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Phosphorus Deficiency in *Lupinus albus*[1]

Altered Lateral Root Development and Enhanced Expression of Phospho*enol*pyruvate Carboxylase

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The development of clustered tertiary lateral roots (proteoid roots) and the expression of phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) in roots were studied in white lupin (*Lupinus albus* L.) grown with either 1 mM P (+P-treated) or without P (−P-treated). The +P-treated plants initiated fewer clustered tertiary meristems and the emergence of these meristems was delayed compared with −P-treated plants. Proteoid root zones could be identified 9 d after emergence in both P treatments. Amounts of PEPC mRNA, PEPC specific activity, and enzyme protein were greater in proteoid roots than in normal roots beginning at 10, 12, and 14 d after emergence, respectively. The increases in PEPC mRNA, PEPC enzyme, and PEPC specific activity suggest that this enzyme is in part under transcriptional regulation. Recovery of organic acids from root exudates coincided with the increases in PEPC specific activity. The −P-treated plants exuded 40-, 20-, and 5-fold more citrate, malate, and succinate, respectively, than did +P-treated plants. Data presented support the hypothesis that white lupin has concerted regulation of proteoid root development, transcriptional regulation of PEPC, and biosynthesis of organic acids for exudation in response to P deficiency.

Proteoid root formation is enhanced by P deficiency in white lupin (*Lupinus albus* L.) (Gardner et al., 1981; Marschner et al., 1986; Dinkelaker et al., 1989; Johnson et al., 1994; Braum, 1995). The amount of citrate exuded by proteoid roots ranges from 6 to 23% of total plant dry weight (Dinkelaker et al., 1989; Braum, 1995). These amounts are phenomenal when compared with amounts in alfalfa (*Medicago sativa* L.), which exudes the equivalent of 0.3% of the total dry weight as citrate when P deficient (Lipton et al., 1987). Citrate exudation into the rhizosphere increases the P available to the plant by mobilizing the sparingly soluble mineral P and, possibly, organic P sources (Gardner et al., 1982a; Dinkelaker et al., 1989; Gerke et al., 1994; Jones and Darrah, 1994; Braum, 1995).

The anatomical features of proteoid roots were described originally for species in the Proteaceae family by Purnell (1960) and were recently reviewed by Dinkelaker et al. (1995). Similar to typical lateral roots, proteoid roots are initiated from the pericycle opposite protoxylem poles at about the time of metaxylem differentiation. In the Proteaceae and Fabaceae the formation of proteoid roots is affected predominantly by the P status of the plant (Dinkelaker et al., 1995). Proteoid roots synthesize increased amounts of citrate and malate for exudation compared with normal roots, with a portion of the exuded C being derived from nonphotosynthetic C fixation (Johnson et al., 1994, 1996). The enhanced synthesis of organic acids coincides with elevated PEPC activity (Johnson et al., 1994).

P deficiency has been shown to stimulate the activity of C_3 PEPC (EC 4.1.1.31) in leaves (Theodorou et al., 1991; Hoffland et al., 1992, Pilbeam et al., 1993) and nonphotosynthetic PEPC in roots (Landsberg, 1986; Pilbeam et al., 1993; Johnson et al., 1994). In addition to supplying anaplerotic C to replenish TCA-cycle intermediates, elevated PEPC caused by P limitation may be a response to increased demands for pyruvate and/or P recycling (Theodorou et al., 1991). Enhanced PEPC activity in the roots could provide a means of maintaining cation uptake by increasing the uptake of HCO_3^- (Pilbeam et al., 1993). Studies in our laboratory provide evidence that PEPC in the roots of P-deficient plants provides as much as 25% of the C for citrate and 34% of the C for malate for exudation, thus alleviating some of the C burden associated with citrate excretion (Johnson et al., 1996). In contrast to C_3 and nonphotosynthetic PEPC, P deficiency in maize (*Zea mays*) decreases the activity of photosynthetic C_4 PEPC in leaves (Usuda and Shimogawara, 1992).

PEPC catalyzes the carboxylation of PEP using HCO_3^- as the C source to form oxaloacetate and to release Pi. In C_4 and CAM plants, PEPC's role in concentrating CO_2 has been well established. In C_3 species, PEPC has been implicated in numerous physiologic functions including: (a) replenishment of tricarboxylic acid cycle intermediates; (b)

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Abbreviations: DAE, days after emergence; PEPC, PEP carboxylase.

recapture of respired CO_2; (c) nitrogen assimilation and amino acid synthesis; and (d) pH maintenance (Latzko and Kelly, 1983). PEPC is regulated by transcription, posttranslational phosphorylation, oligomerization, and protein turnover (Lepiniec et al., 1994). Of the C_3 nonphotosynthetic PEPCs in roots or nodules, only the root nodule form in legumes has been investigated in detail (Lepiniec et al., 1994). Malate inhibits PEPC activity in both roots and nodules of legumes (Marczewski, 1989). During the development of legume root nodules, PEPC activity increases as a result of enhanced expression (Pathirana et al., 1992). All plant forms of PEPC (C_4, CAM, and C_3) studied to date have been regulated by phosphorylation (Lepiniec et al., 1994). The effects of P deficiency on nonphotosynthetic C_3 PEPC in roots have not been investigated thoroughly.

The objectives of this study were to determine if proteoid root initiation and development is coincident with PEPC activity and organic acid exudation, and to assess whether the increased PEPC activity in proteoid roots is the result of increased synthesis of PEPC enzyme and PEPC mRNA.

MATERIALS AND METHODS

Lupinus albus L. var Ultra plants were grown in a growth chamber (Conviron, model no. E15, Controlled Environments, Pembina, ND) at 20/15°C and 16-/8-h day/night cycles, 300 μmol photons m^{-2} s^{-1} at shoot height, and 70% RH. Surface-sterilized seeds were planted in pots containing 1.0-mm-diameter silica sand. Pots were watered every 2 d with 1.0 L of the appropriate nutrient solution (Johnson et al., 1994). The nutrient solutions differed in P concentration: the +P treatment group received 0.5 mM $Ca(H_2PO_4)_2$, whereas the −P treatment group received 0.5 mM $CaSO_4$ and no P. The pH of both treatments was adjusted to 6.0.

Histological Protocols

Intact Root Systems

Twenty-five intact root systems were harvested from each P-treatment group 8, 10, and 12 DAE. The root systems were fixed, cleared, and stained. Roots were observed with a dissecting microscope (10× to 32×). Our working definition of a proteoid root zone was 10 or more meristems or tertiary roots per centimeter of secondary root. The total number of proteoid root zones (meristems and emerged) were determined on each plant.

Serial Sections

Root segments were harvested from 2 cm below the shoot to the root transition zone (defined as where the stem changed from green to white). Segments were cut to about 1 cm in length and fixed in 5% formalin, 5% propionic acid, and 63% ethanol, vacuum infiltrated overnight, and dehydrated with a tertiary butyl alcohol series (Berlyn and Miksche, 1976). The samples were embedded with paraffin and serial sectioned (10 μm). Sections were stained with a common histological stain and counterstain series, safranin and fast green, to visualize cellular structure in paraffin sections (Berlyn and Miksche, 1976).

Clearing and Staining Method

Whole root systems were fixed in an absolute ethanol: glacial acetic acid (3:1, v/v) solution at 4°C for at least 24 h, rinsed for 5 min under running deionized water (Hinchee and Rost, 1992), and cleared by immersion in sodium hypochlorite solution (12.5% active chlorine) diluted with distilled water (50%, v/v) (Truchet et al., 1989) under vacuum (0.03–0.05 MPa) for 10 min and for an additional 10 min at atmospheric pressure. After fixing and clearing, roots were stained with methylene blue (0.01% in distilled water) to visualize the nucleolar material in the meristems (Truchet et al., 1989).

Biochemical Protocols

Root Harvest for Enzyme Assays, Total Protein, Immunoanalysis for PEPC, and RNA Extraction

Secondary lateral roots from +P-treated and −P-treated plants were harvested 5 and 8 DAE. The proteoid root segments were visible from 9 DAE. Root systems were separated into proteoid and normal root segments at 9, 10, 11, 12, 14, 16, and 23 DAE. Proteoid root segments were portions of the secondary root displaying proteoid root morphology (defined above); normal root segments were comparable portions of secondary roots with no or few (fewer than five) tertiary roots per centimeter, both excluding the most basal and apical portions. Root material from 4 to 20 plants per treatment was pooled to provide at least 5 g of fresh tissue.

Preparation of Root Extracts for PEPC Assay and Western Blot Immunoanalysis for PEPC

The chilled tissue was placed in a prechilled mortar and pestle, frozen with liquid N_2, and pulverized to a fine powder. The tissue powder was transferred to a glass homogenizer and blended with 1 mL of cold Tris-HCl (0.25 mM, pH 8.0, with 0.5 M Suc and 0.001 M DTT) for every 0.25 g of fresh tissue (Johnson et al., 1994). After homogenization, 0.1 M PMSF in isopropanol was added (10 μL mL^{-1} buffer). The homogenate was centrifuged for 10 min at 14,000*g* and the supernatant was saved for PEPC activity, total protein, and protein immunoblot assays.

In Vitro PEPC Activity

The supernatant was used to determine PEPC activity spectrophotometrically by monitoring the disappearance of NADH at A_{340} for 2 min in a malate dehydrogenase (EC 1.1.1.37) coupled reaction (Vance et al., 1983). Total protein was determined with a bicinchoninic acid protein assay reagent kit (Pierce) after precipitation with acetone (Johnson et al., 1994).

SDS-PAGE and Protein Immunoblot Analysis for PEPC

Root cell-free protein extract was precipitated by mixing a 1:2 ratio of sample to 10% TCA. The solution was stored for at least 30 min at −20°C before centrifuging (15 min, 14,000*g*), discarding the supernatant, and allowing the pellet to dry. Soluble protein (50 μg) from cell-free extracts was separated by SDS-PAGE (10% acrylamide) and electrophoretically transferred to nitrocellulose. Rabbit polyclonal antibodies to alfalfa nodule PEPC were used to detect lupin root PEPC protein on western blots (Miller et al., 1987). Alfalfa nodule protein (10 μg) was used as a positive control. It has been demonstrated previously that this antibody cross-reacts with the 100-kD lupin nodule PEPC protein, the 100-kD alfalfa root PEPC protein (Miller et al., 1987), and the lupin root PEPC protein (Johnson et al., 1994). Immunoblot analysis was repeated on protein extracts from all three replications.

RNA Extraction and Gel Blot Analysis

RNA was isolated from root tissue using guanidinium buffer (Strommer et al., 1993). Total RNA (20 μg lane^{-1}) or alfalfa nodule RNA (9 μg lane^{-1}) was separated electrophoretically on formaldehyde-1.5% agarose gels and transferred to Zetaprobe (Bio-Rad) in 10× SSC (1× SSC = 140 mM NaCl, 15 mM trisodium citrate) overnight. RNA blots were probed with denatured ^{32}P-labeled PEPC cDNA from alfalfa (*Medicago sativa* L. var Saranac) or lotus (*Lotus corniculatus* L.) (Pathirana et al., 1992; R. Gregerson and C. Vance, unpublished data). Prehybridization and hybridization methods were described by the manufacturer. After hybridization, blots were washed sequentially with 2× SSC at room temperature for 10 min; 2× SSC with 0.1% SDS at room temperature for 10 min; and 2× SSC with 0.1% SDS at 42°C for 15 min. These low-stringency conditions have been shown to be effective for heterologous probes (Haser et al., 1992). Radioactivity on blots was quantified (AMBIS Radioanalytic Imaging System, San Diego, CA), and then filters were exposed to x-ray film. Lane loading was verified by probing the 28S rRNA subunit with a *Lotus* rDNA-PCR product (R. Gregerson and C. Vance, unpublished data). The coefficient of variation for loading was 15%; differences were not due to P treatment or to root type.

Analysis of Exudates for Organic Acids

Plants were watered with 1.0 L of nutrient solution and the eluted solution was collected 6, 8, 10, 12, 14, 16, 18, 20, and 22 DAE. A 25-mL aliquot of well-mixed eluant was stored at −20°C. The acidic fraction was separated from the basic and neutral fractions using strong anion-exchange chromatography (SAX Bond Elute columns, capacity 2.8 mL 500 mg^{-1}; Analytichem International, Varian, Harbor City, CA) (Johnson et al., 1996). The acidic fraction containing organic acids was dried in an autoevaporator (Speedvac, Savant Instruments, Farmington, NY). Individual organic acids were separated by HPLC (Johnson et al., 1994). The accumulation of individual organic acids in the root exudates is the mathematical sum of each acid detected at each collection date during the developmental period. An average recovery after ion-exchange chromatography and HPLC was 96% for citrate, 66% for α-ketoglutarate, 98% for malate, 50% for succinate, and 81% for fumarate, as determined with external standards.

Statistical Analysis

The histological experiments were conducted once for each sample date. The 25 individual plants from each treatment were treated as observations. A general linear model for analysis of variance was used to determine the effect of the two levels of P on the initiation and emergence of proteoid roots.

The amount of total RNA, radioactivity hybridized to PEPC mRNA, PEPC activity, and total protein were determined from three different time-course experiments that were subsampled to provide a minimum of five observations on each date. Each of the time-course experiments used a split-plot design with subsampling; P treatment was the main effect and root type was the subplot effect. A general linear model for analysis of variance was used to determine statistical differences. The LSD values calculated for the interaction terms used weighted SD and *t* values as described by Gomez and Gomez (1984).

The exudation experiment was conducted once in a completely randomized design with three replications. A general linear model for analysis of variance was used to determine statistical differences between the two P treatments.

RESULTS

Proteoid Root Development

Our working definition of a proteoid root is a secondary lateral root with 10 or more meristems or tertiary laterals per centimeter. These clustered tertiary laterals arise from the pericycle at opposite xylem poles (Fig. 1). Light microscopy showed that both proteoid and normal root primordia arise opposite xylem poles in the pericycle (data not shown). Meristems on secondary lateral roots (harvested 2 cm from the transition zone) could occasionally be seen by 5 DAE, but were usually seen easily on cleared material by 6 DAE (Fig. 2). These tertiary roots quickly grew though the cortex and began to emerge through the epidermis by 8 DAE (Fig. 2). The earliest date that roots could be designated normal or proteoid without a microscope was 9 DAE. The density of meristems in proteoid root zones ranged from 11 to 35 meristems per centimeter of root for both P treatments.

Clearing the entire root system revealed that all plants from both P treatment groups had at least one proteoid root zone. However, on +P-treated plants fewer proteoid root zones were initiated and fewer tertiary roots in those zones emerged, resulting in fewer proteoid roots compared with −P-treated plants (Table I). The −P-treated plants had 1.6-fold more secondary laterals with proteoid root zones than did +P-treated plants on 8, 10, and 12 DAE (Table I). Root systems from −P-treated plants had 2.4-fold more lateral roots with emerged tertiary roots in the proteoid root zone than did +P-treated plants 12 DAE (Table I). The percentage of emergence of meristems from the proteoid

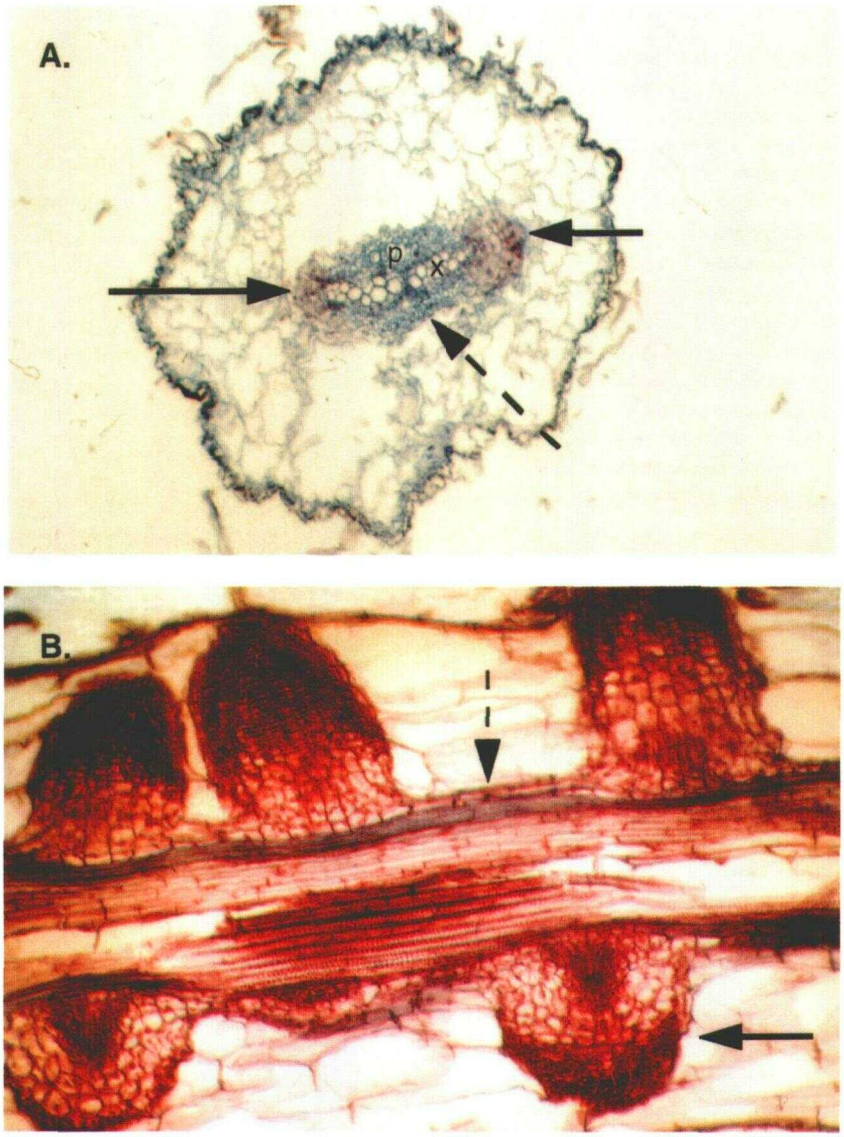

Figure 1. A, Cross-section through a proteoid root segment excised 1 cm behind the apical tip; B, *longitudinal thin-section through a proteoid* root segment excised 2 cm behind the apical tip. Both sections were from a −P-treated white lupin plant. Sections were stained with safranin and fast green. Note the diarch arrangement of the xylem and the numerous meristems arising from the pericycle. Solid arrows, Developing tertiary root meristems; dashed arrows, pericycle; x, xylem; p, phloem. The diameter across the root is about 1 mm; magnification is 100×.

root zones was less in +P-treated plants compared with −P-treated plants, regardless of whether emergence was expressed as a percentage of proteoid segments with emerged tertiary roots or as a percentage of plants with emerged proteoid segments (Table I).

Secondary lateral roots developed tertiary roots in both the +P-treated and the −P-treated plants. The majority of tertiary roots from the +P-treated plants were randomly arranged and occurred at a density less than 10 tertiary roots per centimeter of secondary lateral root; therefore, they were not classified as proteoid roots (Fig. 3). The presence of a small percentage of proteoid roots on +P-treated plants is consistent with our previous results (Johnson et al., 1994, 1996). About two-thirds of the secondary roots from a −P-treated root system could be classified as proteoid by 14 DAE (Fig. 3). Secondary and tertiary roots from +P-treated plants tended to be longer than lateral roots from −P-treated plants, especially after 10 DAE. Tertiary roots in the proteoid root zone had a maximum length of about 1 cm at 22 DAE (Fig. 3). It was demonstrated previously by Johnson et al. (1994) that root systems of −P-treated plants 23 DAE have fewer secondary roots than do those of +P-treated plants.

Exudation of Organic Acids

Malate, citrate, and succinate were first detected in the exudates of −P-treated plants at 12 DAE (Fig. 4). Citrate and malate were detected 14 DAE and succinate was detected 16 DAE in exudates from +P-treated plants. By 14 DAE 17-fold more citrate and 28-fold more malate was recovered in exudates from −P-treated plants than from +P-treated plants. During the 22-d developmental period a total of 40-fold more citrate, 20-fold more malate, and 5-fold more succinate was recovered in exudates from −P-treated plants than from +P-treated plants. By 22 DAE the equivalent of 12% of the total plant dry weight (1.12 g at 22 DAE) of −P-treated plants was recovered in root exudates as organic acids, 5.6% as citrate, 4.7% as malate, and 2.0% as succinate. This calculation is based on an assumed plant moisture content of 85% and fresh

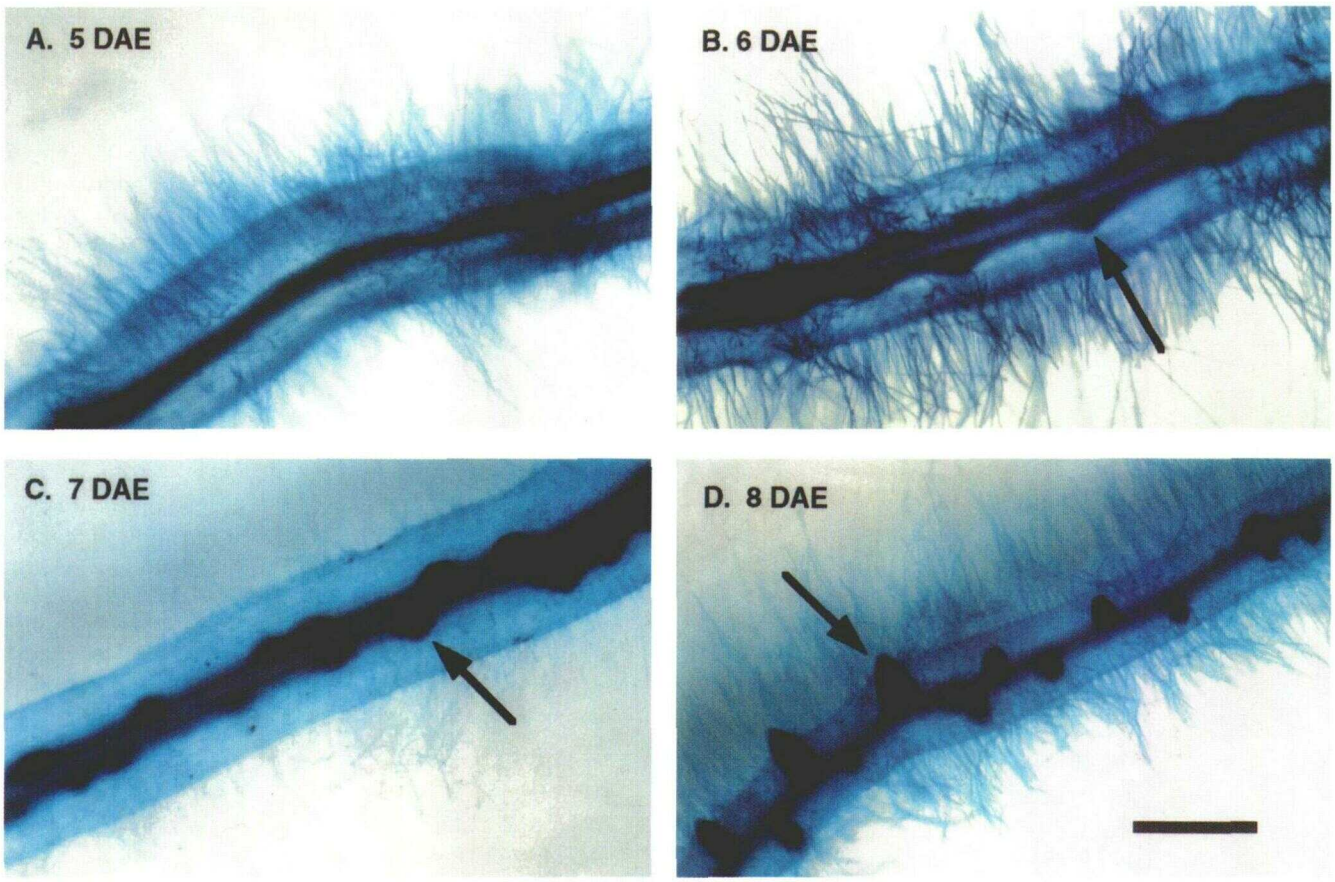

Figure 2. Secondary lateral roots harvested 2 cm from the transition zone on white lupin plants grown without P (−P-treated), cleared with sodium hypochlorite, and stained with methylene blue 5 (A), 6 (B), 7 (C), and 8 (D) DAE. Note the numerous meristems (arrows) 6, 7, and 8 DAE. Meristems begin to emerge through the epidermis by 8 DAE (arrow). Scale bar = 1 mm; magnification is 30×.

weights reported previously by our laboratory (Johnson et al., 1994). The amount of organic acids recovered from root exudates underestimates the actual amounts exuded because plants were grown in a nonsterile environment and because recovery during ion-exchange chromatography and HPLC was less than 100%.

PEPC Specific Activity and Enzyme

The in vitro specific activity of PEPC was higher in −P-treated plants than in +P-treated plants, irrespective of root type, beginning at 11 DAE (Table II). Beginning at 12 DAE, specific activity of PEPC in proteoid roots increased

Table I. *Incidence of proteoid root segments (meristems or emerged roots) on L. albus root systems*

Intact root systems from 25 plants were cleared with sodium hypochlorite and then stained with methylene blue. Secondary roots with 10 or more meristems or tertiary roots per centimeter were classified as proteoid. Plants were grown with 1 mM P (+P-treated) or without P (−P-treated).

Parameter	DAE								
	8			10			12		
	+P	−P	LSD[a]	+P	−P	LSD	+P	−P	LSD
Total number of proteoid segments (meristems or emerged roots) per plant	11.6	18.1	3.6	10.7	18.0	3.2	12.2	20.2	3.6
Number of emerged proteoid segments per plant	0.0	2.5	1.3	1.5	5.2	1.6	3.6	8.8	2.8
Percentage of proteoid segments with emerged tertiary roots	0	14	—[b]	14	29	—	30	44	—
Percentage of plants with emerged proteoid segments	0	50	—	40	84	—	68	100	—

[a] P = 0.05. [b] —, Not applicable.

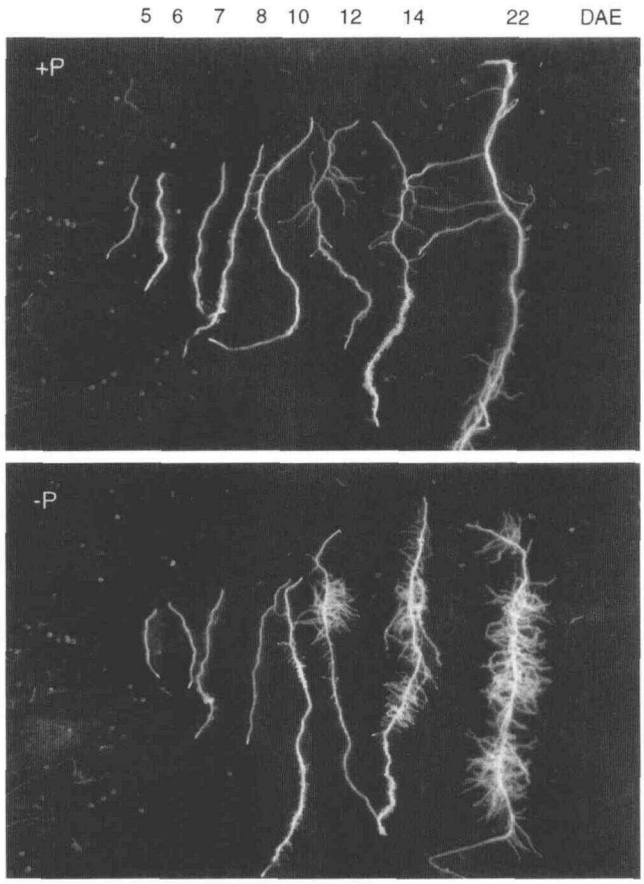

Figure 3. Intact secondary lateral roots harvested 2 cm below the shoot-to-root transition zone from *L. albus* plants grown with either 1 mM P (top) or without P (bottom). The top frame is typical of normal secondary roots from +P-treated plants. The bottom frame is typical of proteoid secondary roots from −P-treated plants. Roots were harvested 5, 6, 7, 8, 10, 12, 14, and 22 DAE. Root systems from +P-treated plants are <10% (w/w) proteoid and root systems from −P-treated plants are >50% (w/w) proteoid by 23 DAE (Johnson et al., 1994).

1.2- to 2.1-fold compared with normal roots. The largest difference between root types was observed in the −P-treated plants 14 DAE. Comparable results were obtained when PEPC activity was expressed on a fresh weight basis (data not shown). During the developmental period the protein concentration was 2.8 mg protein g^{-1} fresh weight in proteoid roots, compared with only 1.8 mg protein g^{-1} fresh weight in normal roots. These concentrations were different ($P \leq 0.01$).

Protein immunoblot analysis specific for the PEPC enzyme showed little to no difference between P treatments or root types up to 12 DAE (data not shown). In all three replications of the immunoblot analysis, beginning 14 DAE, proteoid roots consistently had a more intense band than the corresponding normal roots (Fig. 5). The difference between root types was more striking in the −P-treated plants; on this gel it was particularly noticeable 14 and 23 DAE (Fig. 5). On other immunoblots this tendency was also observed 16 DAE (data not shown). The only band visualized was at 104 kD (Fig. 5) and corresponded in M_r to the alfalfa nodule PEPC used as a positive control (data not shown).

PEPC mRNA Expression

The size of lupin PEPC mRNA is about 3.5 kb (Fig. 6), which corresponds to the size of alfalfa nodule PEPC mRNA (Pathirana et al., 1992). From 10 to 16 DAE, proteoid roots from both P treatments had from 1.0- to 2.5-fold more PEPC mRNA than did normal roots (Table III). There was a tendency for the −P-treated proteoid roots to have more PEPC mRNA than all other tissues (Table III; Fig. 6), but only at 14 DAE were net ^{32}P counts statistically different (Table III). There was a tendency for PEPC mRNA to be enhanced in −P-treated proteoid roots at 23 DAE (Table III). The higher amount of PEPC mRNA in proteoid roots was not due to differences in the amount of total RNA loaded into wells, as shown by hybridization of blots to a *Lotus* 28S rRNA subunit cDNA (data not shown).

Proteoid roots had 1.4- to 3.3-fold more total RNA than did normal roots between 10 and 16 DAE (Table IV). However, by 23 DAE total RNA was reduced nearly 4-fold in the −P-treated plants compared with +P-treated plants. The total RNA concentration and the amount of PEPC mRNA were not different between P treatments or root types before 10 DAE.

DISCUSSION

Proteoid Root Development

Proteoid roots developed on plants grown in both P treatments; however, the incidence of proteoid root zones was enhanced markedly in −P-treated plants compared with +P-treated plants (Table I; Fig. 3). The −P-treated plants had more secondary roots that initiated clustered

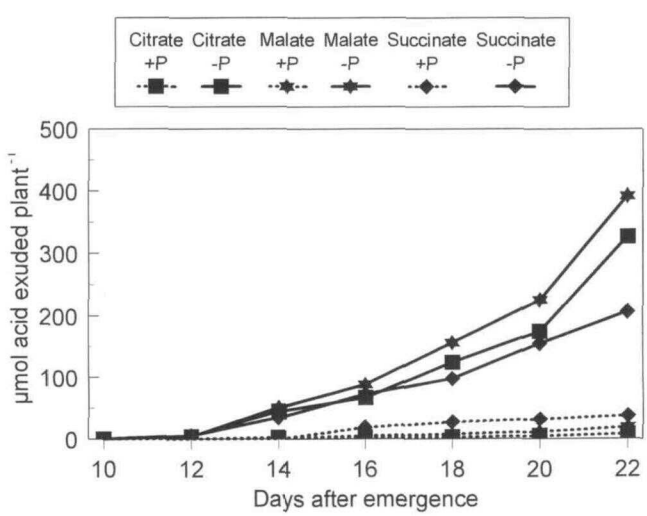

Figure 4. Amount of citrate, malate, and succinate exuded per plant (µmol acid exuded $plant^{-1}$) in root exudates. Root exudates were collected every 2 d from *L. albus* plants grown with 1 mM P (+P-treated) or without P (−P-treated). Organic acids were determined by HPLC and values were accumulated over time. Each point represents the treatment mean (n = 3).

Table II. *In vitro specific activity (μmol NADH min^{-1} mg^{-1} protein) on a mg protein basis of PEPC*

L. albus plants were grown with 1 mM P (+P-treated) or without P (−P-treated) for 5 to 23 DAE. PEPC activity was determined spectrophotometrically at A_{340} for normal and proteoid root segments.

Treatment	Root	DAE								
		5	8	9	10	11	12	14	16	23
		μmol NADH min^{-1} mg^{-1} protein								
+P	Normal	0.08	0.11	0.14	0.17	0.12	0.10	0.08	0.07	0.07
	Proteoid	—[a]	—	0.15	0.14	0.14	0.14	0.11	0.11	0.11
−P	Normal	0.08	0.10	0.13	0.17	0.16	0.14	0.12	0.08	0.08
	Proteoid	—	—	0.14	0.16	0.17	0.17	0.25	0.13	0.14
Number of observations		7	7	8	7	10	12	7	7	6
Significance of treatment		NS[b]	NS	*[c]	NS	**[d]	**	**	**	**
Significance of root		NA[e]	NA	NS	NS	NS	*	**	**	**
Significance of interaction		NA	NA	NS	NS	NS	NS	NS	NS	NS

[a] —, No proteoid root segments present. [b] NS, Not significant. [c] *, Significant at P = 0.05. [d] **, Significant at P = 0.01. [e] NA, Not applicable.

meristems, and more of those meristems emerged to form proteoid root zones compared with +P-treated plants (Table I). Previously we (Johnson et al., 1994) demonstrated that the proteoid root zones constitute 50% (dry weight) of the −P-treated root system, but only 10% of the +P-treated root system. The enhancement of proteoid root development by P deficiency appears to result from an increase in both the number of meristems initiated and the percentage of meristems emerged (Table I).

Formation of proteoid roots at sufficient levels of P and Fe has been reported previously (Gardner et al., 1982b; Marschner et al., 1987). In our experiments P concentration in plant tissue was 0.5% (w/w) in +P-treated plants at 37 DAE (data not shown), more than twice the amount considered adequate for this species (Snowball and Robson, 1986). The concentrations of other nutrients in the +P-treated plants were also well within the adequate range; therefore, it is unlikely that the +P-treated plants used in the experiments presented here were P deficient. The concentration of internal P necessary to completely inhibit proteoid roots in white lupin is not known (Dinkelaker et al., 1995). It appears that nutrient concentrations (especially P and Fe) needed to completely inhibit proteoid root development may be considerably higher than necessary for optimal shoot growth and yield. Another possibility is that a small percentage of the root system in white lupin may always develop clustered meristems regardless of nutritional status.

Similar to typical lateral roots, proteoid roots are initiated from the pericycle at opposite protoxylem poles (Fig. 1) at about the time of metaxylem differentiation. Unlike typical lateral roots, which emerge at random along the axis of primary and secondary roots (Charlton, 1983), proteoid roots emerge nearly simultaneously, crowded together in a longitudinal row along the axis (Purnell, 1960; Dinkelaker et al., 1995) and cause a dramatic increase in root surface area (Fig. 3). In many species, formation of proteoid roots is affected principally by the P status of the plant (Dinkelaker et al., 1995). White lupin will develop cluster roots when grown aseptically, although the presence of bacteria enhances their development. In a number of other species, microbes are necessary for proteoid root formation (Dinkelaker et al., 1995).

Although plant growth regulators have been implicated in proteoid root development (Lamont et al., 1984; Klepper, 1992), currently there is no direct evidence to support this hypothesis. In pea (*Pisum sativum* L.), auxin transport inhibitors and an auxin antagonist blocked normal lateral root development and increased the initiation of lateral root primordia. These primordia tended to be initiated simultaneously at all three protoxylem points of the primary axis (Hinchee and Rost, 1992). Several plant species exhibit aspects of proteoid root development such as

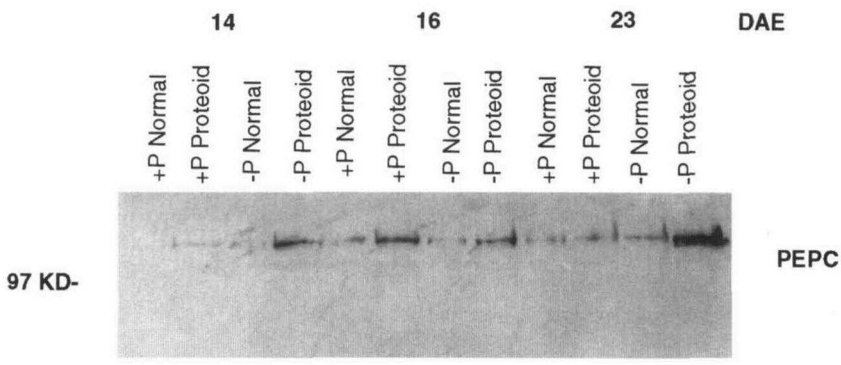

Figure 5. Immunoblot of root protein for PEPC protein using rabbit polyclonal antibodies developed against alfalfa nodule protein. All lanes were loaded with 50 μg of protein. *L. albus* plants were grown with 1.0 mM P (+P-treated) or without P (−P-treated) 14, 16, or 23 DAE.

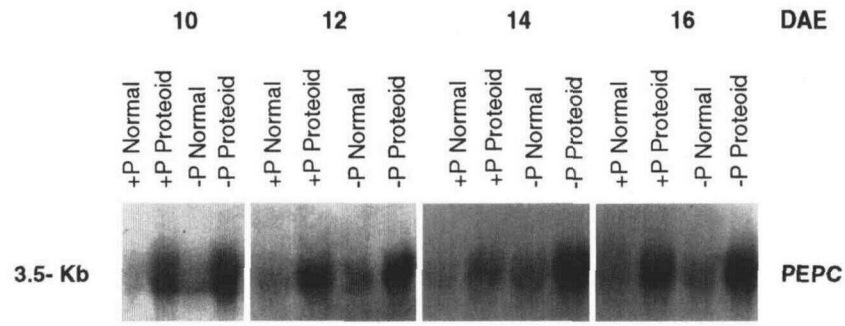

Figure 6. RNA blot of normal and proteoid roots from *L. albus* plants grown with 1.0 mM P (+P-treated) or without P (−P-treated) 10, 12, 14, or 16 DAE. All lanes were loaded with 20 μg of RNA. Blots were probed with *L. corniculatus* PEPC cDNA and washed under low-stringency conditions. The PEPC band is about 3.5 kb; no other bands were apparent on blots under these conditions.

increased numbers of laterals, reduced length of laterals, and reduced length of primary axes when exposed to natural and synthetic auxins (Wightman et al., 1980; Blakely et al., 1988; Hinchee and Rost, 1992).

Lateral root development is influenced strongly by environmental factors and is under distinct hormonal control, particularly by IAA and cytokinin (Marschner, 1995). P deficiency can affect auxin production and perhaps transport (Russell, 1977; Jacobs and Rubery, 1988). It is plausible that P nutrition interacts with plant growth regulators in white lupin to control the development of proteoid roots, although the mechanism has yet to be elucidated.

Organic Acid Exudation

By 22 d the equivalent of 6% of the −P-treated plant's total dry weight was recovered in root exudates as citrate. The quantity of citrate exuded by P-deficient lupin can represent 6 to 11% of the total plant dry weight after 6 weeks (Gardner et al., 1983; Braum, 1995) and 23% after 13 weeks (Dinkelaker et al., 1989). There appears to be a positive relationship between the relative amount of citrate detected in exudates and the duration of P stress or plant age. The increase in citrate relative to plant weight could be due to an increase in the excretion of citrate from proteoid root tissue, a higher percentage of proteoid roots, an increase in the specific activity of the enzymes involved, or a longer period of time for citrate to accumulate in the rhizosphere. In a nonsterile environment the amount of organic acids detected represents the net accumulation that has not been degraded, and thus may underestimate actual amounts exuded (Braum, 1995).

Although citrate, malate, and succinate were recovered in root exudates (Fig. 4), citrate is typically the dominant acid exuded from P-deficient white lupin roots (Gardner et al., 1983; Braum, 1995). Citrate, malate, and succinate were exuded from 24-d-old alfalfa, but only the exudation of citrate was increased significantly by P deficiency (Lipton et al., 1987). There is variability in the composition of organic acids recovered from root exudates of white lupin, ranging from only malate (Braum, 1995), to nearly equal amounts of citrate and malate with small amounts of succinate (Johnson et al., 1996), to almost exclusively citrate (Gardner et al., 1983). The cause of these differences in organic acid composition is unknown, but may be affected by such factors as the age of the plant, microbial degradation, exudate accumulation interval, or genomic variability.

Expression and Regulation of PEPC

By 16 DAE, total RNA concentration was drastically reduced in roots from −P-treated plants compared with +P-treated plants (Table IV). At that time, −P-treated shoots were showing classic P-deficiency symptoms (Marschner, 1995), including necrotic spots. Because P is necessary for nucleotide synthesis, severe P deficiency can result in decreased total RNA biosynthesis. In addition, P defi-

Table III. *Concentration of PEPC mRNA (net ^{32}P counts)*

L. albus plants were grown with 1 mM P (+P-treated) or without P (−P-treated) for 5 to 23 DAE. PEPC mRNA for normal and proteoid root segments was estimated by direct count of net ^{32}P radioactivity on RNA blots after hybridization with PEPC cDNA.

Treatment	Root	DAE								
		5	8	9	10	11	12	14	16	23
		Net ^{32}P counts								
+P	Normal	252	310	218	353	471	443	546	527	364
	Proteoid	—[a]	—	222	564	824	670	1036	1123	657
−P	Normal	252	464	301	489	698	531	731	685	591
	Proteoid	—	—	367	787	1122	790	1832	1440	1009
Number of observations		5	4	5	7	8	8	7	8	6
Significance of treatment		NS[b]	NS	*[c]	NS	NS	NS	NS	NS	NS
Significance of root		NA[d]	NA	NS	**[e]	*	**	**	**	NS
Significance of interaction		NA	NA	NS	NS	NS	NS	*	NS	NS

[a] —, No proteoid root segments present. [b] NS, Not significant. [c] *, Significant at P = 0.05. [d] NA, Not applicable. [e] **, Significant at P = 0.01.

Table IV. *Concentration of total RNA (μg RNA g^{-1})*

L. albus plants were grown with 1 mM P (+P-treated) or without P (−P-treated) for 5 to 23 DAE. RNA was extracted from normal or proteoid root segments using guanidinum buffer.

Treatment	Root	DAE								
		5	8	9	10	11	12	14	16	23
		μg RNA g^{-1} fresh wt								
+P	Normal	117	115	125	102	105	91	60	126	82
	Proteoid	—[a]	—	174	241	166	182	136	174	113
−P	Normal	114	103	80	75	92	79	30	47	17
	Proteoid	—	—	164	138	149	120	100	106	33
Number of observations		5	4	5	7	8	8	7	8	6
Significance of treatment		NS[b]	NS	NS	*[c]	NS	NS	NS	**[d]	**
Significance of root		NS	NS	NS	**	*	*	**	**	NS
Significance of interaction		NA[e]	NA	NS	*	NS	NS	NS	NS	NS

[a] —, No proteoid root segments present. [b] NS, Not significant. [c] *, Significant at P = 0.05. [d] **, Significant at P = 0.01. [e] NA, Not applicable.

ciency induces a specific RNase in tomato (*Lycopersicon esculentum*) and Arabidopsis (Nurnberger et al., 1990; Bariola et al., 1994). This RNase functions to liberate Pi from RNA, facilitating its remobilization (Bariola et al., 1994). The decrease in total RNA concentration due to P deficiency may be the result of decreased biosynthesis, increased degradation, or a combination of both. It should be noted that although the concentration of total RNA was reduced by P deficiency (Table IV), proteoid roots continued to have enhanced expression of PEPC mRNA (Table III; Fig. 6), which suggests preferential synthesis and/or stability of PEPC mRNA.

By 10 DAE, the concentration of PEPC mRNA had increased 1.6-fold (Table III) and by 12 DAE, the specific activity of PEPC had increased 1.2-fold (Table II) in proteoid roots compared with normal roots. The largest difference in both the PEPC mRNA concentration (2.5-fold) (Table III; Fig. 6) and the specific PEPC activity (2.1-fold) (Table II) between proteoid and normal roots of −P-treated plants occurred 14 DAE, consistent with the first detectable differences in organic acid exudation (Fig. 4). The immunoblot analysis for PEPC showed that there was more PEPC enzyme in proteoid roots compared with normal roots on 14, 16, and 23 DAE (Fig. 5). Thus, increased PEPC specific activity was related directly to an increase in PEPC mRNA and PEPC enzyme. These data provide strong evidence that this nonphotosynthetic PEPC is in part under transcriptional regulation.

PEPC activity in C_4 and CAM species is regulated through both transcriptional and posttranslational events. Light induces an increase in the amount of C_4, PEPC mRNA, and enzyme in maize and sorghum (*Sorghum bicolor* L.) (Harpster and Taylor, 1986; Cretin et al., 1991). In the CAM species ice plant (*Mesembryanthemum crystallinum*), salt stress, water stress, and ABA induce PEPC mRNA and PEPC enzyme, but cytokinin suppresses the accumulation of PEPC mRNA (Ostrem et al., 1987; Chu et al., 1990; Schmitt and Piepenbrock, 1992). The shift from C_3 to CAM is partially controlled by ontogeny, but is also tightly regulated by salt stress, light, and circadian rhythms (Lepiniec et al., 1994). Posttranslational regulation is achieved through phosphorylation and oligomerization as affected by PEP, malate, Glc-6-P, and protein turnover (Lepiniec et al., 1994). Increased PEPC activity in C_3 plants has been associated with increases in internal cellular pH or a high demand for C skeletons, such as during amino acid biosynthesis, N assimilation, and exudation of organic acids (Hoffland et al., 1992; Latzko and Kelly, 1983; Pilbeam et al., 1993; Johnson et al., 1994). Nitrate may activate cytosolic protein kinase, modulating the phosphorylation status of PEPC in wheat (*Triticum aestivum* L.) (Champigny and Foyer, 1992). It has been suggested that changes in cytosolic pH may modulate PEPC activity by directly or indirectly regulating its phosphorylation status (Rajagopalan et al., 1993). The phosphorylation status of PEPC in soybean (*Glycine max* L.) root nodules appears to be modulated by photosynthate transported from shoots (Zhang et al., 1995).

CAM, C_4, and photosynthetic C_3 PEPCs are subject to reversible phosphorylation. Furthermore, malate sensitivity depends on the phosphorylation status of the protein (Champigny and Foyer, 1992). In legumes, root nodule C_3 nonphotosynthetic PEPC is also regulated by transcriptional and translational events, as well as by phosphorylation (Miller et al., 1987; Pathirana et al., 1992; Schuller and Warner, 1993). Phosphorylation of root nodule PEPC reduces the sensitivity of the enzyme to malate inhibition (Schuller and Warner, 1993; Zhang et al., 1995). This is a fundamental characteristic of an enzyme that functions in an environment in which malate synthesis is high, such as root nodules and proteoid roots.

P deficiency enhances proteoid root development (Table I; Fig. 3), and these roots synthesize large amounts of citrate and malate. Enhanced synthesis of organic acids is dependent on continued high PEPC activity (Johnson et al., 1994). However, if proteoid root nonphotosynthetic PEPC is modulated by phosphorylation, the plant would expend limited internal Pi to regulate PEPC. Regulation of PEPC in proteoid roots by phosphorylation has yet to be established. Lepiniec et al. (1994) have suggested that phosphorylation is a general regulatory feature of higher-plant PEPCs.

Morphology and Physiology

Recent reviews have suggested that a set of genes coregulated by Pi and comparable to the *Pho* regulon observed in microorganisms such as *Escherichia coli* (Goldstein et al., 1989; Duff et al., 1994) exists in higher plants (a Pi regulon or stimulon). Activation of the *Pho* regulon leads to the synthesis of many proteins involved in the metabolic integration of Pi: phosphatases, phosphate transport proteins, Pi-binding proteins, and membrane-associated proteins (Torriani-Gorini, 1987). Higher plants have several strategies to cope with P deficiency, including enhanced root growth, increased density and length of root hairs, development of proteoid roots, and increased rates of Pi uptake (Marschner, 1995). P starvation in plants induces some RNases (Nurnberger et al., 1990; Loffler et al., 1992; Bariola et al., 1994) and has been reported to induce both phosphatases and phosphodiesterases in plant cells (Ueki and Sato, 1971; Goldstein et al., 1989; Loffler et al., 1992). Budicky et al. (1993) isolated a cDNA clone for a putative Pi starvation-inducible protein kinase from black mustard (*Brassica nigra*). Synthesis of the α subunit of the PPi-dependent phosphofructokinase (EC 2.7.1.90) in black mustard was induced by withholding P (Theodorou et al., 1992). P starvation-inducible metabolism in higher plants involves integrated responses of cells, tissues, and the whole plant (Goldstein et al., 1989). The results reported here are consistent with a model of multiple and integrated responses to P deficiency by white lupin.

SUMMARY

The enhanced development of proteoid roots, enhanced PEPC expression and activity, and citrate exudation are adaptive responses to P deficiency in white lupin. Plants from both P treatments developed proteoid roots and excreted citrate, but the phenomenon was enhanced dramatically by P deficiency (Table I; Fig. 4). Clustered tertiary meristems in the proteoid root zone were visible on very young secondary roots (6 DAE) (Fig. 2). These tertiary meristems continued growing through the cortex to emerge by 9 DAE into proteoid root zones that were visible without a microscope. Within 1 d of proteoid roots becoming visible, PEPC mRNA expression was enhanced in proteoid roots compared with normal roots (Fig. 6; Table III). Shortly thereafter, the amounts of PEPC enzyme (Fig. 5) and specific activity of PEPC (Table II) were higher in proteoid roots compared with normal roots. Citrate and malate exudation was first recovered in root exudate from −P-treated plants on 12 DAE (Fig. 4). Citrate and malate could be detected in the rhizosphere within 3 d after tertiary roots had emerged within proteoid root segments (Fig. 2), and was concomitant with enhanced PEPC expression (Tables II and III; Figs. 5 and 6). The activity of citrate synthase (EC 4.1.3.7) and malate dehydrogenase (1.1.1.37), also involved in citrate and malate biosynthesis, are increased in proteoid roots compared with normal roots (Johnson et al., 1994). These data support the hypothesis that white lupin has concerted regulation of proteoid root development, transcriptional regulation of PEPC, and subsequent biosynthesis of organic acids for exudation in response to P deficiency.

White lupin provides a model for examining root adaptation to P deficiency. The suite of developmental and biochemical changes that result in proteoid root development and citrate exudation provide a convenient and novel system in which to identify the plant factors regulating these responses.

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