*Plant and Soil* **248:** 99–116, 2003.
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# Acclimation of white lupin to phosphorus deficiency involves enhanced expression of genes related to organic acid metabolism

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Received 15 December 2001. Accepted in revised form 22 March 2002

*Key words:* cluster root, *Lupinus albus* L., malate dehydrogenase, phosphorus deficiency, phospho*enol*pyruvate carboxylase, proteoid root

## Abstract

White lupin (*Lupinus albus* L.) acclimates to phosphorus deficiency (−P) by the development of short, densely clustered lateral roots called proteoid (or cluster) roots. These specialized plant organs display increased exudation of citric and malic acid. The enhanced exudation of organic acids from P stressed white lupin roots is accompanied by increased *in vitro* phospho*enol*pyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) activity. Here we report the cloning of full-length white lupin PEPC and MDH cDNAs. RNA blot analysis indicates enhanced expression of these genes in −P proteoid roots, placing higher gene expression at the site of organic acid exudation. Correspondingly, macroarray analysis of about 1250 ESTs (expressed sequence tags) revealed induced expression of genes involved in organic acid metabolism in −P proteoid roots. *In situ* hybridization revealed that PEPC and MDH were both expressed in the cortex of emerging and mature proteoid rootlets. A $C_3$ PEPC protein was partially purified from proteoid roots of P deficient white lupin. Native and subunit Mr were determined to be 440 kD and 110 kD, respectively. Citrate and malate were effective inhibitors of *in vitro* PEPC activity at pH 7. Addition of ATP partially relieved inhibition of PEPC by malate but had little effect on citrate inhibition. Taken together, the results presented here suggest that acclimation of white lupin to low P involves modified expression of plant genes involved in carbon metabolism.

## Introduction

Phosphorus (P) is an essential macronutrient that plays a key role in many plant processes such as energy metabolism and the synthesis of nucleic acids and membranes (Raghothama, 1999). Low availability of P is a limiting factor in crop cultivation in many soils (Marschner, 1995). In response to low P availability, higher plants have evolved highly specialized physiological and biochemical mechanisms, including formation of mycorrhizae and proteoid or cluster roots (Marschner, 1995; Raghothama, 1999). White lupin (*Lupinus albus* L.), a legume capable of hosting nitrogen fixing bacteria, is a plant with extreme tolerance for low P availability and one of few agronomically important species that develop proteoid roots in response to P deficiency (Avio et al., 1990; Gardner et al., 1982; Trinick, 1977). Proteoid roots excrete large amounts of the organic acids citrate and malate (Johnson et al., 1996a; Marschner, 1986, 1987;

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Neumann et al., 1999), which help increase the availability of mineral bound phosphates (Dinkelaker et al., 1989; Gardner et al., 1983) and the release of phosphates from humic substances (Braum and Helmke, 1995). Acid phosphatases that may aid in the release of organic P from soil (Tadano and Sakai, 1991) are excreted coincident with the exudation of organic acids from proteoid roots (Gilbert et al., 1999; Miller et al., 2001; Neumann et al., 1999).

Root and shoot $^{14}C$-labeling studies showed that a significant portion of the C excreted as malate and citrate was fixed by PEPC in roots (Johnson et al., 1996b). Excretion of organic acids from proteoid roots is accompanied by increased activity of malate dehydrogenase (MDH) and phosphoenolpyruvate carboxylase (PEPC) (Johnson et al., 1996a). Phosphoenolpyruvate carboxylase plays an important role in photosynthetic reactions in $C_4$ and CAM species. In $C_3$ species, PEPC has an anaplerotic function providing additional carbon for the formation of citric acid cycle intermediates (Melzer and O'Leary, 1987). The amount of C supplied via PEPC can be significant. PEPC can provide up to 25% of the carbon required for assimilation of nitrogen derived by symbiosis (Maxwell, 1982; Vance et al., 1983) and provides 25–34% of the C excreted as citrate and malate by P deficient proteoid roots of white lupin (Johnson et al., 1996b). Plant PEPC expression is controlled by environment (light, salt) and internal stimuli (hormonal, developmental) and has been shown to be under both positive (sugar phosphates) and negative (malate) allosteric control (Lepiniec et al., 1994).

Many of the physiological functions of PEPC in $C_3$ and $C_4$ plants are coupled to malate dehydrogenase (Lance and Rustin, 1984; Latzko and Kelly, 1983). MDH catalyzes the readily reversible conversion of carbon between oxaloacetic acid and malate (Gietl, 1992). MDH is ubiquitous in plants, playing key roles in the citric acid cycle, glyoxylate bypass, amino acid synthesis, gluconeogenesis and facilitation of the exchange of metabolites between cytoplasm and subcellular organelles (Musrati et al., 1998). Plant mitochondrial, cytosolic, glyoxysomal, chloroplast, and nodule-enhanced forms of the enzyme have been cloned and characterized (Cretin et al., 1988; Gietl, 1992; Guex et al, 1995; Kim and Smith, 1994; Metzler et al., 1989; Miller et al., 1998). While MDH can use either NADH or NADPH as reductant, only the chloroplast form of MDH utilizes NADPH (Gietl, 1992). Plant mitochondria use the concerted action of MDH, NAD-dependent malic enzyme (ME), and pyruvate dehydrogenase to provide citrate in the anaplerotic function of the TCA cycle (Gietl, 1992). Carbon can enter the TCA cycle in the form of cytosolic oxaloacetic acid or malate, the latter produced by the successive operation of PEPC and MDH in the cytosol (Gietl, 1992).

Objectives of this research were to analyze expression of genes related to organic acid metabolism in −P proteoid roots, with special focus on PEPC and MDH, and to examine factors that might affect PEPC activity in proteoid roots. To achieve this goal we wanted to isolate and characterize cDNAs encoding PEPC and MDH from white lupin, examine their expression over time, in specific tissues and in response to P deficiency. Our strategy for isolating PEPC and MDH cDNA was to use heterologous PEPC and MDH cDNAs from alfalfa to screen a cDNA library prepared from −P proteoid roots of white lupin. One full-length PEPC and two full-length MDH cDNA clones were isolated and characterized. The three cDNAs were sequenced and the deduced amino acid sequences were compared to other reported PEPC and MDH sequences. Transcript abundance was evaluated in different plant tissues and at different stages of proteoid root development in RNA gel blots. *In situ* hybridizations were performed to assess the cellular localization of PEPC and MDH transcripts in just emerged and developed proteoid rootlets. Macroarrays with ESTs from −P proteoid roots were performed to analyze expression patterns of other genes related to organic acid metabolism. A PEPC isoform predominant in P deficient proteoid roots was partially purified and characterized. The activity of phosphorylated and dephosphorylated PEPC was determined in response to several possible effectors at different pH in order to analyze regulation of PEPC activity.

## Materials and methods

### *Plant material*

White lupin (*Lupinus albus* L. var Ultra) plants were grown in the growth chamber in sand culture under growth conditions as previously described (Johnson et al., 1996a). P sufficiency or deficiency was defined by the presence or absence of 0.5 m*M* $Ca(H_2PO_4)_2$ in the nutrient solutions, which were replenished every 2 d (Gilbert et al., 2000). To maintain appropriate Ca concentrations, the nutrient solutions for the −P plants contained 0.5 m*M* $CaSO_4$.

*Preparation and screening of a cDNA library*

Proteoid roots of white lupin grown under P deficient conditions were harvested 7 and 10 DAE (days after emergence), and poly(A)$^+$ RNA was isolated. The poly(A)$^+$ RNA obtained from plants 7 and 10 DAE was combined in a 1:1 ratio and 7 $\mu$g total RNA was used for construction of a proteoid root cDNA library in the phage ZAPII vector according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The original library represented approximately $2.6\times10^6$ transformants.

A 2 kb *Eco*RI fragment containing the 5′ portion of a *Medicago sativa* PEPC cDNA (Pathirana et al., 1992) and 2 *Sal*I fragments, which together contained the full-length cDNA encoding for a cytosolic MDH of *Medicago sativa* (Miller et al., 1998) were $^{32}$P labeled and used for screening the cDNA library of white lupin proteoid roots (−P) harvested at 7 and 10 DAE.

*RNA gel blots*

Fifteen $\mu$g total RNA from different tissues were separated electrophoretically on 1.6% denaturing agarose gels and transferred to Hybond N+ nylon membrane following standard capillary blotting procedures (Sambrook et al., 1989). Blots were hybridized to a $^{32}$P-labeled 937bp *Bgl*IIB fragment of LaPEPC1 cDNA and to the full-length LaMDH1 cDNA clone (1290 bp), respectively. Both probes were prepared by random primer-labeling. Hybridizations were performed in 0.5 *M* $Na_2HPO_4$, 7% SDS, and 10 m*M* EDTA at 65 °C. Blots were washed with three subsequent washes (2× SSC, 0.5% SDS; 1×SSC, 0.5% SDS; and 0.5×SSC, 0.5% SDS) before autoradiography. Equivalent loading of each lane was assessed by probing blots with ribosomal RNA and determining counts in the ribosomal bands. Loading variability was no greater than 20%.

*Generation of ESTs*

Proteoid roots of white lupin grown under P-deficient conditions were harvested 12 and 14 DAE. A proteoid root cDNA library was constructed in the phage λZAPII vector according to the manufacturers instructions (Stratagene). 5′ single strand sequences were edited and compared with the non-redundant database at the National Center for Biotechnology (NCBI) using the BlastX program.

*Sequencing and computational analyses*

Sequencing was carried out at the Advanced Genetic Analysis Center (St. Paul, MN, USA). Sequences were processed with the UW-GCG program (Devereux et al., 1984).

*Macroarray assays*

A 0.5 $\mu$l (about 0.2 $\mu$g aliquot) of PCR amplified cDNA was spotted in parallel onto Gene Screen Plus membranes (NEN$^{TM}$ Life Science Products, Boston, MA, USA). $^{32}$P-labeled first strand cDNA probe was synthesized by reverse transcription of 30 $\mu$g total RNA using SuperScriptII reverse transcriptase according to manufacturers instructions (Stratagene). The RNA was isolated from −P proteoid roots, +P and −P normal roots and +P and −P leaves at 14 DAE. Hybridizations were performed in 50% formamide, 0.5 *M* $Na_2HPO_4$, 0.25 *M* NaCl, 7% SDS, and 1 m*M* EDTA at 42 °C. Blots were washed with three subsequent washes (1× SSC, 0.1% SDS; 0.5×SSC, 0.1% SDS; and 0.1×SSC, 0.1% SDS). Radioactivity of each spot was quantified using a Phosphor Screen imaging system (Molecular Dynamics, Sunnyvale, CA, USA).

In situ *hybridization*

A 937bp *Bgl*IIB fragment of LaPEPC1 and the 1290 bp LaMDH1 cDNA, both subcloned into pBluescript, were used to obtain RNA probes for *in situ* hybridization. Linearized constructs were transcribed *in vitro* with T3 or T7 RNA polymerases (Stratagene) using [$^{35}$S]UTP according to the manufacturer's instructions. The probes were degraded partially to a length of about 150 nucleotides by heating at 60 °C in a 0.06 *M* $Na_2CO_3$/0.04 *M* $NaHCO_3$ solution.

*In situ* hybridization was carried out as described by Fleming et al. (1993). Tissues were fixed under mild vacuum at 4 °C in 4% paraformaldehyde and 0.25% glutaraldehyde in 50 m*M* phosphate buffer, pH 7.2. The tissues were then rinsed three times in deionized water, and dehydrated in a degraded ethanol series. After the absolute ethanol was replaced with tertiary butanol, tissues were embedded in Paraplast (Oxford Labware, St. Louis, MO). The embedded tissues were sectioned at a thickness of 10 $\mu$M and affixed onto poly-L-Lysin-coated slides. Hybridizations were performed in mineral oil and the final wash conditions were 0.1 × SSC including 1 m*M* DTT at 50 °C for 20 min. The slides were exposed to photo emulsion for 10–14 d. After development, the sections were

stained with 0.05% toluidine blue O, dehydrated and mounted with Permount (Fisher Scientific). Sections were viewed and photographed with a Labophot microscope (Nikon) equipped with dark- and bright-field optics.

*Enzyme purification*

Proteoid roots were collected, quick frozen in liquid $N_2$ and stored at −80 °C until used for PEPC extraction. All subsequent steps were conducted at 4 °C. Frozen tissue was pulverized in liquid $N_2$ in a pre-chilled mortar and pestle. Phosphoenolpyruvate carboxylase was extracted from the pulverized tissue (2.5 mL buffer $g^{-1}$ tissue) with potassium phosphate ($KP_i$) buffer, pH 7.3 (100 m*M* KP*i*, 10 m*M* $MgCl_2$, 10 m*M* malate, 1 m*M* EDTA, 0.3 m*M* sucrose, 20% v/v glycerol, 4% PEG-2000, 15 m*M* 2-mercaptoethoanol, 2 m*M* PMSF (phenylmethylsulfonyl fluoride), 1 mg $L^{-1}$ trans epoxysuccinyl-*L*-leucylamindo (4-guaninino)-butane, 10 mg $L^{-1}$ chrymostatin, 4 mg $L^{-1}$ 3,4, dichloroisocoumarin, 0.5 mg $L^{-1}$ leupeptin) based on Zhang et al. (1995) in a Waring blender for 1 min. The 2-mercaptoethanol, PMSF and protease inhibitors were added fresh on the day of use to prevent oxidation. Homogenate was filtered through several layers of miracloth before centrifuging at 10 000 ×*g* for 30 min. The supernatant containing the crude PEPC extract was fractionated with 50% polyethylene glycol-8000 in 100 m*M* KP*i*, 2 m*M* EDTA (pH 7.3) to 24% (Schuller, et al., 1990). The 24% fraction was separated by centrifugation at 10 000 ×*g* for 30 min. The resulting pellet was suspended in buffer A (20 m*M* KP*i*, 5 m*M* malate, 10% glycerol, 1 m*M* DTT (dithiothreitol) fresh, 1 m*M* PMSF (fresh) and 5 mg $L^{-1}$ chrymostatin (pH 7.8)) and loaded onto a DEAE-sepharose (1.5 × 7.5 cm). The sample was eluted with a 120-mL linear gradient of 0–40% v/v of buffer A with 1 *M* KCl at a flow rate of 1.0 mL $min^{-1}$. Samples were collected in 1.5 mL aliquots and assayed for PEPC activity. Peak activity fractions were pooled and loaded on a hydroxylapatite column (BioRad Econopac 1 × 5 HT). The sample was eluted with a 40-mL linear gradient 0–100% from buffer A to buffer C (400 m*M* KP*i*, 5 m*M* malate, 10% glycerol, 1 m*M* fresh DTT) followed by 10 mL isocratic buffer C, at a flow rate of 0.5 mL $min^{-1}$. Ten mL aliquots were collected to assay for activity. The active peaks were desalted and concentrated with a 30 K filter microseparation device into buffer A, unless sample was to be used for malate kinetics, in which case it was desalted into buffer A without malate. The partially purified and concentrated PEPC could be stored for several weeks at −80 °C without loss of activity.

*Protein electrophoresis and immunoblotting*

Total soluble proteins were separated by SDS–PAGE or Native–PAGE using PhastGel 10–15% gradient acrylamide gels and transferred to Immobilon P (Millipore, Bedford, MA) (Braun and Abraham, 1989). Rabbit polyclonal antibodies to alfalfa nodule PEPC were used to detect PEPC on protein immunoblots. SDS-PAGE gels with FPLC-purified PEPC were silver-stained to check for protein purity (Heukeshovewn and Dernick, 1988). Phosphoenolpyruvate carboxylase was detected on Native-Page with fast violet B (Scrutton and Fatebene, 1975).

*PEPC assay*

The activity of PEPC was determined spectrophotometrically by monitoring the disappearance of NADH at A340 for two min in a MDH (EC 1.1.1.37) coupled reaction (Vance et al., 1983). Standard assay conditions were 100 m*M* bicine at pH 8, 5 m*M* $MgCl_2$, 10 m*M* $H_2CO_3$, 2 m*M* PEP (phosphoenolpyruvate), 0.16 m*M* NADH and 5 units MDH. The final volume was 1 mL; the reaction was initiated with the addition of the extract or partially purified PEPC. Total protein concentration was determined by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) for micro amounts of protein, using bovine serum albumin standards.

*PEPC kinetic studies and metabolite regulation*

Partially purified PEPC that had been stored at −80 °C in 5 m*M* malate and 10% glycerol was used for kinetic studies. The reaction was initiated with the addition of 10 $\mu$l partially purified PEPC enzyme. The $K_m$ and $V_{max}$ were determined for PEP using a range of 0.05 to 30 m*M* and $Mg^{2+}$ using a range of 0.1–10 m*M* $MgCl_2$, with the remaining reagents maintained at standard assay conditions and pH 8.0. Kinetics were repeated four times using different PEPC preparations.

*Test for regulatory properties of PEPC*

The regulatory effects of several metabolites on PEPC isolated from P-deficient proteoid roots were assessed in the presence and absence of ATP, at pH 7 and at pH 8. The standard assay conditions were maintained with

the exception of addition of metabolites, ATP and pH. ATP activation of PEPC was adapted from Schuller et al. (1993). Briefly, 5 g of roots were ground in 15 mL buffer (25 m*M* Tris-HCl, 0.5 *M* sucrose, 0.01 *M* DTT, pH 7.0). Extract was filtered through 4 layers of gauze and 1 layer of miracloth. The tubes were centrifuged at 26 000 $\times g$ for 10 min. Ten mL of supernatant fraction was desalted over a 10 cm Sephadex G-25 column (1 $\times$ 8 cm) and equilibrated in the grinding buffer. Fifty $\mu$L of crude extract were used for each assay. ATP was added at room temperature and samples were taken every 3 min and assayed for PEPC activity using standard assay conditions described above. Assays were repeated at least three times for each component and condition.

## Results

### *Identification of a PEPC and two MDH cDNAs*

In order to identify a cDNA encoding for PEPC in white lupin, a PEPC cDNA of *Medicago sativa* (Pathirana et al., 1992) was used as a probe for screening a cDNA library prepared from −P proteoid roots of white lupin 7 and 10 DAE. A cDNA-containing clone was identified and designated pLaPEPC1. Double-stranded sequencing confirmed a full-length PEPC cDNA.

To identify cDNA encoding for MDH in white lupin, the full-length cDNA of a cytosolic MDH from *Medicago sativa* (Miller et al., 1998) was used to screen the white lupin proteoid root cDNA library. Two cDNA containing clones were identified, designated pLaMDH1 and pLaMDH2. Double-stranded sequencing of the identified clones revealed two full-length MDH cDNAs.

The 3.3 kb cDNA inserted in pLaPEPC1 contains a 2904 bp ORF, designated *LaPEPC1*, which encodes a deduced protein of 967 amino acids with a Mr of 104 kD and a pI of 5.99. The deduced amino acid sequences of LaPEPC1 display the phosphoenolpyruvate carboxylase active sites, PEPCASE_1 and PEPCASE_2 (Figure 1A) characteristic for PEPCs. A putative phosphorylation site typical for plant PEPCs (Huber et al., 1994; Lepiniec et al., 1994; Zhang et al., 1995) is present in the N-terminus (Figure1A).

Both cDNA inserted in pLaMDH1 and pLaMDH2, respectively, contain 999 bp ORFs, designated *LaMDH1* and *LaMDH2*, which encode deduced proteins of 332 amino acids. Both proteins display a Mr of 35.6 and similar pI (6.1 for LaMDH1, 6.0 for LaMDH2). The deduced amino acid sequences of LaMDH1 and LaMDH2 are 95% identical. Both sequences contain an MDH active site signature (Figure 1B). There are no significant variations in the amino acid sequence between the two clones. Even the 3′ untranslated regions of *LaMDH1* and *LaMDH2* display 78% homology. The nucleotide sequences for *LaPEPC1, LaMDH1* and *LaMDH2* can be accessed through GenBank accession numbers AF459644, AF459645, and AF459646, respectively.

*Table 1.* Percentage of amino acid sequence identity between LaPEPC1 from white lupin and PEPCs from other organisms

| | GenBank Accession # | % identity to *Lupinus albus* LaPEPC1 |
|---|---|---|
| *Glycine max*, housekeeping form | Q02909 | 91 |
| *G. max*, nodule enhanced | BAA23419 | 89 |
| *Medicago sativa*, nodule enhanced | AAB46618 | 88 |
| *Pisum sativum*, nodule enhanced | BAA10902 | 86 |
| *Zea mays*, root form | CAA33663 | 81 |
| *Z. mays*, $C_4$-form | QYZM | 75 |
| *E. coli* | P00864 | 42 |

### *Homology to other known PEPC and MDH*

Protein sequence alignments of LaPEPC1 revealed 91% sequence homology to a $C_3$ isoform of PEPC from *Glycine max* and over 80% to root or nodule enhanced isoforms of *Glycine max, Medicago sativa, Solanum tuberosum* and *Zea mays* but only 75% homology to a $C_4$ form of *Zea mays* (Table 1). There was only 42% sequence homology with the bacterial PEPC form from *E. coli*.

Both putative MDH proteins, LaMDH1 and LaMDH2, display about 90% homology to other NAD-dependent MDH proteins (EC 1.1.1.37), e.g. from *M. sativa*, *N. tabacum*, common iceplant (*Mesembryanthemum crystallinum*), *Z. mays* and *A. thaliana* (Table 2). Both forms are only about 40% homologous to an NADP-dependent form of MDH from *A. thaliana* chloroplasts.

### *Expression of PEPC and MDH is induced under P deficiency*

To assess the expression of PEPC and MDH in various tissues of white lupin, RNA gel blots were performed

**A**

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LaPEPC1  MANRNLEKMASIDAQLRQLAPAKVSEDDKLVEYDALLLDRFLDILQDLHGEDMKETVQEI
         :::::::::::::::: :.:::::::::::::::::::::::::::::::::::::::::
G. max   MANRNLEKMASIDAQLRLLVPAKVSEDDKLVEYDALLLDRFLDILQDLHGEDLKETVQEV
PEPC

         YELSAEYEGKHDPKKLEELGNLITSLDAGDSIVFAKAFSHMLNLANLAEEVQIAHRRRNN
         :::::::::::::::::::::::::::::: ::.::::::::::::::::::::: :::.
         YELSAEYEGKHDPKKLEELGNLITSLDAGDSIVVAKSFSHMLNLANLAEEVQIAHSRRNK

         LKKGDFADENNATTESDIEETFKRLVRELKKSPQEVFDALKNQTVDLVLTAHPTQSIRRS
         :::::::::::::::::::::::.:.:: ..::::::::::::::::::::::::::::::
         LKKGDFADENNATTESDIEETLKKLVVDMKKSPQEVFDALKNQTVDLVLTAHPTQSVRRS

         LLQKHGRIRNNLTELYAKDITPDDKQELDEALQREIQAAFRTDEIRRTPPTPQDEMRAGM
         :::::::::::::.::::::::::::::::::::::::::::::::::::::::::::::
         LLQKHGRIRNNLTQLYAKDITPDDKQELDEALQREIQAAFRTDEIRRTPPTPQDEMRAGM

         SYFHETIWKGVPQFLRRVDTALKNIGINERVPYNAPLIQFSSWMGGDRDGNPRVTPEVTR
         :::::::::::: ::::::::::::::::::::::::::::::::::::::::::::::
         SYFHETIWKGVPTFLRRVDTALKNIGINERVPYNAPLIQFSSWMGGDRDGNPRVTPEVTR

         DVCLLARMMAANLYYSMIDDLMFELSMWRCNDELRVRADEISRSSKKDAVAKHYIEFWKI
         ::::::::::::::::::.:.::::::::::::::::::::::..::::::..::::::::::
         DVCLLARMMAANLYYSQIEDLMFELSMWRCNDELRVRADELNRSSKKNSVAKHYIEFWKA

         IPSSEPYRVILGDVRDKLYRKSRGSCHLLAQGYSEIPGEEILTSVDGFLELLELCYRSLC
         :: .::::::.::.::..::.   . : ::::.::::.:: :: .:.:. ::: :::::::::
         IPPNEPYRVLLGEVRNRLYQTRERSRHLLAHGYSDIPEEETFTNVEEFLEPLELCYRSLC

         VCGDRAIADGTLLDFLRQVSTFGLSLVRLDIRQESDRHTDVLDAITQHLGIGSYQEWSEE
         .::::::::::.::::::::::::::::::::::::::::::::::.:: ::::::::::
         ACGDRAIADGSLLDFLRQVSTFGLSLVRLDIRQESDRHTDVLDAITKHLEIGSYQEWSEE

         KRQQWLLSELSGKRPLFGPDLPQTEEIKDVLDTLHVIAELPPDNFGAYIISMATAASDVL
         :::::::::::::::::::::::::::::::::.::::.:.::::::: :::::::::::::: ::::
         KRQQWLLSELSGKRPLFGPDLPQTEEIRDVLETFHVIAELPLDNFGAYIISMATAPSDVL

         AVELLQRECRIKHPLRVVPLFEKLADLESAPAALARLFSIDWYRNRINGKQEVMIGYSDS
         ::::::::::..::::::::::::::::::::::::::::::::::::::::::::::::
         AVELLQRECHVKHPLRVVPLFEKLADLEAAPAALARLFSVDWYRNRINGKQEVMIGYSDS

         GKDAGRFSAAWQLYKAQEELIEVAKKFGVKLTMFHGRGGTVGRGGGPTHLAILSQPPETI
         :::::::::::::::::::::: :::::::::::::::::::::::::::::::::::::
         GKDAGRFSAAWQLYKAQEELIMVAKQYGVKLTMFHGRGGTVGRGGGPTHLAILSQPPETI

         HGSLRVTVQGEVIEQSFGEQHLCFRTLQRYTAATLEHGMHPPISPKPEWRALMDQMAVIA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
         HGSLRVTVQGEVIEQSFGEQHLCFRTLQRFTAATLEHGMHPPISPKPEWRALMDEMAVIA

         TEEYRSIVFQEPRFVEYFRLATPELEYGRMNIGSRPAKRRPTGGIETLRAIPWIFAWTQT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
         TEEYRSIVFKEPRFVEYFRLATPELEYGRMNIGSRPAKRRPSGGIETLRAIPWIFAWTQT

         RFHLPVWLGFGAAFRQVVPKDVRNLNMLQEMYNQWKFFRVTIDLVEMVFAKGDPGIAALY
         ::::::::::::::::..:. :::::::...:::::::: :::::::::::::::::::::::::
         RFHLPVWLGFGAAFKHVIEKDVRNIHVLQEMYNQWPFFRVTIDLVEMVFAKGDPGIAALY

         DRLLVSKNLWSFGEQLRTNFEETKKLLLQVAAHKDLLEGDPYLKQKLRLRDSYISTLNVC
         :::::::..::::::::::: .::::.::::::::.:::::::::::::.::::::::::::
         DRLLVSEDLWSFGEQLRTMYEETKELLLQVAGHRDLLEGDPYLKQRLRLRDSYITTLNVC

         QAYTLKRIRDPNYDVKLRPHISKECIEISKVADELITLNPTSEYAPGLEDTFILTMKGIA
         :::::::::::::.::::::::::: ::::: :::::::::::::::::::::.::::::::
         QAYTLKRIRDPNYNVKLRPHISKESIEISKPADELITLNPTSEYAPGLEDTLILTMKGIA

         AGLQNTG
         :::::::
         AGLQNTG
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*Figure 1.* (A) Alignment of the predicted amino acid sequence of LaPEPC1 and a PEPC isoform of *G. max* that displayed highest sequence homology (GenBank accession number Q02909; Sugimoto et al., 1992). The phosphorylation motif is shown in bold, the phosphoenolpyruvate carboxylate active sites, PEPCASE_1 and PEPCASE_2 are underlined. Nucleotide sequence of LaPEPC1 is deposited with GenBank under accession number AF459644. (B) Alignment of the predicted amino acid sequences of LaMDH1 and LaMDH2. Nucleotide sequences are deposited with GenBank under accession number AF459645 and AF459646, respectively. The malate dehydrogenase active site signatures are identified by lines underneath. Identity between amino acids for both sequences are indicated by double, similarity by single dots.

B

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LaMDH1 MAKNPVRVLVTGAAGQIGYALVPMIARGVMLGSDQPVILHLLDIPPAAESLNGVKMELVD
       ::.:::::::::::::::::::::::::::::::::::::::::::::::::::::::::
LaMDH2 MARDPVRVLVTGAAGQIGYALVPMIARGVMLGADQPVILHLLDIPPAAESLNGVKMELVD

       AAFPLLKGVVATTDVVEACTGVNIAVMVGGFPRKEGMERKDVMSKNVSIYKSQAFALEKH
       ::::::::::::::.::::::::::.::::::::::::::::::::::::::::: ::::.
       AAFPLLKGVVATTDAVEACTGVNIAVLVGGFPRKEGMERKDVMSKNVSIYKSQASALEKY

       AAANCKVLVVANPANTNALILKEFAPSIPEKNISCLTRLDHNRALGQISERLNVQVSDVK
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
       AAANCKVLVVANPANTNALILKEFAPSIPEKNISCLTRLDHNRALGQISEKLNTQVSNVK

       NVIIWGNHSSSQYPDVNHATVTTPAGEKPVRELVADDAWLNSEFIATVQQRGAAIIKARK
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
       NVIIWGNHSSTQYPDVNHATVTTPAGEKPVRELVCDDAWLNSEFISTVQQRGAAIIKARK

       LSSALSAASAACDHIRDWVLGTPEGIWVSMGVYSDGSYNVPAGLIYSFPVTTQNGEWKIV
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
       LSSALSAASAACDHIRDWVLGTPEGTWVSMGVYSDGSYNVPAGLIYSFPVTTQNGEWKIV

       QGLGIDEFSRKKLDLTAEELSEEKALAYSCLT
       ::::::::::::::::::::::::::::::.
       QGLAIDEFSRKKLDLTAEELSEEKALAYSCLS
```

*Figure 1.*

*Table 2.* Percentage of amino acid sequence identity between LaMDH1 and LaMDH2 from white lupin and MDHs from other organisms

| | GenBank Accession # | % identity to LaMDH1 | % identity to LaMDH2 |
|---|---|---|---|
| *L. albus* LaMDH1 | AF459645 | 100 | 95 |
| *L. albus* LaMDH2 | AF459646 | 95 | 100 |
| *M. sativa*, cytosolic | AAB99756 | 93 | 92 |
| *N. tabacum* | CAC12826 | 93 | 93 |
| Common iceplant | 12229778 | 85 | 84 |
| *Z. mays* | AAB64290 | 89 | 88 |
| *A. thaliana*, cytosolic (NAD) | 15239843 | 89 | 88 |
| *A. thaliana*, chloroplast (NADP) | 15237170 | 40 | 41 |

(Figure 2). Total RNA was isolated from leaves (L), stems (S), cotyledons (C), normal roots (N) and proteoid roots (Pr) of white lupin plants 14 DAE grown under sufficient P (+P). Flowers (F) and pods (Pd) were harvested from +P plants when available. In addition, total RNA was isolated from leaves, cotyledons, normal roots, and proteoid roots of white lupins grown under P deficiency (−P). Nodules (Nd) were harvested from +P and −P plants 21 DAE (Figure 3).

The conserved LaPEPC1 probe hybridized most strongly to a 3.3 kb RNA from −P proteoid roots. Strong hybridization was also detected in −P nodules, leaves and, to a lesser degree in −P normal roots. Under +P conditions only proteoid roots showed strong expression, though to a lesser extent than under −P conditions. All other tissues tested displayed only weak or non-detectable signals when grown under sufficient P. Hybridization with a specific LaPEPC1 probe derived from the 3′ untranslated region resulted in the same expression pattern (data not shown).

The LaMDH1 probe hybridized to a 1.5 kb RNA to a varying degree in all tissues tested, but most strongly in −P proteoid roots. Due to the high degree of homology between LaMDH1 and LaMDH2, hybridizations were performed only with LaMDH1. Under +P conditions, expression was strongest in proteoid roots but low in normal roots. Expression was induced in all P deficient tissues tested (leaves, cotyledons, nodules, normal and proteoid roots), compared to +P conditions.

To examine the relationship of PEPC and MDH expression to timing of proteoid root formation, RNA gel blots were performed with RNA isolated from both +P and −P normal and proteoid roots at different stages of root development (4, 10, and 14 DAE). At 4 DAE, proteoid roots were not visible so only normal roots were collected. Phosphoenolpyruvate carboxylase displayed only weak expression in −P normal and proteoid roots at 10DAE, but expression was induced strongly in normal and proteoid roots

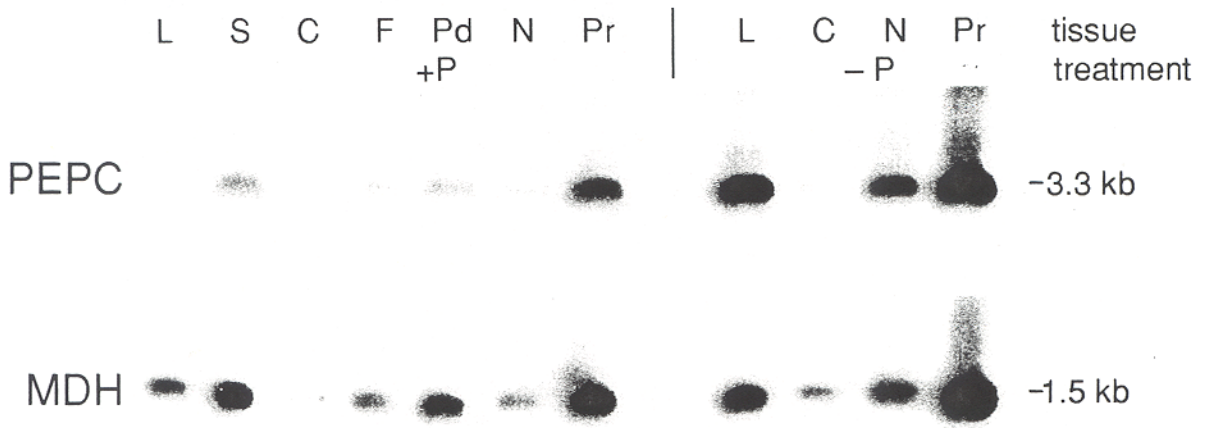


*Figure 2.* Gel blot analysis of RNA isolated from different tissues of +P and −P treated white lupin. LaPEPC1 cDNA was used as a probe in the upper panel while in the lower panel LaMDH1 was used as a probe. Plants grown in presence of sufficient P are designated by (+) and those grown under P stress are designated by (–). Letters above each lane represent: L = leaves; S = stems; C= cotyledons; F = flowers; Pd= pods; N= normal roots; Pr = proteoid roots. Flowers and pods did not form on −P plants. Each lane contains 15 $\mu$g of total RNA.

under −P conditions by 14 DAE (Figure 3). A similar expression pattern was found for MDH. Induction of MDH in −P normal and proteoid root tissue was not apparent at 10 DAE but MDH was strongly expressed by 14 DAE (Figure 3).

### In situ *hybridization*

Single stranded sense and antisense RNA, derived from LaPEPC1 and LaMDH1 cDNAs, were used as probes for *in situ* hybridization of just emerging (9 DAE) and developed (12 DAE) proteoid roots as well as of +P normal roots (12 DAE) to ascertain the cellular localization of the corresponding transcripts. *In situ* hybridization with the LaPEPC1 antisense probe, seen in the dark field as bright silver grain, revealed expression in the just emerging rootlet (Figure 4A) but stronger expression throughout the proteoid rootlet 12 DAE (Figure 4C). Transverse sections of the rootlet displayed no detectable transcript at the root cap (Figure 4E), but high transcript abundance in the cortex of a cross section about 500 $\mu$M distant to the tip (Figure 4G). Normal roots showed high transcript abundance in the apical meristem and to a lesser degree in the more distal cortex (Figure 4I).

After hybridization with the LaMDH1 antisense probe, dark-field microscopy revealed high transcript abundance throughout an emerging rootlet 9 DAE (Figure 5A). Proteoid rootlets 12 DAE display strong expression over a region of several hundred $\mu$M along the rootlet but less so at the tip or base (Figure 5C). Accordingly, no hybridization was detected in a cross section from the root tip (Figure 5E). Dark field photography of a transverse section approximately 500 $\mu$M distant to the proteoid rootlet tip revealed high transcript abundance that seems most pronounced in the cortex (Figure 5G). Normal roots have lower expression overall but a similar expression pattern, displaying highest transcript abundance in the cortex at some distance from the root tip (Figure 5I). No significant hybridization was detected when normal roots were probed with LaPEPC1 (Figure 4K) and LaMDH1 (Figure 5K) sense probes, respectively.

### *Macroarray analysis*

To assess C-metabolism related gene expression in proteoid roots on a wider scale, we performed macroarray analyses of about 1250 ESTs derived from −P proteoid roots of white lupin. A total of 55 ESTs (4.4%), displayed significant homology (E-value $\leq 10^{-10}$) to genes involved in the glycolytic pathway. Two ESTs each displayed homology to PEPC and MDH. Alignments of the corresponding ESTs with the complete PEPC and MDH cDNAs revealed sequence identity to LaPEPC1 and LaMDH2, respectively.

Gene expression was compared in −P proteoid roots, +P normal roots, −P normal roots, +P leaves and −P leaves at 14 DAE. Macroarray analyses revealed at least 2-fold induction in −P proteoid roots,

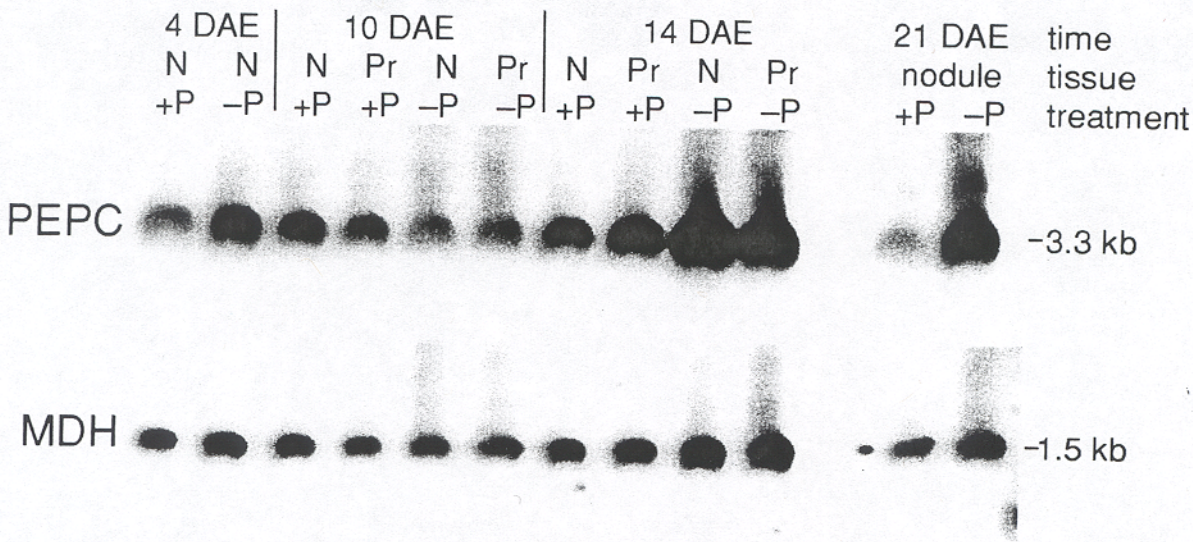


*Figure 3.* Gel blot analysis of RNA isolated from +P and −P normal (N) and proteoid (Pr) roots at 4, 10, and 14 DAE and nodules (Nd) at 21 DAE. LaPEPC1 cDNA (upper panel) and LaMDH1 cDNA (lower panel) served as a probe. Each lane contains 15 $\mu$g of total RNA.

compared to +P normal roots, of the majority of glycolytic pathway-related genes (Table 3, Figure 6). In contrast, the majority of these genes did not show induced expression in −P leaves compared to +P leaves, nor in −P normal roots compared to +P normal roots (Table 3). RNA gel blots performed with selected ESTs confirmed the results obtained by macroarray analysis. Figure 7 shows induced expression of a glyceraldehyde-3P dehydrogenase in −P proteoid roots but not in +P or −P normal roots or leaves.

*Partial purification and characterization of a proteoid root enhanced PEPC isoform*

Phospho*enol*pyruvate carboxylase enzyme protein was purified about 70 fold from −P proteoid roots (Table 4). After desalting and concentrating, the specific activity was 18.3 units. A unit of PEPC specific activity is defined as the conversion of 1 $\mu$mol NADH to $NAD^+$ $min^{-1}$ $mg^{-1}$ protein.

The purified PEPC from −P proteoid roots displayed a $K_m$ PEP of 0.055 m*M* (±0.01) and a $K_m$ $Mg^{2+}$ of 0.24 m*M* (± 0.095) with $V_{max}$ of 0.07 (±0.028) and 0.046 (±0.019) units, respectively. These measurements were done at standard assay conditions at pH 8 using PEPC stored at −80 °C in the presence of 10% glycerol and 5 m*M* malate. Only 10 $\mu$L of PEPC was used in each assay; therefore the concentration of malate and glycerol in the assay were negligible.

Native protein immunoblots and native PAGE activity stainings indicated a native molecular weight of about 440 kD for the partially purified PEPC (Figure 8). SDS-PAGE as well as Superose 6 analysis indicated a subunit size of 110 kD (data not shown).

*Metabolite regulation of partially purified proteoid root-enhanced PEPC*

Several metabolites were analyzed in regard to inhibition or activation of PEPC at pH 7 and pH 8, with and without preincubation with ATP (Table 5) to assess possible effects of endogenous phosphorylation (Schuller and Warner, 1993). Hexose phosphates showed strongest activation in absence of ATP at pH 7. Citrate and 2-oxoglutarate were strong inhibitors independent of pH and independent of the presence of ATP. Malate was the strongest inhibitor at pH 7; inhibition was reduced by preincubation with ATP or by increasing the pH to pH 8. The metabolites 3-phosphoglycerate and 2-phosphoglycerate displayed 20–40% inhibition after preincubation with ATP and at pH 8, but showed no effect at pH 7 in the absence of ATP. DHAP increased activity 7% at pH 7 and 15% at pH 8 when assayed without preincubation, but had no effect when PEPC was preincubated with ATP. The amino acids Asp and Gln caused less than a 15% decrease in PEPC activity at pH 7 and had marginal effects at pH 8. Addition of $P_i$ in concentrations ranging from 10 to 30 m*M* showed some activation at pH 7 in

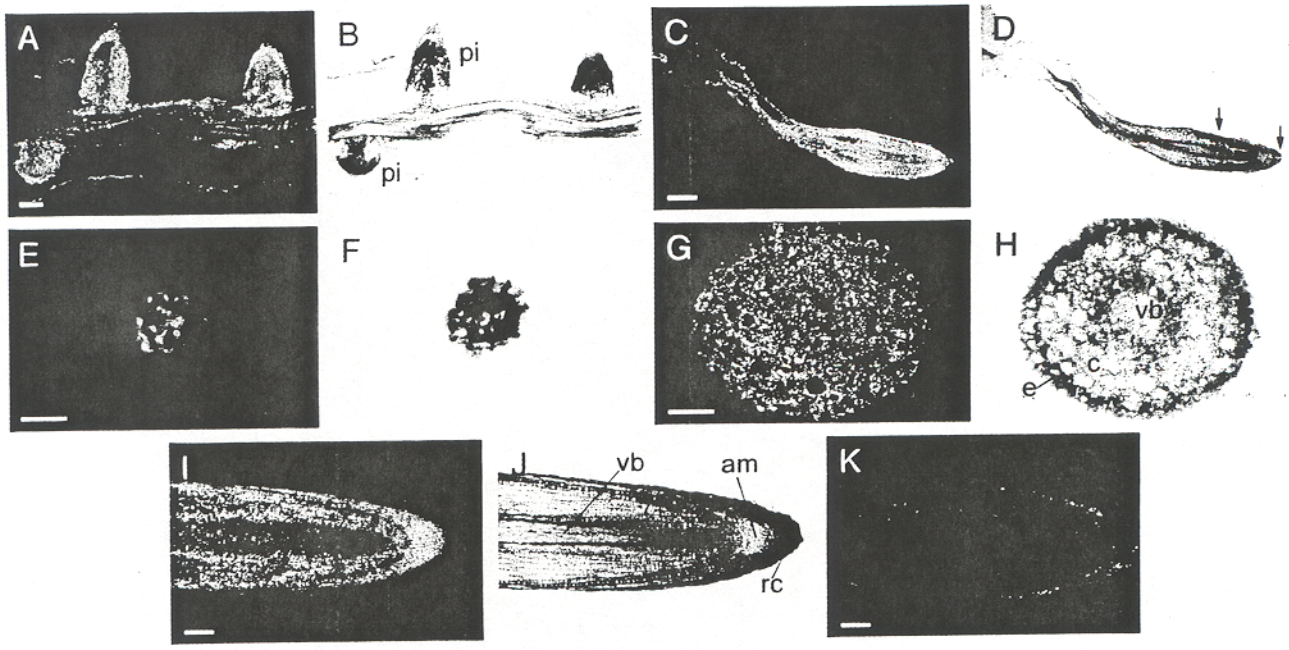


*Figure 4.*

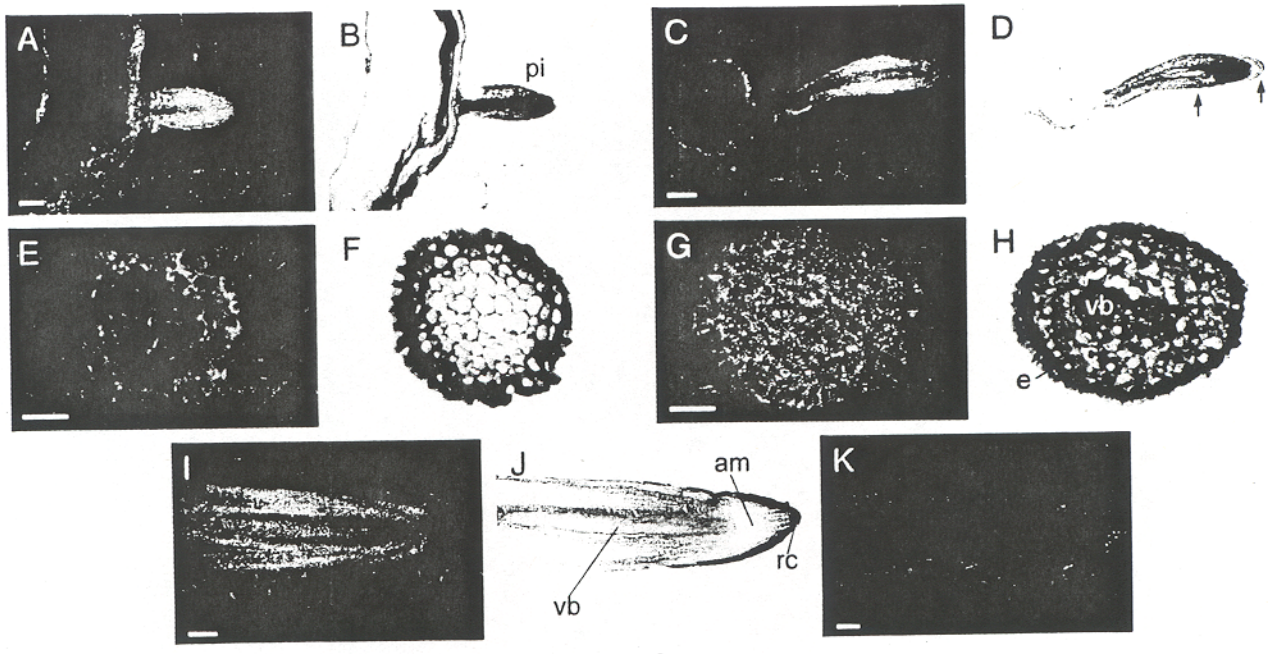


*Figure 5.*

*Table 3.* Macroarray analysis revealed −P proteoid root-induced expression of genes with putative function in the glycolytic pathway. Expression in leaves and normal roots was not induced under P deficiency

| Annotation | Number of ESTs[b] | Ratio of gene expression[a] | | |
|---|---|---|---|---|
| | | −P proteoid/ +P normal root | −P leaf/ +P leaf | −P normal root/ +P normal root |
| Sucrose synthase | 6 | 3 | 1 | 0.8 |
| Fructokinase | 1 | 2.2 | 0.5 | 0.8 |
| Phosphoglucomutase | 2 | 2.1 | 0.7 | 0.9 |
| Glucose-6P isomerase | 1 | 2.1 | 1.3 | 0.8 |
| PPi-dependent phosphofructokinase | 5 | 7.3 | 0.9 | 1.9 |
| Fructose-1,6-bisP aldolase | 12 | 4.2 | 0.8 | 1 |
| Triose-phosphate isomerase | 3 | 3.2 | 0.9 | 1.4 |
| Glyceraldehyde-3P dehydrogenase | 12 | 3 | 0.8 | 1.3 |
| Phosphoglycerate kinase | 4 | 3 | 1.7 | 1 |
| Enolase | 4 | 2.5 | 0.9 | 1.1 |
| Phospho*enol*pyruvate carboxylase | 2 | 6 | 0.5 | 1.4 |
| Malate dehydrogenase | 2 | 4.9 | 0.6 | 1.4 |
| Malic enzyme | 1 | 1.2 | 2 | 1.6 |

[a] Ratios are the average of corresponding ESTs from two independent macroarray experiments.
[b] Number of ESTs with corresponding BLASTX annotation.

*Table 4.* Partial purification of PEPC from 94 g of −P proteoid root tissue collected from plants 14 DAE

| Sample | mg protein $ml^{-1}$ | Total protein mg[a] | PEPC specific activity | Protein total U[b] $mg^{-1}$ | Purification | PEPC yield | Protein yield % |
|---|---|---|---|---|---|---|---|
| Crude extract | 0.478 | 119.55 | 0.27 | 32.75 | 1.00 | 100.00 | 100.00 |
| 4–24% PEG fraction | 0.919 | 27.56 | 0.70 | 19.38 | 2.57 | 59.18 | 23.06 |
| DEAE | 0.142 | 2.35 | 7.97 | 18.69 | 29.09 | 57.08 | 1.96 |
| HT | 0.093 | 0.56 | 13.06 | 7.28 | 47.67 | 22.22 | 0.47 |
| Desalted and concentrated | 0.123 | 0.32 | 18.28 | 5.86 | 66.72 | 17.89 | 0.27 |

[a] Protein determined with Bio-Rad assay for micro amount of protein based on Bradford (1976).
[b] One unit of PEPC specific activity is the conversion of 1 $\mu$mol NADH to $NAD^+$ $min^{-1}$ $mg^{-1}$ protein at $A_{340}$ in an MDH coupled assay.

←

*Figure 4. In situ* localization of PEPC in −P proteoid roots of white lupin. Sections A–J were hybridized with $^{35}$S-labeled antisense RNA derived from LaPEPC1 cDNA, whereas section K was hybridized with the corresponding sense RNA probe. The dark field (A, C, E, G, I, K) and bright field (B, D, F, J) images were longitudinal and transverse sections through proteoid rootlets 9 DAE (A, B) and 12 DAE (C-H) as well as through 12 DAE +P normal roots (I, J, K). Arrows in panel D indicate the approximate location of the two transverse sections shown in panel E–H. Bar sizes represent 50 $\mu$M (E, G), 100 $\mu$M (A), and 200 $\mu$M (C, I, K), respectively. Letters represent: pi = proteoid initials, vb = vascular bundle, c = cortex, e = epidermis, am = apical meristem, rc = rootcap.

*Figure 5.* Localization of MDH in −P proteoid roots of white lupin by *in situ* hybridization. Sections A–J were hybridized with $^{35}$S-labeled antisense RNA, derived from LaMDH1 cDNA, section K was hybridized with the corresponding sense RNA probe. The dark field (A, C, E, G, I, K) and bright field (B, D, F, J) images were longitudinal and transverse sections through proteoid rootlets 9 DAE (A, B) and 12 DAE (C–H) as well as through 12 DAE +P normal roots (I, J, K). Arrows in panel D indicate the approximate location of the two transverse sections shown in panel E–H. Bar sizes represent 50 $\mu$M (E, G), 100 $\mu$M (A, K), and 200 $\mu$M (C, I), respectively. Letters represent: pi = proteoid initials, vb = vascular bundle, c = cortex, e = epidermis, am = apical meristem, rc = rootcap.

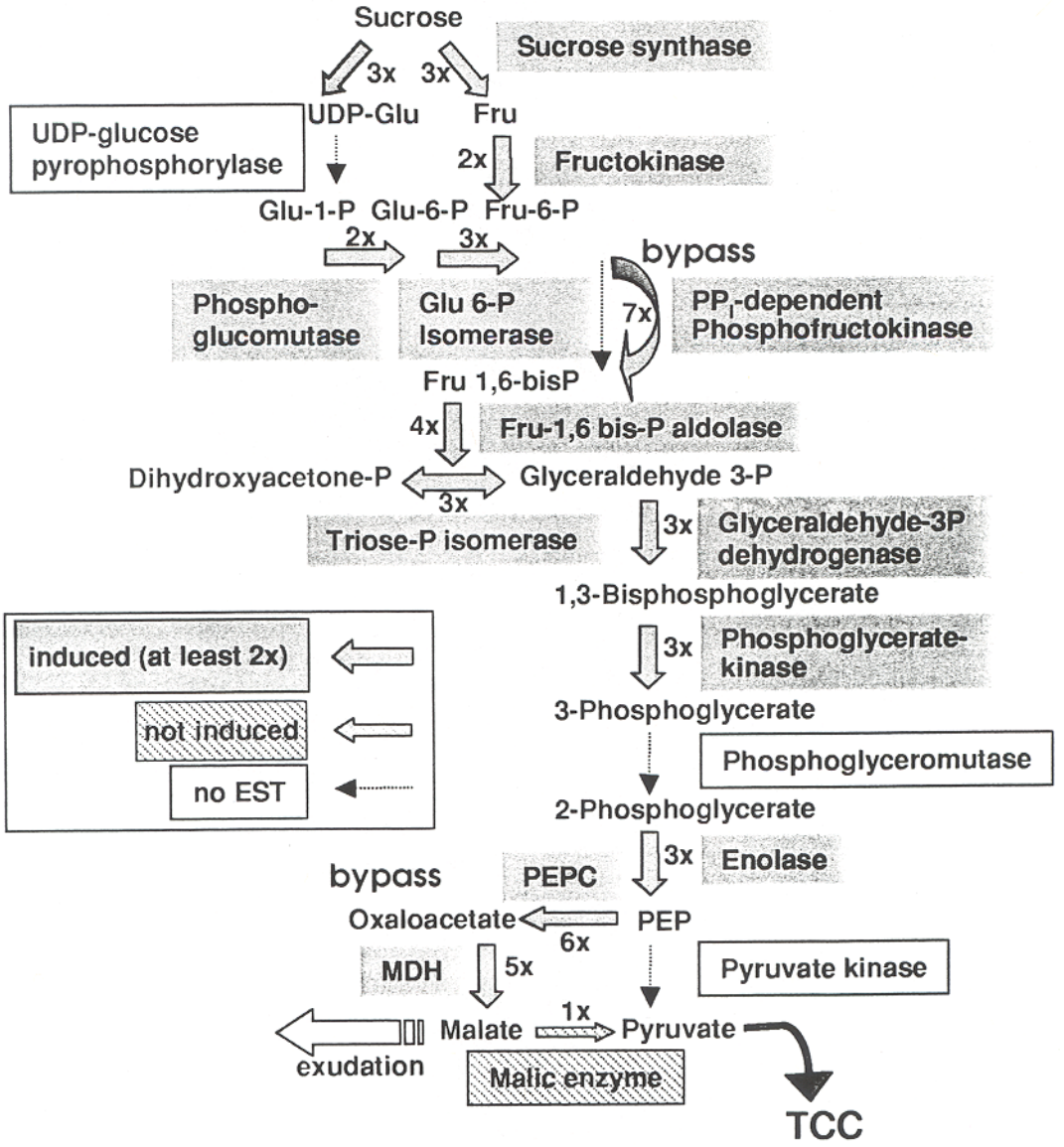


*Figure 6.* Schematic representation of the glycolytic pathway. ESTs with induced expression in −P proteoid roots, compared to +P normal roots, are represented in gray boxes. The average of gene induction as determined by two independent macroarrays is indicated at the corresponding arrows (e.g. 2x). Enzymes of the glycolytic pathway that were not found in the collection of 1250 ESTs are shown in white boxes and are represented by dotted arrows. The majority of ESTs with possible function in the glycolytic pathway displayed increased expression in −P proteoid roots, compared to +P normal roots.

the absence of ATP but inhibition to various degrees under all other conditions tested.

## Discussion

In this report, we have advanced the understanding of how enzymes involved in organic acid synthesis, particularly MDH and PEPC, participate in the process of acclimation to P deficiency in white lupin. This was done by (1) isolating and sequencing cDNAs with high homology to PEPC and MDH; (2) demonstrating induced expression of PEPC and MDH under P deficiency in different tissues but most pronounced in proteoid roots; (3) revealing different expression patterns of PEPC and MDH in just emerged and developed proteoid rootlets by *in situ* hybridization; (4) show-

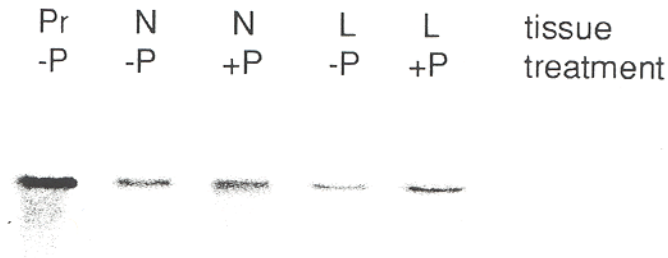


*Figure 7.* RNA gel blot analysis to confirm the differential expression pattern indicated by macroarray analysis. RNA was isolated from −P proteoid roots (Pr), +P and −P normal roots (N) and +P and −P leaves (L) at 14 DAE. Each lane contains 15 $\mu$g of total RNA. An EST with homology to NAD-dependent glyceraldehyde-3P dehydrogenase was used as a probe.

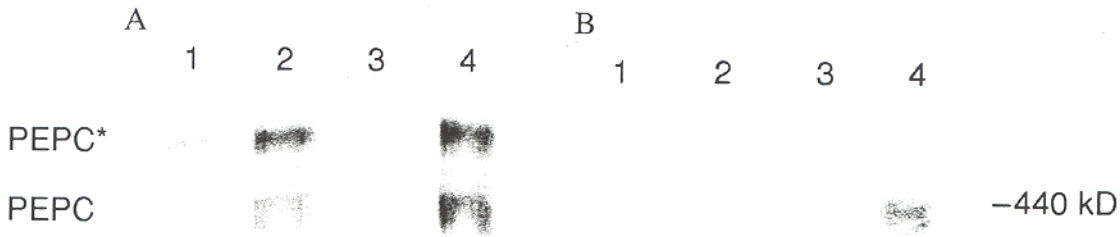


*Figure 8.* Native protein immunoblot (A) and native PAGE activity staining (B) of various samples obtained during the partial purification of PEPC from proteoid roots of white lupin. The band detected by activity staining corresponds to the lower band of the protein gel blot and represents the 440 kD tetramer of PEPC. The upper band visible in the protein gel blot was not detected in activity stainings and might reflect a different phosphorylation status of PEPC. Lane 1 = crude extract, 482 ng total protein/lane; Lane 2 = PEG cut, 729 ng total protein/lane; Lane 3 = DEAE cut, 72 ng total protein/lane, Lane 4 = HT column concentrated, 131 ng/lane.

ing differential expression of genes with homology to enzymes of the glycolytic pathway by macroarray analysis of proteoid root derived ESTs; and (5) partially purifying and characterizing a proteoid root-enhanced PEPC isoform.

Both PEPC and MDH exist in small multigene families. Each member encodes a distinct isoform associated with its respective metabolic pathway. The different isoforms play different physiological roles depending on location and function in the plant (Ting and Osmond, 1973). Root forms of PEPC are known to have various anaplerotic functions that include providing the carbon skeletons for nitrogen assimilation, pH maintenance, and osmolarity regulation (Latzko and Kelly, 1983). Like PEPC, MDH is a widely distributed enzyme playing a crucial role in many metabolic pathways.

To identify cDNAs of PEPC and MDH isoforms that are expressed in proteoid roots, we screened a proteoid root cDNA library of white lupin with heterologous PEPC and MDH cDNAs from *M. sativa*. With this approach we isolated a putative PEPC cDNA and two putative MDH cDNAs. Protein sequence alignments of LaPEPC1 revealed higher sequence homology (80–90%) to root or nodule enhanced $C_3$ isoforms than to a $C_4$ form of *Z. mays* (75%) (Table 1). LaMDH1 and LaMDH2 both displayed high homology (about 90%) to cytosolic MDH forms of *M. sativa*, *Z. mays* and *A. thaliana* but only relatively low homology (40%) to a NADP-dependent form from *A. thaliana* chloroplasts (Table 2).

RNA gel blot experiments revealed induced expression of PEPC in −P root and leaf tissues with highest expression in −P proteoid roots. The high expression in −P proteoid roots is consistent with results

*Table 5.* Comparison of regulatory properties of PEPC in −P proteoid roots in absence and presence of 5 m*M* ATP

| Metabolite | Relative activity in %[a] | | | |
|---|---|---|---|---|
| | pH 7 | | pH 8 | |
| | − ATP | + ATP | − ATP | + ATP |
| 4 m*M* Glu-6-P | 135 | 127 | 113 | 123 |
| 4 m*M* Glu-1-P | 120 | 105 | 110 | 108 |
| 4 m*M* Fru-6-P | 134 | 112 | 104 | 106 |
| 4 m*M* DHAP | 107 | 99 | 115 | 101 |
| 5 m*M* 3-PGA | 102 | 72 | 65 | 58 |
| 5 m*M* 2-PGA | 121 | 81 | 69 | 78 |
| 1.25 m*M* Malate | 26 | 54 | 82 | 86 |
| 1.25 m*M* Asp | 88 | 86 | 97 | 95 |
| 1.25 m*M* Glutamine | 87 | 94 | 104 | 106 |
| 2.5 m*M* Citrate | 18 | 24 | 30 | 45 |
| 5 m*M* 2-OG | 55 | 58 | 67 | 61 |
| 10 m*M* $P_i$ | 121 | 92 | 83 | 81 |
| 20 m*M* $P_i$ | 133 | 82 | 67 | 62 |
| 30 m*M* $P_i$ | 117 | 72 | 80 | 60 |

[a]Enzyme activity is expressed relative to the respective control set at 100%. Each value represents the average of at least three separate measurements. Standard errors did not exceed 15%.

from previous work showing increased in vitro activity and enzyme protein level in P deficient proteoid roots (Johnson, 1996b; Neumann et al., 1999, 2000). Expression of MDH follows a similar pattern with some induction under P deficiency in leaves and normal roots but highest expression in −P proteoid roots. Induction of PEPC and, to a lesser degree MDH, under P deficiency has also been reported in tomato (Pilbeam et al., 1993) and *Sesbania rostrata* (Aono et al., 2001).

Time course experiments revealed greater expression of PEPC and MDH in mature proteoid roots (14 DAE) than in just emerged proteoid roots (10 DAE). This timing corresponds to the timing of organic acid exudation and increased PEPC activity previously reported for white lupin (Johnson et al., 1996b). Watt and Evans (1999a), however, reported maximal PEPC activity 1 day before the onset of citrate efflux and 2 days before its peak, followed by a 75% decrease of PEPC activity over a period of 3 days. The observation that maximal PEPC activity precedes citrate efflux might be explained by the increase of internal citrate concentrations prior to excretion (Neumann et al., 1999). The early decrease in PEPC activity is not reflected in the transcript abundance and might be directly related to maximum inhibition by increased concentrations of internal citrate. The time discrepancy between maximum *in vitro* PEPC activity and citrate efflux led to the authors' postulation that another factor, possibly an organic acid transporter, might be responsible for the timing of organic acid efflux (Watt and Evans, 1999a). Maximum acid phosphatase activity and strong expression of a putative high affinity phosphate transporter have also been observed in mature proteoid roots at 14 DAE (Gilbert et al., 1999; Liu et al., 2001), indicating a precise coordination of the different components of the proteoid root response to P deficiency. In this regard, it is noteworthy that plant uptake of P hydrolyzed by acid phosphatase is thought to be improved by the presence of citrate, a chelate that effectively binds metals that would otherwise compete for released P (Braum and Helmke, 1995; Watt and Evans, 1999b).

To reveal the spatial and temporal distribution of PEPC and MDH expression in proteoid roots, *in situ* hybridizations were performed on −P proteoid rootlets and +P normal roots. Mature proteoid rootlets display a differentiated apex, a loosely anchored root cap, an endodermis with a Casparian band, a diarch stele, and numerous root hairs (for reviews see Dinkelaker et al., 1995; Skene et al., 1998b). Citrate efflux has been shown to begin shortly after the rootlets reach their final length (Watt and Evans, 1999a). *In situ* hybridization revealed expression of PEPC in the just emerging proteoid rootlets but as the proteoid rootlet matured the transcript abundance increased (Figure 4). These findings are consistent with the RNA gel blot time course experiments reported here that displayed low PEPC expression for juvenile (10 DAE) but high expression in mature (14 DAE) proteoid roots. As can be seen in Figure 4, PEPC expression is localized primarily in the cortex and in the meristem. This first report of the site of organic acid synthesis in proteoid roots is consistent with the likely routes of organic acid excretion including secretion into the cortical apoplast or cell to cell transport and excretion from the epidermis.

*In situ* hybridization of MDH also revealed expression in the cortex but not in the meristem of mature rootlets. Malate dehydrogenase displayed expression throughout the just emerging proteoid rootlet; however, in mature proteoid rootlets expression was restricted to a central zone along the rootlet with no detectable expression at the tip and base (Figure 5). This finding might indicate that part of the oxaloacetate that is produced by PEPC is not converted into malate by the subsequent action of MDH. An alternative fate of oxaloacetate is its conversion into citrate by citrate synthase in the mitochondria. Thus, in mature proteoid root tissues that display PEPC but not MDH, a flux of

oxaloacetate might be directed to citrate rather than to malate. These findings correspond to reports that show a significant increase of citrate and decrease of malate exudation during the development of proteoid roots (Neumann et al., 1999).

To gain insight into expression patterns of other genes related to organic acid metabolism, we performed macroarray analyses with about 1250 ESTs from −P proteoid roots. The majority of ESTs with homology to enzymes of the glycolytic pathway, including ESTs with homology to LaPEPC1 and LaMDH2, were significantly induced in P deficient proteoid roots (Table 2; Figure 6). These results correspond well to findings from Massonneau et al. (2001) who reported increased expression and enzyme activity of sucrose synthase, fructokinase and phosphoglucomutase in P deficient proteoid roots of white lupin.

An interesting aspect of organic acid metabolism under P deprivation in plants is the induction of alternative glycolytic pathways (reviewed by Theodorou and Plaxton, 1993). In P-stressed proteoid roots, PEPC not only provides C for the synthesis of organic acids but may also recycle $P_i$. Phosphorus stress can severely limit the activity of pyruvate kinase (PK), an enzyme requiring $P_i$ and ADP. PEPC and MDH can bypass PK (Figure 6) and thus maintain the flow of carbon from glycolysis to the TCA cycle by avoiding the use of ADP but generating free $P_i$ (Theodorou et al., 1992).

Another alternative glycolytic pathway known to occur in P deficient plants is catalyzed by a $PP_i$-dependent phosphofructokinase (PFP) that can bypass the ATP-dependent phosphofructokinase (PFK). PFK was not represented in our EST collection, whereas the alternative PFP was found redundantly. Moreover, macroarrays revealed strong induction of the PFP gene in −P proteoid roots, compared to +P normal roots, indicating the significance of this bypass in P deficient proteoid roots of white lupin (Table 3, Figure 6).

Though these results emphasize the role of P-conserving alternative pathways in the carbon metabolism of −P proteoid roots from white lupin, we did not find evidence for the induction of a third alternative glycolytic pathway known in plants that is catalyzed by a non-phosphorylating NADP-dependent glyceraldehyde-3P dehydrogenase. None of the 1250 ESTs displayed homology to the alternative NADP-dependent form of this enzyme whereas the NAD-dependent form was found redundantly and, moreover, showed induction in −P proteoid roots (Table 3).

RNA gel blot analyses and macroarray data give an insight into the expression patterns of genes involved in organic acid synthesis. To address the question whether PEPC in roots of P deficient white lupin is also regulated at the post-translational level by phosphorylation or effectors of enzyme activity, PEPC was partially purified from P-deficient proteoid roots. Immunoblot analysis indicated a total protein of 440 kd with a subunit mass of 110 kd, indicative of a homotetramer structure for the enzyme. These findings are consistent with native and subunit masses of PEPC from soybean nodules (Schuller et al., 1990).

Known isoforms of PEPC, ($C_4$-photosynthetic, CAM-photosynthetic, $C_3$-photosynthetic and non-autotrophic forms) are distinguishable by $K_m$ and $V_{max}$ values, ion exchange chromatography, and sensitivity to malate inhibition (Ting and Osmond, 1973). The $K_m$ PEP and $K_m$ $Mg^{2+}$ of *Zea mays* are lower in root isoforms than in the $C_3$ and $C_4$ leaf forms (Dong et al., 1998; Ting and Osmond, 1973). The low $K_m$ PEP of 0.05 m*M* for the PEPC isoform from proteoid roots of white lupin corresponds well to the kinetic data determined for several other root and nodule forms of PEPC, e.g. a root form from *Z. mays* ($K_m$ PEP 0.04 m*M*; Dong et al., 1998), a nodule form from soybean ($K_m$ PEP 0.06 m*M*; Schuller et al., 1990) and a nodule form from yellow lupin (PEPC I, $K_m$ PEP 0.09 m*M*, Marczewski, 1989).

In yellow lupin, a total of three different isoforms have been isolated from nodules and roots. We found little evidence for an additional PEPC isoform in roots of white lupin. When screening a proteoid root cDNA library, we identified a partial cDNA clone with high homology to LaPEPC1 in the coding region but distinct differences in the 3′ non-coding region. Hybridization with the 3′ non-coding region displayed extremely weak expression in 14 DAE −P normal and proteoid roots but not in nodules (data not shown). Activity stainings at various steps of the PEPC purification, however, displayed only single bands. Though protein gel blots did show two bands, they most likely reflect a change in phosphorylation state of the enzyme, since only the 440 kb band displayed PEPC activity. If there is an additional isoform of PEPC in roots of white lupin, extremely weak expression and high similarity to LaPEPC1 might make it difficult to detect.

It is well established that PEPC in leaves of $C_4$ and CAM plants is regulated by protein kinase-mediated phosphorylation of a specific serine residue near the N terminus (Schuller and Werner, 1993; Wang and

Chollet, 1993). This phosphorylation is known to decrease the sensitivity of PEPC to malate inhibition (Chollet et al., 1996; Jiao and Chollet, 1991; Schuller and Werner, 1993). PEPC- kinase and an opposing phosphatase have been isolated and characterized (Bakrim et al., 2001; Dong et al., 2001; Hartwell et al., 1999; Taybi et al., 2000; Zhang and Chollet, 1997). A time-dependent increase in PEPC activity due to phosphorylation by endogenous kinase *in vitro* has been shown when desalted nodule extracts were preincubated with ATP (Schuller and Werner, 1993). Preincubation of the partially purified PEPC from white lupin proteoid roots with ATP resulted in a comparable decrease of sensitivity to malate inhibition, suggesting *in vitro* phosphorylation of this PEPC isoform by an endogenous protein kinase. Correspondingly, a putative phosphorylation motif was present in the N-terminus of the proteoid root induced LaPEPC1 (Figure 1). Phosphorylation of PEPC had a strong influence on its response to several activating or inhibiting metabolites tested, indicating an important role of phosphorylation in the regulation of this isoform (Table 5). However, the phosphorylation state of the enzyme had little effect on the inhibition by citrate and 2-oxoglutarate. Inhibition and activation of a number of metabolites was influenced by pH. For example, Glu-6-P, Glu-1-P and Fru-6-P were stronger activators at pH 7 compared to pH 8, while malate and citrate were stronger inhibitors at pH 7 than at pH 8. Similar influence of pH has been reported for the effect of Glu-6-P and malate on PEPC from maize leaves and soybean nodules (Frank et al., 2001; Israel and Jackson, 1982; Schuller et al., 1990), a finding that led to the suggestion of a pH stat function of soybean nodule PEPC (Israel and Jackson, 1982).

Even though there are some differences in the degree of activation and inhibition, the effects of the analyzed metabolites in regard to pH and phosphorylation state of PEPC are similar to those described in PEPC nodule forms of soybean and lupin (Marczewski, 1989; McCloud et al., 2001; Schuller et al., 1990). These findings indicate that regulation of proteoid root-enhanced PEPC activity occurs in a general metabolic manner in response to metabolites, pH and phosphorylation and does not respond specifically to P-availability. This result is consistent with findings from Moraes and Plaxton (2000), who reported that an increase of PEPC activity under P deficiency in *Brassica napus* suspension cells was not correlated with a change in regulatory properties of PEPC.

The results presented here indicate that regulation of a proteoid root-enhanced PEPC isoform in response to P deficiency might occur mainly at the level of gene expression. Correspondingly, most genes encoding for enzymes involved in the glycolytic pathway, including two isoform(s) of MDH, displayed significant induction in −P proteoid roots on the level of transcription. Furthermore, *in situ* hybridization revealed a fine-tuning of the coordinated expression of PEPC and MDH in a tissue-dependent manner that might reflect changes in the composition of excreted organic acids during proteoid root development.

## Acknowledgements

This research was supported in part by US Department of Agriculture National-Research Initiative Competitive Grant No.USDA-NRI-CGP 98-35100-6098. The authors wish to thank Kate Plaisance for advice on use of the FPLC, enzyme purification, and analysis of enzyme kinetic and inhibition data.

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