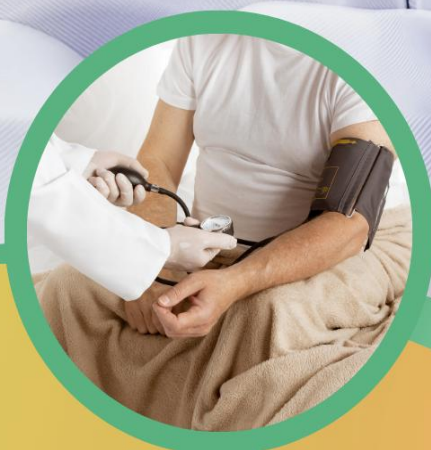


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There are differences in the microRNA 399-Mediated PvPHO2 Regulation within the PvPHR1 Signaling Pathway between two common bean genotypes that respond differently to phosphorus deficiency.

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Abstract: Low phosphate (P) in the soil often limits crop yield of the significant legume, the common bean (*Phaseolus vulgaris*). The common bean's BAT477 and DOR364 genotypes react differently to P shortage. Under P deprivation, plants with the BAT477 P shortage tolerant genotype displayed more phosphate content and root biomass than DOR364 plants. An important part of the reaction to P shortage is the PvPHR1 transcription factor-signaling pathway. The target gene of PvmiR399 is PvPHO2, a negative regulator of this pathway that produces a ubiquitin E2 conjugase that facilitates the breakdown of P-responsive proteins. When P is lacking, PvPHO2 is downregulated in BAT477 plants, but this reaction is not seen in P-starved DOR364 plants. In the 5' UTR region of both genotypes, five potential PvmiR399 binding sites were found. The fifth location (the binding site of PvPHO2) had three base alterations and greater complementarity scores in DOR364 compared to BAT477, but the other four sites displayed the same DNA sequence. Modified 5'RACE tests showed that DOR364 P-starved plants have altered PvmiR399 binding and/or processing. We suggest that the DOR364 genotype would have a stronger PvPHO2-mediated degradation of P-responsive proteins with less P deficit tolerance due to a less effective cleavage of the PvPHO2 mRNA mediated by PvmiR399 itself.

Keywords: *Phaseolus vulgaris*; common bean; phosphorus deficiency; post-transcriptional regulation by microRNAs; PvPHR1/PvmiR399 signaling pathway

1. Introduction

An important macronutrient for the growth and development of plants is phosphorus (P). It is essential for energy transfer and the control of enzyme activity, and it makes up a significant portion of basic macromolecules [1–3]. P is obtained by plants from the soil as phosphates, however in over 30% of the world's arable land, phosphate availability limits crop productivity and plant development [4]. Low P availability in agricultural systems may be made up for by using P fertilizer, however excessive phosphate input can have detrimental effects on the ecosystem. Millions of tons of phosphate are extracted annually from limited ground reserves that are progressively being exhausted. It has been estimated that the world's P reserves will last between 50 and 100 years, or maybe just until 2050, given the anticipated rising need for fertilizers to feed an expanding population [5].

In order to get sufficient P levels under restricted circumstances, plants have developed a variety of morphological, physiological, and biochemical mechanisms [4]. Transcriptional regulation governs a number of P-deficiency responses [6–8]. Numerous responsive genes implicated in adaptation to low P of several plant species, including *Arabidopsis thaliana* (*Arabidopsis*), *Oryza sativa* (rice), *Lupinus albus* (white lupin), and *Phaseolus vulgaris* (common bean), have been identified by global transcriptome studies [9–15]. Furthermore, it has been shown that many transcription factors (TF) regulate P starvation

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signaling pathways by genetic and molecular methods. At least five TF have been identified in Arabidopsis that play crucial roles in low P signaling pathways: PHR1, WRKY75, ZAT6, BHLH32, and MYB62 [16–19].

The best-characterized TF linked to vascular plant P deficit signaling is the Arabidopsis PHR1 gene (PHOSPHATE STARVATION RESPONSE 1) [20], which is similar to PSR1 (PHOSPHORUS STARVATION RESPONSE 1) in *Chlamydomonas reinhardtii* [21] and encodes a MYB protein. PHR1 is only marginally activated after detecting P deficit, and it is expressed under P adequate circumstances. The imperfect palindrome GNATATNC (also known as PHR1-binding sequence) is present in the promoter regions of PHR1-controlled P-responsive genes, such as those involved in P remobilization (like RNases and acid phosphatases), P transport (like PTH1 and PHO1), P homeostasis, and anthocyanin biosynthesis [20,22–24]. PHR1 is located in the nucleus and recognizes this palindrome.

Three PHR1 signaling pathway regulatory genes that encode microRNA 399 (miR399), *At4*, and PHO2/UBC24 have been identified. These genes are important for controlling P homeostasis. Dicer-like 1 (DCL1) and Argonaute 1 (AGO1) proteins process a miR399 precursor that PHR1 positively regulates [25], resulting in mature miR399 that is significantly elevated in P-depleted

vascular tissues, shoots and roots [26]. Also, miR399 has been detected in the phloem sap of rapeseed (*Brassica napus*) and pumpkin (*Cucurbita maxima*) [27,28] and, thus, may serve as a systemic signal for P starvation [26]. During P starvation, miR399 recognizes and degrades its target mRNA, *PHO2/UBC4*, an ubiquitin E2 conjugase, that is a negative regulator of P-responsive genes, such as the high affinity P transporter, PHT1 [29–32]. PHR1 is also a positive regulator of the *At4* gene, from the *IPSI* gene family, that negatively regulates miR399 activity through a target mimicry mechanism [33]. A region within the *At4* sequence is complementary to miR399, but the pairing is interrupted by a mismatch loop at the miRNA cleavage site; thus, *At4* is not cleaved, but instead sequesters miR399, preventing the degradation of its target *PHO2*. This elegant mechanism enables the transient downregulation of *PHO2* by miR399 during P starvation, allowing not only a rapid response to starvation, but also a quick return to normal levels afterwards to prevent P toxicity [33]. Homologous genes of miR399, *PHO2* and *At4/IPSI* have been identified in other plant species, including rice, *Medicago truncatula* and the common bean [34–37]. The regulation of miR399 and *PHO2* is conserved among plants, thus highlighting the evolutionary importance of the PHR1 signaling pathway in response to P deficiency.

The common bean (*Phaseolus vulgaris*) is the world's most important grain legume for direct human consumption. It is widely grown in poor soils of Latin America and Africa, where P deficiency is perhaps the most limiting factor for symbiotic nitrogen fixation, resulting from the association with rhizobial bacteria, and for crop productivity [38]. Research from our group has identified a plethora of genes and metabolites that respond to P deficiency in common bean roots and root nodules [14,15]. In addition, a reverse genetic approach has led to demonstration of the essential role of the PvPHR1 signaling pathway in P-deprived common bean roots [38].

The biodiversity of *P. vulgaris* species is well documented, and genotypes that show differences in the ability to efficiently acquire and utilize P under P-limiting conditions have been characterized [38–42]. The latter includes the BAT477 and DOR364 genotypes that show contrasting responses to P deficiency. Both genotypes were developed at the International Center for Tropical Agriculture (CIAT) in Cali, Colombia. BAT477 is a drought tolerant breeding line that when grown in P-deficient field conditions, shows a good symbiotic N fixation capacity. Instead, DOR364, a

disease-resistant genotype, when grown under P deficiency, forms smaller nodules and shows a much-reduced symbiotic N fixation capacity, due to low P use efficiency [40]. The latter is reflected in lower seed production of DOR364 plants compared with BAT477 plants grown under similar P starvation conditions [41,42]. Different reports suggest that the difference in P acquisition efficiency between these genotypes may be related to their ability to alter rhizosphere conditions that are known to influence the bioavailability of soil P, via the release of protons organic acids or phosphatase-like enzymes [7,43,44].

In this work, we aimed to explore possible factors that may influence or determine the contrasting P deficiency response in the BAT477 vs. DOR364 *P. vulgaris* genotypes. Our comparative analysis focused in the PvPHR1/PvmiR399 signaling pathway that, as we demonstrated, plays an essential role in P deficiency signaling in common bean roots. Our rationale was that a variation in the regulation/function of one or more of the key components from this pathway may be related to the differences in tolerance to P starvation between the two genotypes. Therefore, we did a comparative analysis of the expression levels of key regulatory genes of the PvPHR1/PvmiR399 signaling pathway. We then performed an *in silico* analysis of the *PvPHO2* gene DNA sequence from both genotypes to identify potential PvmiR399 binding sites, as well as their degree of complementarity. Furthermore, we experimentally validated the miR399-dependent cleavage position of *PvPHO2* from two of the five identified putative PvmiR399 binding sites: binding site 3, which is identical, and binding site 1, which shows differences in the degree of PvmiR399/*PvPHO2* complementarity between the two genotypes. The different regulation of *PvPHO2*, a negative regulator of the PvPHR1 pathway, between the BAT477 and DOR364 plants presented here, provides insights related to the contrasting P response of these *P. vulgaris* genotypes.

2. Results and Discussion

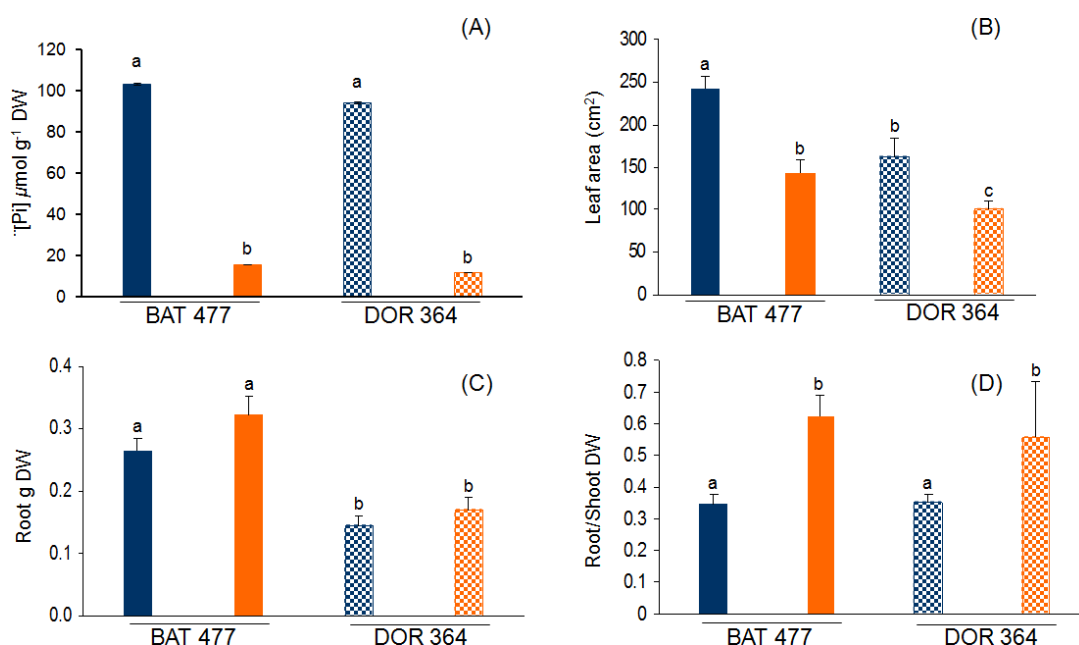
2.1. Comparative Phenotypic Analysis of BAT477 vs. DOR364 Plants under P Deficiency

Compared to the full-nutrient (control) condition, the P shortage (–P) treatment resulted in a 200-fold decreased Pi level in the nutrient solution used to irrigate common bean plants. Both the BAT477 and DOR364 bean genotypes showed signs of P starvation after three weeks of treatment. In comparison to the control condition, the leaf Pi concentration was 6.8 times lower in BAT477 plants and 8.5 times lower in DOR364 plants cultivated in –P (Figure 1A). Bean BAT477 plants had 27.5% more Pi in their leaves than bean DOR364 plants grown under –P circumstances, even though the Pi concentration in the leaves of the control plants from the two genotypes was equal (Figure 1A). Reduced leaf area and increased root development, which raises the root to shoot weight ratio, are typical and well-documented reactions to P deprivation of several plant species [7,45–47]. For the two common bean genotypes, we conducted a comparative study of these responses. In comparison to control plants, both genotypes under –P treatment showed a 1.6-fold decrease in leaf area (Figure 1B). While both genotypes growing under –P conditions had larger root biomass, BAT477 had a 1.9-fold higher root dry weight than DOR364 ($p < 0.05$) (Figure 1C). BAT477 and DOR364 plants had a 1.7-fold and 1.5-fold ($p < 0.05$) larger root to shoot dry weight (DW) ratio, respectively, as a consequence of the elevated root biomass and the halted shoot development seen in the P deficient treatment (Figure 1D).

In terms of growth, nodulation, and nitrogen fixation capacity, genotypes BAT477 and DOR364 under P deficiency during bean-rhizobia symbiotic nitrogen fixation have been reported to exhibit similar outcomes as those shown in Figure 1 in both field and greenhouse conditions [41,42]. In contrast to our growth conditions, where the concentration of Pi was only 16 and 13% for BAT477 and DOR364, respectively, Tang et al. [42] found that the concentration of Pi in plants grown under –P conditions was

only 12% to 30% of those grown in optimal P supply. According to Tang et al. [41,42], both genotypes displayed an increase in the root/shoot ratio under $-P$ circumstances (Figure 1D); however, we also observed an increase in the dry weight of the roots in both genotypes when compared to the control plants (Figure 1C). Our findings (Figure 1) are in line with how many plant species often react to restricted P, where an increase in root development enables the plant to occupy a greater region and increases the chance of obtaining soil P. [7]

Figure 1. Effect of phosphorus (P) deficiency on common bean BAT 477 and DOR 364 genotypes. (A) soluble P_i content in leaves; (B) leaf area from fully expanded leaves; (C) root dry weight; (D) root to shoot dry weight ratio. Plants were grown for three weeks under P sufficient (blue bars) or in P deficient (orange bars) conditions. Values are the mean (\pm SE) from two independent experiments with eight replicates per experiment. Within each panel, bars marked with different letters represent significantly different means according to the statistical analysis ($p \leq 0.05$).

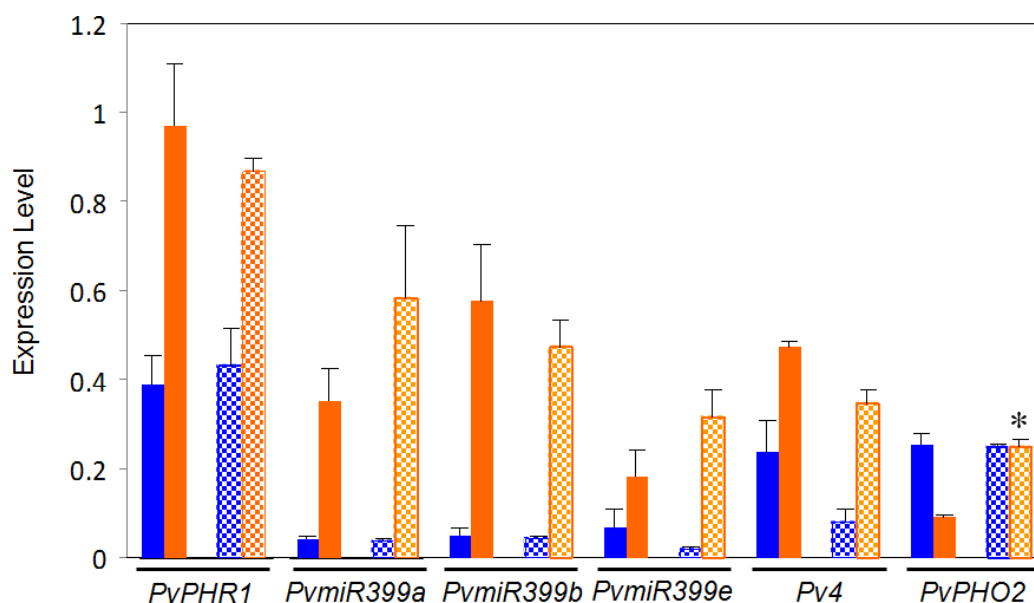


2.2. Comparative Expression Analysis of Regulatory Genes from the P_vPHR1 Signaling Pathway, BAT477 vs. DOR364 P-Deficient Roots

This work aimed to investigate if possible variations in the P_vPHR1 signaling pathway between the BAT477 and DOR364 common bean genotypes could be a factor related to the contrasting P deficiency response observed for these two genotypes [41]. To this end, we first performed a comparative gene expression analysis, through qRT-PCR, of the regulatory genes from this pathway that have been identified in *P. vulgaris* [36]. The regulatory genes tested were those coding for: the MYB family TF *PvRHR1*, *Pv4*, *PvPHO2* (*PvUBC4*) and three isoforms of P_vmiR399, hereby named P_vmiR399a, P_vmiR399b and P_vmiR399e [48]. *PvRHR1* is a positive regulator of P_vmiR399 and P-responsive genes [20,36]. *Pv4* is a negative regulator of P_vmiR399 by means of the target mimicry mechanism [33]. *PvPHO2* (*PvUBC4*) is a negative regulator of P-responsive proteins, and it is the target gene of P_vmiR399 [29,36]. The analysis was performed on roots from plants grown in the P deficiency treatment and in the control condition. As expected, both genotypes showed significant increases in the transcript levels of *PvPHR1*, *Pv4* and *PvmiR399* (isoforms a, b and e) in roots from

plants under $-P$ as compared to control plants (Figure 2). The increase in *PvPHR1* expression ranged from 2.5- to 3-fold. *Pv4* increased by 4.6-fold in DOR364 and 2.0-fold in BAT477 in $-P$ roots. A dramatic increase in the expression levels of the three isoforms of *Pvmir399*, ranging from 9.3- to 15-fold, was observed in both genotypes. Previously, we analyzed the expression of only the *PvmiR399b* isoform in P-deficient bean roots [36]; however, the other two recently detected isoforms [48] also showed a similar trend of induction under P deficiency. As expected, the transcript levels of the negative regulator *PvPHO2* decreased 3.7-fold in P-deficient roots from BAT477 plants; however, a significant difference was observed in the DOR364 genotype that showed similar transcript levels of *PvPHO2* in stress vs. control conditions. DOR364 $-P$ roots showed a 2.7-fold more ($p \leq 0.05$) *PvPHO2* transcript level than BAT477 P-deficient roots (Figure 2).

Figure 2. Expression of regulatory genes from the *PvPHR1* signal pathway in roots of common bean BAT477 (solid bars) and DOR364 (hatched bars) genotypes. Plants were grown for three weeks under P sufficient (blue bars) or in P deficient (orange bars) conditions. Transcript levels were determined by qRT-PCR. Values are the mean (\pm SE) from three independent experiments with nine replicates per experiment. * Significantly different response to P deficiency between BAT477 and DOR364 ($p \leq 0.05$).



The ubiquitin E2 conjugase activity of *PHO2* results in ubiquitination and subsequent degradation of target proteins, such as *PHT1*, a high affinity P transporter. The P deficiency response of *PHO2* transcript degradation mediated by *miR399* allows the required increase in concentration of P transporters during the stress condition [3]. Transgenic plants of *Arabidopsis* expressing a *PHO2* gene where the 5' UTR (un-translated region) was eliminated and, hence, eliminating complementary *miR399* binding sites, resulted in a high and stable *PHO2* transcript levels in P-limited plants. In addition, the transgenic plants expressing the *miR399*-deregulated *PHO2* showed less *PHT1* transcript as compared to the wild-type under $-P$ condition [29].

Our data show a variation in *PvPHO2* regulation of gene expression in the $-P$ sensitive DOR364 genotype: the transcript level was as high in $-P$ as in the control condition (Figure 2). Higher *PvPHO2* content in DOR364 $-P$ roots might lead to an increased degradation of relevant $-P$ responsive

proteins, such as the PHT1 transporter, that in turn result in a less efficient P transport and lower P content in P-deficient conditions. As shown in Figure 1, DOR364 -P roots showed less P_i content as compared to -P roots from BAT477 plants. We postulated that the increased expression of *PvPHO2* is a relevant factor related to DOR364 phenotype of higher sensitivity to P starvation.

Based on the results obtained on the contrasting expression levels of *PvPHO2* between BAT477 and DOR364 in P-deficient roots, we aimed to explore factors that could explain the observed variation in the regulation of gene expression.

In the common bean, as well as in other plants, *PvPHO2* transcript levels are negatively regulated by PvmiR399, and the action of this miRNA is in turn modulated by *Pv4* through a target mimicry mechanism [33,36]. Our data showed that PvmiR399 and *Pv4* are induced in similar levels in -P roots from both genotypes (Figure 2); however, the effectiveness of the target mimicry regulation would not only depend in the amount of PvmiR399 and *Pv4*, but also on the level of base pair complementarity between these transcripts. To explore a possible difference related to the target mimicry mechanism, we cloned the *Pv4* gene from both genotypes and analyzed its DNA sequence. This gene is highly conserved in both genotypes, showing 99% homology. As in common bean Negro Jamapa 81 [36], the *PvmiR399* target site was identified in the *Pv4* coding region from both BAT477 and DOR394 genes, showing 100% identity between the two genotypes (Figure 3). The sequence of the *Pv4* target site for PvmiR399a, b and e has characteristics expected for the target mimicry, showing base pairing and a mismatch loop required for this mechanism [33,36]. Therefore, we can propose that the target mimicry mechanism is regulating PvmiR399 action in a similar manner in both common bean genotypes, and we cannot consider this mechanism as a significant factor contributing to their contrasting P deficiency response.

Figure 3. Alignment of all miR399 isoforms with the complementarity sequence of *Pv4* gene (blue) from BAT477 and DOR364 genotypes. Nucleotides displaying full base pairing are shown in black, and those displaying a mismatch are shown in red. * Corresponds to positions involved in interfering with cleavage through the mimicry mechanism (loop).

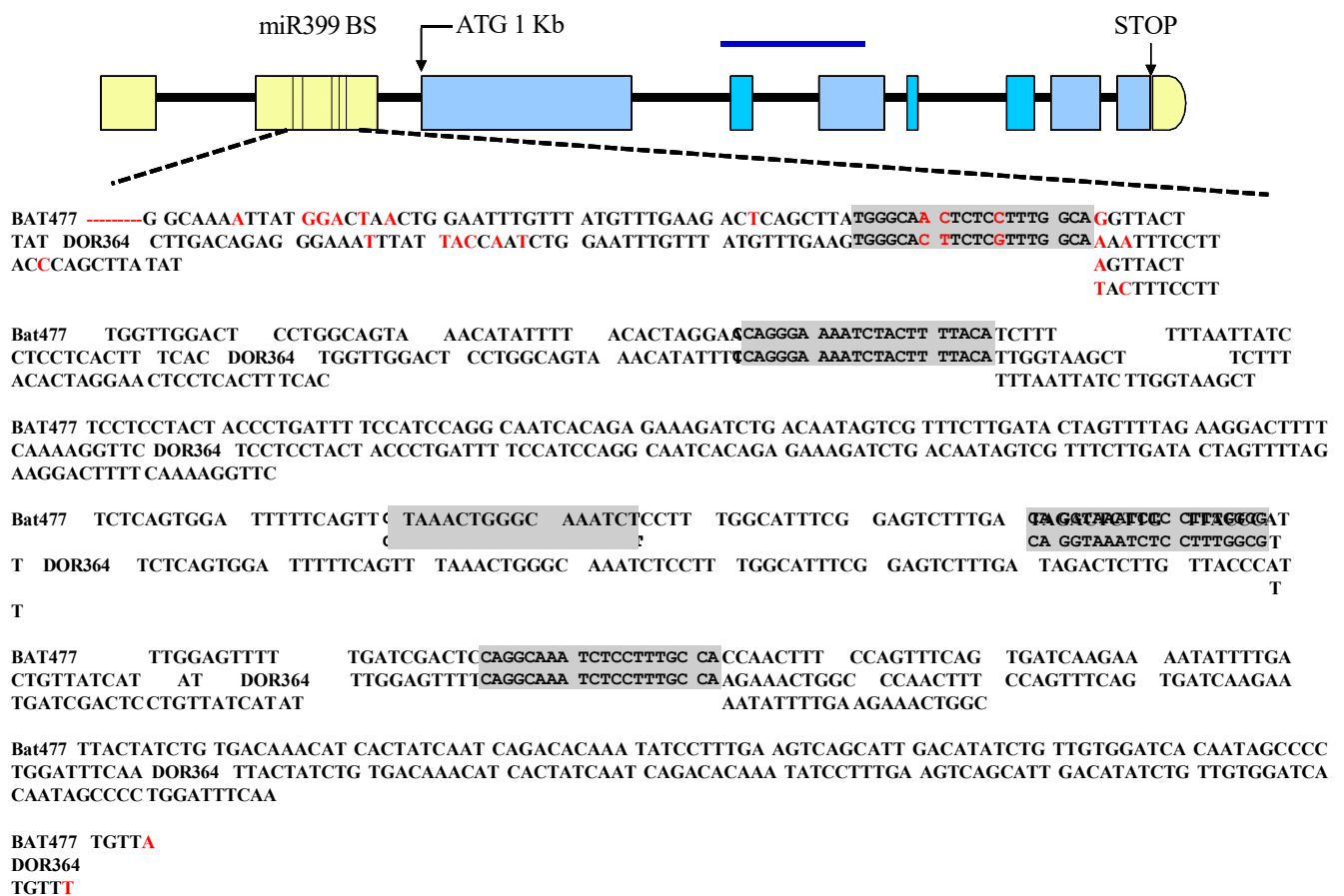
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BAT 477  AAGTTTGCTGGGAATGAACCGTCCTTCTTAAGGGCGATTTCTCTTGAAAGGGCAACTTCCATCCTTTGGCAATTTTCCT
DOR 364  AAGATTGCTGGGAATGAACCGTCCTTCTTAAGGGCGATTTCTCTTGAAAGGGCAACTTCCA TCCTTTGGCAATTTTCCT
                    5'
                    Pvu miR399a  GTCCCGTTAG *** AGGAAACCGT 3'
                    Pvu miR399b  GTCCCGTTGAG *** AGGAAACCGT
                    Pvu miR399c  GACCCGTTAG *** AGGAAACCGT
    
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We then aimed to identify and analyze the sequence of the predicted target site(s) of PvmiR399 in the *PvPHO2* gene. This was done based on the *P. vulgaris* genome sequence recently made available in Phytozome (www.phytozome.net) [49,50]. As depicted in Figure 4, the *PvPHO2* gene is organized into nine exons and eight introns; two exons are contained in the 5' UTR, and the rest in the coding region. Five predicted PvmiR399 binding sites were identified within the second exon of the *PvPHO2* 5' UTR (Figure 4). In order to compare the DNA sequence of the PvmiR399 binding sites from the two genotypes, DNA fragments of 1.625 kb and 1.635 kb from the 5' UTRs of BAT477 and DOR364, respectively, were cloned and sequenced. Sequence alignment of the 5' UTR of *PvPHO2* for BAT477 and DOR364 common bean genotypes showed a number of single nucleotide polymorphisms (Figure 4).

After analysis with the psRNATarget program, five 20–21 nucleotide motifs complementary to the PvmiR399 isoforms were identified, located at positions 1084, 1175, 1345, 1399, 1453 in BAT477 and 1094, 1185, 1355, 1409 and 1463 in DOR364 *PvPHO2* 5' UTRs (Figure 4). For comparison, these predicted PvmiR399 sites will be referred to as 1 to 5, starting with the site at the 5' end.

Figure 4. Gene structure of *PHO2* gene from the common bean. Introns are represented by black lines, and exons are represented by yellow boxes for UTRs and by blue boxes for coding regions. The ticks in the second exon of the 5' UTR depict the position of the five predicted PvmiR399 binding sites. The 605 bp DNA sequence of the region flanking the predicted *PvmiR399* binding sites of the BAT477 and DOR364 genotypes is shown; nucleotides that differ among the two genotypes are marked in red, and the sequences complementary to PvmiR399 are shaded gray.



Our results agree with those from the *Arabidopsis PHO2* gene that contains five binding sites complementary to miR399 in its 5' UTR region, approximately 200–400 nucleotides upstream of the start codon; miR399-dependent *PHO2* transcript cleavage at these sites has been experimentally verified [51]. Putative *PHO2* orthologs containing five miR399 binding sites in their 5' UTRs have also been identified in rice, *Medicago truncatula* and poplar (*Populus trichocarpa*) [31]. In addition, *PHO2* gene structure is fully conserved in these diverse species, except that the fifth exon was split into two in rice [31].

In plants, the degree of base complementarity between the mRNA target site and miRNA determines the stability of miRNA:mRNA duplexes in the “RNA-induced silencing complex (RISC)”, and therefore, it is a critical characteristic for the miRNA-mediated target degradation. Based on this

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characteristic, a scoring system for miRNA-mRNA stability that considers mismatches, single-nucleotide bulges or gaps has been established and is widely used to predict miRNA negative regulation over the target genes. A score up to 5 is considered as functional for a stable duplex that will result in miRNA-mediated target gene degradation [25]. We analyzed the alignment score of each of the PvmiR399 isoforms with each of the *PvPHO2* putative target sites identified in the BAT477 and

DOR364 genotypes (Table 1). The 30 miRNA:mRNA pairing scores obtained indicated that the five sites identified may be functional for PvmiR399 mediated *PvPHO2* mRNA degradation, except site 2 with PvmiR399b, which showed a score of 6 (Table 1). The DNA sequence and scores observed for the 2, 3, 4 and 5 PvmiR399 predicted binding sites were identical between the two genotypes (Figure 3, Table 1). However, site 1 showed three different nucleotides in the sequence (Figure 3) and clearly different PvmiR399:*PvPHO2* pairing scores when comparing BAT477 to DOR364 (Table 1).

Table 1. Pairing of PvmiR399 isoforms a, b and e with the predicted binding sites of the 5' UTR of the *PvPHO2* target gene from BAT477 and DOR364 genotypes. The sequence of the predicted binding sites is shown in Figure 3. Watson-Crick base pairing is indicated by “I”; G:U base pairing by “:”; and “-” indicates a mismatch. Each miRNA:mRNA alignment score is shown in parenthesis: mismatch penalty = 1, G:U pair penalty = 0.5 [25]. Differences between BAT477 and DOR364 in binding site 1 are highlighted in red.

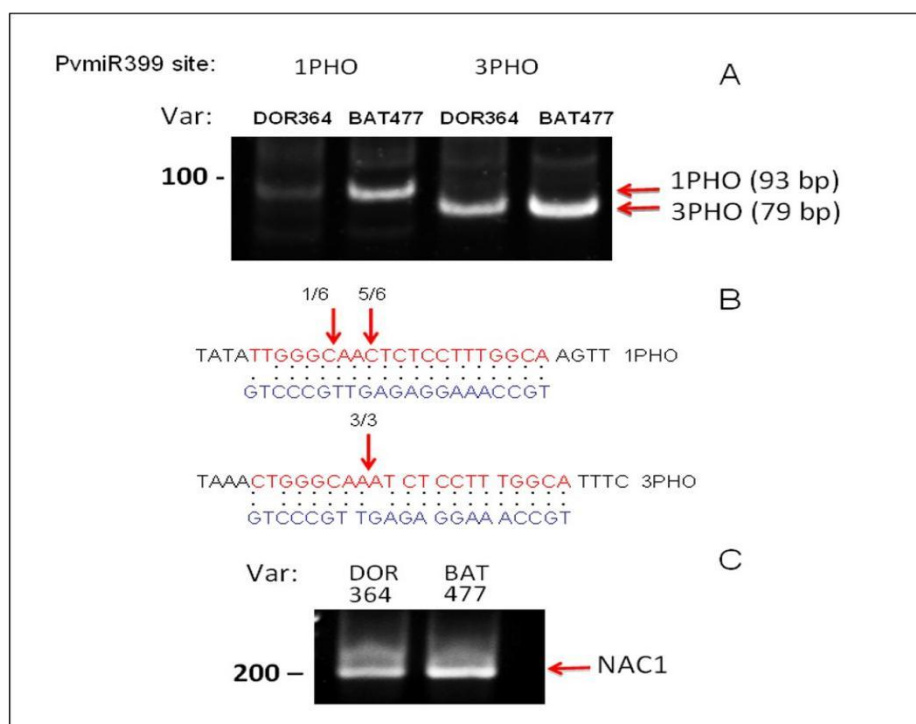
miRNA	PvmiR399 binding site PHO2 5' UTR region	BAT477 miRNA:mRNA pairing (score)	DOR364 miRNA:mRNA pairing (score)
PvmiR399a	1	I-III-III-III-III-III (2.0)	I-III-III-III-III-III (4.0)
PvmiR399b	1	I-III-III-III-III-III (1.0)	I-III-III-III-III-III (3.5)
PvmiR399e	1	III-III-III-III-III-III (1.0)	III-III-III-III-III-III (3.0)
PvmiR399a	2	III-III-III-III-III-III (4.0)	III-III-III-III-III-III (4.0)
PvmiR399b	2	III-III-III-III-III-III (6.0)	III-III-III-III-III-III (6.0)
PvmiR399e	2	I-III-III-III-III-III (5.0)	I-III-III-III-III-III (5.0)
PvmiR399a	3	I-III-III-III-III-III (1.0)	I-III-III-III-III-III (1.0)
PvmiR399b	3	I-III-III-III-III-III (2.0)	I-III-III-III-III-III (2.0)
P-miR399e	3	III-III-III-III-III-III (0.0)	III-III-III-III-III-III (0.0)
PvmiR399a	4	I-II-III-III-III-III (3.0)	I-II-III-III-III-III (3.0)
PvmiR399b	4	I-II-III-III-III-III (4.0)	I-II-III-III-III-III (4.0)
PvmiR399e	4	I-II-III-III-III-III (3.0)	I-II-III-III-III-III (3.0)
PvmiR399a	5	I-III-III-III-III-III (3.0)	I-III-III-III-III-III (3.0)
PvmiR399b	5	I-III-III-III-III-III (4.0)	I-III-III-III-III-III (4.0)
PvmiR399e	5	I-III-III-III-III-III (3.0)	I-III-III-III-III-III (3.0)

While BAT477 showed very high complementarity of binding site 1 with the three PvmiR399 isoforms (score 1–2); the equivalent site in DOR364 genotype showed lower complementarity (score 3–4) (Table 1). The latter may indicate that PvmiR399:*PvPHO2* mRNA duplexes are less stable or affect processing, thus leading to decreased miR399-mediated degradation and higher transcript level of *PvPHO2* in the DOR364 plants under P deficiency (Figure 2).

As mentioned before, four out of five PvmiR399 putative binding sites showed identical DNA sequence, while site 1 showed differences at three nucleotide positions that can potentially affect miR399 binding and/or processing. In order to explore the possibility that miR399 binding/processing is affected specifically in the DOR364 genotype, we performed modified 5'RACE experiments to assess the extent of cleavage at this site. In parallel, we also determined cleavage at the third site, which shows identical sequence between DOR364 and BAT477 genotypes, as a reference for this analysis. When compared, the abundance of a 5'RACE product at the third site, which shows identical

sequence between both varieties, is similar for *PvPHO2* mRNAs in both bean genotypes (Figure 5A). In contrast, cleavage detection at PvmiR399 recognition site 1 was easily detected for BAT477, but much less so for the DOR364 genotype, which contains a less conserved sequence complementarity to miR399 (Table 1). Amplification products were purified and sequenced to confirm their identity. For binding site 1, we recovered six independent clones, where the majority (five out of six) corresponded precisely to the expected cleavage site, while for the third site, we recovered three independent clones containing the *PvPHO2* sequence cleaved exactly where predicted for miR399 recognition (Figure 5B) This result suggests that the difference in sequence at the first site corresponds to a less efficient cleavage of the *PvPHO2* mRNA directed by PvmiR399 in DOR364 genotype that can be detected by lower levels of the 5'RACE product.

Figure 5. microRNA-directed cleavage of *PvPHO2* mRNA. 5'RACE analysis was performed for *PvPHO2* using oligonucleotides to specifically determine cleavage directed by PvmiR399 at two recognition sites (1 and 3) in the BAT477 and DOR364 genotypes. (A) PCR products corresponding to cleaved *PvPHO2* fragments from BAT477 variety were amplified by nested PCR and resolved in a 6% PA gel (arrows indicate size and position of predicted fragments; for procedure details, see the Experimental Section). The corresponding PCR fragments from BAT477 were cloned and sequenced to confirm their identity; (B) The alignment between *PvPHO2* binding sites 1 and 3 and PvmiR399b is shown; arrows indicate site of cleavage recovered, and numbers refer to number of independent clones analyzed. C. PCR fragments corresponding to cleavage of *NAC1* by miR164 that is unrelated to the P -deficiency response.



3. Experimental Section

3.1. Plant Material and Growth Conditions

The common bean (*Phaseolus vulgaris*) BAT477 and DOR364 genotypes were used in this study [39]. Surface-sterilized seeds were germinated, and plants were grown in pots with vermiculite under glasshouse conditions with natural light and controlled temperature (26–28 °C). Pots were watered 3 days per week with the plant nutrient solution reported by Summerfield *et al.* [52]. For P-deficient condition (–P), cotyledons from each plant were cut 1 week after planting and K₂HPO₄ concentration of the plant nutrient solution was reduced from 1 mM to 5 μM. Plants were grown for 3 weeks before harvesting. Roots for nucleic acids isolation were immediately frozen in liquid nitrogen and preserved at –80 °C until used.

3.2. Phenotypic Characterization

The effect of P deficiency on common bean plants was assessed by measuring soluble P_i content in leaves, leaf area of fully expanded leaves, root dry weight and root to shoot dry weight ratio in BAT477 and DOR364 bean plants grown for 3 weeks in –P or control (full-nutrient) conditions. Soluble P_i content was determined using the colorimetric assay previously reported [14] on leaves that were harvested, weighed and immediately homogenized in 10 N TCA. For each parameter, 16 replicates from two independent experiments (8 replicates per experiment) were analyzed.

3.3. Real-Time Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from roots of bean plants grown –P or control conditions using Trizol reagent (Life Technologies, Carlsbad, CA, USA), following manufacturer instructions. For quantification of transcript levels of selected regulatory genes, cDNA was synthesized from 2 μg of total RNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Copenhagen, Denmark). For quantification of mature miRNA levels, cDNA was synthesized using the NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen, California, CA, USA). For qRT-PCR analysis, SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) and the Applied Biosystems 7500 Real-Time PCR System were used, and the thermocycler settings were: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The sequences of oligonucleotide primers used for each of the genes and each amplicon size are the following. For *PvPHO2*: 5'-CAGCTGCCGAAGTTTGGAA-3' (forward) and 5'-GGGCCTGAAGAGAAAGAAGGA-3' (reverse), 65 bp amplicon size. For *PvPHRI*: 5'-TCTGGATGCCATGGTGGTT-3' (forward) and 5'-GCCGTTGCTTCTTGGTTGAT-3' (reverse), 67 bp amplicon size. For *Pv4*: 5'-GCTGGGAATG AACCGTCCTT-3' (forward) and 5'-GATGGAAGTTGCCCTTTTCAAG-3' (reverse), 59 bp amplicon size. For amplification of each miR399, 21-mer oligonucleotides were synthesized. For PvmiR399a: 5'-TGCCAAAGGAGATTTGCCCTG-3' (forward). For PvmiR399b: 5'-TGCCAAAGGAGAGTT GCCCTG-3' (forward). For PvmiR399e: 5'-TGCCAAAGGAGATTTGCCAG-3' (forward). The ubiquitin *UBC9* (TC34057) gene, which showed a constant transcript level in all the conditions tested, was included for normalization in every qRT-PCR run using the primers: 5'-GCTCTCCATTGCT

CCCTGTT-3' (forward) and 5'-TGAGCAATTTTCAGGCACCAA-3' (reverse), amplicon size 66 bp. The qRT-PCR efficiency for each amplicon was ≥ 1.8 . Average expression ratios ($-P/C$) were calculated with the $\Delta\Delta C_T$ method, as reported [14], and the fold change value (\log_2) was calculated.

3.4. Cloning and DNA Sequence Analysis of *Pv4* Gene and of the 5' UTR from *PvPHO2*

The genes encoding for *Pv4* (*PvIPSI*) and for the ubiquitin E2 conjugase *PvPHO2* were identified from the common bean genome sequence deposited in Phytozome [49,50] after a BLAST search based on a EST sequence assigned to TC43701 and to TC34730, respectively (Bean Gene Index DFCI, [53]). Two primers were designed for the amplification of a 536 bp fragment from *Pv4*: 5'-CAACACTCCTTCTCAAATCCTCTC-3' forward and 5'-AGTAAGAAGCAATTTTGTTTTG-3' reverse. For the amplification of a 1.6 kb fragment from the 5' UTR region of *PvPHO2* (locus ID Phvul.006G185400), two primers were designed: 5'-CAAACCTGAAACCAAGCTTTGGGATTGACCCTTTTC-3' forward and 5'-AAACATTGAAATCCAGGGGTATTGTGATCC-3' reverse. The PCR products obtained after PCR amplification from genomic DNA of the BAT477 and DOR364 genotypes were cloned in a pCR 2.1-TOPO TA vector (Invitrogen, California, CA, USA). Plasmid DNA from at least two clones from each genotype was purified and sequenced at least three times (replicates).

The 1.6 kb sequence from the *PvPHO2* 5' UTR from each of the bean genotypes was analyzed to identify putative target sites complementary to miR399. The program psRNATarget [54] was used for such analysis. This program also allows predicting the level of matching (score) from each of the microRNA complementary sites [55].

3.5. Target Validation by 5'RACE of *PvPHO2* mRNA

To experimentally validate targets for PvmiR399, we used a modified 5'-end rapid amplification of cDNA ends (5'RACE) approach. The 5'RACE experiment was performed, as previously described [56], using the First Choice RLM-RACE kit (Ambion, Austin, TX, USA). Total RNA (2.5 μ g) was ligated to an RNA adapter using T4 RNA ligase. An aliquot was then reverse transcribed using random oligonucleotides to prime the reaction. Two rounds of nested PCR using RNA adapter-based primers and oligonucleotides designed to amplify products specific for cleavage at the first or third miR399 binding site in *PvPHO2* mRNA were used: Rev1-1PHO 5'-ATGTTTACTGCCAGGAGTCC-3' reverse; Rev2-1PHO 5'-TGAAAGTGAGGAGTTCCTAG-3' reverse; Rev1-3PHO 5'-GGGTAACAAGAGTCTATCAAAGACTC-3' reverse; Rev2-3PHO 5'-CAAGAGTCTATCAAAGACTCCG-3' reverse. Amplification products were resolved in a 6% polyacrylamide gel in 1xTBE and EtBr stained for visualization. Only after the second round of nested PCR, we obtained specific fragments after 30 or 35 cycles. For PCR product quantitation, the amplification products (after 30 cycles, in Figure 5) were analyzed using the ImageQuantTL software (GE Healthcare Bio-Sciences, Uppsala, Sweden). The PCR fragments obtained were purified by elution and ethanol precipitation from the gel and were cloned using the TOPO-TA system (Invitrogen, California, CA, USA). Several independent clones were sequenced to confirm their identity (Unidad de Secuenciación, IBT, UNAM).

4. Conclusions

The significance of the PvPHR1/PvmiR399 signaling pathway in the reaction to this stress is supported by our examination of two *P. vulgaris* genotypes with different reactions to P deprivation. The negative regulator PvPHO2 was downregulated in BAT477 but not in the -P-sensitive DOR364 genotype, although the transcript levels of the regulators PvPHR1, Pv4, and PvmiR399 exhibited equivalent overexpression in P-deficient roots from both genotypes. The target gene of PvmiR399 that causes its mRNA cleavage is PvPHO2 (E2 ubiquitin conjugase). Site 1 had three nucleotide changes between the BAT477 and DOR364 genotypes, but the other four of the five potential PvmiR399 binding sites found in the PvPHO2 5' UTR region of both genotypes displayed identical DNA sequences. In the DOR364 plants under P deficit, the latter was shown by a significant difference in pairing scores between the two genotypes, suggesting that PvmiR399:PvPHO2 mRNA duplexes are less stable and result in reduced PvmiR399-mediated binding and/or degradation of PvPHO2 transcript. Cleavage products from PvPHO2 site 3 (same in both genotypes) and NAC1, the target of miR164-independent to P deficit, were comparable in BAT477 and DOR364, however the product from PvPHO2 site 1 was almost three times lower in DOR364, according to modified 5'RACE tests. Based on these findings, we hypothesize that the BAT477 and DOR364 genotypes' different responses to P deprivation are attributable to differences in PvmiR399-mediated control of PvPHO2. Increased PvPHO2-mediated degradation of P-responsive proteins, including P transporter PHT1, would lead to a reduction in P content and usage efficiency in the sensitive DOR364 bean plants. This would be the consequence of higher PvPHO2 in DOR364 due to less effective PvmiR399-mediated mRNA degradation.

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