

# *PRD*, an *Arabidopsis AINTEGUMENTA*-like gene, is involved in root architectural changes in response to phosphate starvation

Juan José Camacho-Cristóbal · Jesús Rexach ·  
Geneviève Conéjéro · Yves Al-Ghazi ·  
Philippe Nacry · Patrick Doumas

Received: 12 February 2008 / Accepted: 9 May 2008 / Published online: 28 May 2008  
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**Abstract** Changes in root architecture are one of the adaptive strategies used by plants to compensate for local phosphate (Pi) deficiency in soils. Root architecture variables triggered by Pi availability are well documented in *Arabidopsis* (*Arabidopsis thaliana*), but the molecular mechanisms behind these adaptive responses remain to be elucidated. By the use of transcriptomic and quantitative RT-PCR analysis, we observed that an *AINTEGUMENTA*-like gene, named *PRD* for Phosphate Root Development, was rapidly repressed in roots under low Pi conditions. The physiological function of the *PRD* gene was analyzed through the null allele mutant *prd*, which displayed less development of primary and lateral roots under Pi-starvation

**Electronic supplementary material** The online version of this article (doi:10.1007/s00425-008-0754-9) contains supplementary material, which is available to authorized users.

J. J. Camacho-Cristóbal · J. Rexach  
Departamento de Fisiología, Anatomía y Biología Celular,  
Facultad de Ciencias Experimentales,  
Universidad Pablo de Olavide, 41013 Sevilla, Spain

G. Conéjéro · Y. Al-Ghazi · P. Nacry · P. Doumas  
Laboratoire de Biochimie et Physiologie Moléculaire des Plantes,  
INRA, 34060 Montpellier cedex 1, France

G. Conéjéro  
Plateau d'Histocytologie et Imagerie Cellulaire Végétale,  
CIRAD TA A96/02, 34398 Montpellier cedex 5, France

**Present Address:**

Y. Al-Ghazi  
CSIRO Plant Industry, GPO Box 1600, Canberra,  
ACT 2601, Australia

P. Doumas (✉)  
UMR DIAPC (INRA/IRD/SupAgro/UM2), IRD,  
BP 64501, 34394 Montpellier cedex 5, France  
e-mail: doumas@mpl.ird.fr

conditions than wild-type plants. Complementation of the *prd* mutant with the wild-type gene led to a similar response to Pi starvation as wild-type plants, indicating the complete rescue of the mutant phenotype. These results suggest that *PRD* gene is involved in the regulation of root architectural responses to Pi starvation by controlling primary and lateral root elongation. This model is in agreement with the tissue-specific pattern of *PRD* gene expression, which was observed to occur specifically in the apex in both the primary and lateral roots. However, Pi influx, anionic profiles and root expression of genes typically induced by Pi starvation, such as high affinity Pi transporters (*PHT1;1* and *PHT1;4*) and an acid phosphatase (*AtACP5*), were similar in wild type and *prd* plants in response to Pi starvation. These results support the hypothesis that the *PRD* gene is not a checkpoint for Pi-starvation responses, but acts specifically as a regulator of root architectural responses to Pi starvation.

**Keywords** *Arabidopsis* · Phosphate starvation · Root development · Transcription factor

## Abbreviations

BSA	Bovine serum albumin
EST	Expressed sequence tag
PBS	Phosphate buffer saline
QTL	Quantitative trait locus
RT-PCR	Real-time PCR
SSC	Sodium sodium citrate

## Introduction

Phosphorus is an essential structural constituent of many bio-molecules and plays a pivotal role in energy conservation

and metabolic regulation. Paradoxically, phosphorus bioavailability is one of the most challenging problems in crop nutrition and is often a limiting factor even in fertile soils. Although inorganic orthophosphate (Pi), the assimilated form of phosphorus, may be present in sufficient amounts, the high soil binding capacity of Pi leads to very low Pi concentrations available to plants (Hinsinger 2001). As a consequence, under Pi limiting conditions, plants have evolved sophisticated metabolic and developmental strategies to maintain Pi homeostasis and to maximize its acquisition from the rhizosphere. These adaptive responses to increase the availability of internal or external Pi include increased expression of high-affinity Pi transporters, enhanced production of enzymes such as phosphatases and nucleases, and secretion of organic acids (Raghothama 1999; Raghothama and Karthikeyan 2005). Besides allowing roots to explore a larger volume of soil in search of nutrient-rich patches, changes in the root system architecture play a central role in the plant's adaptive response to Pi starvation. In recent years, this adaptive response has been widely studied and several studies have described the effects of Pi deprivation on root architecture. In *Arabidopsis*, Pi starvation affects primary root growth and lateral root initiation and elongation (Williamson et al. 2001; Linkohr et al. 2002; López-Bucio et al. 2002, 2005; Al-Ghazy et al. 2003; Nacry et al. 2005). In fine, Pi deprivation alters cell division and elongation at the primary root tip and induces an irreversible shift from an indeterminate to a determinate root growth program in which the quiescent center seems to play a central role (Sánchez-Calderón et al. 2005). However, despite recent extensive studies on global gene expression during Pi deficiency, little is known about the molecular components of the signal transduction pathway that trigger the Pi-starvation responses (Hammond et al. 2003; Uhde-Stone et al. 2003; Wasaki et al. 2003, 2006; Wu et al. 2003; Misson et al. 2005; Muller et al. 2007). To date, only few transcription factors have been shown to be required for Pi-starvation-dependent responses in higher plants. The MYB transcription factor PHR1 was first reported to control a small subset of Pi-starvation responses that including only a few genes whose expression is activated by Pi limitation, such as *AtACP5*, *AtIPS1/3*, *At4*, and *RNS1* (Rubio et al. 2001). In addition, *phr1* mutants showed reduced anthocyanin accumulation, fresh weight, intracellular Pi, and, to a lesser extent, a reduced root-to-shoot growth ratio in response to Pi starvation, indicating that PHR1 is a positive regulator of Pi-starvation responses. However, other changes in root architecture typically associated with Pi deficiency were unaffected in *phr1*. Recently, the bHLH transcription factor OsPTF1 was shown to provide tolerance to Pi starvation in rice and to be involved in the efficient utilization of Pi in plants (Yi et al. 2005). The WRKY75 transcription factor has also been characterized as a regulator of Pi stress

responses, but its regulatory effect on root architecture was independent of the Pi status of the plant (Devaiah et al. 2007). Moreover, several groups have also isolated mutants impaired in their Pi response including *pdr2*, which disrupts sensing of low external Pi availability that lead to alterations in root growth (Ticconi et al. 2004), and *lpi* that fails to arrest primary root growth when grown under low Pi conditions (Sánchez-Calderón et al. 2006). The molecular function of these genes is still unknown and none of these mutations specifically triggered changes in root architectural responses to Pi starvation. By the use of an elegant QTL approach, Reymond et al. (2006) and Svistoonoff et al. (2007) recently identified an *LPR* gene encoding a multicopper oxidase specifically expressed in the root tip and involved in the sensing of the external Pi concentration.

Thus, the aim of this work was to identify and characterize regulatory genes specifically involved in root architectural responses to Pi deprivation in *Arabidopsis*. Using transcriptome analysis, we identified a transcription factor named PRD for Phosphate Root Development, belonging to the *AINTEGUMENTA*-like (*AIL*) gene family (Nole-Wilson et al. 2005), that was regulated by Pi starvation. In-depth physiological, genetic, histological, and molecular analyses show that this gene regulates root growth responses to Pi deprivation.

## Materials and methods

### Plant material and growth conditions

*Arabidopsis thaliana*, ecotype Columbia (Col-0) and T-DNA mutant seeds were obtained from the Nottingham Arabidopsis Stock Centre (Scholl et al. 2000). Seeds were surface-sterilized in 4% (v/w) Bayrochlor (Bayrol, Mundolsheim, France), 50% (v/v) ethanol for 15 min, and washed three times with 100% ethanol and three times in sterile water. Sterile seeds were sown on 12 × 12 cm Petri dishes (Greiner Bio-One, Frickenhausen, Germany) containing 40 ml of sterile culture medium and sealed with Parafilm. The high Pi culture medium contained 0.5 mM CaSO<sub>4</sub>, 2 mM KNO<sub>3</sub>, 0.5 mM MgCl<sub>2</sub>, 0.05 mM NaFe-EDTA, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM Mes, 50 μM H<sub>3</sub>BO<sub>3</sub>, 12 μM MnCl<sub>2</sub>, 1 μM CuCl<sub>2</sub>, 1 μM ZnCl<sub>2</sub>, and 30 nM NH<sub>4</sub>Mo adjusted to pH 5.7 with 1 N KOH and solidified with 0.8% (w/v) Bactoagar (DIFCO, BD Bioscience, Sparks, MD, USA). All chemicals were purchased from Sigma Chemicals (St. Quentin, France). In the low Pi medium, KH<sub>2</sub>PO<sub>4</sub> was replaced by 1 mM KCl and the Pi content was estimated at 3 μM due to the slight Pi content of Bactoagar. After sowing on either high or low Pi medium, Petri dishes were cold treated at 4°C for 24 h in darkness to promote and synchronize germination,

subsequently transferred in a vertical position to a growth chamber at a temperature of 21.5°C, and subjected to a photoperiod of 16 h of light (150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) from fluorescent and metal halogen lamps.

### Morphological analysis

A desktop scanner (resolution: 450 dpi) was used to record images of the root system directly from plants growing in Petri dishes. Images corresponding to different growth times were analyzed using Optimas software version 6.1 (Media Cybernetics, Silver Spring, MD, USA). The length of primary and lateral roots, and the number of lateral roots were determined manually. Data were exported to an Excel work-sheet for final processing. All results were statistically analyzed using Statistica software version 7.1 (Statsoft, Tulsa, OK, USA). For the analysis of variance, 2- and 3-factors ANOVA with an LSD post hoc test were used to measure differences ( $P = 0.05$ ).

### Identification of a T-DNA insertion mutant for the PRD gene

The homozygote plants of the T-DNA insertion line (SALK\_046920) for the At1g79700 gene were identified by PCR with the following primer sets: 5'-TTCGAATGGGCTTAGAATCG-3' and 5'-AGGGAACAAAG AAGGTCACG-3' from the gene and the primer LBb1, from the T-DNA region. Individual homozygous mutants were back-crossed twice with the wild-type Col-0. The genotype of the F<sub>2</sub> individuals was checked by PCR using gene-specific primers and T-DNA primers.

### Plant transformation

For complementation of the *prd* mutant, a 5.1-kb fragment, containing the PRD coding region and the 1.34 kb of the 5' flanking DNA upstream of the starting codon, was amplified by PCR with 5'-AATGGATCCATTCCGGCATGTAAAGATCG-3' and 5'-GGCAAGCTTAGACGTCACGGATGACGAA-3' primers and DNA genomic BAC F20B17 as the template. The PCR product was cloned into the binary vector pCAMBIA1300 (Cambio, Canberra, Australia) using *Bam*HI and *Hind*III restriction sites (underlined in the sequence primers). The construct and empty vector control were used to transform *Arabidopsis* plants using the floral-dip method (Clough and Bent 1998) and transgenic seedlings were selected by spraying 50  $\mu\text{g l}^{-1}$  Basta.

### RNA extraction and analysis

Frozen roots were ground in liquid nitrogen and total RNA was isolated using TRIzol reagent (Invitrogen, Cergy

Pontoise, France) according to the manufacturer's protocol. The quality and quantity of RNA were measured with OD<sub>280</sub>/OD<sub>260</sub> and gel electrophoresis. RNA extracts were stored at -80°C prior to hybridization onto macroarrays or PCR quantification.

### Macroarray analysis

Macroarrays were prepared, hybridized and analyzed as previously described (Al-Ghazi et al. 2003; Tranbarger et al. 2003). Complex probes were prepared from 5 to 10  $\mu\text{g}$  of total RNA, using anchored oligo-dT (VT<sub>18</sub>) as previously described (Al-Ghazi et al. 2003). Two sets of macroarray hybridizations were performed in two replicate biological experiments and three membranes were used for each hybridization.

### Quantitative real-time PCR

Three micrograms of RQ-DNase (Promega, Madison, WI, USA) digested total RNA was used to prepare cDNA by reverse transcription using M-MLV reverse transcriptase (Promega) and oligo-dT (V<sub>18</sub>) primers, according to the manufacturer's protocol. Gene expression was determined by quantitative RT-PCR (LightCycler, Roche Diagnostics, Mannheim, Germany) using gene-specific primers (see sequences below) and LightCycler FastStart DNA Master SYBR GREEN I (Roche Diagnostics). Specific primers were designed to generate PCR gene products between 100 and 250 bp. Expression levels of tested genes were normalized to expression levels of the *EF1a* (At1g07920, At1g07930, At1g07940) or *TUBULIN* (At5g12250) gene. Gene-specific primer sequences were [5'-3' and corresponded to forward (F) or reverse (R) gene sequences] for *PRD* (At1g79700): F, CAAATGCGCTTCGTATCTCC; R, TAAAACAGCGTCTGCGTCTG; for *AtACP5* (At3g17790): F, TCATCGATCCTACCTAATTCATCA; R, ACCCCAA TCTCCGATGACTA; for *PHT1;1* (At5g43350): F, AAAA CCAAACATCGCACTCC; R, CCAGGCATCGGAGTTA AGAA; for *PHT1;4* (At2g38940): R, TGATAAGCTCGG GAGGAAGA; F, TGGTTGCGGATAAAGGGTA G; for *EF1 $\alpha$*  (At1g07940): F, GTCGATTCTGGAAAGTCCGAC; R, AATGTCAATGGTGATACCACGC; for *TUBULIN* (At5g12250): F, ATAGCTCCCCGAGGTCTCTC; R, TCC ATCTCGTCCATTCTTC.

### Whole-mount in situ hybridization

Sense and antisense probes were labeled with UTP-digoxigenin (Roche Diagnostics) during transcription with the T7 MAXIscript kit (Ambion, Applied Biosystems, Courtaboeuf, France). Nucleic probes for *PRD* and *PLT1* (At3g20840) were specially designed to reach a length

between 150 and 200 bp. Probes for ribosomal 18S were used as controls. Whole-mount in situ hybridization was performed manually according to Friml et al. (2003) with the following modifications: 8-day-old *Arabidopsis* seedlings were fixed for 30 min in nylon biopsy bags in a 1:1 (v/v) mixture of fixation solution (4% paraformaldehyde, 15% DMSO, 0.08 M EGTA, 0.1% Tween 20 in PBS pH 7.4) and heptane, with shaking. The fixed seedlings were then incubated in methanol (2 × 5 min), ethanol (3 × 5 min), ethanol/safesolv (Labonord, Templemars, France) mixture (1:1, 30 min), washed with ethanol (2 × 5 min), and rehydrated in ethanol/PBS series (75, 50 and 30%, 10 min each). The rehydrated seedlings were post-fixed in fixation solution (20 min) and washed in PBS (3 × 5 min). To remove proteins from RNA, the samples were treated by proteinase K digestion (40 µg ml<sup>-1</sup>) for 20 min at 37°C and the reaction was stopped with glycine (0.2% in PBS) for 5 min. The samples were washed in PBST (PBS, 0.1% Tween 20, 2 × 10 min). The treated samples were incubated in 2 ml Eppendorf tubes in a 1:1 mixture (PBST/hybridization mix) for 15 min, pre-hybridized in 400 µl of hybridization mix (50% formamide, 10% dextran sulfate, 5 × Denhardt's solution, 2 × SSC and 1 mg ml<sup>-1</sup> tRNA) for 2 h at 45°C, and hybridized in 400 µl of hybridization mix with 0.5–1 ng µl<sup>-1</sup> denatured riboprobes overnight at 45°C. After hybridization, seedlings were washed for 10, 60, and 20 min in a solution containing 50% formamide, 2 × SSC, and 0.1% Tween 20, at 50°C, and then incubated in NTE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl) twice at room temperature. RNaseA digestion (40 µl ml<sup>-1</sup> in NTE) was carried out for 30 min at 37°C. The samples were finally washed in 2 × SSC-0.1% Tween 20 for 20 min at 50°C and in 0.2 × SSC-0.1% Tween 20 for 20 min at 50°C.

For signal detection, samples were equilibrated in PBST for 10 min, blocked for 2 h in 2% BSA in PBST and incubated with gentle agitation in an anti-digoxigenine alkaline phosphatase-conjugated Fab fragment antibody (Roche Diagnostics) 1/1000 in PBST, 2% BSA, overnight at 4°C. After five washes in PBST for 20 min each, the tissues were equilibrated in detection buffer (0.1 M Tris-HCl, pH 8.2, 2 mM Levamisole) for 10 min and in the substrate from Vector Blue (Vector Laboratories, Burlingame, CA, USA) for 2 h. The reaction was stopped by immersion in 0.1 M Tris-HCl, pH 8.2, follow by water. The seedlings were mounted in Mowiol on ESCO slides. The microscope imaging was performed at the Montpellier RIO Imaging Platform ([www.mri.cnrs.fr](http://www.mri.cnrs.fr)) with a Leica DM6000 microscope (Leica Microsystems, Rueil-Malmaison, France) and images were processed through Volocity 4.0.1 (Improvision, Lexington, MA, USA). The experiment was repeated four times, and for each experiment, 20 seedlings were used for each treatment.

## Pi influx measurements

Pi influx was measured on whole plants grown on vertically oriented agar plates for 12 days. Roots of whole plants were placed for 2 min at 25°C in a 0.05 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> solution adjusted to pH 4.5 with Mes buffer, transferred in an identical solution containing 7.4 kBq ml<sup>-1</sup> of [<sup>33</sup>]Phosphorus radiotracer (GE Healthcare, Aulnay sous Bois, France). After 20 min of incubation, the roots were washed three times for 30 s in ice-cold 0.2 mM CaSO<sub>4</sub> solution, harvested, and blotted with absorbent paper. Their soluble content extracted in a 100 mM HCl solution by heating for 30 min at 70°C. The radioactivity of the acidic extract was determined after addition of scintillation liquid (Ultimas Gold, Packard, Rungis, France) using a scintillation counter (Tri-Carb 2101 TR, Packard). Pi influx was measured on plants grown in similar conditions as described earlier for root measurements.

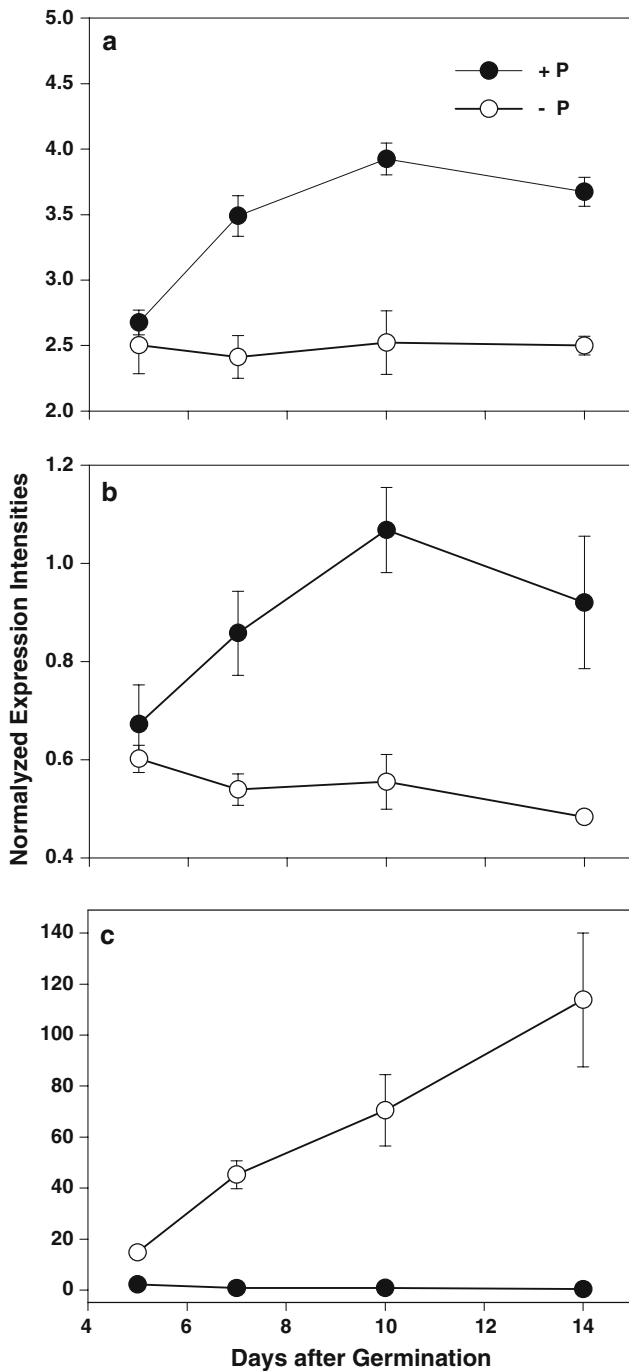
## Soluble anionic profiles

Soluble anions were extracted by incubating 20 mg of fresh tissues in 1 ml 70°C water for 30 min, and these water soluble extracts were filtered (0.45 µm, cellulose acetate, Nalgene) to remove particular matter. Pi and major inorganic and organic anions were analyzed by high-performance ion chromatography (LC20, Dionex, Voisins Le Bretonneux, France) using a Dionex IonPac AS11 column and a sodium hydroxide (1–22 mM) linear gradient as described by El Kassis et al. (2007).

## Results

### The *PRD* expression is repressed by Pi starvation in *Arabidopsis* roots

In order to identify transcription factors involved in root architectural responses to Pi-deprivation, a set of 126 putative transcription factor ESTs were arrayed on nylon membranes (Tranbarger et al. 2003). Hybridizations were performed with RNA extracted at 5, 7, 10, and 14 days after germination in both the control and Pi-starved plants. These time points were selected according to the time-course analyses of the root adaptive responses to Pi-deprivation reported by Al-Ghazi et al. (2003). This transcriptomic approach led to the selection of 19 transcription factors up- or down-regulated in response to Pi starvation (data not shown). Among them, one gene (*At1g79700*) showed a unique expression profile under Pi-starvation conditions characterized by global repression of expression (Fig. 1a). After 5 days of culture on high Pi medium, the root expression of this gene rapidly increased to reach a plateau from



**Fig. 1** Kinetic effects of Pi availability on the root expression profiles of *PRD* and *AtPT2* genes in *Arabidopsis* roots. Wild-type Columbia seedlings were grown for 14 days in the presence of low (3  $\mu$ M; open circles) or high (1 mM; full circles) concentrations of Pi on vertically orientated agar plates. **a** Macroarray analysis of *PRD* expression. **b** Quantitative RT-PCR analysis of *PRD* expression. **c** Quantitative RT-PCR analysis of *AtPT2* (*At2g38940*) expression. Relative expression levels are expressed in arbitrary units. Data correspond to the average values ( $\pm$ SE) of three independent measurements

day 10. In contrast, on low Pi medium, the expression remain unchanged and led to a maximum fold change (+P/-P) of 1.7 at day 10 after germination. This expression

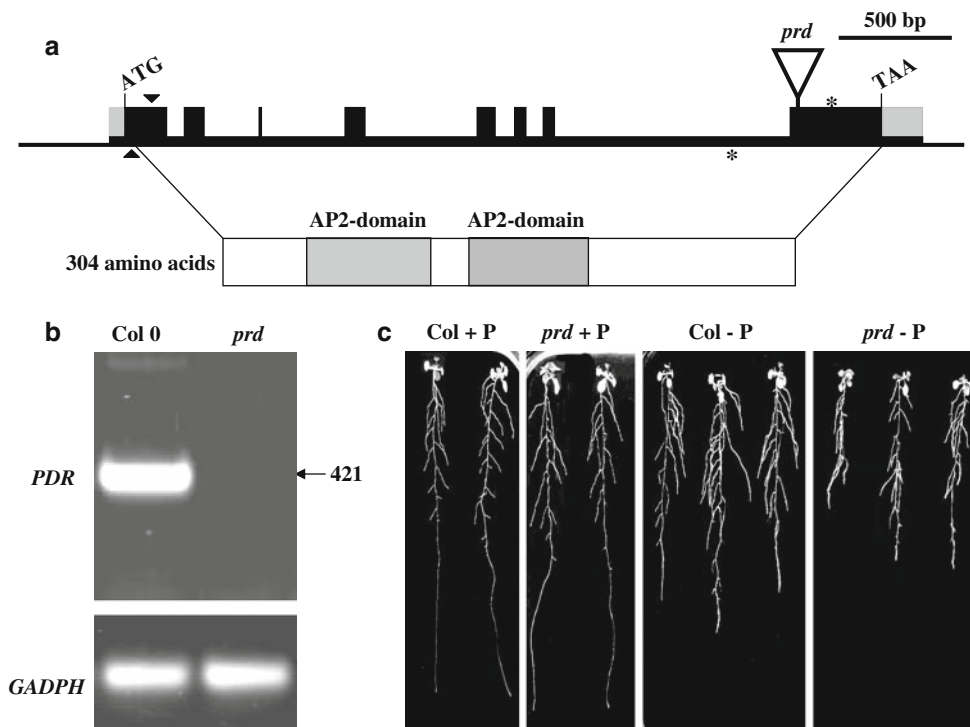
profile was confirmed by quantitative RT-PCR analysis in three independent experiments (Fig. 1b). In parallel, the expression of a high-affinity Pi transporter gene (*PHT1;4*) was analyzed as a root-specific marker of Pi starvation and, as expected, under Pi-starvation conditions, the expression of this gene increased continuously throughout the growth time course (Fig. 1c).

These data showed that the level of *At1g79700* mRNA accumulation in *Arabidopsis* roots was dependent of the Pi content of the growth medium. This gene, named *PRD* for Phosphate Root Development, encodes a putative AP2/ERF transcription factor and belongs to the family of *AINTEGUMENTA*-like (AIL) genes as defined by Nole-Wilson et al. (2005). The AP2 subgroup of this family is comprised of 18 genes including the floral developmental gene *AINTEGUMENTA* (*ANT*; Elliott et al. 1996; Klucher et al. 1996) and the root developmental meristem genes *PLETHORAI* (*PLT1*) and *PLT2* (Aida et al. 2004).

The *PRD* gene is involved in the regulation of architectural root responses to Pi starvation

To determine the physiological function of *PRD* in *Arabidopsis*, one mutant line that harbors the T-DNA insertion in the *PRD* gene (Salk\_046920) was isolated in the T-DNA insertion collection generated at the Salk Institute (Alonso et al. 2003). The T-DNA insertion was localized in the eighth exon (Fig. 2a). The presence and the location of the T-DNA insertion were verified by junction PCR and sequencing. Nucleotide sequencing of the T-DNA::genomic borders of the homozygous *prd* mutant revealed that the T-DNA insertion site was 2872-bp downstream of the translation start. Individual homozygous mutants were back-crossed twice with the wild-type Col-0. To ensure that the T-DNA insertion altered *PRD* gene expression in this mutant, PCR analyses were carried out regardless of Pi growth conditions. No amplification was detected in the *prd* mutant indicating that it is probably a null allele (Fig. 2b). Under greenhouse conditions with normal fertilization, the aerial parts of *prd* mutants were phenotypically normal. Their vegetative and floral development and seed production were similar to that of wild-type plants. In contrast, under low-Pi (3  $\mu$ M) conditions, root growth pattern of *prd* mutants were found to be distinguishable from the wild-type plants (Fig. 2c).

To precisely evaluate the consequences of *PRD* gene disruption on root architectural responses to Pi starvation, we examined the root growth parameters for mutant plants grown under high- and low-Pi conditions (Fig. 3). Wild type and *prd* seeds were germinated on medium with high Pi content during 7 days and then transferred to fresh agar plates on media with low- or high-Pi concentrations during 5 days. Temporal analysis of the root system architecture of



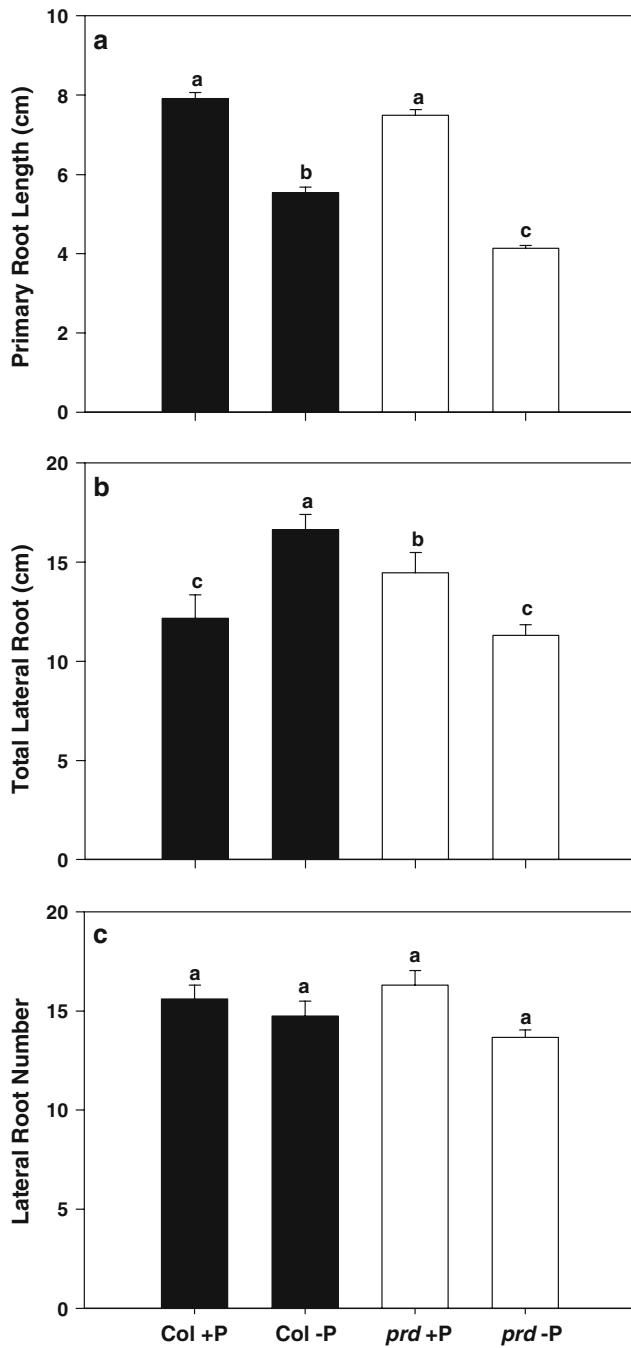
**Fig. 2** Isolation of the *prd* T-DNA insertional mutant. **a** Intron–exon organization of the *Arabidopsis PRD* gene (At1g79700) and T-DNA location. Gray shaded boxes indicate untranslated regions, and solid black boxes and the solid line indicate coding regions and the intron, respectively. The position of the T-DNA insertion in the *prd* allele is indicated by a triangle (not to scale). **b** PCR analysis of *PRD* transcript in wild type (Col-0) and mutant alleles. Expression of *GAPDH*

(At3g26650) was analyzed as a control. **c** Root architecture of *prd* mutant. Wild type and *prd* plants were grown for 7 days at high Pi concentration (1 mM) and then transferred for 5 days on media with high (1 mM) or low (3  $\mu$ M) Pi concentrations on vertically orientated agar plates. Primer locations are indicating in Fig. 2a by asterisks for homozygous mutant line screening and by dark triangles for quantitative RT-PCR analyses and whole-mount RNA in situ hybridization

wild type and *prd* seedlings was determined 7–12 days after germination (i.e. from day 0 to day 5 after transfer; Supplementary Fig. S1). As confirmed at day 5 after transfer (Fig. 3), when grown on high Pi medium, both *prd* and wild-type plants showed a similar root phenotype suggesting that, under optimal culture conditions, *PRD* plays no role in root growth. In contrast, low Pi availability reduced the length of primary roots in wild-type plants (30%), while the reduction was more drastic (45%) in *prd* plants and their primary root growth was almost arrested (Fig. 3a). Furthermore, low Pi availability promoted lateral root elongation in wild-type plants (37%), while it clearly reduced it in *prd* mutant plants (22%, Fig. 3b). Regardless of Pi treatment, there were no differences in the lateral root number between wild type and *prd* mutant plants (Fig. 3c). To assess whether Pi deficiency affected other morphological responses, leaf size was measured on wild type and *prd* mutant plants grown under high- and low-Pi conditions for 10 days (Table 1). This variable was drastically reduced by almost fivefold under low-Pi conditions for both wild type and *prd* mutant plants, while no difference was observed between mutant and wild-type plants suggesting a specific role for the *PRD* gene at the root level.

To confirm that the altered root responses to Pi starvation were the result of the *PRD* gene disruption, complementation analysis of the *prd* mutant was performed using *Agrobacterium tumefaciens*-mediated transformation. A 5.2-kb BAC genomic DNA fragment containing the *PRD* ORF, 1.4-kb upstream and 0.6-kb downstream of the sequence was cloned into the pCAMBIA 1300 binary vector for transformation (Hajdukiewicz et al. 1994). Transgenic lines were able to respond to Pi starvation in a similar way to wild-type plants, indicating the complete rescue of the mutant phenotype (Fig. 4).

To explore the tissue-specific pattern of expression of the *PRD* gene in more detail, endogenous *PRD* transcripts were observed in wild type and *prd* mutant seedlings using whole-mount in situ hybridization (Fig. 5). This technique allows direct observations of tissues without samples embedding and sectioning, and it is particularly adapted to the localization of gene expression at the root tip level (Friml et al. 2003). The tissue was hybridized with digoxigenin-labeled single-stranded *PRD* RNA in either the antisense (Fig. 5a) or the sense orientation (Fig. 5d). The sense RNA is a negative control and reveals a weak background, nonspecific staining. The antisense probe specifically



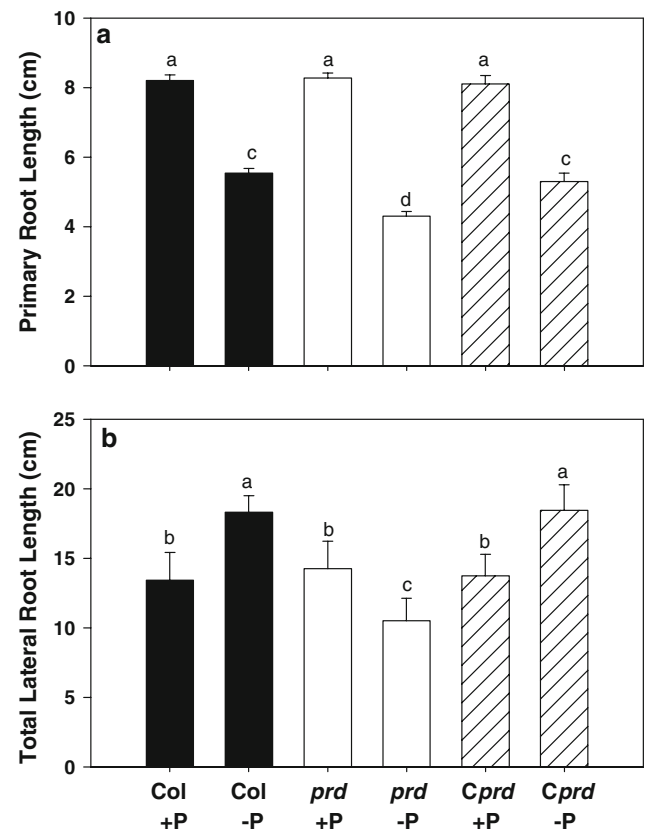
**Fig. 3** Effects of Pi availability on root architecture variables. Wild type (black bars) and mutant (white bars) seedlings were grown for 7 days at high Pi concentration (1 mM) and then transferred for 5 days on media with high (1 mM) or low (3 μM) Pi concentrations on vertically orientated agar plates. **a** Primary root length, **b** Total lateral root length and **c** the number of emerged lateral roots were measured at day 12. All results are the average value (±SE) of 16 seedlings. The letters represent statistically homogenous subgroups using the LSD post hoc test at a  $\alpha = 0.05$  significance level

hybridizes with the *PRD* mRNA present in the tissue, thereby revealing its spatial distribution. Several other controls were included in the whole-mount experiment such as

**Table 1** Effects of Pi availability on leaf growth

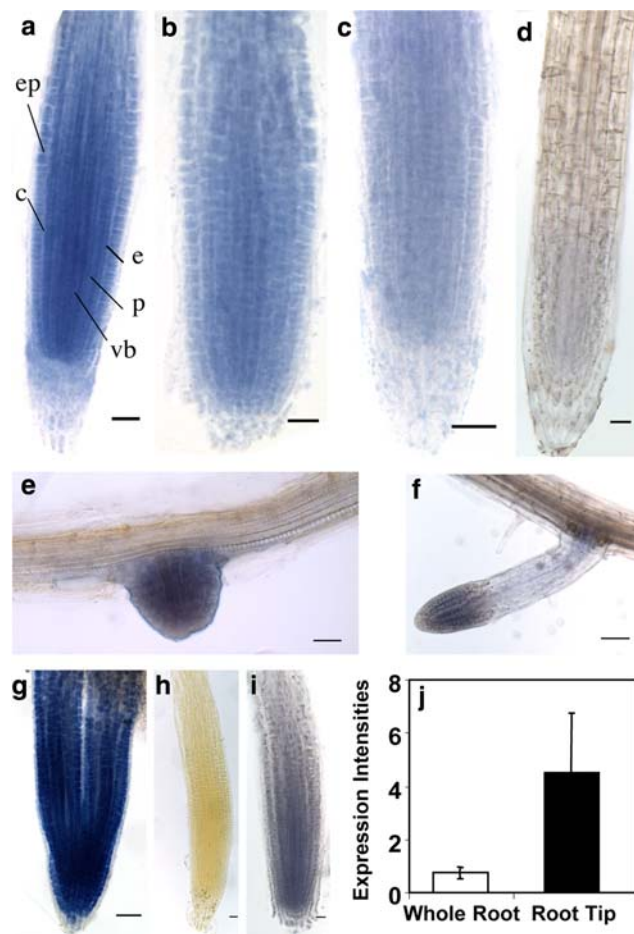
<i>Arabidopsis</i> line	Leaf area (cm <sup>2</sup> )		
	High Pi	Low Pi	-P/+P (%)
Wild type	1.409 (±0.119) <sup>a</sup>	0.307 (±0.012) <sup>b</sup>	21.8
<i>prd</i>	1.284 (±0.064) <sup>a</sup>	0.356 (±0.013) <sup>b</sup>	27.8

Wild type and *prd* mutant seedlings were grown for 10 days in the presence of high (1 mM) or low (3 μM) Pi concentrations on vertically orientated agar plates. Whole leaf areas were measured at day 10. All results are the average value of 16 seedlings (±SE); letters in superscript represent statistically homogenous subgroups using an LSD post hoc test at a  $\alpha = 0.05$  significance level



**Fig. 4** Architectural root analyses of complemented *prd* mutant. Wild type (black bars), *prd* mutant (white bars) and complemented *prd* mutant (*Cprd*, hatched bars) seedlings were grown for 7 days at high Pi concentration (1 mM) and then transferred for 5 days on media with high (1 mM) or low (3 μM) Pi concentrations on vertically orientated agar plates. **a** Primary root length and **b** total lateral root length were measured at day 12. All results are the average value (±SE) of 16 seedlings. The letters represent statistically homogenous subgroups using LSD post hoc test at a  $\alpha = 0.05$  significance level

the 18S ribosomal RNA antisense (Fig. 5g) and sense probe (Fig. 5h). Thus, only primary and lateral root tips showed a specific signal, strong and detectable in all the cells and in the thickness of root apex, whereas no signal was observed with the sense probe, validating our analysis at the root apex level. The *PLT1* probe was used as a positive control



**Fig. 5** Localization of *PRD* expression in root apex of *Arabidopsis* by whole-mount RNA in situ hybridization. *PRD* antisense probe on primary root of wild-type plants grown in the presence of high (1 mM, **a**) or low Pi (3 μM, **b**) Pi concentrations, and on primary root of *prd* mutant plants grown in the presence of high Pi concentration (**c**). **d** *PRD* sense probe on primary root of wild-type plants grown in the presence of high Pi concentration. **e**, **f** *PRD* antisense probe on lateral roots of wild-type plants grown in the presence of high Pi concentration. **g** 18S ribosomal RNA antisense probe. **h** 18S ribosomal RNA sense probe. **i** *PLT1* antisense probe. Seedlings were grown for 8 days in the presence of high or low Pi concentrations. *c* cortex, *e* endodermis, *ep* epidermis, *p* pericycle, *vb* vascular bundle. Scale bar = 20 μm. **j** Quantitative RT-PCR analysis of *PRD* expression from whole-root (white bars) or dissected root tips (black bars) of wild-type seedlings grown for 12 days at high Pi concentration on vertically orientated agar plates

because of its well described expression pattern in the root apex using similar techniques (Aida et al. 2004) and it belongs to the same AIL family as the *PRD* gene (Fig. 7i). The *PRD* hybridization signal was specifically observed in the root apex, mainly in the central cylinder and the cortex (Fig. 5a). A weak signal was also observed in the epidermal cells. Under similar conditions, *PLT1* presented a similar expression pattern but the hybridization signal was strongest in the central cylinder (Fig. 5i). Quantitative RT-PCR showed an accumulation of *PRD* mRNA in the apex part of the root compared to the whole root confirming the in situ

localization of the *PRD* gene in the root apex (Fig. 5j). Even if the whole-mount in situ hybridization technique was not the adequate technique to quantify gene expression, a slight decrease in the hybridization signal of *PRD* gene was observed in Pi-starvation plants, especially at the vascular tissue level, compared to those from the control conditions (Fig. 5b). Moreover, the weak and diffuse hybridization signal observed with *PRD* antisense probe in the *prd* mutant plants confirmed the tissue-specific pattern of *PRD* gene expression (Fig. 5c). The weak and diffuse hybridization signal observed in Fig. 5c may reveal weak cross-hybridizations with some of the other genes of the AIL family or the presence of *PRD* truncated mRNA. All these converging data confirmed that *PRD* gene expression was tightly linked with meristem activity, in both the primary and lateral roots, and reinforced its functional role in response to Pi privation.

Together, these results clearly showed that *prd* mutation reduced growth of both primary and especially lateral roots under Pi starvation. These data suggest that the *PRD* gene plays a role in the regulation of root architectural responses to Pi starvation through its involvement in root elongation.

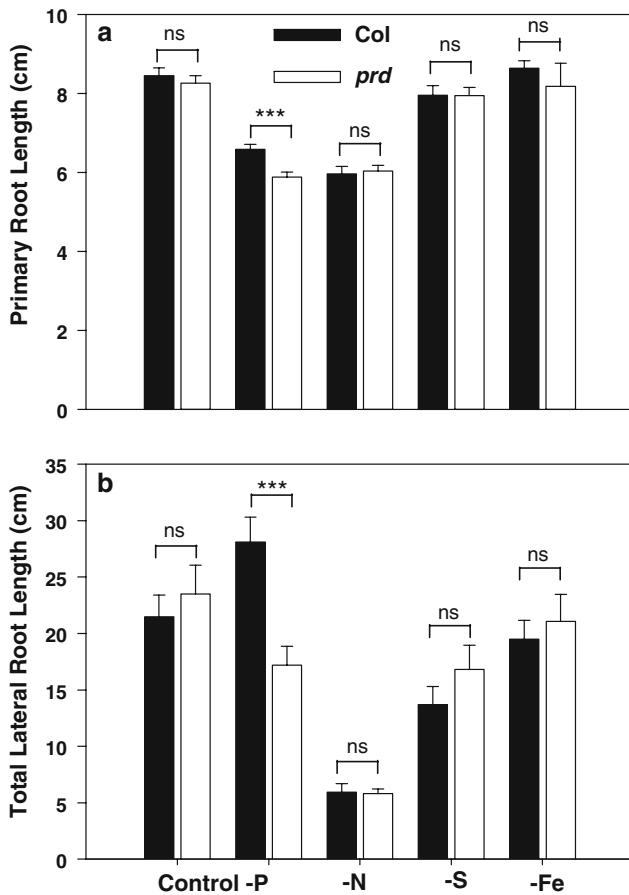
Root development defect in *prd* mutant was not observed under other nutrient deprivations.

To investigate whether *PRD* was specifically involved in root architectural responses to Pi starvation, we assessed the effects of other nutrient limited conditions including nitrogen (–N), sulfur (–S), and iron (–Fe) on the length of the primary and lateral roots. Nutrient starvation affected either primary root or lateral root growth, or both (Fig. 6). As expected, the Pi-starved medium inhibited primary root growth (Fig. 6a) and stimulated lateral root growth (Fig. 6b) in wild-type plants. Primary root length was mainly affected by Pi- or N-starvation and lateral root length was strongly reduced by Pi-, N-, or S-starvation. However, no significant difference was observed between wild type and *prd* mutant plants under starvation conditions at the exception of Pi starvation where highly significant differences were measured for both the primary and lateral roots growth. These results suggest that *PRD* is involved in the regulation of root architecture in responding specifically to Pi-starvation signal.

The *PRD* gene is not a checkpoint for responses to Pi starvation

To check if the loss of *PRD* affects other characteristic responses to Pi starvation, we conducted several experiments including analysis of gene expression, Pi uptake, and anionic profile.





**Fig. 6** Effects of nutrient availability on root architecture variables. Wild type (black bars) and *prd* mutant (white bars) seedlings were grown for 7 days at high Pi concentration (1 mM) and then transferred for 5 days on media with high Pi concentrations (1 mM) or medium lacking a mineral element (Pi, N, S, Fe) on vertically orientated agar plates. **a** Primary root length and **b** total lateral root length were measured at day 12. All results are the average value ( $\pm$ SE) of 16 seedlings. Comparison of means (*t* test) are indicated as non-significant (NS) or very highly significant (\*\*\*) responses ( $P < 0.001$ )

To study whether the genetic defects in *PRD* had an effect on the expression of Pi-responsive genes, we used quantitative RT-PCR to analyze the expression level of several Pi-starvation-induced genes such as *PHT1;1* and *PHT1;4* (two high-affinity Pi transporters), and *AtACP5* (an acid phosphatase). As previously described, the steady-state transcript levels of these three genes increased noticeably in wild-type plants subjected to Pi starvation (Fig. 7). Similar increases were also observed in *prd* mutants that show no significant difference from the wild-type plants grown under Pi deficiency.

With the same rationale, Pi uptake was monitored in wild type and *prd* mutants using  $^{33}\text{P}$  labeling (Supplementary Fig. S2). The results showed that 12 day-old wild-type plants Pi-starved for 5 days increased their Pi uptake rate about fourfold compared to plants supplied with Pi.

Phosphate starvation induced a similar increase in Pi uptake by *prd* mutants.

Finally, anionic profiles of wild type and *prd* mutant plants were analyzed in roots and leaves after 12 days of Pi treatments (Supplementary Fig. S3). In both the roots and leaves, Pi starvation caused a significant reduction in nitrate and Pi contents, and a significant increase in the accumulation of some organic acids such as malate and citrate. Similar changes were observed in wild type and mutant plants indicating that *PRD* mutation did not alter anion accumulation in response to Pi starvation, but that the changes in root development in *prd* mutants were not caused by a general or local decrease in Pi content in the root of this mutant with respect to wild-type plants.

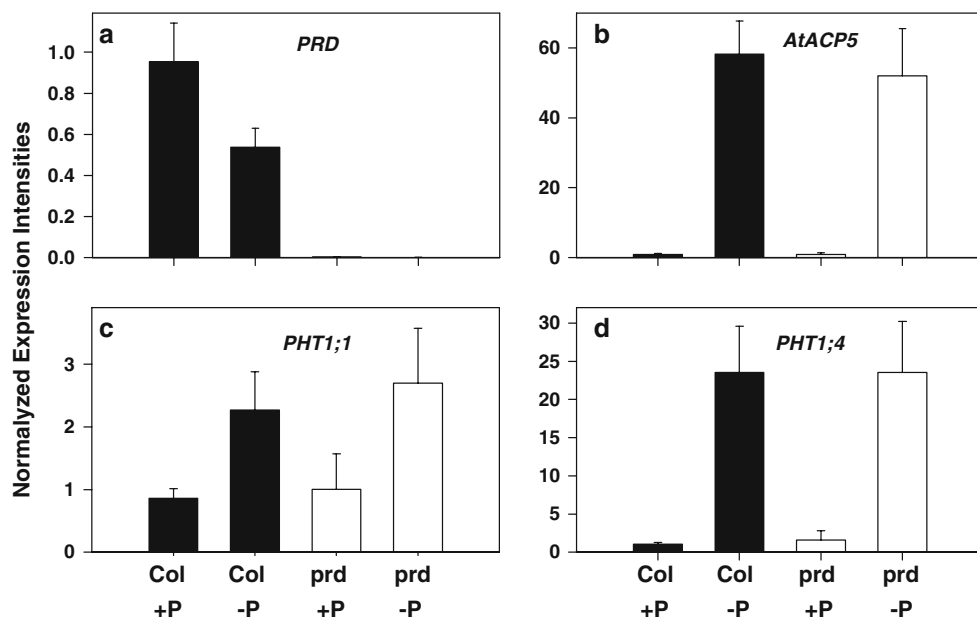
Taken together, these results support the hypothesis that the *PRD* gene does not function as a checkpoint for Pi-starvation responses, but acts specifically as a regulator of root architectural response to Pi starvation.

### Discussion

Since roots often determine the capacity of plants to efficiently explore and exploit the spatially heterogeneous composition of the soil, changes of root architecture are one of the major adaptive responses of plants to Pi starvation. Two of the most consistent root architectural changes that occur during Pi starvation are the reduction in primary root growth and the prolific growth of lateral roots (Williamson et al. 2001; Al-Ghazi et al. 2003; Nacry et al. 2005; Sánchez-Calderón et al. 2006). As expected, we obtained similar results under our conditions with wild-type plants (Figs. 2c, 3). Recently, it was reported that the reduction in primary growth is a consequence of a determinate program induced by low Pi that inhibits cell division in the primary root meristem and promotes differentiation within the root tips (Sánchez-Calderón et al. 2005). However, low Pi conditions did not lead to complete permanent loss of meristem activity. When plants were transferred from low to high Pi medium, primary root growth was rapidly stimulated (Chen et al. 2000). This result suggests a high degree of meristem plasticity in response to external Pi status and an adjustment of root architecture via changes in meristem activity. Moreover, the maintenance of a basal level of meristem function under low Pi conditions could be a basic process to reorient root growth in *Arabidopsis* and is probably under the control of multiple genes, to increase the flexibility of root plasticity responses.

Despite their important implications, little is known about the molecular events controlling for Pi sensing and the effect of Pi starvation on root system architecture. Several studies have provided new insights into the genetic components involved in root architectural adaptive

**Fig. 7** Effect of Pi availability on the expression profiles of the *Arabidopsis PRD*, *AtACP5*, *PHT1;1* and *PHT1;4* genes. **a** Quantitative RT-PCR analyses of *PRD*, **b** *AtACP5*, **c** *PHT1;1*, and **d** *PHT1;4* gene expression. Relative levels of expression are expressed in arbitrary units. Wild type (black bars) and *prd* mutant (white bars) seedlings were grown for 7 days at high Pi concentration (1 mM) and then transferred for 5 days on media with high (1 mM) or low (3  $\mu$ M) Pi concentrations on vertically orientated agar plates. Root gene expressions were measured at day 12. Data correspond to the average values ( $\pm$ SE) of three independent measurements



responses to Pi availability (Ticconi et al. 2004; Sánchez-Calderón et al. 2006; Svistoonoff et al. 2007). The data presented in this work showed that mRNA accumulation of *PRD*, an AP2/ERF transcription factor, in *Arabidopsis* roots was regulated by the Pi content of the medium and strongly repressed by Pi deficiency (Figs. 1, 7). The physiological function of the *PRD* gene was analyzed through the null allele mutant line *prd*. The effects of Pi availability on architectural root variables were compared between wild type and *prd* mutant plants (Figs. 2c, 3). Mutants displayed shorter primary roots under Pi-starvation conditions than wild-type plants. Moreover, low Pi availability clearly reduced lateral root elongation in *prd* mutant plants, whereas it promoted it in wild-type plants. These results suggest that the *PRD* gene is involved in the regulation of root architectural responses to Pi starvation by controlling primary and lateral root elongation. This is in agreement with the tissue-specific expression pattern of the *PRD* gene which was specifically observed in the apex in both the primary and lateral roots (Fig. 5). In addition, no difference was observed in the emergence of lateral roots between *prd* and wild-type plants suggesting that the *PRD* gene was not directly involved in the initiation and emergence of root primordia, but in the constitutive control of root growth. It should be emphasized that both the *prd* and wild-type plants showed similar root architectural responses to the lack of other nutrients, including nitrate, sulfur, and iron (Fig. 6), and thus, conferring a specific role for *PRD* in the regulation of root architecture under Pi-starvation conditions. Other typical Pi-starvation responses such as leaf size reduction (Table 1), induction of high-affinity Pi transporters and acid phosphatase gene expression (Fig. 7), and Pi influx, as well as endogenous Pi content were similar in *prd*

mutant and wild-type plants (Supplementary Figs. S2, S3). Overall, these data suggest that the *PRD* gene does not function as a checkpoint in the cascade of Pi-starvation responses, but acts specifically as a modulator of root growth in response to Pi deprivation. A complex series of signaling cascades that control plant responses to Pi starvation is emerging to include several transcription factors such as AtWRKY75, a WRKY transcription factor (Deviah et al. 2007), or PHR1, a myb transcription factor (Rubio et al. 2001), but these genes appear to control a wide range of Pi-starvation responses. To our knowledge, *PRD* is the first identified molecular element that can act specifically on the root architecture in responses to Pi deprivation and does not seem to be involved in other starvation responses (Fig. 7; supplementary data). Our results underline the complexity and the diversity of the signaling cascades involved in the responses to Pi privation. Nevertheless, it is important to notice that public microarray database analysis through Genevestigator (<https://www.genevestigator.ethz.ch/>) show that At1g79700 gene expression was affected in the shoots by different stress suggesting a more general role of *PRD* in the adaptive responses of the plant to fluctuant environments.

Interestingly, *PRD* belongs to the *AINTEGUMENTA*-like (*AIL*) gene family (Nole-Wilson et al. 2005). *AIL* genes are mainly expressed in actively growing and developing tissues and may specify meristematic or division-competent states (Nole-Wilson et al. 2005). Several genes of the *AIL* family have been functionally characterized. *APETALA2* (*AP2*), one of the founding members of the *AP2* family, is a floral homeotic gene that specifies sepal and petal identity (Bowman et al. 1991). *AINTEGUMENTA* (*ANT*) is required for integument initiation and promotion of growth within

developing floral organs (Elliott et al. 1996; Klucher et al. 1996). BABY BOOM (BBM) has been reported to be involved in a variety of critical plant cellular functions and is preferentially expressed in seeds (Boutillier et al. 2002). *BBM* is likely to promote cell proliferation and morphogenesis during embryogenesis. Recently, a clade of four *PLT* homologs was found to be required for specification and maintenance of stem cells within the root apical meristem and was necessary for the root formation (Aida et al. 2004; Galinha et al. 2007). On the other hand, all the *AIL* genes were expressed in multiple plant tissues, and at higher levels in young tissues (seedlings, roots, inflorescences, seeds, siliques) compared to older tissues (mature rosette leaves, stems). These data confirm that *AIL* genes are important regulators of floral and root development and are consistent with the possible role of *AIL* genes in the specification of meristem states. In this context, *PRD* should be considered as a constitutive element necessary for primary and lateral roots growth. *PRD* should be considered as a root factor functioning as a fine tune to adjust root growth and architecture in response to low external Pi availability. However, even in *prd* mutants, we did not observe a completely growth-arrested or truncated root system in response to Pi privation but rather a slowing down of root growth, suggesting that *PRD* is one of the components of the signaling pathway by which Pi starvation modulates primary and lateral roots growth. The lack of drastic phenotype also raised the issue of the functional redundancy. Based on the Needleman-Wunsch global alignment algorithm to find the optimum alignments, *PRD* gene presents 72% of similarity with At1g16060, another member of the *AIL* family (Nole-Wilson et al. 2005). These high sequence similarities may imply a partial functional redundancy. Future studies will define at a cellular level the role of *PRD* gene in cell division and/or cell elongation processes and will focus on dissecting the interacting components involved in the regulation of root architecture.

**Acknowledgments** This work was supported in part by the INRA ECOGENE programme. JJCC and JR received a research fellowship from Consejería de Educación y Ciencia (Junta de Andalucía, Spain).

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