RESEARCH PAPER

A phosphoenol pyruvate phosphatase transcript is induced in the root nodule cortex of *Phaseolus vulgaris* under conditions of phosphorus deficiency

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Abstract

Although previous studies on N$_2$-fixing legumes have demonstrated the contribution of acid phosphatases to their phosphorus (P) use efficiency under P-deficient growth conditions, localization of these enzymes in bean nodules has not been demonstrated. In this study, phosphoenol pyruvate phosphatase (PEPase) gene transcripts were localized within the nodule tissues of two recombinant inbred lines, RIL115 (P-deficiency tolerant) and RIL147 (P-deficiency sensitive), of *Phaseolus vulgaris*. Nodules were induced by *Rhizobium tropici* CIAT899 under hydroaeroponic conditions with a sufficient versus a deficient P supply. The results indicated that PEPase transcripts were particularly abundant in the nodule infected zone and cortex of both RILs. Analysis of fluorescence intensity indicated that nodule PEPase was induced under conditions of P deficiency to a significantly higher extent in RIL147 than in RIL115, and more in the inner cortex (91%) than in the outer cortex (71%) or the infected zone (79%). In addition, a significant increase (39%) in PEPase enzyme activity in the P-deficient RIL147 correlated with an increase (58%) in the efficiency of use in rhizobial symbiosis. It was concluded that nodule PEPase is upregulated under conditions of P deficiency in the P-deficiency-sensitive RIL147, and that this gene may contribute to adaptation of rhizobial symbiosis to low-P environments.

Key words: nitrogen, nodule, *Phaseolus vulgaris*, phosphoenol pyruvate phosphatase, phosphorus, transcript.

Introduction

Phosphorus (P) is an essential, but limiting macronutrient that plays critical roles in plant metabolism and development. It is the least accessible macronutrient in many soils because it readily forms insoluble calcium salts in alkaline soils, or complexes with iron and aluminum oxides in acidic soils, rendering it inaccessible for root uptake (Richardson et al., 2009). The available P in many soils usually ranges from 1 to 10 µM (Hinsinger, 2001). This is far lower than the intracellular P concentrations of 5–20 mM that are required for optimal plant growth (Vance et al., 2003; Fang et al., 2009) and legume nodule development (Bargaz et al., 2011a). The legume nodules where symbiotic nitrogen fixation (SNF) takes place are particularly sensitive to low P availability, and N$_2$-fixing legumes usually require more P than plants dependent on mineral nitrogen (N) fertilizers (Serraj and Adu-Gyamfi, 2004).
Two major mechanisms are involved in P nutrition of legumes under P-deficient conditions: (i) increasing P acquisition using such mechanisms as root morphology, root exudation, and P uptake systems; and (ii) enhancing P utilization by internal mechanisms associated with efficient use of P at the cellular level (Raghothama, 1999; Vance et al., 2003). Secretion of acid phosphatases (APases) that hydrolyse esterified P is among the intricate array of adaptations used to enhance P acquisition and utilization from their environment (Vance et al., 2003; Richardson et al., 2009). Induction of intracellular and secreted APases appears to be a universal plant response to nutritional P limitation that participates in systemic P mobilization from soil organic matter-localized P, including nucleic acids (Tran et al., 2010). Likewise, several studies have suggested that high levels of APases in the rhizosphere may hydrolyse P from external organophosphates, which can comprise up to 80% of total soil P (Radersma and Grierson, 2004).

Generally, most APases are non-specific and may hydrolyse P from a broad spectrum of P_{i}, mono-esters over a wide pH range. These enzymes may constitute an adaptive mechanism for N_{2}-fixing legumes to tolerate P deficiency, as attested by the increase in the activities of APases and phytase in nodules of P-deficient Phaseolus vulgaris (Araújo et al., 2008). Moreover, a report on APases purified from soybean (Glycine max) nodules suggests that these enzymes are involved in the conversion of purines into ureides during SNF (Penheiter et al., 1997). Moreover, an APase recently identified as PvPAP3 in the roots of P-deficient P. vulgaris plants was most active with ATP as a substrate, suggesting that it might function in acclimation of these plants to P starvation through the use of extracellular ATP as a P_{i} source from the environment (Liang et al., 2010). Likewise, results on the examination of the vegetative vacuole proteome of Arabidopsis thaliana rosette leaf tissue have confirmed that APAP26 is the predominant vacuolar APase exhibiting high intracellular APase activities with phosphoenol pyruvate (PEP) as a substrate (Veljanovski et al., 2006, Tran et al., 2010).

The results of the above studies, the marked activation of PEP phosphatase (PEPase) in P. vulgaris roots under conditions of P deficiency (Juszczuk and Rychter, 2002), and the characterization in embryonic axes of P. vulgaris of an APase encoding a dimer enzyme that presents its highest activity against PEP (Yoneyama et al., 2004) led us to assume that PEPase may play a key role in internal nodule P metabolism and thus may contribute to P use efficiency for SNF. Thus, the overall aims of this study were to utilize biochemical and molecular approaches to analyse the effect of P deficiency on both PEPase activity and transcript abundance in nodules of P. vulgaris–rhizobia symbiosis.

**Materials and methods**

**Plant material and growth conditions**

Experiments were conducted in a glasshouse under natural light with day/night temperatures of 28/20 °C and a 16 h photoperiod with additional illumination of 400 µmol photons m−2 s−1 and 70% relative humidity during the day. This study was carried out using two recombinant inbred lines (RILs) originating from the International Centre of Tropical Agriculture (CIAT), RIL115 and RIL147. The RIL115 and RIL147 lines were characterized previously under conditions of P deficiency as tolerant and sensitive genotypes, respectively, based on plant growth and seed yield in relation to P availability (Drevon et al., 2011). Seeds were surface sterilized with 3% calcium hypochlorite and then washed thoroughly in ten successive baths of sterile distilled water. Thereafter, seeds were germinated for 4 d at 28 °C in rolls of germination paper soaked in sterile distilled water.

Seedling roots were inoculated with the reference strain Rhizobium tropici CIAT899 grown in liquid yeast extract mannitol medium at 28 °C for 3 d to an approximate cell density of 10^{10} ml^{-1}. Thereafter, the seedlings were transferred into hydroaeroponic culture consisting of vats filled with 40 l of nutrient solution, which were aerated intensely and arranged in a fully randomized block design. The roots of selected uniform seedlings were passed through the hole of a rubber stopper on the vat cover. Cotton wool was fitted at the hypocotyl level to maintain the root system suspended in the nutrient solution (Vadez et al., 1999) with either 75 or 250 µmol P per plant per week, defined as P-deficient or P-sufficient supplies, respectively. Urea was supplied at 2 mM per plant into the nutrient solution during the initial 2 weeks of growth to avoid N deficiency during nodule development. Thereafter, the plants were grown in N-free nutrient solution. Each week, the nutrient solution pH was adjusted to around pH 7 by the addition of 0.2 g 1^{-1} of CaCO_{3} and the medium was aerated by an air flow of ambient air at 400 ml min^{-1} during the experiment.

**Design of PEPase gene primers with nodule DNA and RNA**

Nodules of approximately 3 mm diameter for each RIL corresponding to 50 ± 5 mg of nodule fresh weight were carefully detached at 42 d after transplantation (DAT) and immediately frozen in liquid N_{2} and stored at −80 °C until use. Prior to nucleic acid extraction, all solutions and glassware were rendered RNase free by diethyl pyrocarbonate (DEPC) treatment, and only certified RNase- and DNase-free plastic ware was used. Total RNA extraction was performed using a FastDNA Spin kit for soil, an RNase kit, and a Fast Prep Instrument (MP Biomedicals, Santa Anca, CA, USA). Extraction of DNA and RNA was confirmed and quantified using a Quan-iTRM Pico Green DNA Assay kit and a Quan-iTRM Ribof Green RNA Assay kit (Molecular Probes, Carlsbad, NM, USA) and visualized in an RNase-free agarose gel (1.5%). Yields of approximately 25 µg of DNA and 4 µg of total RNA g^{-1} of fresh weight of nodule were observed in the total extracts.

Design of the PEPase gene primers was performed online with the only PEPase found in GenBank, an enzyme characterized in Allium cepa (Shinano et al., 2001; GenBank accession no. AB052619.1). The protein sequence was analysed using BLAST against enzymes of plants in the family Fabaceae in the National Center for Biotechnology Information protein database. Subsequently, several primer pairs were designed for use in an in situ RT-PCR approach.

Total nodule RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase H (Promega, Madison, WI, USA) following the manufacturer’s recommendations. The different primer pairs were used to amplify gene products using 30 cycles of 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 45 °C, with final extension at 72 °C for 2 min, using cDNA and genomic DNA as templates. The amplified bands were ligated into a pGEM-T Easy vector (Promega) using bacteriophage T4 DNA ligase, and recombinant plasmids were transformed into Escherichia coli grown on Luria–Bertani/ampicillin/ IPTG/X-Gal plates at 37 °C for 24 h. The PCR products were sequenced (Beckman Coulter Genomics, UK) to verify the amplification of the desired gene.

Finally, we used the primers PEPdir (5'-ATGTCATCGTTTATGAAAGATC-3’) and PEPrev (5'-CTTGGGCTACATCGTGGCGT-3’) to quantify and localize the P vulgaris gene transcript of PEPase. These primers show 100% nucleotide identity to a P vulgaris mRNA for APase (GenBank accession no. AB116720.1; Yoneyama et al., 2004).

**In situ RT-PCR of PEPase transcripts**

Sample preparation and fixation for in situ RT-PCR were carried out according to the protocol described by Molina et al. (2011). The method involves in situ amplification of specific nucleic acid sequences on nodule sections, followed by fluorescence detection (Van Aarle et al., 2007) of the localized
PCRs were performed to visualize the PEPase transcript and ten images per RIL and P treatment were used for statistical analyses of the signal quantification.

**PEPase and APase assays**

Samples of nodules of 3 mm diameter corresponding to 100 ± 5 mg of nodule fresh weight were carefully detached from roots at 42 DAT and immediately frozen at −80 °C. Each nodule sample was ground with an extraction mixture consisting of 500 µl of 0.1 M sodium acetate buffer (pH 5.6) containing 1 mM DEPC-treated water, and freed from residual agarose by three washes with DEPC-treated water heated at 60 °C. For reverse transcription, the first cDNA strand was synthesized from total RNA of the 50 µm nodules slices using MMLV reverse transcriptase following the manufacturer’s recommendations. Nodules slices were incubated in 40 µl reverse transcriptase mix containing the PEPase gene-specific reverse primer PEPRev. Negative controls were prepared by omitting the reverse transcriptase. The reverse transcriptase mix was removed and 40 µl of PCR mix was added including 0.25 mM each of the gene-specific PEPdir and PEPrev primers. Thermocycling was performed using 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, with extension at 72 °C for 2 min.

The amplified cDNA in the fixed tissue was then detected after removing the PCR mix. Samples were washed and incubated in 100 µl of blocking solution under gentle agitation in the dark at 37 °C. The blocking solution was replaced by 100 µl of alkaline phosphatase-conjugated anti-dioxigenin Fab fragment (Roche Diagnostics, Basel, Switzerland) diluted 1:1000 in 2% BSA. The samples were incubated at room temperature for 90 min and then washed three times to remove excess antibody. Detection of alkaline phosphatase was carried out using an ELF-97® endogenous phosphatase detection kit (Molecular Probes, Leiden, The Netherlands). Observations were made with an Olympus BX61® microscope (Olympus, Hamburg, Germany) equipped with an epifluorescence condenser, a Hoechst/DAPI longpass filter set configured at an excitation filter of 360–370 nm, a dichroic mirror of 400 nm and an emission longpass of 420 nm. Images were photographed with a grey View II® camera (ORCA AG; Hamamatsu) using Analysis® software (Soft Imaging System, Munster, Germany). Image analysis and signal quantification were performed using ImageJ software as an image analysis program. Overall, 16 in situ RT-PCR experiments were performed to visualize the PEPase transcript and ten images per RIL and P treatment were used for statistical analyses of the signal quantification.

**Results**

**Localization of the PEPase transcript**

The PEPase transcript was detected in the infected zone, nodule cortex, and vascular traces for both RIL115 and RIL147 and under both P treatments (Fig. 1). Under conditions of P deficiency, the PEPase transcript was more abundant in nodules of the sensitive RIL147 (Fig. 1G) than in the tolerant RIL115 (Fig. 1C), most clearly in the infected zone. More precisely, at a higher magnification, the PEPase transcript was detected mainly in infected cells (Fig. 1I), particularly those localized next to the inner cortex (Fig. 1D, E and H), while there was no transcript in uninfected cells. Moreover, it could clearly be seen that more PEPase transcript was localized in the outer cortex than in the inner cortex (Fig. 1D, E and H).

**Quantification of PEPase transcripts and phosphatase activities**

The results indicated that the PEPase transcript signal intensity increased significantly in the sensitive RIL147 under conditions of P deficiency (Fig. 2A). This sensitive RIL147 markedly increased the PEPase transcript abundance by 79, 91 and 71% in the infected zone, inner cortex, and outer cortex, respectively (Fig. 2B–D). Whatever the RIL and the P supply, the PEPase transcript signal intensity was higher in the outer cortex than in the inner cortex. By contrast, the signal intensity did not vary significantly in vascular traces (Fig. 2E).

Although P deficiency increased the PEPase activity for both RIL115 (29%) and RIL147 (44%), the increase was only significant in RIL147 (Fig. 3A). Likewise, pNPP APase activity showed the same trend as PEPase (Fig. 3B), with a significant increase under P deficiency in the sensitive RIL147 (39%) compared with RIL115 (23%).

Overall, considering both P treatments and the two common bean RILs, the proportion of PEPase was approximately 15% of the total APases measured in this study.
Fig. 1. *In situ* localization of PEPase transcripts (green spots) in nodules of common bean RIL115 and RIL147 (as indicated) inoculated with *R. tropici* CIAT899 and grown under a sufficient (250 P) versus a deficient (75 P) P supply. (A, A’) Negative controls without reverse transcriptase; (B, D) P-sufficient RIL115; (C, E) P-deficient RIL115; (F, H) P-sufficient RIL147; (G, I) P-deficient RIL147. InC, infected cell; IC, inner cortex; IZ, infected zone; OC, outer cortex; UC, uninfected cell; VT, vascular trace parenchyma. Bars, 500 µm (A, A’, B, C, F, G); 200 µm (D, E, H, I). (This figure is available in colour at *JXB* online.)
Nodule and shoot P and N content, and efficiency of use in rhizobial symbiosis

The results indicated that P deficiency significantly decreased the total nodule P content by 26 and 30% for RIL115 and RIL147, respectively (Fig. 4A), while the inorganic nodule P was significantly decreased by about 10% for both RILs (Fig. 4B).

Although shoot P content was decreased in both RILs, this parameter was not statistically different for the tolerant RIL115, whereas, for the sensitive RIL147, shoot P content was almost twice as high under P-sufficient compared with P-deficient conditions (Fig. 4C).

P deficiency decreased the nodule and shoot N contents for both RILs tested (Fig. 5). However, the decrease in nodule N content was not significant for RIL115 (31%) or RIL147 (14%). By contrast, shoot N content decreased significantly for RIL147 (46%). Overall, the decrease in N content was more pronounced in shoots than in nodules for plants grown under conditions of P deficiency.

The decrease in both P and N content was associated with a significant reduction in growth and nodulation of the tested RILs.

Fig. 2. PEPase transcript signal (pixel number of the green spots) in (A) total nodule sections, (B) infected zone, (C) inner cortex, (D) outer cortex, and (E) vascular traces of common bean RIL115 and RIL147 inoculated with *R. tropici* CIAT899 and grown under a sufficient (open columns) versus deficient (filled columns) P supply. Data are means ±SD of ten images of nodules harvested at 42 DAT. Mean values labelled with the same letter were not significantly different at *P* <0.01.

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The decrease in both P and N content was associated with a significant reduction in growth and nodulation of the tested RILs.
under conditions of P deficiency (Fig. 6). However, the decrease in both nodule and shoot biomass was higher for the sensitive RIL147 (41 and 29%, respectively) than for the tolerant RIL115 (14 and 15%, respectively). The biomass of shoots and nodules of the RIL115 and RIL147 were positively correlated (up to $r^2 = 0.6$) under both P treatments (Fig. 6). The slope value of the regression curve, namely the EURS, was higher under conditions of P deficiency than under P sufficiency, by 58 and 14% for RIL147 and RIL115, respectively.

Fig. 4. Nodule (A, B) and shoot (C) P content of common bean RIL115 and RIL147 inoculated with *R. tropici* CIAT899 and grown under a sufficient (open columns) versus deficient (filled columns) P supply. Data are means ±SD of six replicates harvested at 42 DAT. Mean values labelled with the same letter were not significantly different at $P < 0.01$.

Fig. 5. Nodule (A) and shoot (B) N contents of common bean RIL115 and RIL147 inoculated with *R. tropici* CIAT899 and grown under a sufficient (open columns) versus a deficient (filled columns) P supply. Data are means ±SD of six replicates harvested at 42 DAT. Mean values labelled with the same letter were not significantly different at $P < 0.01$.

**Discussion**

The development of a successful *in situ* RT-PCR methodology provides an interesting opportunity to link localization and distribution of candidate genes within organs such as nodules of N$_2$-fixing legumes. In addition, the technique allows quantification of the effects of environmental constraints on gene expression. Our work holds significant promise in addressing the effect of P deficiency on the nodule localization of a PEPase transcript that very probably correlates with the activity of PEP hydrolysis and presents the highest sequence homology with an APase gene expressed specifically in the embryonic axes of *P. vulgaris* (Yoneyama *et al.*, 2004).
The detection of a PEPase transcript in common bean nodules is, to our knowledge, the first observation of this gene expression among APase genes that are known to be overexpressed in legume nodules in response to P deficiency (Figs 1 and 2). Increased transcript levels for PEPase under conditions of P deficiency in nodules of RIL147 (Figs 1G and I and A), in contrast to that in nodules of the tolerant RIL115, which exhibited a similar level under both P treatments, suggest that PEPase may be involved in the tolerance of rhizobial symbiosis to P deficiency. This is consistent with a previous conclusion that P deficiency specifically affects SNF by limiting growth and survival of bacteroids (O'Hara et al., 1988), nodule function (Tang et al., 2001), and host plant growth (Tsvetkova and Georgiev, 2003).

Although the high level of PEPase transcripts under conditions of P deficiency is linked to a rise in nodule PEPase and total APase activities (Fig. 3), transcript abundance does not always predict intra- or extracellular proteome remodelling or in the gene products that result from nutrient limitations (Li et al., 2008; Tran and Plaxton, 2008). Nevertheless, the increases in these phosphatase activities were linked to a decrease in nodule P content, which may subsequently serve for ATP generation via O_{2} respiration, with at least 16 ATPs consumed per N_{2} reduced (Salsac et al., 1984). The link of PEPase expression to EURS, particularly for the sensitive RIL147 under P deficiency (Fig. 6), suggests tight regulation between EURS and the nodule P requirement (Fig. 4), probably in relation to the high energy requirement of the SNF process. In agreement with this, Li et al. (2011) reported that some members of the purple acid phosphatase gene family in soybean are possibly involved in the response of the host plant to symbiosis with rhizobia or arbuscular mycorrhizal fungi under P-deficient conditions.

Regarding the differential expression of the PEPase gene among nodule tissues (Figs 1 and 2), the high level of transcripts in the infected zone (Figs 1C and G and B) is consistent with a large P requirement for optimum metabolism of bacteroids, as well as for multiplication and survival of bacteroids. All of this is consistent with the high P content of nodules compared with other plant organs (Schulze and Drevon, 2005; Bargaz et al., 2011a). The increase in PEPase transcripts in the nodule cortex might be an adaptive response to P deficiency. Furthermore, PEPase expression in the inner cortex may play a role in the relation of SNF to O_{2} flux (Hunt and Layzell, 1993), which is postulated to be a regulator of osmotic conditions (Schulze and Drevon, 2005). The higher level of transcripts in the outer cortex compared with the inner cortex (Figs 1D, E and H and D) may be a response to the high P demand of the infected zone and the P mobilization from senescing tissues in the nodule external cortex. The production of pyruvate in P-deficient nodules may have an essential role in limiting the production of active oxygen species, as pyruvate has been proposed to be an antioxidant undergoing a non-enzymatic oxidative decarboxylation by hydrogen peroxide (Nath et al., 1995).

We conclude that PEPase can be classified as a P-starvation-inducible gene involved in the tolerance of nodulated legumes to P deficiency. The marked increase in PEPase transcripts in the nodule cortex of P-deficient nodules not only suggests an increase in intracellular P scavenging but also opens up a challenge to understand whether such sublocalization is involved in the scavenging of P in extracellular organophosphates. Although PEPase appears to be transcriptionally induced during P deficiency, additional studies are needed to understand whether this phosphatase plays a secondary role in response to constraints other than P deficiency, such as oxidative stresses, which may occasionally occur.

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