



RESEARCH PAPER

Phosphate deficiency regulates phosphoenolpyruvate carboxylase expression in proteoid root clusters of white lupin

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Received 25 June 2004; Accepted 13 August 2004

Abstract

Proteoid roots play a major role in enabling white lupin (*Lupinus albus* L.) to adapt to phosphate (Pi) deficiency. Such roots release citrate from proteoid rootlets, which allows this species to mobilize Pi from sparingly soluble Pi sources. Release of citrate is preceded by a significant accumulation of organic acids, in which a Pi deficiency-inducible phosphoenolpyruvate carboxylase (PEPC) activity has been involved. To gain an insight into this adaptive mechanism, the expression of three different transcripts coding for PEPC was examined in proteoid rootlets of Pi-starved and Pi-starved-and-rescued white lupin. Semi-quantitative reverse transcriptase (RT)-PCR experiments performed with gene-specific primers targeted to the 3'-end region of the corresponding cDNAs revealed that the transcripts for these three PEPCs differentially accumulate in both Pi-starved and Pi-starved-and-rescued proteoid rootlets. Semi-quantitative RT-PCR analysis in Pi-starved proteoid rootlets sampled at different times after being rescued from Pi deficiency showed that Pi levels differentially down-regulated the three PEPC transcripts. RT-PCR experiments were further extended to Pi-starved and Pi-fed whole roots, cotyledons, and leaves on which a tissue-specific, Pi-dependent PEPC expression was observed. These results indicate that there exists at least three different transcripts coding for PEPC in proteoid root clusters of white lupin, whose expression are differentially regulated by Pi.

Key words: *Lupinus albus*, PEPC activity, PEPC expression, proteoid rootlets.

Introduction

A plant's response to phosphate (Pi) deficiency involves a series of co-ordinated morphological, physiological, and metabolic changes that enable the plant to adapt to this unfavourable condition, thereby increasing its survival (Raghothama, 1999). Such changes have been shown to occur in white lupin (*Lupinus albus* L.), in which the foremost morphological adaptation to Pi deficiency is the development of proteoid roots (Gardner *et al.*, 1981). Proteoid roots are entire primary lateral roots that develop one or more clusters of rootlets along their axes (Watt and Evans, 1999a, b). In this species, proteoid roots accumulate citrate, which is subsequently released into the rhizosphere of Pi-starved plants (Keerthisinghe *et al.*, 1998; Neumann *et al.*, 1999; Peñaloza *et al.*, 2002a). Citrate release increases the bioavailability of Pi within the rhizosphere, thereby providing the white lupin with additional soluble Pi (Gardner *et al.*, 1982).

The accumulation of citrate in proteoid roots is accompanied by metabolic changes induced by Pi deficiency that alter the activity of several enzymes involved in organic acid synthesis and catabolism (Johnson *et al.*, 1994, 1996b; Neumann *et al.*, 1999, 2000). One such enzyme is phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31). This enzyme plays a major role as an anaplerotic carbon source for the biosynthesis of organic acids in response to Pi

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deficiency in many C_3 species (Hoffland *et al.*, 1992; Pilbeam *et al.*, 1993; Johnson *et al.*, 1994). In white lupin, non-photosynthetic CO_2 fixation mediated by increasing PEPC activity in Pi-deficient proteoid roots can provide 25% and 34% of the C excreted as citrate and malate, respectively (Johnson *et al.*, 1996b). Such PEPC activity varies along the proteoid root axis with the greatest activity occurring at sites of citrate exudation (Keerthisinghe *et al.*, 1998). Furthermore, *in situ* hybridization studies revealed PEPC expression in the cortex of emerging and mature proteoid rootlets, consistent with the sites of organic acid synthesis and excretion (Uhde-Stone *et al.*, 2003b).

The temporal and spatial organization of metabolic events occurring within proteoid roots of white lupin provides a unique system to study the molecular aspects of adaptation to Pi deficiency. To date, a number of genes related to organic acid synthesis that are induced or repressed in such a model tissue have been identified (Massonneau *et al.*, 2001; Peñaloza *et al.*, 2002b; Uhde-Stone *et al.*, 2003a, b). Recently, a full-length PEPC cDNA (*LaPEPC1*) from white lupin proteoid roots was isolated and characterized (Uhde-Stone *et al.*, 2003b). Due to the lack of evidence for additional PEPC isoforms, Uhde-Stone *et al.* (2003b) proposed that *LaPEPC1* corresponds to the predominant PEPC activity found in proteoid roots of white lupin. In this study, evidence that there exists at least three different transcripts coding for PEPC in this type of root is presented. Using a Pi-starved-and-rescue system, which allows the examination of molecular and metabolic changes in white lupin under varying Pi conditions (Peñaloza *et al.*, 2002a), it is demonstrated that the expression of these PEPC transcripts are differentially regulated by Pi in proteoid root clusters of this species.

Materials and methods

Plant material

Lupinus albus cv. Victoria-Baer was grown in a greenhouse in springtime with a mean temperature of 24/14 °C (day/night) and 60% relative humidity. Seed treatments and the basal nutrient solution used in these experiments were as described previously (Peñaloza *et al.*, 2002a). Twenty 5-d-old seedlings were planted into six 42 l containers filled with aerated basal nutrient solution without Pi. The solution was changed every 7 d and the pH adjusted daily to 5.8. Upon formation of mature proteoid roots, plants from two containers were supplied with 0.1 mM Pi (as KH_2PO_4). These plants are referred to as Pi-starved-and-rescued plants. When required, plants were maintained with 0.1 mM Pi throughout the experiment. These plants are referred to as Pi-fed plants.

Proteoid rootlets and root cluster axes were collected from 21–24-d-old Pi-starved and Pi-starved-and-rescued plants as described previously (Peñaloza *et al.*, 2002b). Whole roots and leaves were sampled from 24-d-old Pi-starved and Pi-fed plants, whereas cotyledons were sampled from 3-d-old seedlings. Plant tissues were harvested, frozen in liquid nitrogen, ground to a fine powder, and stored in microcentrifuge tubes at -80 °C. Frozen tissues were used for total RNA extractions and for quantifying PEPC activity and inorganic Pi levels.

Total RNA extraction

Total RNA was extracted from frozen tissues with phenol-containing Chomczynsky solution according to the manufacturer's instructions (W&Z, Santiago, Chile). To ensure the quality of total RNA, samples were visualized on denaturing agarose gels stained with ethidium bromide. To eliminate amplification of genomic DNA in RT-PCR analyses, RNA samples were treated with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA) at 37 °C for 1 h. Each RNA sample was quantified spectrophotometrically (Sambrook *et al.*, 1989).

Construction of a cDNA library and isolation of PEPC cDNA clones

A directional cDNA library was constructed from proteoid rootlets of 24-d-old Pi-starved white lupin. Poly(A)⁺ RNA was purified from total RNA using PolyATtract mRNA Isolation System III (Promega, Madison, WI, USA). The first cDNA strand was synthesized from 5 µg poly(A)⁺ with the SuperScript Lambda System for cDNA Synthesis and λ Cloning (Gibco BRL, Gaithersburg, MD, USA). Synthesized cDNAs were ligated into the λ derived phage λ ZIPLIX and subsequently packaged *in vitro* using the Packagene Lambda DNA Packing System (Promega) with *E. coli* Y1090 as a host strain. The original library of approximately 3.9×10^5 phages was amplified, aliquotted, and stored at -80 °C. A 313 bp *Hind*III fragment from the A427 clone, which contains the 3'-end region of a putative PEPC gene from *L. albus* (Peñaloza *et al.*, 2002b) was labelled with [$\alpha^{32}P$]dCTP (NEN Life Science Products, Boston, MA, USA) with the Random Primer Labeling kit (Promega) and used to screen the library. Hybridizations were carried out as described by Sambrook *et al.* (1989), but the temperature was decreased to 60 °C to reduce stringency. Positive clones were purified and the phage vector transformed to the plasmidial form by *in vivo* excision as indicated by the manufacturer. Plasmids were isolated and sequenced by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and the ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Representative cDNA clones were subsequently used to obtain the full-length PEPC cDNA by the GeneRacer kit (Invitrogen) according to the manufacturer's specifications.

Reverse transcriptase (RT)-PCR

Semi-quantitative reverse transcriptase (RT)-PCR experiments were carried out using total RNA extracted from proteoid rootlets, whole roots, cotyledons, and leaves. First strand cDNA was synthesized from 1 µg of the DNase-treated RNA in a final volume of 25 µl. The reaction mixture containing 0.2 µg of anchored oligo(dT) 23-mers, 10 mM DTT, 0.25 mM dNTPs, and 100 units RNasin (Promega), was incubated at 42 °C for 2 min before adding 200 units SuperScript II RNase H⁻ reverse transcriptase (Invitrogen). The reaction was incubated for an additional 50 min at 42 °C, before heat inactivation of the enzyme at 70 °C for 15 min. A second 1 µg aliquot of each RNA sample was subjected to the same conditions, but without the addition of RT (the minus RT control).

Primers for the PCR reactions were designed to amplify a specific region at the 3'-end of each PEPC cDNA. Specific PCR amplifications were obtained using anti-sense primers in the 3'-untranslated region (3'-UTR) and sense primers in either the 3'-end region of the open reading frame or the 3'-UTR (Fig. 1). The specific primer pairs were as follows: C36R, 5'-TATCTCACATTCCTTCCAACAA-3' and C36F, 5'-TTTCCGTCTCTTCTTTTATTC-3' for *LaPEPC2*; C41R, 5'-TTTTGGTGAGTGCAACTATGAT-3' and C41F, 5'-TCGTGACCCGAACCTTAATGTG-3' for *LaPEPC3*; C54R, 5'-AGCAGCATAATGACATAAAAAG-3' and C54F, 5'-CACTGAATCCAACAAGTGAATA-3' for *LaPEPC4*. Specificity of the primers was demonstrated by amplifying plasmids harbouring *LaPEPC2* (pLaPEPC2), *LaPEPC3* (pLaPEPC3), and *LaPEPC4*

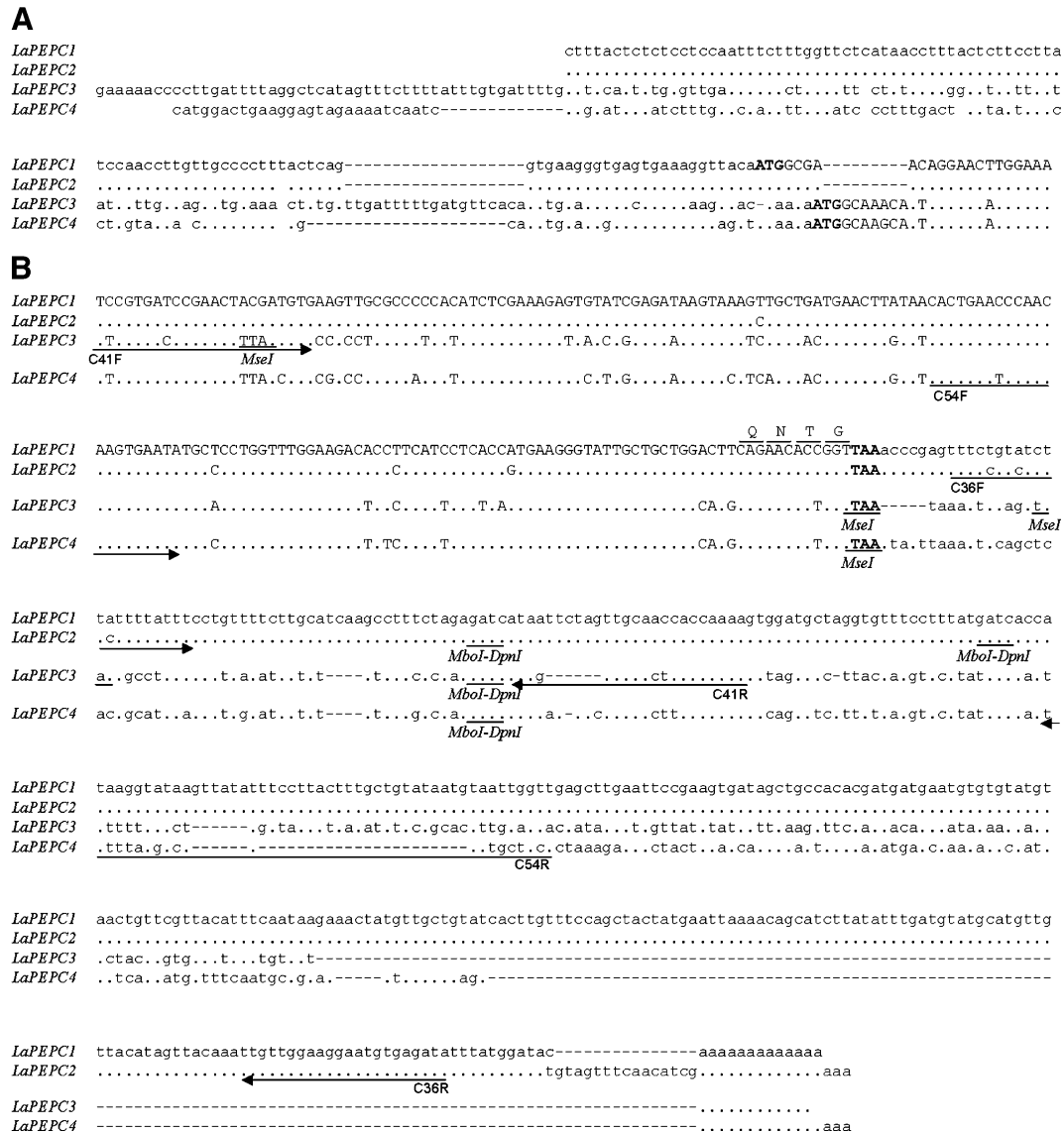


Fig. 1. Partial alignment of *LaPEPC2*, *LaPEPC3*, and *LaPEPC4* 5'-end (A) and 3'-end (B) cDNA sequences. Conserved nucleotides are indicated by a dot, and gaps introduced for alignment purposes by a dash. Untranslated 5'-end and 3'-end regions are shown in lower case whereas coding regions are in upper case lettering. *MboI*, *MseI*, and *DpnI* used to demonstrate primers specificity are indicated below their corresponding restriction sites. Nucleotides encoding the initiator codon and the stop codon are shown in bold letters. The location of gene-specific primers used for RT-PCR is marked with arrows indicating polarity 5 to 3. The motif QNTG is indicated above the nucleotide sequence. Partial alignment of *LaPEPC1* (accession AF459644) is included on (A) and (B). The nucleotide sequence of *LaPEPC2*, *LaPEPC3*, and *LaPEPC4* cDNAs were deposited in the GenBank under the accession numbers AY663386, AY663387 and AY663388, respectively.

(pLaPEPC4) cDNAs, and by digesting the amplified PCR products with *MboI*, *MseI*, and *DpnI*. The DNA methylation-dependent *DpnI* was used as a negative control of digestion. The restriction sites along the sequence of the three PEPC cDNAs for *MboI*, *MseI*, and *DpnI* are indicated in Fig. 1B. Control experiments showed that the selected primers specifically amplified a nucleotide sequence of the expected size for identifying *LaPEPC2* (346 bp), *LaPEPC3* (249 bp), and *LaPEPC4* (228 bp). These three products correspond to different unique cDNAs since no cross amplification with the gene-specific set of primers was observed (Fig. 2A). Likewise, restriction enzyme analysis shows the expected digestion pattern, demonstrating the specificity of the primer sets (Fig. 2B). Cyclophilin, a gene that does not change its expression pattern under the conditions analysed, was used as an internal control in the semi-quantitative RT-PCR experi-

ments. A 194 bp fragment of cyclophilin was amplified using primers 5'-AAAACCTAAACGAAGAC-3' and 5'-GTGAGGGACATAGAGAAG-3'.

PCR reactions were performed in a final volume of 30 μ l containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1.5 units *Taq* DNA polymerase (Invitrogen), 40 ng of cDNA template and 0.2 μ M of each primer. PCR reactions were conducted at 94 $^{\circ}C$ for 5 min to denature the RT product, followed by 16–30 PCR cycles of 94 $^{\circ}C$ for 40 s, 54 $^{\circ}C$ for 30 s, 72 $^{\circ}C$ for 30 s, and a 5 min final extension step at 72 $^{\circ}C$. PCR products were separated on 1.8% (w/v) agarose gels and visualized by ethidium bromide staining. No amplification products were detected with the specific primer sets when DNaseI-treated RNA was used as the template, confirming that the results of the semi-quantitative reactions represent mRNA transcript levels.

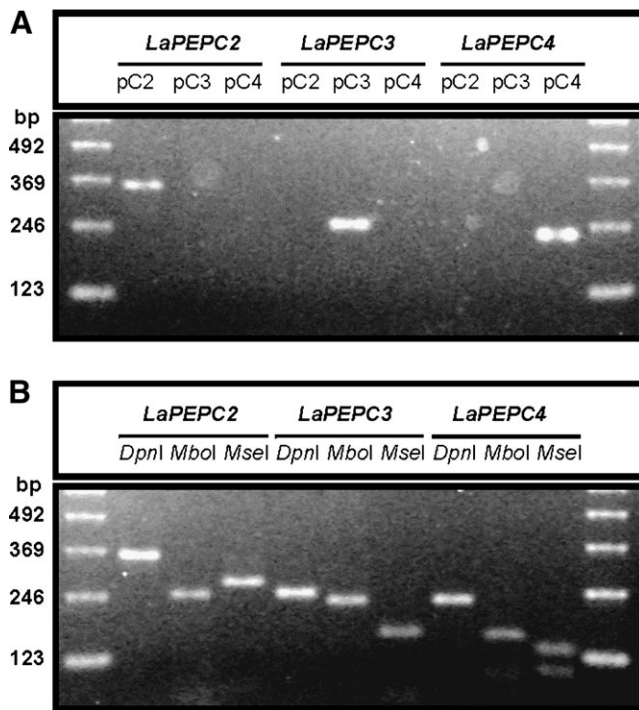


Fig. 2. Analysis of the specificity of the primers used in the semi-quantitative RT-PCR experiments. (A) Plasmids pLaPEPC2 (pC2), pLaPEPC3 (pC3), and pLaPEPC4 (pC4) cDNAs were used as templates for PCR amplification with gene-specific primer pairs for *LaPEPC2*, *LaPEPC3*, and *LaPEPC4*. (B) *LaPEPC2*, *LaPEPC3*, and *LaPEPC4* cDNA fragments amplified by PCR with gene-specific primer pairs were digested with *MboI*, *MseI*, and *DpnI*. The DNA-methylation dependent *DpnI* was included as negative control of digestion.

Reproducibility of the semi-quantitative RT-PCR experiments was demonstrated by using different RNA preparations from the same experiment as well as from an independent experiment that was established during the autumn. PCR products were digitalized using ethidium bromide staining intensity (in pixels) corrected for background, and quantified with the Gen Tools Analysis Software version 3.02.00.

Extraction and assay of PEPC activity

PEPC specific activity was determined in proteoid rootlets and root cluster axes sampled at different times after supplying 0.1 mM Pi to 21-d-old Pi-starved plants. Proteins were extracted from -80°C frozen samples that were ground to a fine powder with liquid nitrogen. The powder was homogenized with 50 mM HEPES/KOH pH 7.8, 1 mM EDTA, 0.1 M KCl, 2 mM DTT, 1% (w/v) PVP, and 10% glycerol. The homogenate was centrifuged at 10 000 g for 15 min at 4°C and the supernatant was used for PEPC activity and total protein quantification. PEPC activity was determined spectrophotometrically by monitoring NADH oxidation at 340 nm in a malate dehydrogenase coupled reaction. Standard conditions for PEPC activity were 0.1 M TRIS-HCl pH 8.0, 3 mM PEP, 10 mM NaHCO_3 , 10 mM MgCl_2 , 0.20 mM NADH, 10 units malate dehydrogenase (Boehringer Mannheim GmbH, Mannheim, West Germany), and 30 μl crude extract in a total volume of 1 ml. The reaction was initiated by adding PEP after a 5 min preincubation. Total protein was quantified according to Bradford (1976) using BSA as standard. PEPC activity was also assayed from an independent experiment established during the autumn. Both spring and autumn assays showed the same results.

Pi content in tissue

Inorganic Pi was extracted from frozen tissues with 2% (v/v) acetic acid (Bollons and Barraclough, 1997) and determined by the colorimetric procedure of Murphy and Riley (1962).

Statistical analysis

Variance analysis was used to determine the significance of Pi treatments, sampling dates, root tissues, and the corresponding interaction effects for PEPC activity. Means and standard deviations are provided where the *F*-test indicates significant main effects and two- or three-way interactions. Significant differences among means were estimated by using LSD at the 0.05 probability level.

Results

Isolation of PEPC cDNA clones

Approximately 1×10^6 phages of an amplified rootlets cDNA library were screened by using a 313 bp PEPC fragment as probe. Ten positive clones ranging in size from 0.6 kb to 1.8 kb were isolated. Comparisons of the 3'-end sequences showed that the ten clones could be sorted into three groups comprising three, three, and four clones each. Sequence analyses indicated that they all share a high degree of protein coding similarity to PEPC ($E < 10^{-30}$). Representative clones from each group were selected for further studies. These clones were designated pLaPEPC2 (0.9 kb), pLaPEPC3 (1.7 kb), and pLaPEPC4 (1.5 kb). Of these cDNA clones, pLaPEPC2 shares 97% identity over a 900 nucleotide stretch with the 3'-end PEPC cDNA from *L. albus* previously reported (*LaPEPC1*, GenBank accession AF459644) (Uhde-Stone *et al.*, 2003b). The nucleotide sequence of pLaPEPC3 shows 100% identity with accession BQ441944, a differential display product from proteoid rootlets of this species (Peñaloza *et al.*, 2002b). Sequences identical to pLaPEPC4 have not been reported previously. Clones pLaPEPC2, pLaPEPC3, and pLaPEPC4 were further used to isolate and identify their corresponding full-length cDNAs, which were designated *LaPEPC2*, *LaPEPC3*, and *LaPEPC4*, respectively. The main features and GenBank accession numbers of each full-length cDNA are presented in Table 1. Sequence alignment of the 5'-end and 3'-end of *LaPEPC2*, *LaPEPC3*, and *LaPEPC4* with *LaPEPC1* reveals the nucleotide residues conserved and divergent between these genes. This alignment shows the regions in which gene-specific primers were designed for RT-PCR experiments (Fig. 1).

Expression analysis of *LaPEPC2*, *LaPEPC3*, and *LaPEPC4*

The expression of PEPC genes was first assessed by analysing the pattern of mRNA accumulation in Pi-starved and Pi-starved-and-rescued proteoid rootlets. Semi-quantitative RT-PCR conducted at different numbers of PCR cycles revealed a significant difference in the kinetics of mRNA accumulation under both Pi conditions, when the PCR

Table 1. Characteristics of the full-length PEPC cDNAs isolated from Pi-starved proteoid rootlets of white lupin

PEPC cDNAs	GenBank accession	Full-length (bp)	Coding region (bp)	Identity coding region (%)		3'-UTR (bp)	Identity 3'-UTR (%)		5'-UTR (bp)	Identity 5'-UTR (%)	
				<i>LaPEPC3</i>	<i>LaPEPC4</i>		<i>LaPEPC3</i>	<i>LaPEPC4</i>		<i>LaPEPC3</i>	<i>LaPEPC4</i>
<i>LaPEPC2</i>	AY663386	3404	2904	87.7	87.3	397	27.3	32.2	103	32.9	45.7
<i>LaPEPC3</i>	AY663387	3320	2907		93.2	239		60.2	174		45.5
<i>LaPEPC4</i>	AY663388	3288	2907			248			133		

products specific for each of the three PEPC transcripts were within the linear range of amplification (Fig. 3A, B). These results indicate that the relationship between the transcript levels of the PEPC genes changes with changes in Pi availability. Similar quantities of amplified products of *LaPEPC3* and *LaPEPC4* in Pi-starved proteoid rootlets were obtained at 20 PCR cycles, whereas four more cycles were needed to reach equivalent quantities for *LaPEPC2*. Accordingly, 24, 20, and 20 PCR cycles were used for studying *LaPEPC2*, *LaPEPC3*, and *LaPEPC4* expression further, along with 22 PCR cycles for cyclophilin. Under these conditions, the absence of an amplification product was interpreted as a lack of or undetectable gene expression in the treatments and tissues being analysed. At 22 cycles of PCR amplification, cyclophilin mRNA was detected in all tissues and Pi treatments, thereby confirming that cyclophilin gene expression is not significantly affected by Pi (Peñaloza *et al.*, 2002b) and may, therefore, be used as an internal control for the RT-PCR reactions.

To examine the causal relationship between PEPC expression and Pi supply, semi-quantitative RT-PCR was performed in proteoid rootlets sampled at different times after adding 0.1 mM Pi. Results from this analysis demonstrate additional differences in the accumulated transcript levels between the PEPCs (Fig. 4A). The addition of exogenous Pi was reflected in the proteoid rootlets as an increase in the Pi content within this tissue (Fig. 4B). As Pi increases from 1.1 to 3.3 $\mu\text{mol Pi g}^{-1}$ fresh weight, *LaPEPC3* and *LaPEPC4* were differentially repressed while *LaPEPC2* appears unaffected. A further increase to 11.6 $\mu\text{mol Pi g}^{-1}$ fresh weight at 24 h did not affect the expression of *LaPEPC2* and *LaPEPC4* transcripts. However, *LaPEPC4* was strongly repressed 72 h after applying exogenous Pi, even though no significant difference in the steady-state of endogenous Pi was detected. This response to exogenously applied Pi indicates that subsequent alterations in endogenous Pi levels affect *LaPEPC3* expression significantly, whereas both *LaPEPC2* and *LaPEPC4* were less responsive to changes in Pi content within the tissue.

In order to determine tissue-specific differences in the transcript levels of the three PEPCs, semi-quantitative RT-PCR experiments were extended to different plant tissues. Transcripts of *LaPEPC2* were detected in whole roots and leaves, whereas transcripts of *LaPEPC3* and *LaPEPC4* accumulated at very low levels, primarily in Pi-starved tissues (Fig. 5A, C). Running PCR cycles at near saturation

(26 cycles of amplification) also showed detectable *LaPEPC2* transcripts in cotyledons of both Pi-starved and Pi-fed tissues. Under these PCR conditions, *LaPEPC3* and *LaPEPC4* transcripts were detected at significant levels in whole roots and Pi-starved leaves but not in cotyledons and Pi-fed leaves (Fig. 6B, C). This tissue-specific expression is further evidence that the three PEPC transcripts differ from each other.

PEPC activity in proteoid root clusters

In order to correlate PEPC expression with PEPC activity in mature proteoid root clusters, specific PEPC activity was assayed in the same tissues used for RT-PCR analyses. As expected, the highest specific activity was observed in Pi-starved proteoid rootlets, whereas it was reduced by 45% in Pi-starved proteoid root axes (Fig. 6). In order to determine how much of this activity is affected by Pi supply, PEPC activity was measured in rootlets and axes at 8, 24, and 72 h after adding 0.1 mM Pi to 21-d-old Pi-starved plants. PEPC activity remained essentially constant in Pi-starved proteoid rootlets, whereas it was significantly reduced by exogenous Pi, but only in Pi-starved-and-rescued proteoid rootlets. In these experiments, Pi concentration in leaves of Pi-starved-and-rescued plants increased from $1.6 \pm 0.6 \mu\text{mol g}^{-1}$ fresh weight to the level of Pi-fed plants ($29 \pm 3.6 \mu\text{mol g}^{-1}$ fresh weight) within 24 h after exogenous Pi was supplied.

Discussion

Proteoid roots are a suitable model tissue to examine gene expression in response to Pi starvation in white lupin (Massonneau *et al.*, 2001; Peñaloza *et al.*, 2002b; Uhde-Stone *et al.*, 2003a, b). Studies on such roots allowed the identification of a partial PEPC cDNA, which represents a gene that is preferentially expressed in Pi-deficient proteoid rootlets (Peñaloza *et al.*, 2002b). By using this cDNA as probe, three different full-length PEPC cDNAs were isolated. All three cDNAs encode the highly conserved QNTG plant motif at the C terminal of the deduced protein, which indicates that none of them correspond to the PEPC of bacterial origin recently reported in *Arabidopsis* (Sánchez and Cejudo, 2003). These PEPC cDNAs share high nucleotide identity within the coding region. However, their corresponding 5'-UTRs and 3'-UTRs differ in nucleotide sequence and length (Table 1; Fig. 1). Since 3'-UTRs may

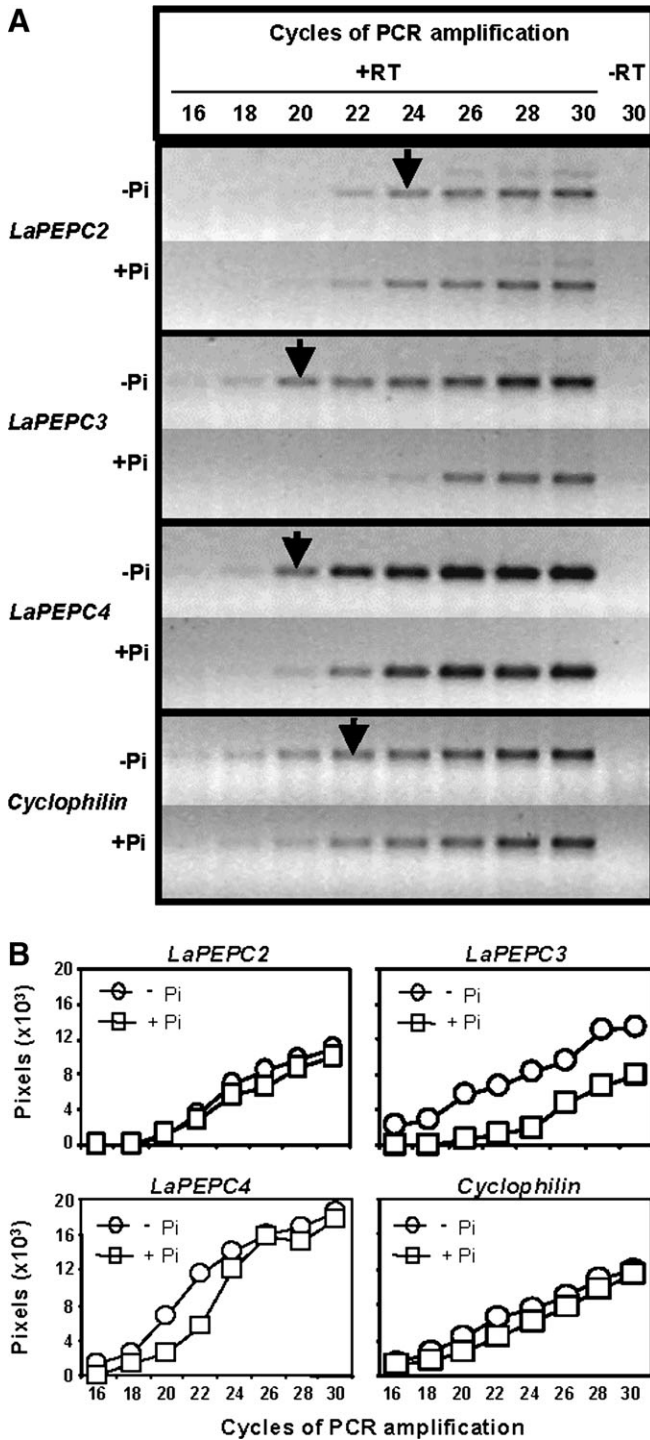


Fig. 3. Semi-quantitative RT-PCR analysis of *LaPEPC2*, *LaPEPC3*, and *LaPEPC4* transcripts in proteoid rootlets from Pi-starved (–Pi) and Pi-starved-and-rescued (+Pi) white lupin using gene-specific primers for each cDNA. (A) Ethidium bromide-stained gel of the amplified products. Arrows indicate the cycle number resulting in a similarly intense amplification product. Cyclophilin transcript serves as the internal control. The last column from the left corresponds with the minus RT control. (B) Signal intensity of amplification products assessed by digitalized analysis of ethidium bromide-stained gels.

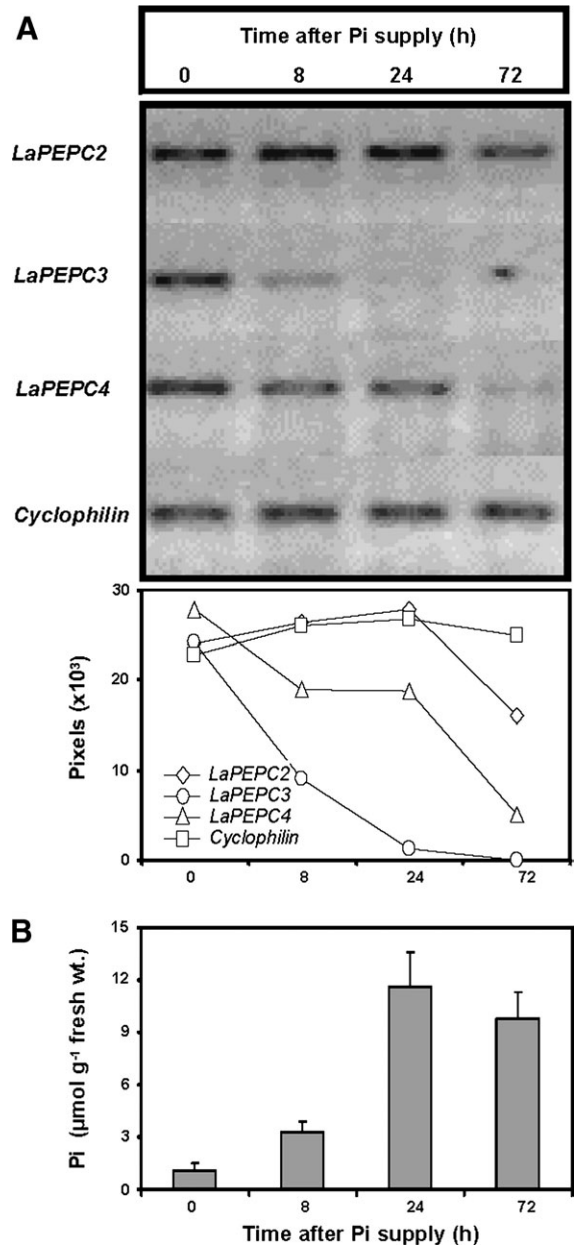


Fig. 4. Semi-quantitative RT-PCR analysis of *LaPEPC2*, *LaPEPC3*, and *LaPEPC4* transcripts in proteoid rootlets using gene-specific primers for each cDNA. Proteoid rootlets were sampled at 0, 8, 24, and 72 h after adding 0.1 mM Pi to 21-d-old Pi-starved plants. (A) Ethidium bromide-stained gel of the amplified products. Cyclophilin transcript serves as the internal control. The minus RT control is not included. Signal intensity of the amplification products assessed by digitalized analysis of ethidium bromide-stained gels is shown at the bottom. (B) Inorganic phosphate (Pi) in tissue at each sampling time point. Each Pi value represents the mean \pm SD of four replicates.

host important regulatory elements affecting gene expression and message stability (Ingelbrecht *et al.*, 1989; Johnson *et al.*, 1998; Graber *et al.*, 1999), it was suggested that these three cDNAs would represent distinct PEPC genes.

Preliminary studies using pLaPEPC2, pLaPEPC3, and pLaPEPC4 cDNAs as probes did not allow any distinction

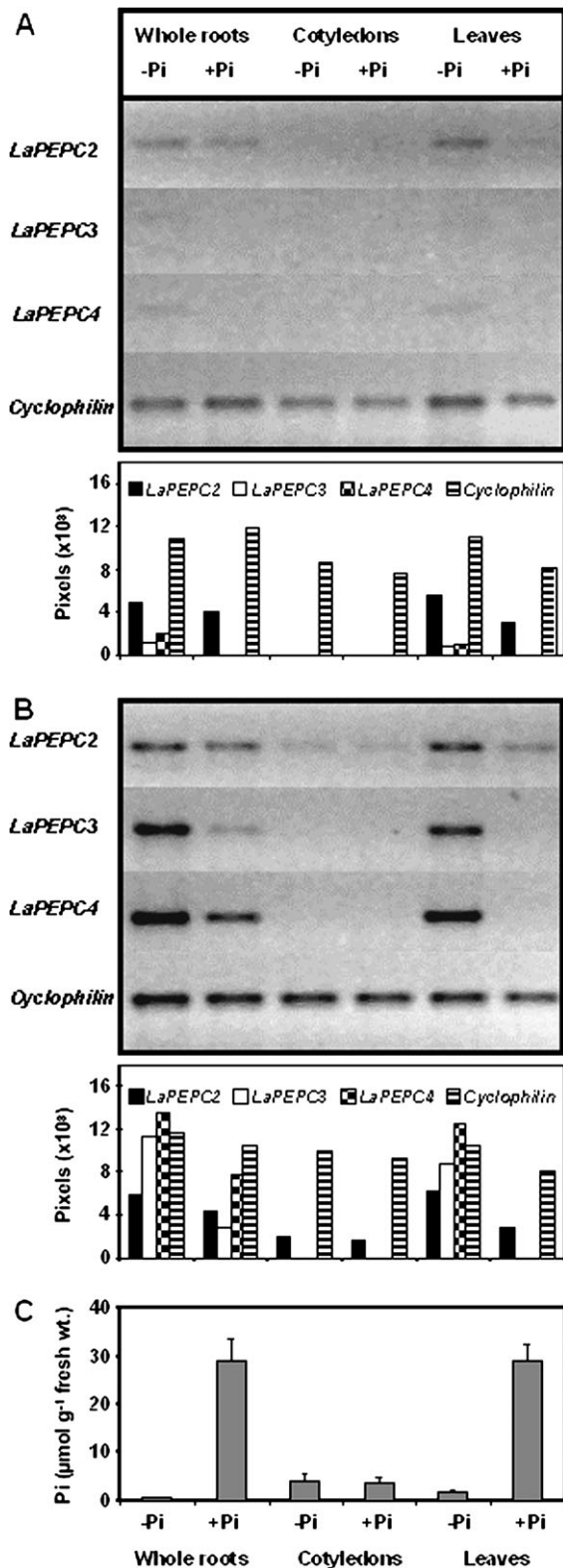


Fig. 5. Semi-quantitative RT-PCR analysis of *LaPEPC2*, *LaPEPC3*, and *LaPEPC4* transcripts in whole roots, cotyledons, and leaves from Pi-starved (-Pi) and Pi-fed (+Pi) white lupin using gene-specific primers for each cDNA. Cotyledons were sampled from 3-d-old plants whereas whole roots and leaves were from 24-d-old plants. Ethidium bromide-stained gels of PCR products amplified under standard conditions and at

between *LaPEPC2*, *LaPEPC3* and *LaPEPC4* transcripts by northern hybridization (not shown). Therefore, the expression of the three PEPCs was addressed by performing RT-PCR with gene-specific primers targeted to the divergent 3'-end region of the corresponding cDNAs. Semi-quantitative RT-PCR analyses carried out in proteoid rootlets revealed a pattern of mRNA accumulation that differentiated the three PEPC transcripts from each other. An obvious difference under Pi deficiency was the comparatively high levels of *LaPEPC3* and *LaPEPC4*, whereas a striking difference under Pi sufficiency was the strong effect of Pi supply in down-regulating *LaPEPC3* (Fig. 3). Sampling at different times after Pi supply demonstrated that the timing of down-regulation for the three PEPC transcripts was dependent on Pi content in tissues (Fig. 4). Although down-regulation of *LaPEPC2* and *LaPEPC4* transcripts by Pi seems to follow what would be expected for a Pi-dependent gene expression (Martin *et al.*, 2000; Abel *et al.*, 2002), the timing of down-regulation of *LaPEPC3* precedes the increase in Pi content in the tissue. This would suggest that Pi itself does not seem to be involved in modulating the response of *LaPEPC3* transcripts, as observed for several Pi-starvation-inducible genes which are systemically repressed in roots well before these roots reach a high Pi level (Burleigh and Harrison, 1999). These patterns of PEPC regulation in proteoid rootlets mimic what has been observed for phosphatases and Pi transporter genes, whose expression depends on differential sensitivity to changing Pi concentration in the medium (Baldwin *et al.*, 2001; Mukatira *et al.*, 2001; Kai *et al.*, 2002).

Because of the various physiological roles that have been postulated for C₃ PEPC in higher plants (Latzko and Kelly, 1983), it may be expected that a constitutive expression of PEPCs in many tissues is necessary. This criterion was met by the *LaPEPC2* transcript, whereas *LaPEPC3* and *LaPEPC4* were barely detectable in tissues other than proteoid rootlets (Fig. 5A). Although at a low level, *LaPEPC3* and *LaPEPC4* were expressed in leaves of Pi-starved plants, which suggest that the three PEPC gene products might be involved in organic acid synthesis, not only in proteoid roots but also in the aerial part of the plant. It is known that shoots accumulate large amounts of malate (Peñaloza *et al.*, 2002a), and that about one-third of C released as root exudates by Pi-starved white lupin comes from shoots (Johnson *et al.*, 1996a). Accordingly, PEPC gene products in leaves may contribute to a portion of the

near saturation are shown in (A) and (B), respectively. Cyclophilin serves as the internal control. The minus RT control is not included. Signal intensity of the amplification products assessed by digitalized analysis of ethidium bromide-stained gels is shown at the bottom of (A) and (B). (C) Inorganic phosphate (Pi) content of each tissue at the time of sampling. Each Pi value represents the mean \pm SD of four replicates.

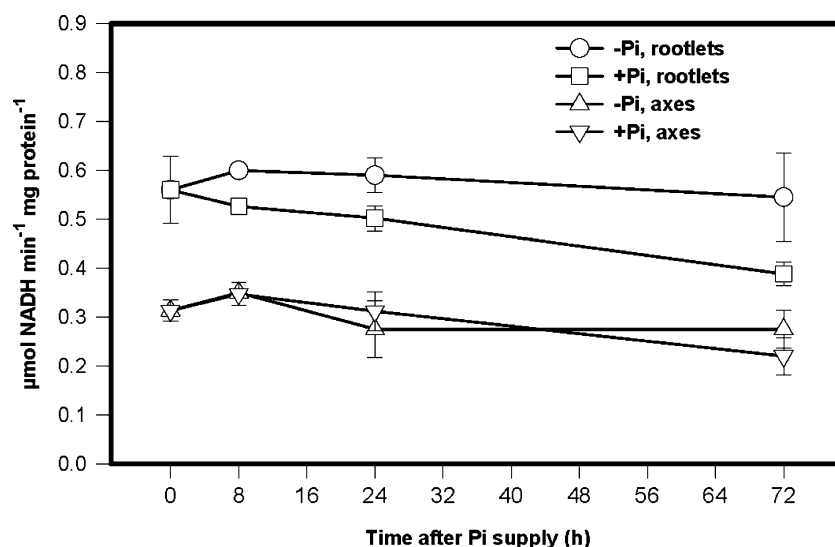


Fig. 6. PEPC specific activity in proteoid rootlets and root cluster axes of mature root clusters from Pi-starved (–Pi) and Pi-starved-and-rescued (+Pi) white lupin. Rootlets and axes were separated from proteoid root clusters sampled at 0, 8, 24, and 72 h after adding 0.1 mM Pi to 21-d-old Pi-starved plants. Each value represents the mean \pm SD of three replicates.

C released from the roots of Pi-starved plants. Increasing the number of PCR cycles, to near saturation, allowed *LaPEPC2* to be detected in all tissues examined (Fig. 5B). This would imply that the *LaPEPC2* gene product might have a housekeeping function in white lupin.

Evidence indicates that the PEPC enzyme plays a major role in the adaptation of white lupin to Pi deficiency (Johnson *et al.*, 1994, 1996a, b, Neumann *et al.*, 1999). PEPC enzyme activity increases at sites of citrate accumulation and exudation in proteoid root clusters of this species (Keerthisinghe *et al.*, 1998; Neumann *et al.*, 2000). Sectioning a proteoid root cluster into rootlets and axis showed that most of the PEPC activity occurs in rootlets (Fig. 6). Pi supply reduced PEPC activity in such a tissue, but the effect was lower than expected for a Pi-deficiency-inducible enzyme. Since PEPC activity has been correlated with PEPC mRNA and protein (Johnson *et al.*, 1996b), these results suggest that PEPC activity observed after Pi supply may reflect the expression of just some specific PEPC isoforms. Based on the semi-quantitative RT-PCR experiments (Fig. 4), the *LaPEPC2* gene product appears to represent the predominant PEPC activity that remains 72 h after Pi supply. Because PEPC activity was assayed under *in vitro* conditions, it is possible that the *in vivo* activity is modified by allosteric effectors or by phosphorylation/dephosphorylation reactions (Chollet *et al.*, 1996), an alternative option that should also be considered.

In conclusion, this study demonstrate that there exists at least three different PEPC transcripts in proteoid root clusters of white lupin, whose expression is enhanced by Pi deficiency. Altogether, these results suggest that more than one PEPC isoform is responsible for the Pi-deficiency-induced PEPC activity in such a root tissue. Since the

existence of a PEPC isoform other than the one coded by *LaPEPC1* in proteoid roots of white lupin was recently questioned (Uhde-Stone *et al.*, 2003b), these results demonstrate that there exist an unexpected complexity in the expression of PEPC in proteoid root clusters of this species.

Acknowledgements

The authors wish to thank Drs Jean Rivoal and Sonia Dorion (University of Montreal, Canada), and Dr Lee Meisel (Universidad de Chile, Santiago, Chile) for their suggestions and critical comments on the manuscript. This research was supported by Grants from the Fundación para la Innovación Agraria (FIA, BIOT 01-A-36), Chile.

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