The phosphate transporters LjPT4 and MtPT4 mediate early root responses to phosphate status in non mycorrhizal roots

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ABSTRACT

Arbuscular mycorrhizal (AM) symbiosis improves host plant phosphorous (P) status and elicits the expression of AMinducible phosphate transporters (PTs) in arbusculecontaining cells, where they control arbuscule morphogenesis and P release. We confirmed such functions for LjPT4 in mycorrhizal Lotus japonicus. Promoter-GUS experiments showed LjPT4 transcription not only in arbusculated cells but also in root tips, in the absence of the fungus: here LjPT4 transcription profile depended on the phosphate level. In addition, quantitative RT-PCR confirmed the expression of Lotus and Medicago truncatula PT4 in the tips of non-mycorrhizal roots. Starting from these observations, we hypothesized that AMinducible PTs may have a regulatory role in plant development, irrespective of the fungal presence. Firstly, we focused on root development responses to different phosphate treatments in both plants demonstrating that phosphate starvation induced a higher number of lateral roots. By contrast, Lotus PT4i plants and Medicago mtpt4 mutants did not show any differential response to phosphate levels, suggesting that PT4 genes affect early root branching. Phosphate starvation-induced genes and a key auxin receptor, MtTIR1, showed an impaired expression in mtpt4 plants.We suggest PT4 genes as novel components of the P-sensing machinery at the root tip level, independently of AM fungi.

Key-words: AM-inducible phosphate transporters; LjPT4; MtPT4; root apex; root branching; phosphate starvation; phosphate sensing; Lotus japonicus; Medicago truncatula.

INTRODUCTION

Phosphorous is an essential plant macronutrient, but its low availability in many soils limits plant growth (Schachtman et al. 1998): as a consequence phosphate (Pi) supply to increase crop yield is a worldwide procedure. However, massive use of Pi fertilizer represents both a considerable expense for farmers and an ecological problem for water bodies downstream of agricultural runoff (Cordell et al. 2009). Moreover, Pi fertilizers come from rock Pi, a nonrenewable natural resource. Consequently, Pi deposits are being depleted rapidly; some analyses indicate that if this rate of consumption continues, the world's reserves should last for only about 125 years (Gilbert 2009). By contrast, the demand of Pi is expected to grow by 2.7% per year (FAO 2015). Facing this environmental limitation and improving Pi uptake require better knowledge of the morphological and physiological adaptations that plants use to acquire nutrients (Raghothama 1999). One of the most ancient and successful adaptations for nutrient acquisition involves the establishment of a symbiosis with arbuscular mycorrhizal (AM) fungi. AM symbiosis improves plant Pi uptake via efficient supply of Pi from the AM fungus, and up to 100% of plant Pi uptake can come from the AM pathway (Ravnskov and Jakobsen 1995). Recently, the distal and local effects of AM colonization on direct root Pi uptake were investigated, suggesting that AM fungi influence the direct root Pi uptake activity locally but not in distal direct pathway activity (Watts-Williams et al. 2015). AM symbiosis specifically induces the expression of some plant Pi transporters (PTs), such as MtPT4 in Medicago truncatula, OsPT11 in rice and AsPT1 in Astragalus sinicus (Harrison et al. 2002; Paszkowski et al. 2002; Xie et al. 2013). Such genes are considered functional markers ofAMsymbiosis. PTproteins accumulate in specific domains of the periarbuscular membrane (Pumplin and Harrison 2009) and give plants access to the Pi absorbed from the extraradical mycelium, via the H+ energy gradient produced byH+-ATPases (Krajinski et al. 2014;Wang et al. 2014). Functional symbiosis requires AM-induced PTs, and Pi acts as a key regulator, as exposure to high Pi abolishes theAM interaction (Balzergue et al. 2011; Breuillin et al. 2010). To optimize nutrient uptake, plants have also evolved root system plasticity and the capacity to sense and react to environmental nutrient conditions; thus, the external Pi level also controls root morphology and branching (Kellermeier et al. 2014; Péret et al. 2011). Studies on root system architecture under different Pi level have been mostly conducted on Arabidopsis plants, where the exposition to low Pi led to a shallow root, an inhibition of primary root (PR) elongation and an induction of lateral root (LR) formation (Gruber et al. 2013). The increase in LRs under low Pi availability is regulated by a nutrient-sensing machinery located in the root tip (Svistoonoff et al. 2007). Plants have developed complex mechanisms to a transfer Pi-starvation signals and maintain Pi homeostasis. Roots perceive the extracellular nutrient status and send signals to the shoot via the xylem; once the shoot has sensed the signals, it sends signals via the phloem both to the shoot apices and roots to adjust developmental processes and nutrient uptake (Liu et al. 2009; Lucas et al. 2013). The Pi-starvation signalling pathway has been well studied inArabidopsis, where theMYB transcription factor PHR1 was identified as a central component of the primary Pi response (Rubio et al. 2001). PHR1 binds to the P1BS cis element (Rubio et al. 2001) and induces the expression of many Pi starvation-inducible genes, such as AtIPS1/At4, miR399, PHF1, and genes encoding SPX domain-containing proteins (Bari et al. 2006; Nilsson et al. 2007; Duan et al. 2008; Bayle et al. 2011; Stefanovic et al. 2011). The SPX/PHR

interaction was recently reported, in both Arabidopsis and rice, as being directly influenced by Pi, providing a link between Pi sensing and signalling (Puga et al. 2014; Wang et al. 2014b). In recent years, many studies have investigated how Pi sensing influences AM symbiosis. In 2010, Branscheid and colleagues correlated miR399 expression, Pi homeostasis, and AM development in plants, speculating thatmiR399 represents a systemic low-Pi signal promoting or required for AM formation. Here we aimed to increase our knowledge of the plant Pi starvation signalling pathway, understanding whether the AM inducible PTs could also be players in the plant Pi-sensing/signalling regulatory machinery. We previously observed that LjPT4, a Lotus japonicus homologue of MtPT4, is expressed not only in arbusculated plant cells, as expected for AM-inducible PTs, but also in root tips of non-mycorrhizal plants (Volpe et al. 2013b). To examine the function of LjPT4 in arbusculated cells and in root tips, we investigated its effect on arbuscule formation, plant P concentration, PR and LR length, and LR formation at high and low Pi levels. Our findings demonstrated that arbusculecontaining cells require LjPT4 for the proper arbuscule formation on the fungal side and for improved Pi uptake on the plant side. In parallel, we used quantitative real time polymerase chain reaction (qRT-PCR) and promoter GUS activity to show that LjPT4 and MtPT4 expression in root tips depends on Pi levels.We also provided direct evidence for a link between AM-induced PTs, early root branching, and transcript levels of Pi-starvation marker genes. These results suggest that AM-responsive PTs play a role in root tips, creating a link among Pi-perception, root branching, and Pisignalling mechanisms.

MATERIALS AND METHODS

Plant growth and mycorrhization

Seeds of L. japonicus (Miyakojima ecotype, MG-20) were treated as described (Volpe et al. 2013b). M. truncatula WT (cv. Jemalong, genotype A17) and the mtpt4-2 mutant (Javot et al. 2011) were scarified with 3min in H2SO4, washed several times in distilled water, sterilized with 2min in diluted commercial bleach (1:5), and finally washed 4 times with sterile distilled water. Seeds were pregerminated on agar plates (0.6%w/v) in dark conditions, at 23 °C, for 48 h. Then seedlings were transferred to the different plates.WT and mutant seeds have been collected and stored in the same condition. Plants used for mycorrhization were inoculated with Gigaspora margarita or Funnelliformis mosseae. G. margarita spores were collected froma vernalized pot of inoculated Trifolium pratense. One hundred spores were used for the inoculation of each pot containing four plants. F. mosseae inoculum (30%) (BEG 12), purchased from MycAgroLab (www.mycagrolab.com), was mixed with a sand/perlite (2:1) mixture and used for colonization.

Plasmid constructs

RNAi constructs were created with a 158 bp fragment of LjPT4. The PCR product, amplified from genomic DNA using the primers listed in Table S1, was recombined into pK7GWIWG2D(II), which allows expression of a hairpin RNA under the control of the CaMV 35S promoter (Karimi et al. 2002) and contains the eGFP gene as a screenablemarker. A control RNAi construct was created, similarly targeting a fragment of the GUS (UidA) gene. A LjPT4 promoter fragment of 1143 bp was PCR-amplified from genomic DNA using the primers shown in Table S1. The promoter fragment was recombined upstream of the GUS gene in the vector pKGWFS7.0,

where the red fluorescent marker DsRED, under the control of the constitutive Arabidopsis UBIQUITIN10 promoter (pUBQ10) (Limpens et al. 2005), was inserted.

Generation of Lotus composite plants

The generation of composite plants, with transgenic roots on a wild-type shoot, was performed as described by Volpe et al. (2013b). Plants with transformed roots were grown in pots, mycorrhized as described in the preceding texts, and fertilized with 4mM NO^{3-} and 20 μM Pi, high N and low P concentrations (Javot et al. 2011). Plants were sampled after 8weeks. Transformed roots were selected under the stereomicroscope, by examining the fluorescence of the reporter gene present in the used plasmid (DsRED for GUS experiments and eGFP for the silenced lines). Plants harbouring transformed roots with the silencing construct were analysed, using the primers reported in Table S1, to measure the LjPT4 mRNA levels, according to Volpe et al. (2013b). The LjPT4 expression level in the non-mycorrhizal plants was too low to be detected by qRT-PCR on the whole root apparatus. Therefore, we quantified the transcript level and the silencing effect in mycorrhizal roots where LjPT4 is expected to reach its highest expression. In detail, at the end of the experiment on root branching (refer to in the succeeding texts), all putative silenced plants were inoculated with F. mosseae and after 2months we evaluated both the mycorrhizal status and the silencing level of LjPT4 by qRTPCR. On this basis, root architecture analysis was performed exclusively on the plants, which a posteriori resulted to be efficiently silenced in the range between 100 and 70%.

RNA isolation, cDNA synthesis, and quantitative RT-PCR

RNA isolation, cDNA synthesis, and qRT-PCR were performed as described (Guether et al. 2009). The sequences of the oligonucleotides used in this experiment are reported in Table S1.

Assessment of the arbuscule phenotype

Transformed root segments of PT4-silenced and GUS lines (PT4i and GUSi lines) were embedded in agarose (8%). Agarose blocks were cut into 200 μM -vibratome slices, which were put on a slide. Slices were treated for 5 min in 0.5%commercial bleach diluted in Pi buffer (pH7), washed again, and then incubated for 2 h with wheat germagglutinin-fluorescein isothiocyanate (WGA-FITC) (Sigma-Aldrich, Milan, Italy), at a final concentration of 10 μg/mL, to detect the chitin of fungal cell walls. Working conditions for the Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) for observations and image acquisition were performed as previously described (Genre & Bonfante, 1997).

Histochemical analysis of root tissue

Lotus composite plants carrying transformed roots were fertilized with 20 μM or 2mM Pi and inoculated with F. mosseae or left as control plants. Root fragments showing DsRed fluorescence were selected under a stereomicroscope and excised. The root segments were covered with freshly prepared GUS buffer and treated according to the method described by Calcagno et al. (2012).

Analysis of root architecture after treatment with high and low phosphate

Lotus or Medicago WT seedlings were grown on square plates (15 × 15 cm) containing Minimal M medium (Bécard and Fortin 1988) with 20 μM KH2PO4 (low Pi level, LP) or 2mM KH2PO4 (high Pi level, HP) and Phytagel 0.4% (Fig. S1a).

Both media had the same pH value, 5.5. Each plate contained seven or five 4-day-old Lotus or Medicago WT seedlings, respectively, with a total number of 49 Lotus and 50 Medicago plants. Moreover, in order to homogenize the plant growth, the seedlings were placed on the plate with the hypocotyl zone at 3 cm from the upper edge and the distance between plants was 1.9 and 2.5cm for Lotus and Medicago, respectively (Fig. S1a). Root system development was monitored over two weeks. Every day each plate was scanned using an Epson Expression 1680 (Epson), and root architecture was analysed using SmartRoot software (Lobet et al. 2011; Fig. S2). For each plant we measured the length of PR and LR and counted the number of LRs, as represented in Fig. S2. In order to demonstrate the involvement of LjPT4 and MtPT4 in the early regulation of LR formation, root architecture analysis on transgenic (Lotus PT4i lines and Medicago mtpt4 mutants) and WT plants was performed with two growth systems in round plates (Ø 9 cm): an agar cube system and a whole-plate system. Round plate is the ideal system to analyse the LR formation, because the single plant is able to produce more LRs compared with the square plate. For the agar cube system, one 4-day-old Lotus or Medicago WT seedling per round plate was grown on plant agar (1%) media, placing the PR tip in contact with an agar cube containing Minimal M medium as described in the preceding texts Fig. S1b). At least 13 Lotus or Medicago plants were used or each treatment, except for PT4i lines, for which we used ine plants. Because we found in the first experiment on T plants that the first time point where there was a difference n the number of LRs between the two different levels f Pi was the seventh day, LR formation was monitored at hat time point, placing the root tip of each newly emerged R in contact with an agar cube. Cubes were replaced every ay with new ones, allowing the root tips to remain in contact ith fresh medium. The same agar cube system was applied o analyse LR formation in PT4i and GUSi Lotus lines and n Medicago mtpt4 mutants. Because both LjPT4i lines and tpt4 mutants gave the same root response to different levels f Pi, we decided to investigate the response in mtp4 mutants, earing a stable mutation and therefore easier to handle. In his experiment, one 4-day-old Medicago WT and mtpt4 mutant eedling per round plate was grown on Minimal M medium t the same Pi concentration, as described in the receding texts. We called this set-up whole-plate system Fig. S1c). Twenty plants were used for each treatment. LR formation was analysed for seven days.

Phosphorous quantification

For P quantification, about 2mg of dried material was digested in 1mL 6MHNO3 for 1 h at 95 °C. The analysis was performed as described (Zouari et al. 2014). In silico analysis PLACE database (Higo et al. 1999) was used to analyse the main plant cis-acting regulatory DNA elements in the LjPT4 promoter.

Statistical analysis

Datasets were analysed using Past v.3.08 (Hammer et al. 2001) for one-way ANOVA with Tukey post hoc tests (where data passed Shapiro–Wilk and Levene tests for normality and homogeneity of variance) or rank correlations and nonparametric Kruskal–Wallis and Mann–Whitney tests.

Accession numbers

LjPT4, chr1.CM2121.10.r2.a; LjUBQ10, chr1.TM0487.4; LjPT1, chr5.CM0341.370.r2.a; LjPT2, chr1.CM0295.140.r2.m; LjPT3, chr6.CM0722.390.r2.m; LjPHO2, chr4.CM0432.1480. r2.m; LjSPX3,

chr3.LjT35O23.80.r2.d; MtPT4, AY116211; MtTEF1a, TC178258; MtPHO2, Medtr2g017250.1; MtSPX, Medtr1g013760.1.

RESULTS

The LjPT4 promoter is activated in arbuscule-containing cells

Previous experiments in L. japonicus demonstrated that the Pi transporter LjPT4 was induced more than 1400-fold in mycorrhizal roots, and laser microdissection analysis revealed LjPT4 transcripts in arbusculated cells (Guether et al. 2009). These observations suggested that LjPT4 has a role in arbusculecontaining cells, similar to MtPT4 (Javot et al. 2007), OsPT11 (Paszkowski et al. 2002), and AsPT1 (Xie et al. 2013). To examine the activity of the LjPT4 promoter, 1143 base pairs of the upstream sequence were isolated. In silico analysis using the PLACE database (http://www.dna.affrc.go.jp/PLACE/ signalscan.html) revealed conserved eukaryotic cis-elements: a TATA-box -70 to -74 bp upstream of the start codon and a CAAT-box between -42 and -46 bp from the start codon. Analysis of the LjPT4 promoter also revealed the presence of a root motif box (ATATT), anOSE1ROOTNODULEmotif (AAAGAT), a CATATGGMSAUR motif (CATATG) involved in auxin responsiveness, and other two conserved regulatory cis-elements, P1BS and MYCS, already reported in Volpe et al. (2013a). The P1BS and MYCS motifs were identified as necessary for the activation of Pi starvation-inducible genes and of AM-responsive PTs, respectively (Chen et al. 2011). For analysis of promoter activity, the LjPT4 upstream sequence was fused to the UidA reporter gene and used to create composite Lotus plants carrying this construct in the roots. Plants were inoculated with F. mosseae and fertilized at LP levels (20 μM), allowing successful AM colonization. Transformed mycorrhizal roots of 8-weeks-old plants were subjected to histochemical GUS staining, which revealed GUS activity in the colonized root area (Fig. 1a,c,d,e), whereas non-colonized cortical cells and epidermal cells were unstained (Fig. 1c). Meristems and caps of the root tips, which cannot be colonized by AM fungi, were unstained (Fig. 1b). At higher magnification, cortical cells showed GUS activity exclusively in arbuscule-containing cells (Fig. 1d,e). In conclusion, GUS experiments confirmed the qRT-PCR results (Guether et al. 2009), showing that LjPT4 is a mycorrhiza inducible gene.

LjPT4 silencing affects arbuscule morphology and P concentration

We next used RNA interference to examine the effect of LjPT4 silencing. In order to analyse arbuscule morphology, eight PT4i and eight GUSi lines, used as controls, were stained with WGA-FITC, a lectin which binds to chitin, a component of the fungal cell wall. The fungal structures in PT4i lines showed an abnormal phenotype, with swollen main arbuscule trunks and reduced branching (Fig. 2a). In PT4i lines, 74% of the arbuscules showed this morphology, while in control GUSi lines, most of the arbuscules showed normal morphology (Fig. 2b,c). The PT4i and GUSi lines had similar levels of colonization, with a fungal frequency around 40% and an arbuscule abundance of 14%(Fig. 2d).We considered PT4i lines with silencing efficiency ranging between 100 and 70% (Fig. 2e). To confirm that the LjPT4 silencing construct specifically affects LjPT4, we measured the transcript levels of other characterized Lotus PTs. In particular, we measured the expression of LjPT3 (Maeda et al. 2006), another PT induced by the AM symbiosis, LjPT1 and LjPT2 (Maeda et al. 2006), which are involved in Pi transport but are not induced by AM symbiosis. We

verified that the silencing construct was specific to LjPT4 and did not affect the expression of the other characterized LjPT genes (Fig. S3). To investigate whether LjPT4 silencing also affected symbiotic Pi transfer, we quantified the root P concentration in mycorrhizal PT4i and GUSi lines. PT4i lines showed lower P concentration than GUSi lines (Fig. 2f). Moreover, the lower P concentration of PT4i lines was confirmed by the analysis of two marker genes for Pi-starvation. Indeed the mycorrhizal PT4i lines showed a higher Pi starvation status than mycorrhizal GUSi lines (Fig. S4), as indicated by the expression levels of both marker genes. Taken as a whole, the results showed that the downregulation of LjPT4 affected arbuscule morphology and Pi absorption but did not affect the expression of other PTs involved in both symbiotic and direct uptake.

LjPT4 is expressed in the root apex in the absence of AM fungi

In a previous study focused on a MYB-like transcription factor, we observed that LjPT4 was also expressed in the root tips in the absence of AM fungi, in a Pi-dependent manner (Volpe et al. 2013b). To confirm these preliminary results, we collected one hundred root apexes (1–2mm) per each biological replicate and quantified LjPT4 transcript levels by qRT-PCR. Furthemore, plants expressing pPT4-GUS were grown in low (LP, 20 μM) and high Pi (HP, 2mM) for 8weeks. Histochemical GUS assays showed strong staining in the root apex of LP plants (Fig. 3a), but weaker staining in the HP plants (Fig. 3b), consistent with the relative expression levels of LjPT4 (Fig. 3c) under these conditions. To validate these findings in another model plant, we selected M. truncatula, because MtPT4 has been well characterized (Harrison et al. 2002; Javot et al. 2007; Javot et al. 2011). Medicago plants were grown and root tips were collected in the same way as Lotus. MtPT4 was expressed in the root apex depending on the Pi level (Fig. 3d), consistent with the molecular and histochemical results from Lotus data. The root apex, not susceptible to AM colonization, is probably involved in nutrient sensing through root cap (Svistoonoff et al. 2007). Therefore, the localization of both PTs in the root apex, as well as their Pi-dependent expression, suggested that PT4 might have a role in Pi sensing, independently of AM fungal presence.

Lotus and Medicago root architecture responses to phosphate

In order to investigate Medicago and Lotus responses to high and low Pi, we grew 5 and 7 seedlings per each squared plate, respectively, and we monitored PR and LR growth and number of LRs over 14 days (Fig. 4). The analysis on a total of 50 Medicago and 49 Lotus plants showed that Pi has a transitory effect on PR growth in both plants, because the differences in PR length between the two treatments were significant at a single time point only (7 days). By contrast, the length of LR showed two opposite trends: in Lotus LR average length increased significantly after 14 days under LP treatment; on the contrary, in Medicago, the average value of LR length did not increase linearly and HP plants had longer LR than LP. To explain this different behaviour, we hypothesized that LP condition promotes a continuous meristematic activity in Medicago roots, leading to new LR formation over time. However, the main effect induced by LP treatment in both plants regarded the number of newLRs: LP treatment induced higher number after 1week and this effect got more significant over time. Therefore, in order to characterize the activity of LjPT4 and MtPT4 in the perception of Pi, we focused on their role in root branching, 7 days after the beginning of the Pi treatment.

Impairment of LjPT4 or MtPT4 affects root responses to Pi in Lotus and Medicago

To understand whether L. japonicus was sensitive to different Pi concentrations and whether the root apex participates in Pi sensing, we used an agar cube system. In this system, each Lotus seedling was grown on plant agar (1%) media on a round plate, placing thePRtip in contactwith an agar cube containing Minimal Mmedium with LP or HP (Fig. S1b). After one week, the Lotus WT plants in contact with the LP agar cubes showed more LRs than plants in contact with HP medium (Fig. 5), confirming previous experiment (Fig. 4). To test whether LR formation depends on LjPT4, we monitored the PT4i and GUSi plants with the same agar cube system, treating the root tips with LP or HP. After one week, the GUSi plants with all root tips in contact with LP showed increased LR formation compared with the plants treated with HP (Fig. 5), in line with the results obtained with Lotus WT plants. By contrast, PT4i plants showed a similar number of LRs in LP orHP treatments (Fig. 5), revealing a role for LjPT4 at the root tip level. To confirm the Lotus results, we performed the same experiment using MedicagoWT and mtpt4 mutant plants. Firstly, we showed that over 7 days of treatment at LP or HP, mutant plants were not affected in their growth compared with WT plants. Moreover, they showed a similar PR growth (Fig. S6). Then, we used the agar cube system to monitor LR formation in Medicago WT seedlings at different Pi concentrations. In contrast to Lotus and to the experiment performed in squared plates (Fig. 4), Medicago roots exposed to HP agar cubes produced more LRs than roots exposed to LP agar cubes (Fig. 6a). However, the AM-induced MtPT4 played a similar key role in determining the number of LRs: mtpt4 mutant plants (Javot et al. 2011) lost their capacity to react to high/low Pi and, as a consequence, showed no change in LR number in HP and LP (Fig. 6a). Secondly, to understand whether the mtpt4 phenotype was maintained in another setup, the experiment was repeated by growingWTand mtp4 mutant seedlings in a whole-plate system, where the plate contained a minimal M medium at LP or HP level (Fig. S1c). After seven days, the mtp4 mutant plants showed a similar level of LR formation in both Pi concentrations (Fig. 6b), whileWT plants showed reduced LR branching in HP (Fig. 6b), in line with the L. japonicus results in both experimental conditions (agar cube and whole-plate systems, Fig. 5 and Fig. S5, respectively). Expression of MtPT4 on 7-day-old Medicago rootlets during branching experiment was investigated by RT-PCR, showing the presence of MtPT4 transcripts in both LP and HP plantlets (Fig. S7). In conclusion, we validated for both legumes that root branching depends on Pi level and that PT4 genes have a relevant role in this response.

The mtpt4 mutants showed a reduced response to Pi status

To assess whether MtPT4 has a role in Pi accumulation in root tips of non-mycorrhizal plants, we quantified the P concentration of 12-day-old WT and mtp4 mutant plantlets grown at LP or HP. The result revealed no significant differences in total P concentration between WT and mtp4 plantlets (Fig. 7). The experiment demonstrated that MtPT4 has no significant role in Pi accumulation in non-mycorrhizal plants under LP or HP condition, differently from mycorrhizal roots where MtPT4 plays a crucial role in the Pi uptake (Javot et al. 2007). By contrast, we found that the impairment of MtPT4 causes aberrant gene expression of the Pi-starvation marker genes MtPHO2, a Pi-homeostasis marker, and MtSPX, a Pi stress marker. As expected, under Pi starvation conditions (LP) the WT plants showed up-regulation of MtSPX and down-regulation of MtPHO2, consistent with plant P concentration (Fig. 8), and in line with expression patterns previously observed by Branscheid et al. (2010). By contrast, in mtpt4 mutants, Pi levels did not significantly affect the expression of MtSPX and MtPHO2. In addition, themutants showed an

impaired expression of a key auxin receptor, MtTIR1, indicating no significant expression difference between LP and HP treatment, differently from WT plants where LP induces an overexpression of MtTIR1 (Fig. 8). Therefore, we concluded that the root systeminWTplants is indeed much more sensitive to Pi availability, compared with mtpt4 mutants, as suggested by the higher responsiveness of the Pi-starvation genes.

DISCUSSION

Plant PTs in the Pht1 group allowmycorrhizal plants to take up Pi from their fungal partners and also regulate arbuscule morphogenesis (Javot et al. 2011; Xie et al. 2013; Yang et al. 2012). Here, we demonstrate that L. japonicus LjPT4 plays similar roles inAMsymbiosis, and we further show that LjPT4 may have an additional role in the root tips, in the absence of the AM fungus. Our results suggest that LjPT4 integrate signals from plant Pi status and regulate relevant developmental programmes, like root branching. This novel function was validated in another model legume, M. truncatula, using mtpt4 mutants, which guarantee a stable impairment of gene function. In addition, the expression of Pi starvation-induced genes and the auxin receptor MtTIR1 was impaired in mtpt4 plants. LjPT4 is an AM-induced Pht1 phosphate transporter essential for functional AM symbiosis LjPT4i composite plants phenocopied ospt11 mutants (Yang et al. 2012) and resembled mtpt4 mutants (Javot et al. 2011), with stunted arbuscules and compromised hyphal branching. Consistent with this effect on arbuscule morphogenesis and perifungal membrane biogenesis (Javot et al. 2007), PT4i plants accumulate less P than WT mycorrhizal plants, confirming the pivotal role of PT proteins in the development of a functional symbiosis and in the accumulation of Pi from the fungal partner. Pi constitutes the main ‘currency’ accepted by the plant to balance the flux of carbon compounds provided to AM fungi by plants (Kiers et al. 2011). However, the network that links Pi availability, success of theAMsymbiosis, and PTs is not fully understood. One open point is whether PTs also sense the amount of symbiotically-delivered Pi in the periarbuscular space and thus regulate arbuscule formation, as proposed for OsPT13 (Yang et al. 2012) and AsPT1 (Xie et al. 2013), or whether the systemic level of Pi in the plant is the main factor governing AM colonization (Balzergue et al. 2011; Breuillin et al. 2010). Impairment of the function of AM PTs usually leads to collapsed or stunted arbuscules (Fig. 2a), but this does not imply minor colonization by the AM fungus, as confirmed in our experiment (Fig. 2d). Taken as a whole, the current data prompted us to ask whether AM-inducible PTs may act as central nodes in regulatory networks involving environmental conditions (P status), biotic components (AM fungi and their arbuscule development), and more general plant processes, such as root development. The study of Lotus and Medicago root development system represents the main focus of this work.

Low phosphate induces LR formation in Lotus and Medicago

Our studies on root systemarchitecture in Lotus and Medicago plants investigate developmental adaptations to different Pi, providing an overview on different root traits, such as PR and LR growth and LR numbers, at LP or HP. We showed that the root apparatus of Lotus and Medicago plants is more complex compared with Arabidopsis, where it is possible to define root branching and LR formation zones (Dubrovsky and Forde 2012). In our system, the two zones cannot be discriminated because the LR formation was spread over the entire root length (Fig. S8). Moreover,

both legume plants, differently from Arabidopsis, did not show a dramatic inhibition of PR growth during the LP treatment, even if we detected a single time point where the PRgrowthwas lower in the plants exposed to LP, suggesting an early and temporary response (Fig. 4). Similar behaviour of Lotus and Medicago plants was reported for monocots, where the PR growth was not affected by LP treatment nor in rice or barley plants (Péret et al. 2014). On the contrary, Pi status affects the formation of LRs, where both legume plants showed a significant change in LRs number. From these results we can conclude that the LR formation parameter, rather than PR growth, is more ideal for the study on developmental adaptation to different Pi level in Lotus MG20 and Medicago A17 genotypes.

LjPT4 and MtPT4 are expressed in root tips where they regulate early root branching

We previously observed that the AM-induced gene LjMAMI was up-regulated in arbusculated cells from mycorrhizal plants, as well as in root meristems, also in the absence of the fungus (Volpe et al. 2013b) and that LjPT4 showed a similar unexpected expression pattern. Here, promoter-GUS experiments confirmed those preliminary data in Lotus root tips. In addition, our qRT-PCR experiments corroborated the expression of LjPT4 and MtPT4 in Lotus and Medicago root tips. In both cases, transcript levels depended on the Pi level, with PT expression up-regulated in LP and downregulated in HP. Because AM symbiosis has a crucial effect on root system architecture (Amor et al. 2003; Fusconi 2013; Gutjahr and Paszkowski 2013), we hypothesized that root branching could be another component of the regulatory network linking PTs, Pi, andAMfungi.Root branching experiments helped us to examine the effect of Pi levels on root architecture of Lotus and the role of the root apex in Pi sensing. Indeed, we found that contact of just the root tip with low Pi media was sufficient to induce LR formation (Fig. 4). This experiment agrees with results described in Arabidopsis, which is a non-mycorrhizal plant, (Svistoonoff et al. 2007) potentially allowing us to use molecular and genetic knowledge from Arabidopsis to support our characterization in legumes. As a second step, we demonstrated that LjPT4 is responsible for the early regulation of LR formation, thus providing a clue about its possible role as a Pi sensor. LjPT4 has a mode of action similar to that of AtNRT1.1, a nitrate transporter of Arabidopsis (Remans et al. 2006): the sensing of Pi levels could activate a transcription factor - yet to be characterized- involved in LR formation. The cells of the root tip have been proposed to receive sensory information from the root cap and trigger a molecular response in the elongation zone (Baluška and Mancuso 2013). A similar mechanism was proposed to act in arbuscule formation, where the Pi delivered to the periarbuscular space constitutes a signal allowing the host to maintain functional colonization (Breuillin-Sessoms et al. 2015; Javot et al. 2007; Yang et al. 2012). This signal is probably detected byAM-PTs that control either the traffic or the concentration of outer Pi. AM-PTs could play a similar role in the root tip, monitoring the cellular Pi, regulating root system architecture according to Pi levels (Figs 5 & 6). Lastly, a higher branched root system eventually increases the chances of meeting an AM fungus. Medicago and Lotus belong to the same family, the Fabaceae, but their ancestors diverged 37-38 MYA (Hyung et al. 2014). Notwithstanding this distance, MtPT4 and LjPT4 seem to act in a similar way, confirming the conservation of the AM symbiosis molecular toolkit among legumes. The findings in this study indicate that our set-up could constitute a novel and reliable tool in order to test the Pi-sensing capacity of AM-induced PTs.

Mtpt4 knockout-impaired Pi-starvation and MtTIR1 auxin receptor gene expression
To obtain further information about the role of MtPT4 in the plant's Pi starvation responses, we quantified transcript levels of two marker genes for Pi stress (MtSPX) and homeostasis (MtPHO2) by qRT-PCR. The mtpt4 mutants showed a reduced response to Pi level, as indicated by the expression levels of both genes, which did not significantly change between LP and HP (Fig. 8). Although further biochemical assays will be needed, it is tempting to speculate that the AM-PTs act as Pi sensors. Because MtPT4 was previously localized in the periarbuscular membrane (Javot et al. 2007) and not inside the nucleus, it is unlikely that MtPT4 directly regulates MtPHO2 and MtSPX transcription. Thus, it could act in other ways: (i) it could participate indirectly in the network that leads the regulation of Pi starvation-responsive genes or (ii) it could directly sense Pi, regulating subsequent signalling pathways. In addition, mutant lines showed an impaired expression of a key auxin receptor, MtTIR1, whose homologue in Pi-starved Arabidopsis plants showed an increased expression, enhancing the auxin sensitivity and the LR formation and emergence (Pérez-Torres et al. 2008). In conclusion, a growing body of evidence indicates the importance of environmental sensors related to nutrient transporters (Holsbeeks et al. 2004; Schothorst et al. 2013). In yeast, a Pi (Popova et al. 2010) and two sulfate transporters (Kankipati et al. 2015), located in the plasma membrane, have been shown to trigger a sensing pathway inside the cell, independently of the translocation of the substrate, and therefore, they have been defined as transceptors, a nutrient transporter that can function also as nutrient sensor. Our data suggest that AM-inducible PTs could act upstreamof the Pi-sensing cellular machinery, representing potentialmembrane Pi transceptors in an AM-independent pathway, even though their mechanism of action remains to be elucidated. However, our results demonstrated that LjPT4 andMtPT4 are expressed in root tips, mediate early root branching and their knockout impairs the expression of Pi-starvation marker genes, opening new lines of research on Pi sensing in mycorrhizal and non-mycorrhizal roots. Currently, in plants, no techniques have been developed to discriminate between effects caused by the transport function of a transporter and those caused by its receptor function. Therefore, a further characterization in yeast could represent the most feasible way to further describe the LjPT4 andMtPT4 signalling cellular role. Lastly, these findings convincingly demonstrate that LjPT4 and MtPT4 are operational in a constitutive way, opening the question of their role in an evolutionary context. During the colonization of land, Devonian plants experienced strong mineral nutrient deprivation and roots were not present yet (Bonfante & Genre, 2008). Plant molecular mechanisms in Pi sensing could have represented driving forces in different nutrient uptake strategies. In this hypothetical scenario, the same PT4 proteins were able to perceive external Pi conditions and regulate both the LR formation and the interaction with AM fungi.

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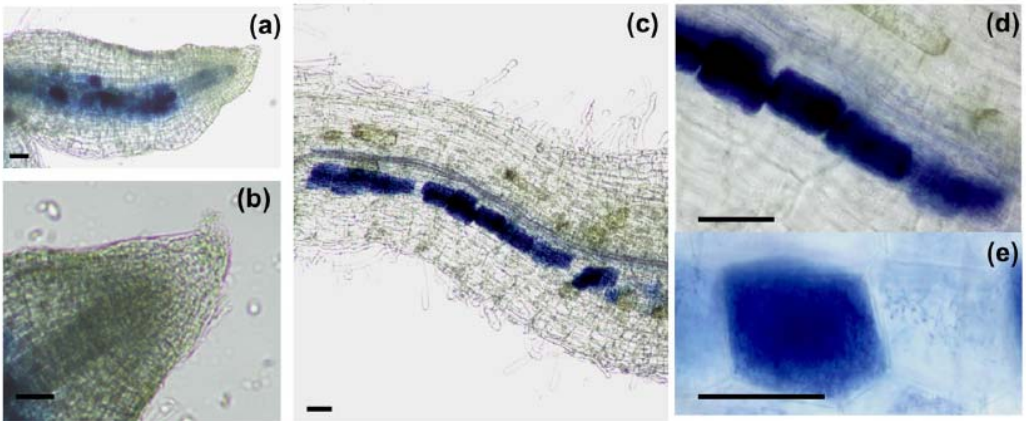

Figure 1. Histochemical GUS staining ofmycorrhizal Lotus japonicus roots expressing pLjPT4:GUS. The localization of GUS activity confirms the exclusive presence of LjPT4 in the arbusculated cells (a-e), whereas the non-colonized cortical cells, the epidermal cells and the root tip were not marked. (b) shows a magnification of the tip, where the root meristem and the cap are unstained, while (d) and (e) show a magnification of arbusculated cells with a strong staining. Scale bars 80 μM.

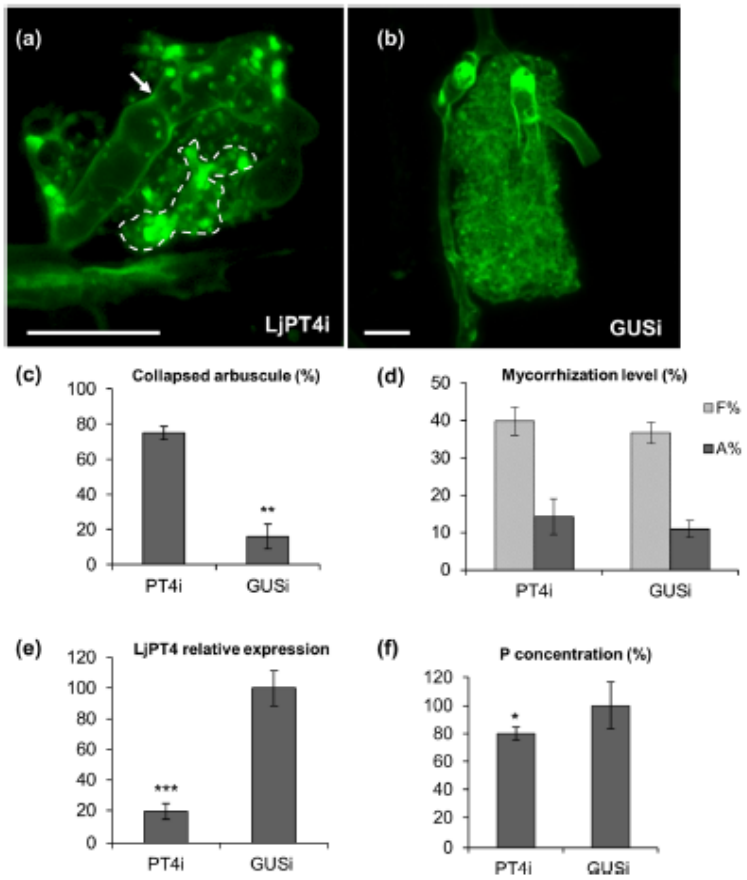

Figure 2. AM phenotype of LjPT4i silenced lines. (a) Cortical cells from PT4i lines contain swollen unbranched hyphae or coiled hyphae with frequent septa (arrow) and a reduced number of loose hyphal branches (dashed line) which clump at highly-fluorescent spots. (b) In control GUSi lines, arbuscules have the expectedmorphology with a basal trunk andmany hyphal branches which fill up the cortical cell lumen. Scale bars: 80 µM (a) and 10 µM (b). (c) Ratio of collapsed arbuscules in PT4i and GUSi lines. PT4i lines had 75%of degenerated arbuscules, whereasGUSi lines only 16% at 3months post inoculation. Data are based on the measurements of 30 infection units for each of three biological replicates. (d) Frequency of mycorrhizal hyphae (F) and arbuscule abundance (A) of PT4i and GUSi lines after trypan blue staining. Both lines showed a similar frequency and arbuscule abundance.One hundred root fragments (1 cm) were analysed for each sample. (e)Validation of LjPT4 downregulation through qRT-PCR in PT4i andGUSi lines. A significant ($P<0.001$) reduction in the expression level is revealed in PT4i lines. (f) Root phosphorous (P) concentration in PT4i andGUSi lines. PT4i lines showed a reduced capacity in the P accumulation compared withGUSi lines, on the basis of P concentration measured in the whole root system. Data are the average of eight plants for each treatment. Error bars represent SE. Asterisks indicate significant differences (ANOVA test, *$P<0.05$, **$P<0.01$, and ***$P<0.001$).

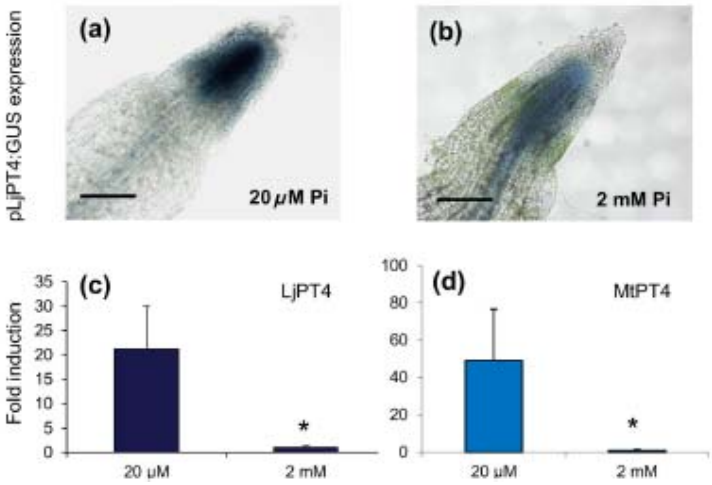

Figure 3. Expression of LjPT4 and MtPT4 in the root apex. (a, b) Histochemical GUS staining of L. japonicus roots expressing pLjPT4:GUS at 20 µM Pi, LP (a), or 2mMPi, HP (b). Vivid blue staining is detected in the root tips of plants grown at LP (a), whereas weaker staining is detected in plants grown at HP (b), revealing that pLjPT4:GUS expression is phosphate-dependent. Cap is never stained. Scale bar: 800 µM. The histochemical results were in agreement with the gene expression in the root apex: quantification of LjPT4 (c) andMtPT4 (d) expression levels by qRT-PCR at LP and HP relative to LjUBQ10 and MtTEF1a, respectively. The expression of both genes was detectable in the root apex with higher level at LP, revealing phosphate-dependence. Black asterisks indicate significant differences for Kruskal-Wallis test with a P value <0.05. Values are the means of four biological replicates and the bars indicate SE.

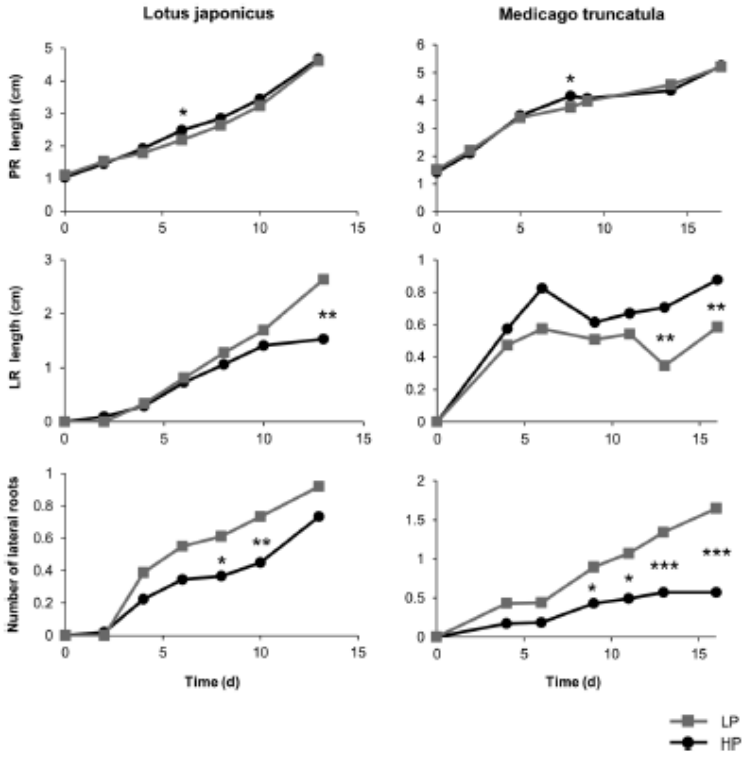

Figure 4. Lotus and Medicago primary and lateral root response to low and high phosphate. Primary root (PR) length was not affected during the exposition to low or high phosphate (LP or HP) in neither plants, except at the seventh day of the treatment when the PR growth was significant inhibited at LP. Lotus plants showed an increase in lateral root (LR) length during the treatment at LP, by contrast Medicago plants showed a more complex response with a decrease in LR length at LP level. Number of new LRs was significantly induced in both plants exposed to LP. Data are the average of 49 Lotus and 50 Medicago plants. Black asterisks indicate significant differences for Anova test, with a P value for ANOVA test (*<0.05, **<0.01, and ***<0.001).

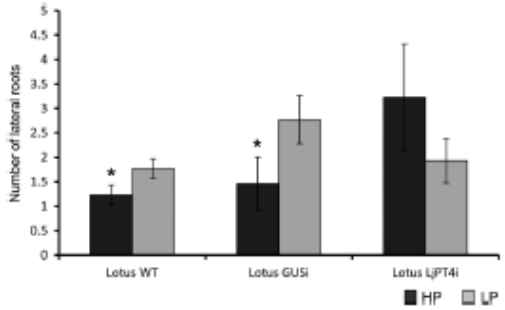

Figure 5. Lotus lateral root production depends on phosphate availability and on LjPT4 activity. The agar cube system was used to test the phosphate sensing by the root tip. Seedlings were grown on plant agar in round plates and the primary root tip was placed in contact with an agar cube containing M-medium with high phosphate (HP) or low phosphate (LP) levels of KH2PO4. When a new lateral root emerged, the root tip was placed in contact with a new agar cube. In Lotus WT plants and GUSi control plants, tip contact with LP media induced higher lateral root formation than tip contact with HP media. By contrast Lotus LjPT4i did not show any difference between HP and LP condition. Data are the average of at least 10 plants per each condition and the bars represent ± SE. Black asterisks indicate significant differences for Kruskal Wallis test, with a P value <0.05.

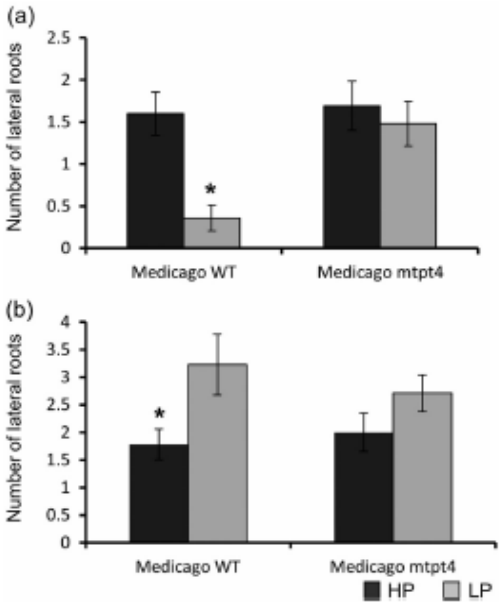

Figure 6. Medicago lateral root production depends on phosphate availability and onMtPT4 activity. The agar cube systemand the whole plate system were used to test lateral root branching with different phosphate availability. (a) In agar cube system, Medicago WT plants induce lateral root production when the tips are in contact with HP media with significant difference ($P<0.05$) compared with LP media. By contrast mtpt4mutants did not showany difference betweenHP and LP conditions. (b) In whole plate system, MedicagoWT plants show a different behaviour than plants in agar cube system, with an increase in lateral root formation in plants grown at LP with significant difference ($P<0.05$) compared withHPmedia.Differently,mtpt4 mutants show a similar lateral root production in both media, maintaining the same behaviour of plants in agar cube system.Data are the average of at least twenty plants and the bars represent ± SE. Black asterisks indicate significant differences for Kruskal Wallis test with a P value <0.05.

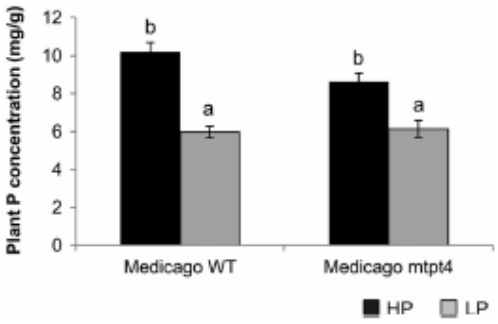

Figure 7. Phosphorous concentration inWTand mtpt4mutant plants. Phosphorous (P) concentration was measured in the whole plantlets. As shown on the chart, bothWTand mtp4mutant plants have the same P concentration in both treatments at lowand high phosphate.Data are the average of five plants for each treatment ± SE. Bars subtended by the same lowercase letter do not differ significantly at $P<0.05$, according to Tukey's test.

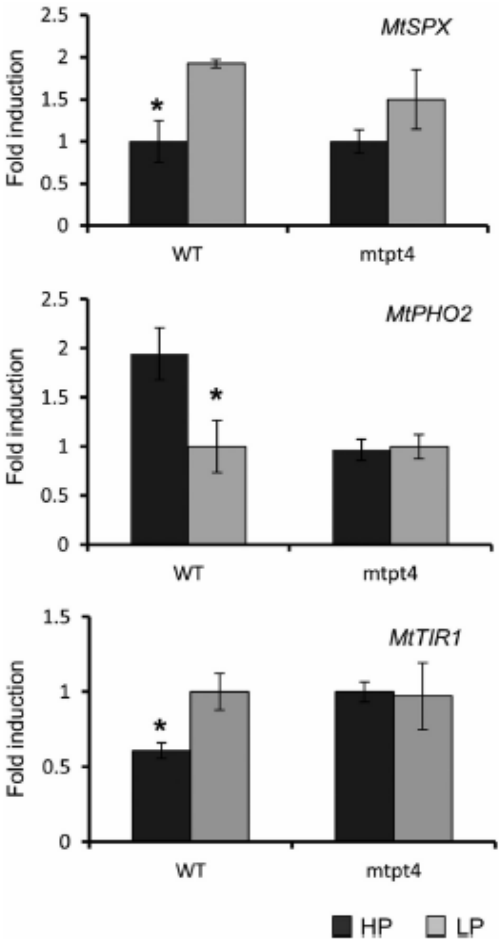

Figure 8. Fold induction of phosphate starvation marker gene, MtSPX and MtPHO2, and auxin receptor, MtTIR1, inWTand mtpt4 mutant plants. During the treatment at low phosphate, WT plants showed an induction of MtSPX and a downregulation of MtPHO2, as opposed to the plants treated at high phosphate. Conversely mtpt4 plants did not show any significant difference between the two phosphate (Pi) levels for neither genes, showing a possible impairment of Pi starvation mechanism. The same regulation was clear with MtTIR1, an auxin receptor whose homologue in Arabidopsis regulates root branching during Pi starvation. Data are the average of at least three biological replicates and the bars represent ± SE. Black asterisks indicate significant differences for KruskalWallis test, with a P value <0.05.