 3b).

The *PvDCL1*-RNAi composite plant with the highest degree of gene silencing (70%, Fig. 3b) was further analysed to evaluate the transcript levels of selected P-responsive genes (Hernández *et al.* 2007; Supporting Information Table S1) as compared with control (empty vector) composite plants under +P and –P (Fig. 4b). The *PvDCL1*-RNAi plant showed a significant reduction in the –P response of *PvAP*, *PvAPC5* and *PvPHT1* genes. In

contrast, a high increase in *PvPHO2* transcript levels was observed in the *PvDCL1* silenced plant grown in –P. This behaviour was independent of *PvPHR1*, because the *PvPHR1* –P induction was not affected by *PvDCL1* silencing. Also, the –P gene expression response of *PvRNS* and *Pv4*, two PvPHR1 target genes, was not affected in the *PvDCL1* silenced plant (Fig. 4b).

Although these results suggest the participation of miR399 in the PvPHR1 signalling pathway, the effect of silencing *PvDCL1* may not be solely mediated through this miRNA. *PvDCL1*-RNAi plants showed reduced accumulation of several miRNAs (Fig. 3b) probably involved in diverse biological processes. The effect of other miRNAs on gene expression of –P-responsive genes in bean roots cannot be excluded.

DISCUSSION

In contrast to *Arabidopsis*, common bean and other legumes are not easily amenable to stable genetic transformation, and hence, protocols for high-throughput generation of transgenic legume plants are not available. On the other hand, alternatively, fast, reproducible and efficient protocols for the generation of composite plants, with untransformed shoots using *A. rhizogenes*-mediated transformation, have been established for several legume species including common bean (Estrada-Navarrete *et al.* 2007). This approach had not been used in common bean. In this work, we used the composite plants approach in conjunction with RNAi-silencing technology and showed its feasibility to carry on functional genomics in common bean.

A model summarizing the crucial molecular events in the P-deficiency signalling controlled by *PvPHR1* in bean roots is presented (Fig. 5). Once P deficiency is sensed – either locally or systemically – by unknown molecule(s), this TF positively regulates the expression of target genes involved in P transport (*PvPHT1* and *PvAQ*), remobilization (*PvAP*, *PvAPC5* and *PvRNS*) and homeostasis (*Pv4* and PvmiR399). Transcript analysis of *PvDCL1* silenced plant suggests that PvmiR399 is a negative regulator of *PvPHO2* that can, in turn, directly or indirectly, regulate the expression of P-responsive genes like *PvAPC5*, *PvPHT1* and *PvAP*. However, these effects may also be mediated by other miRNAs that are reduced in PvDCL1 silenced plants. The action of PvmiR399 is apparently regulated by *Pv4* probably through the mimicry mechanism that regulates P homeostasis (Franco-Zorrilla *et al.* 2007). The bean *At4* ortholog that we identified (*Pv4*, TC7206) has a miR399 target site with the same characteristics of *At4*, showing base pairing and a mismatched loop with the PvmiR399 sequence required for the mimicry mechanism.

Silenced *PvPHR1* transgenic roots showed a 23% reduction on free P content under P deficiency as compared with control roots. This phenotype could be related to the drastic reduction of the high-affinity P transporter (*PvPTH1*) that should impair P uptake in the *PHR1*-RNAi plant during P starvation. In addition, a reduction of free P content was observed in non-transformed stems (90%) and leaves (38%) of the silenced plant as compared with plant organs of control composite plants, which can be related to the drastic reduction of *Pv4* transcript, implicated in P homeostasis. *Arabidopsis At4* loss-of-function mutants are impaired in P distribution between roots and shoots during P starvation (Shin *et al.* 2006).

Under P deficiency, bean and other plants modify their root architecture by decreasing primary root growth and increasing lateral roots and root hair formation, and by modifying their root gravitropism to develop shallower and adventitious roots (Bonser *et al.* 1996; Lynch & Brown 2001; López-Bucio *et al.* 2003). However, one of the caveats of the transgenic roots system is its limitation for morphological/physiological analyses of root phenotypes such as the one expected for –P bean plants. We observed that –P induction of the phytohormone-related genes *PvACCO* and *PvIAAIP* was not altered in *PHR1*-RNAi silenced plant. These genes could be involved in modification of root architecture, known to be regulated through phytohormone gradients, principally involving auxins, cytokinins and ethylene (Nacry *et al.* 2005). In *Arabidopsis*, AtWRKY75, AtZAT6 and AtBHLH32, but not AtPHR1, have been implicated in the regulation of root architecture modification (Chen *et al.* 2007; Devaiah *et al.* 2007a,b).

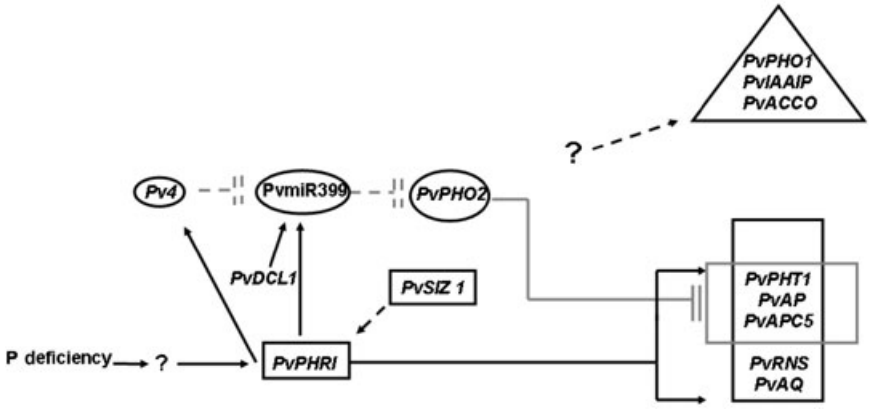

Figure 5. Model for the PHR1 signalling pathway in bean. Positive regulators and the *PvPHR1* target genes are inside the squares. Negative regulators are inside the ovals. The PvmiR399 or *PvPHO2* target genes are inside a grey square. Target genes for an unknown transcription factor (TF) are inside a triangle. Positive regulation is represented with arrows, and negative regulation with grey lines. The dotted lines represent putative positive or negative regulation. Question marks represent unknown receptors or regulators.

The regulation of common target genes by different TF, such as AtPHR1, AtWRKY75 and AtZAT6 (Rubio *et al.* 2001; Devaiah *et al.* 2007a,b), indicates that there is a complex crosstalk occurring during signalling in P-deficient response in *Arabidopsis*. We identified TF candidate genes from different families proposed to be involved in the regulation of P-deficient responses (Graham *et al.* 2006; Hernández *et al.* 2007). Tesfaye *et al.* (2007) have reported that members of the WRKY gene family are P responsive in bean, either by induction or by repression. It is possible that crosstalk among different signalling pathways during P starvation functions in bean, but this remains to be demonstrated.

Recent reviews have emphasized the essential role of TF and miRNAs in gene regulation, representing the largest families of gene regulatory molecules in multicellular organisms. Most of the genes in the eukaryotic genomes are controlled by a combination of transacting factors; miRNAs and TF are linked to one another in gene regulatory networks (Hobert 2008). In this work, we demonstrate the role of a TF, together with a miRNA, in the regulation of P-responsive genes in common bean. It has been proposed that miRNAs are involved in rapid, adaptive changes in gene expression to maintain homeostasis and respond to specific environmental signals (Hobert 2008). In this regard, the role of PvmiR399 could be essential for the regulation of P homeostasis in beans, and its transport into phloem sap, demonstrated in *Brassica napus*, *Cucurbita maxima* and *Oryza sativa* (Buhtz *et al.* 2008; Pant *et al.* 2008; Zhou *et al.* 2008), could be necessary for regulating P homeostasis in different plant organs.

The results presented here set the basis for understanding the signalling events that occur in response to P deficiency in bean, and can be used for improving bean germplasm for tolerance to abiotic stress, either by breeding or by biotechnological approaches.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Diagrams of RNAi constructs and their expression in transgenic roots. Diagrams (not drawn to scale) representing the *PvPHR1*-RNAi (a) and *PvDCL1*-RNAi (c) gene construct Gateway cassettes, cloned in the pTDT-DC-RNAi vector. The constructs consist of inverted repeats of a *PvPHR1* or *PvDCL1* gene fragments, separated by the WRKY intron and driven by the CaMV 35S promoter. Arrows indicate the position of the primers used to verify the orientation of the inverted repeats by PCR amplification. Expression of *PvPHR1*-RNAi (b) and *PvDCL1*-RNAi (d) were determined by sRT-PCR from putative transgenic roots as compared with control (empty vector) roots from composite plants grown in +P and –P conditions. The *TDT* and actin genes were used as internal controls.

Figure S2. Nucleotide sequence of PvmiR399 and *Pv4* and deduced amino acid sequences of PvDCL1 and PvPHO2 and as compared with *Arabidopsis* orthologous genes. (a) Comparison between members of AtmiR399 gene family and homologous PvmiR399 nucleotide sequences. Conserved bases are highlighted in black. (b) Comparison of deduced amino acid sequence of AtDCL1 and PvDCL1; conserved regions corresponding to double-strand RNA-binding domain are highlighted. (c) Alignment of AtmiR399 and PvmiR399 with complementary sequences from *At4* and *Pv4*, respectively. Mispaired nucleotides are shown in red. (d) Comparison of deduced amino acid

sequence of AtPHO2 and PvPHO2; conserved ubiquitin-conjugating enzyme E2 catalytic domain (UBCc) is highlighted.

Table S1. Selected P-responsive common bean genes: annotation, designed primers and sRT-PCR conditions used for expression analysis.

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