Nylon Filter Arrays Reveal Differential Gene Expression in Proteoid Roots of White Lupin in Response to Phosphorus Deficiency

Claudia Uhde-Stone, Kelly E. Zinn, Mario Ramirez-Yáñez, Aiguo Li, Carroll P. Vance, and Deborah L. Allan*

Departments of Soil, Water, and Climate (C.U.-S., K.E.Z., A.L., D.L.A.) and Agronomy and Plant Genetics (C.U.-S., K.E.Z., M.R.-Y., A.L., C.P.V.), University of Minnesota, 1991 Upper Buford Circle, St. Paul, Minnesota 55108; Centre de Investigación Sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México Apdo Postal 565–A, 62210 Cuernavaca Mor., Mexico (M.R.-Y.); and United States Department of Agriculture-Agricultural Research Service, Plant Science Research Unit, 1991 Upper Buford Circle, St. Paul, Minnesota 55108 (C.P.V.)

White lupin (*Lupinus albus*) adapts to phosphorus deficiency (−P) by the development of short, densely clustered lateral roots called proteoid (or cluster) roots. In an effort to better understand the molecular events mediating these adaptive responses, we have isolated and sequenced 2,102 expressed sequence tags (ESTs) from cDNA libraries prepared with RNA isolated at different stages of proteoid root development. Determination of overlapping regions revealed 322 contigs (redundant copy transcripts) and 1,126 singletons (single-copy transcripts) that compile to a total of 1,448 unique genes (unigenes). Nylon filter arrays with these 2,102 ESTs from proteoid roots were performed to evaluate global aspects of gene expression in response to −P stress. ESTs differentially expressed in P-deficient proteoid roots compared with +P and −P normal roots include genes involved in carbon metabolism, secondary metabolism, P scavenging and remobilization, plant hormone metabolism, and signal transduction.

Phosphorus (P) is an essential macronutrient for plant growth and development that plays key roles in many processes, including energy metabolism and synthesis of nucleic acids and membranes (Raghothama, 1999). It is second only to nitrogen as the most limiting nutrient for plant growth (Bieleski, 1973; Raghothama, 1999; Vance, 2001). In many soils, low availability of P is a limiting factor in crop production (Marschner, 1995). Due to the low availability of soluble P in many ecosystems, plants have developed adaptive mechanisms that aid in the acquisition of P from soil. Strategies that lead to better uptake or acquisition include expanded root surface area through increased root growth and root hair development (Lynch and Brown, 1998; Gilroy and Jones, 2000; Williamson et al., 2001), organic acid synthesis and exudation (Dinkelaker et al., 1989; Johnson et al., 1996a; Jones, 1998; Aono et al., 2001; Massonneau et al., 2001; Sas et al., 2001), exudation of acid phosphatases (Duff et al., 1991; del Pozo et al., 1999; Gilbert et al., 1999; Miller et al., 2001), enhanced expression of phosphate transporters (Leggewie et al., 1997; Liu et al., 1998a, 1998b; Chiou et al., 2001; Liu et al., 2001), and mycorrhizal associations (Marschner and Dell, 1994; Smith et al., 1994). Strategies aimed at conserving P involve internal remobilization of P and use of alternative metabolic pathways (Theodorou et al., 1992; Theodorou and Plaxton, 1993; Plaxton and Carswell, 1999).

White lupin (*Lupinus albus*), a species known for its extreme tolerance for low P availability, has proven an illuminating model system for understanding plant adaptations to low P, despite its lack of mycorrhizal symbiosis. Instead, its adaptation to P deficiency (−P) is a highly coordinated modification of root development and biochemistry resulting in proteoid (or cluster) roots—short, densely clustered tertiary roots—that resemble bottlebrushes (Gardner et al., 1982, 1983; Dinkelaker et al., 1995; Johnson et al., 1996b; Neumann et al., 1999; Massonneau et al., 2001). Unlike typical lateral roots which emerge at random along the axes of primary and secondary roots (Charlton, 1983), proteoid roots develop laterals that emerge from every xylem pole within the axis, accompanied by extensive root hair growth, resulting in a more than 100-fold increased surface area (Dinkelaker et al., 1995; Skene, 2001).

Proteoid roots excrete large amounts of the organic acids citrate and malate (Marschner et al., 1986; Marschner et al., 1987; Johnson et al., 1996a, 1996b; Neumann et al., 1999; Massonneau et al.,

[1] This work was supported in part by the U.S. Department of Agriculture National-Research Initiative (Competitive Grant nos. USDA–CSREES/98–35100–6098 and USDA–CSREES/2002–35100–12206).

* Corresponding author; e-mail dallan@soils.umn.edu; fax 612–625–2208.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.102.016881.

2001), which help increase the availability of mineral-bound phosphates (Gardner et al., 1983; Dinkelaker et al., 1989) 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; Gilbert et al., 1999; Miller et al., 2001) are excreted coincident with the exudation of organic acids from proteoid roots (Gilbert et al., 1999; Neumann et al., 1999; Miller et al., 2001). Concurrently, the expression of phosphate transporter genes is strikingly enhanced in P-starved proteoid roots (Neumann et al., 1999; Liu et al., 2001). Because of these adaptations, P uptake is greatly enhanced in proteoid root zones.

Proteoid root formation might be mediated by the coordinated expression of a number of genes. Expression of phosphate transporters (Liu et al., 2001), acid phosphatase (Gilbert et al., 1999; Miller et al., 2001), and genes related to organic acid synthesis (Massonneau et al., 2001; Penaloza et al., 2002; Uhde-Stone et al., 2003) have been reported to be induced in proteoid roots. Analysis of expressed sequence tags (ESTs) is an efficient approach for identifying large numbers of plant genes expressed during different developmental stages and in response to a variety of environmental conditions (Gyorgyey et al., 2000; Ohlrogge and Benning, 2000; White et al., 2000; Dunaeva and Adamska, 2001). In addition, once ESTs are generated, they provide a resource for transcript profiling experiments. In plants, differential profiling has successfully been used for identification and analysis of novel genes involved in diverse aspects of biotic and abiotic stress responses and in development (Girke et al., 2000; Sasaki et al., 2000; Kawasaki et al., 2001; Thimm et al., 2001).

Objectives of this research were to assess genes expressed in proteoid root formation and to analyze global gene expression in −P stress-induced proteoid roots. To achieve this goal, we: (a) identified ESTs from −P proteoid roots at two different developmental stages; (b) performed nylon filter arrays to compare gene expression in −P proteoid roots, +P and −P normal roots, and +P and −P leaves; (c) confirmed expression patterns for differentially expressed ESTs by RNA gel blots and reverse RNA gel blots; and (d) compared gene expression in +P and −P roots of *Medicago truncatula* by heterologous hybridization of white lupin ESTs.

RESULTS

Generation of ESTs from Early and Later Stages of Proteoid Root Development

Two cDNA libraries from proteoid roots of −P-stressed white lupin were constructed. A cDNA library of early stages of proteoid root development contained pooled RNA isolated from P-deficient roots and developing proteoid roots collected at 7 and 10 d after emergence (DAE). At 7 DAE, proteoid roots are not yet visible, so normal roots of −P-stressed plants were collected. At 10 DAE, immature proteoid roots were collected. A cDNA library of the later developmental stages of proteoid roots was generated with pooled RNA isolated from −P proteoid roots collected at 12 and 14 DAE. Average cDNA insert size was found to be approximately 1.6 kb. Single-pass 5′ sequencing resulted in 2,102 sequences of good quality with a length of at least 100 bp. Typical sequence lengths of good quality ranged from 400 to 500 bp. Of these 2,102 ESTs, 843 sequences derived from cDNA from the early developmental stages (7 and 10 DAE) and 1,259 ESTs from the library of more mature proteoid roots (12 and 14 DAE).

Functional Annotation

Using the BLASTX algorithm, DNA sequences were translated into their corresponding amino acid sequence and searched against the nonredundant protein database GenBank. A probability threshold for E values $\leq 10^{-6}$ was used to assign functions, whereas E values greater than 10^{-6} were considered not significant. Based on homology to already known or predicted genes and gene products, the 2,102 ESTs were grouped into four main categories: metabolism (33%), cell cycle and plant development (9%), interaction with the environment (26%), and unknown function (32%). The ESTs were further grouped into 16 subcategories (Fig. 1).

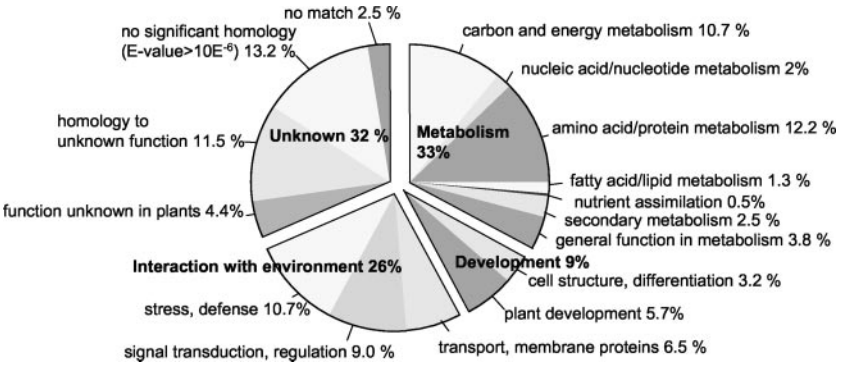

Figure 1. Based on homology (E values $\leq 10^{-6}$), the 2,102 ESTs from proteoid roots of P-deficient white lupin were grouped into four main categories and 16 different subgroups.

Contigs

Redundant ESTs were grouped into contigs using the program Phrap/Consed (University of Washington, Seattle) with a minimum overlap length of 50 bp and a minimum Smith Waterman score of 80. The latter refers to a cumulative score for the alignment that increases for any positive match, whereas it decreases for any mismatch or gap. A total of 322 contigs and 1,126 unique genes compiled to a nearly unigene set of 1,448 different transcripts. The term "nearly" in this context refers to the possibility that two ESTs, though corresponding to the same gene, do not overlap, and in this case would not be grouped into the same contig. We found 35 contigs containing five or more ESTs; the largest (PR-10 protein) consisted of 34 ESTs (Table I). Of the total 322 contigs, 199 contigs (about 60%) contained two ESTs and, together with the singletons, were considered relatively low-copy gene transcripts. Many ESTs that produced identical BLAST hits were grouped into the same contig, but we also found a number of ESTs with similar, but not identical, DNA sequences that may encode different isoforms.

To identify genes that might be primarily expressed in early or later stages of proteoid root development, we compared the representation of contigs in the two cDNA libraries derived from proteoid roots at early (7 and 10 DAE) and later (12 and 14 DAE) developmental stages (Table I). Contigs that were only represented in the 7- and 10-DAE proteoid root library include a contig containing five ESTs with homology to a ribosomal protein S13, a contig of six ESTs with homology to thaumatin, and a contig with seven ESTs with homology to aquaporin. Contigs that were found only in the 12- and 14-DAE proteoid root cDNA library include a contig of five ESTs with homology to cytokinin oxidase, a contig of five ESTs with homology to a transcription factor ERF-1, and a contig of 10 ESTs with homology to a multidrug and toxin extrusion protein (MATE), a

Table I. *Representation of contigs containing five or more ESTs*

Accession No. of GenBank Hit	E Value of Tentative Consensus Sequence	Annotation	Possible Function	Count of ESTs 7 and 10 DAE	Count of ESTs 12 and 14 DAE
NP_187461.1	1E-131	MATE	Transport	0	10
Q9LTS3	8E-24	Cytokinin oxidase	Cytokinin degradation	0	5
AAF63205.1	2E-23	Transcription factor ERF1	Regulation	0	5
Q9M4Y9	3E-20	AP2 domain-containing protein	Development	1	4
METE_MESCR	2E-60	Met synthase	Ethylene synthesis	1	4
Q9SJQ9	4E-47	Fru-bisphosphate aldolase, cytoplasmic	Glycolysis	3	9
Q9XG67	2E-62	Glyceraldehyde 3-P dehydrogenase	Glycolysis	5	10
GTX6_SOYBN	2E-59	Glutathione *S*-transferase	Oxidative stress response	2	5
O65332	1E-53	Polyubiquitin	Protein degradation	3	6
Q9AT55	1E-110	*S*-adenosylmethionine synthetase	Ethylene synthesis	9	11
Q43183	1E-72	Sulfate adenyltransferase	Sulfate assimilation	2	3
SAHH_ARATH	1E-107	Adenosyl-homocysteinase	Ethylene synthesis	2	3
O81361	2E-33	Ribosomal protein S-8	Translation	2	3
TBA_PRUDU	1E-150	Alpha tubulin	Cell structure	2	3
IDHP_MEDSA	5E-65	Isocitrate dehydrogenase	Carbon metabolism	2	3
THI4_CITSI	4E-55	Thiazole biosynthetic enzyme	Thiamin biosynthesis	3	3
P33560	4E-88	Tonoplast membrane integral protein	Transport	3	3
TBB_CICAR	3E-41	Beta tubulin	Cell structure	3	3
Q9LSF3	4E-47	Peptidyl-prolyl cis-trans isomerase	Protein folding	3	2
Q9AT52	8E-17	Extensin	Cell wall structure	3	2
BAB63949	3E-49	PR-10 protein	Defense	20	14
EF1A_LYCES	1E-50	Elongation factor 1-alpha	Translation	6	3
Q9M5M7	5E-79	60 S Ribosomal protein L-10	Translation	5	1
Q9LM03	1E-105	Met synthase	Ethylene synthesis	6	3
ALF_CICAR	1E-65	Fru-bisphosphate aldolase, cytoplasmic	Glycolysis	7	3
Q9SXL7	1E-63	Ribosomal protein L3	Translation	4	1
CAA69353.1	9E-20	Tonoplast intrinsic protein	Transport	4	1
P30172	1E-115	Actin	Cell structure	4	1
Q9FNV7	3E-24	Auxin-repressed protein	Development	5	1
O65357	9E-62	Aquaporin2	Transport	6	1
Q9MAV9	5E-48	Ribosomal protein S-13	Translation	5	0
O81926	6E-70	Thaumatin	Stress response	6	0
CAC01618.1	5E-70	Aquaporin	Transport	7	0
O49874	4E-98	Aquaporin	Transport	13	4
Q9LKJ6	9E-75	Plasma membrane intrinsic protein	Transport	14	1

putative transporter. Two contigs with homology to Fru-bis-P aldolase were differentially represented in both cDNA libraries. One isoform represented by 10 ESTs was found seven times in the cDNA library of early proteoid roots and three times in the cDNA collection from 12- and 14-DAE proteoid roots. A second contig of ESTs with homology to Fru bis-P aldolase was found primarily in the cDNA collection from 12- and 14-DAE proteoid roots (nine of 12 copies). The 5′ sequences of both cDNAs share 71% identity over a region of about 700 bp.

Nylon Filter Arrays

To assess global gene expression in proteoid roots, nylon filter arrays of the 2,102 ESTs from P-deficient proteoid roots were performed. The cDNA of each EST was amplified by PCR, using standard T_3 and T_7 primers, and spotted on nylon membranes. The 1,259 ESTs from later developmental stages of proteoid root development (12 and 14 DAE) were spotted on two sets of filters (Arrays I and II) in replicate (two spots per EST per filter), using a multiple channel pipetter. In addition, all 2,102 ESTs from earlier and later stages of proteoid root development were spotted mechanically in replicate (two spots per EST per filter) on two sets of nylon filters, using an automated Q-bot (Genetix, Boston); these arrays were designated Arrays III and IV. Gene expression was compared in 10-, 12-, and 14-DAE −P proteoid roots, and in +P normal roots, −P normal roots, +P leaves, and −P leaves at 14 DAE. Nylon filter arrays were performed in two to four replicates using RNA from independent plant tests.

To visually estimate the variability of signal intensities from nylon filter arrays, scatter plot analysis proved valuable. Examples for scatter plot analysis are shown in Figure 2. Guide lines at $y = 2x$ and $y = x/2$ indicate a 2-fold increase or decrease of signal intensities between the compared conditions. Parallel-spotted cDNA from the same hybridization (referred to as positions A and B) showed good correlation, except for those with low signal intensities (Fig. 2A). It should be noted, however, that the comparison of gene expression from different tissues was performed in parallel hybridizations and, thus, might show a lesser degree of correlation. Because of the high internal signal variation at low intensities, ESTs with average signal intensities below 10% of the mean array signal intensities were not analyzed further. Figure 2, B and C, display scatter plots from average intensities of the four independent nylon filter arrays (Arrays I–IV). In Figure 2B, arrays were hybridized with first strand cDNA from12-DAE −P proteoid root, and in Figure 2C arrays were hybridized with first strand cDNA from 14-DAE −P proteoid roots. Both test hybridizations are plotted against the same control hybridization, performed with first strand cDNA from +P normal root. Although Figure 2B shows some differential gene expression in 12-DAE −P proteoid roots, compared with +P normal roots, indicated as spots above and below the guide lines, Figure 2C shows much greater changes of gene expression in 14-DAE −P proteoid roots, compared with +P normal roots.

Slopes of approximately 1 in the scatter plots indicate that overall gene expression was quite similar in −P proteoid roots and +P normal roots. In general, expression in roots tended to be higher than in leaves in both −P and +P conditions. Overall expression was lower in −P normal roots compared with +P normal roots and lower in −P leaves compared with +P leaves (data not shown).

To select differentially expressed genes, average intensities from both replicate spots per EST were calculated for each hybridization. Rates for relative expression were calculated for each EST as ratios by dividing the average intensities of the test hybridizations by the average intensities of the control hybridizations. The calculated ratios were compared in all independently replicated arrays. ESTs from the 7- and 10-DAE proteoid root library that displayed at least 2-fold higher intensities in 10 DAE −P proteoid roots compared with +P normal roots (ratio ≥ 2) in

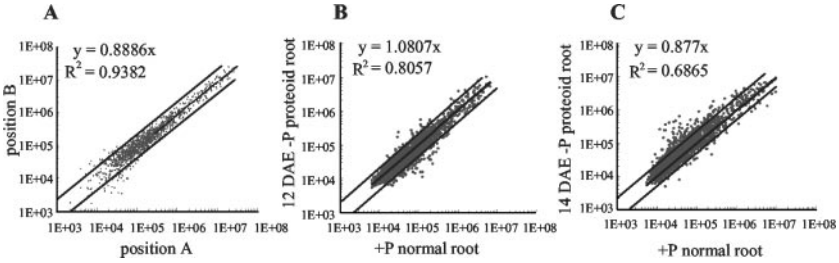

Figure 2. Scatter plot analysis of signal variability. For comparison of signal variation, guidelines were added at $y = 2x$ and $y = x/2$, indicating a 2-fold increase and a 2-fold decrease in signal intensity. A, Average signal intensities of two replicate spots (positions A and B) from the same hybridization (Array I, hybridized with first strand cDNA from −P proteoid roots at 14 DAE). At low intensities, signal variability exceeds a 2-fold increase or decrease. Thus, ESTs with average signal intensities below 10% of the mean array intensities were not included in our EST selections. B and C, Average intensities from four independent nylon filter arrays. In B and C, signal intensities from hybridizations with first strand cDNA derived from −P proteoid roots were plotted against signal intensities from hybridization with first strand cDNA derived from +P normal root. C, Higher signal variation in 14-DAE −P proteoid root, compared with 12-DAE −P proteoid roots (B).

both replicate arrays tested are summarized in Table II. ESTs from the 12- and 14-DAE proteoid root library that displayed at least 2-fold higher intensities in −P proteoid roots from 12 or 14 DAE, compared with +P normal roots (ratio ≥ 2), in all four replicate arrays tested are summarized in Table III. Based on these criteria, we found 35 genes that showed enhanced expression in −P proteoid roots at different developmental stages, compared with +P normal roots (Tables II and III). However, we found only one contig (EST nos. 241, 847, 998, 1,032, and 1,036, homology to cytokinin oxidase) that consistently showed reduced expression (ratio ≤ 0.5) in any stage of proteoid root development, compared with +P normal roots. As can be seen in Table III, ESTs with homology to cytokinin oxidase displayed low transcript abundance in 10- and 12-DAE but enhanced expression in 14-DAE −P proteoid roots, compared with +P normal roots.

Confirmation of Expression Pattern for Selected ESTs by RNA Gel Blots

RNA gel blots (Fig. 3) and reverse gel-blot analysis (data not shown), performed with selected ESTs, confirmed the results obtained by nylon filter arrays. As can be seen in Figure 3, the induction patterns obtained with RNA gel blots correlate well with those revealed in nylon filter arrays. ESTs with homology to MATE (EST no. 449), formamidase (EST no. 187), FDH (EST no. 728), and calmodulin (EST no. 343) displayed enhanced expression in −P proteoid roots at 14 DAE. An EST with homology to cytokinin oxidase displayed some induction in −P proteoid roots at 14 DAE but showed reduced expression in −P proteoid roots at 10 and 12 DAE, compared with normal roots. ESTs with homology to a glyceraldehyde-3P dehydrogenase (EST no. 1,141) and an RNase (EST no. 488) displayed significant induction throughout all developmental stages. An EST with homology to a novel phosphatase (no. 901) displayed enhanced expression in 14-DAE −P proteoid roots and −P normal roots compared with +P roots and in −P leaves compared with +P leaves.

Comparison of Gene Induction in P-Deficient Roots of *M. truncatula*

To determine whether our findings of differentially expressed genes in P-deficient white lupin occurred in another legume species, we searched the *M. truncatula* Gene Index (MtGI; http://www.tigr.org) to identify genes that were primarily (≥60%) represented in a cDNA library derived from roots of P-starved *M. truncatula* (MHRP−, Maria Harrison, Noble Foundation, Ardmore, OK), as compared with all other accessible *M. truncatula* cDNA libraries. The cDNA library MHRP− contains 4,388 ESTs, compiled to 3,936 tentative consensus (TC) sequences and 452 singletons. ESTs that were found in high frequency in the cDNA library MHRP− include two putative acid phosphatases, TC32797 (E value = 1E-150) and TC41065 (E value = 2E-68). TC32797 was found eight times in the −P root cDNA library, compared with only one corresponding EST in the library derived from +P roots, whereas TC41065 was found three of four times in the −P root library. TC32797 displayed about 60% homology to four ESTs encoding secreted acid phosphatase in white lupin (EST nos. 769, 823, 1,085, and 1,146), whereas TC41065 did not show significant homology to any acid phosphatases identified in white lupin. *Medicago* ESTs that were found exclusively in the MHRP− cDNA library also include two contigs with homology to cytochrome P450 (TC40856, five copies; and TC33723, four copies), both displaying about 40% sequence identity with EST number 374 from white lupin (cytochrome P 450, E value = 1E-25). A putative MATE protein (TC38392, E value = 3E-65) was found in two copies in the MHRP− cDNA library; however, the TC sequence did not display significant sequence identity to the ESTs with homology to a putative MATE protein identified in white lupin (EST nos. 131, 449, 471, 584, 685, 729, 1,020, 1,160, 1,281, and 1,297).

Nylon filter arrays were performed to assess: (a) whether a probe made from RNA isolated from −P-stressed *M. truncatula* would show hybridization patterns similar to those obtained with RNA from −P proteoid roots of white lupin, and (b) whether similar genes were induced upon −P stress of *M. truncatula* as those in white lupin. The 1,259 ESTs derived from white lupin proteoid roots at 12 and 14 DAE were hybridized in parallel with first strand cDNA derived from RNA of +P and −P roots of *M. truncatula*. This hybridization was repeated with cDNA from an independent plant test on nylon filter arrays of 100 selected ESTs.

The majority of genes were either not expressed in *M. truncatula* or might not share a sufficient degree of homology. Cross hybridization requires sequence identity of at least about 80% sequence identity (Wu et al., 2001). As can be seen in Table IV, a number of white lupin ESTs showed enhanced expression in −P roots of *M. truncatula* compared with +P roots. A BLASTn search against the *M. truncatula* Gene Index revealed that these lupin ESTs displayed at least 79% sequence identity with TC sequences of *M. truncatula*, whereas a lupin EST corresponding to an acid phosphatase and sharing about 60% sequence identity with an *M. truncatula* TC did not show cross hybridization.

Five ESTs identified as more highly expressed in roots of −P *M. truncatula* showed homology to enzymes of the glycolytic pathway: a Suc synthase (EST no. 183), a triose-P isomerase (EST no. 1,211), an NAD-dependent glyceraldehyde 3-P dehydrogenase (EST no. 1,441), an enolase (EST no. 508), and a phospho*enol*pyruvate carboxylase (EST no. 1035).

Table II. *White lupin ESTs derived from a cDNA library of proteoid roots at 7 and 10 DAE were hybridized against first strand cDNA of different tissues*

Listed are ESTs that displayed induced expression in −P proteoid roots, compared to +P normal roots.

EST Identification No.	GenBank Accession No. of EST	Accession No. of GenBank Hit	BLASTX E Value	Annotation	Possible Function	Ratio of Expression[a]				
						−P Proteoid Root/+P Normal Root			Normal Root	Leaf
						10 DAE	12 DAE	14 DAE	−P/+P	−P/+P
E22, E99, E372	CA410752, CA526338, CA526349	AAC16012.1	9E-76	Polyubiquitin	Here, serves as control	1 ± 0.2	0.9 ± 0.2	1.3 ± 0.3	1 ± 0.2	1 ± 0.2
E170, E292, E304	CA526339, CA411008, CA411018	Q9SJQ9	4E-47	Fru-1,6-bisP aldolase	Glycolysis	1.7 ± 0.7	2.2 ± 0.9	2.5 ± 1.4	0.9 ± 0.4	1.1 ± 0.3
E100, E111, E147, E440, E549, E625, E790	CA410825, CA410836, CA410872, CA411147, CA411156, CA411327, CA411489	Q9XG67	2E-62	NAD-dependent glyceraldehyde-3P dehydrogenase	Glycolysis	2.2 ± 0.8	2.2 ± 0.7	2.4 ± 0.8	1.3 ± 0.5	1.3 ± 0.4
E241	CA410959	O42908	1E-62	2,3-Bisphosphoglycerate mutase	Glycolysis	2.1 ± 0.2	3.7 ± 1.6	4.2 ± 1.2	1.3 ± 0.2	1.2 ± 0.7
E18	CA410748	CAB75428.1	1E-68	Enolase	Glycolysis	1.7 ± 0.1	2.4 ± 0.1	2.4 ± 0.3	1.4 ± 0.1	1.1 ± 0.5
E171, 447	CA410895, CA411154	AAL11502.1	5E-63	Malate dehydrogenase	Glycolysis	2.1 ± 0.2	2.4 ± 0.3	2.5 ± 0.4	1.5 ± 0.3	1.2 ± 0.8
E112, E423, E677	CA410837, CA411130, CA411379	AF520576	5E-27	Extensin-like protein	Cell wall structure protein	2.3 ± 0.6	1.6 ± 0.6	1.3 ± 0.7	1.4 ± 0.3	1.3 ± 0.4
E81, E757, E796	CA410808, CA411457, CA411495	BAB33421.1	2E-25	Putative senescence-associated protein	Unknown	2.1 ± 0.5	1.7 ± 0.8	2.2 ± 0.5	1.4 ± 0.4	0.8 ± 0.4
E183	CA410907	NP_198080.1	5E-17	Putative protein	Unknown	4.4 ± 0.2	3.8 ± 0.9	1.4 ± 0.8	0.4 ± 0.3	1.3 ± 0.3
E711	CA411412			No significant match	Unknown	1.2 ± 0.2	1.6 ± 1.1	5.1 ± 0.6	4.9 ± 0.1	0.8 ± 0.1

[a] Values represent averages from two independent nylon filter arrays.

Table III. *White lupin ESTs derived from a cDNA library of proteoid roots at 12 and 14 DAE were hybridized against first strand cDNA derived from different tissues*

Listed are ESTs that displayed induced expression in −P proteoid roots, compared to +P normal roots.

EST Identification No.	GenBank Accession No. of EST	Accession No. of GenBank Hit	BLASTX E Value	Annotation	Possible Function	Ratio of Expression[a]				
						−P Proteoid Root/+P Normal Root			Normal Root	Leaf
						10 DAE[a]	12 DAE[b]	14 DAE[b]	−P/+P[b]	−P/+P[b]
518, 587, 787, 820, 1,359	CA410255, CA410317, CA410522, CA410554, CA409802	AAC16012.1	9E-76	Polyubiquitin	Here, serves as control	1.1 ± 0.3	0.9 ± 0.3	1 ± 0.3	1 ± 0.5	1 ± 0.4
769, 823, 1,085, 1,146	CA410503, CA410557, CA409546, CA409608	AAK51700.1	1E-99	Secreted purple acid phosphatase	P_i acquisition	2.1 ± 0.8	6.7 ± 6.5	11.3 ± 15.8	3 ± 5.7	1.2 ± 1.2
901	CA410634	AAG40473.1	2E-68	Novel acid phosphatase	P_i recycling	1.2 ± 0.2	4.4 ± 5	12.9 ± 16	9.5 ± 13	2.1 ± 0.9
488	CA410221	JC4867	5E-35	Ribonuclease	P_i recycling	2.6 ± 0.1	2.9 ± 1	3.3 ± 0.8	1.3 ± 0.6	1.4 ± 0.7
649, 657	CA410377, CA410386	AAK38196.1	3E-80	Phosphate transporter	P_i uptake	0.8 ± 0.3	1.3 ± 0.6	2.3 ± 0.5	1.5 ± 0.3	1.3 ± 0.2
131, 449, 471, 584, 685, 729, 1,020, 1,160, 1,281, 1,297	CA409769, CA410182, CA410204, CA410314, CA410416, CA410461, CA409484, CA409623, CA409730, CA409746	NP_187461.1	1E-131	MATE	Transport, extrusion	1.6 ± 0.9	3.3 ± 2.1	8.6 ± 6.7	1.3 ± 0.9	1.3 ± 1.6
872, 1,421	CA410603, CA409864	AAF80647.1	7E-39	Nodulin 21-like protein	Integral membrane protein	1.5 ± 0.3	2.2 ± 0.6	3.3 ± 0.8	1.4 ± 0.9	1.1 ± 0.7
197, 904	CA409931, CA410636	CAC32462.1	3E-83	Sucrose synthase	Glycolysis	1.8 ± 0.6	1.5 ± 0.6	2 ± 0.4	1.2 ± 0.4	0.9 ± 0.3
245	CA409981	G6PD_MEDSA	8E-33	Glc-6P dehydrogenase	Glycolysis	2.4 ± 1.1	1.8 ± 0.7	2.6 ± 0.5	0.9 ± 0.5	0.9 ± 0.5
103, 1,097	CA840666, CA409559	T06011	1E-140	PP_i dependent phosphofructokinase	Glycolysis	1.5 ± 0.2	2.2 ± 0.9	4.3 ± 3	1.6 ± 1.5	1 ± 0.5
133, 350, 538, 647, 786, 834, 836, 896, 1,399	CA409784, CA410092, CA410100, CA410375, CA410521, CA410569, CA410571, CA410627, CA409843	Q9SJQ9	4E-47	Fru-1,6-bisP aldolase	Glycolysis	2.1 ± 1	4 ± 1.3	3.1 ± 2.4	1.1 ± 0.5	0.9 ± 0.4
1,098, 1,211	CA409560, CA409671	TPIS_COPJA	3E-54	Triose-P isomerase	Glycolysis	2.3 ± 0.4	2.3 ± 1	2.7 ± 0.6	1.5 ± 0.5	1 ± 0.7
14, 199, 263, 418, 470, 578, 799, 1,141, 1,265, 1,293	CA409888, CA409932, CA410001, CA410155, CA410203, CA410309, CA410531, CA409604, CA409716, CA409742	Q9XG67	2E-62	Glyceraldehyde-3P dehydrogenase (NAD)	Glycolysis	1.9 ± 0.5	2 ± 1.1	2.4 ± 1	1.2 ± 0.5	1 ± 0.5

(*Table continues on following page.*)

Table III. (*Continued from previous page.*)

EST Identification No.	GenBank Accession No. of EST	Accession No. of GenBank Hit	BLASTX E Value	Annotation	Possible Function	Ratio of Expression[a]				
						−P Proteoid Root/+P Normal Root			Normal Root	Leaf
						10 DAE[a]	12 DAE[b]	14 DAE[b]	−P/+P[b]	−P/+P[b]
33, 415, 490, 1,000	CA410080, CA410152, CA410224, CA409465	PGKY_WHEAT	1E-130	Phosphoglycerate kinase	Glycolysis	2.2 ± 0.5	1.9 ± 0.7	2.4 ± 0.8	1.3 ± 0.6	1.7 ± 1.4
508	CA410244	CAB75428.1	1E-68	Enolase	Glycolysis	2.6 ± 0.2	2.4 ± 1	3.3 ± 0.4	1.3 ± 0.3	0.9 ± 0.3
626, 1,394	CA410354, CA409839	AAL11502.1	5E-63	Malate dehydrogenase	Glycolysis	2.1 ± 0.4	1.9 ± 0.7	2.9 ± 1	1.4 ± 0.7	0.7 ± 0.2
1,035, 1,156	CA409498, CA409618	CAC28225.1	8E-60	Phospho*enol*pyruvate carboxylase	Glycolysis	1.9 ± 0.4	2.3 ± 0.8	3.1 ± 1.6	1.2 ± 0.7	0.9 ± 0.5
771, 1,407	CA410506, CA409851	P45458	8E-66	Malate synthase	Glyoxysomal pathway	2.8 ± 0.8	4.7 ± 2.7	3.9 ± 2.8	0.8 ± 0.4	1 ± 0.5
218, 728	CA409954, CA410460	NP_196982.1	8E-53	Formate dehydrogenase	Anaerobic metabolism	1.1 ± 0.2	2.4 ± 0.9	4.7 ± 3.4	0.8 ± 0.4	1.2 ± 0.5
41, 61, 109, 187	CA410157, CA410348, CA409562, CA409923	BAB63595	1E-105	Formamidase-like protein	Function unknown in plants	1.5 ± 0.3	4 ± 3.4	7.3 ± 10.6	1 ± 0.8	1.2 ± 0.4
241, 847, 998, 1,032, 1,036	CA409977, CA410582, CA410728, CA409495, CA409499	CAA77151.1	7E-23	Cytokinin oxidase	Cytokinin degradation	0.4 ± 0.2	1 ± 0.4	2.5 ± 1.5	1.3 ± 0.7	0.9 ± 0.7
865	CA410597	AAD38147.1	1E-42	1-Aminocyclo propane-1 carboxylate oxidase	Ethylene biosynthesis	1.2 ± 0.1	1.9 ± 0.6	2.9 ± 0.8	1.2 ± 0.3	1 ± 0.6
723	CA410456	NP_194614.1	7E-56	Xyloglucan endotransglycosylase	Root hair development	1.1 ± 0.2	1.5 ± 0.4	2.8 ± 1.2	1 ± 0.6	0.9 ± 0.6
374	CA410116	AF139532_1	1E-25	CytochromeP 450	Flavonoid biosynthesis	1.4 ± 0.4	2 ± 1.4	3.3 ± 1.9	1 ± 0.8	0.9 ± 0.6
789, 1,112, 1,129	CA410523, CA409576, CA409592	T09399	4E-59	Caffeoyl-CoA 3-O-methyl transferase	Lignin synthesis	2.4 ± 0.8	2.4 ± 1	2 ± 1	0.9 ± 0.4	1 ± 0.4
955	CA410689	F19G10.5	9E-13	Similar to CotA (laccase)	Lignin synthesis	2.4 ± 1	3 ± 0.9	4.4 ± 0.9	1.2 ± 0.4	0.7 ± 0.4
342, 343	CA410083, CA410084	T08585	3E-53	Calmodulin	Signal transduction	1 ± 0.4	3.1 ± 2.9	4.9 ± 5	0.6 ± 0.2	0.8 ± 0.2
210	CA409946	AAF86307.1	2E-25	Ca^{2+}-binding protein	Signal transduction	1 ± 0.2	2.2 ± 0.7	2.6 ± 0.5	0.8 ± 0.3	1.1 ± 0.1
702	CA410435	T49815	6E-11	Cyclin-dependent kinase PHO85-like	Response to P_i starvation	1.6 ± 0	1.7 ± 0.4	2.7 ± 0.7	2.4 ± 2.3	1.6 ± 0.5
65, 1,059, 1,090	CA410389, CA409520, CA409552	T45939	3E-35	Early nodulin ENOD18	ATP-binding motif	1.7 ± 1.2	1.7 ± 0.9	2.7 ± 1.7	1.2 ± 0.4	1.2 ± 0.5
674	CA410405	NP_197145.1	6E-32	Unknown protein		1.9 ± 0.3	3 ± 0.8	3.1 ± 1.1	0.8 ± 0.2	1.2 ± 0.6
315	CA410054			No match		2.5 ± 1.6	2.3 ± 0.7	2.8 ± 0.8	1.5 ± 1	1 ± 0.6

[a] Values represent averages from two independent nylon filter arrays. [b] Values represent averages from four independent nylon filter arrays.

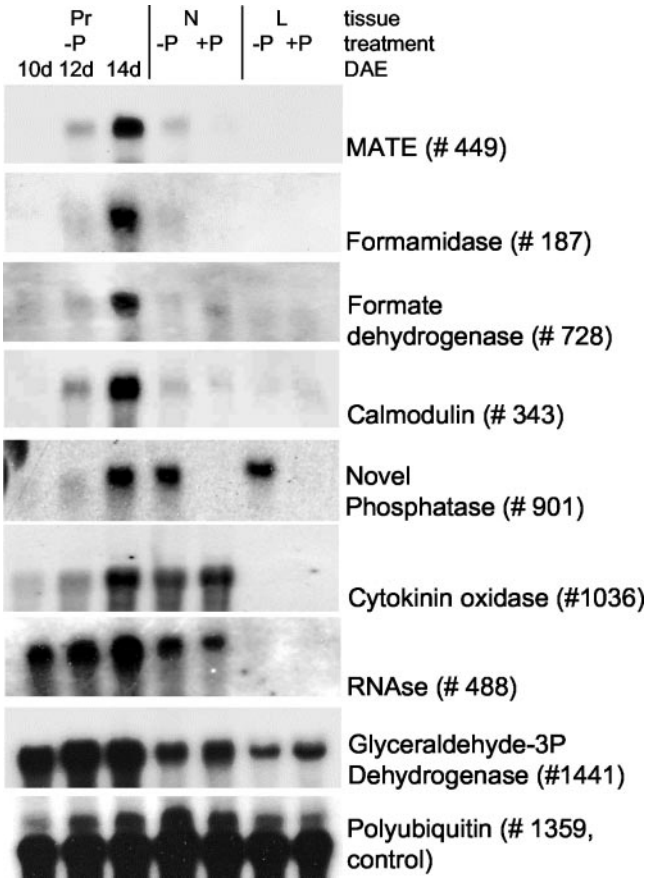

Figure 3. RNA gel analysis of selected ESTs confirmed enhanced expression patterns similar to those determined by nylon filter array analysis. ESTs with homology to MATE (EST no. 449), formamidase (EST no. 187), formate dehydrogenase (FDH; EST no. 728), and calmodulin (EST no. 343) displayed enhanced transcript abundance in 14-DAE −P proteoid roots. EST number 901 with homology to a novel acid phosphatase also showed enhanced expression in −P normal roots and −P leaves. An EST with homology to a cytokinin oxidase (EST no. 1,036) displayed some induction in −P proteoid roots at 14 DAE but showed a decrease of expression in proteoid roots at 10 and 12 DAE, compared with +P and −P normal roots. ESTs with homology to extracellular ribonuclease (RNAse; EST no. 488) and NAD-dependent glyceraldehyde 3-P dehydrogenase (EST no. 1,441) displayed induction at all stages of proteoid root development, compared with +P or −P normal roots. All RNA gel blots were performed in replicate. Polyubiquitin (EST no. 1,359) served as control for equal loading. Pr, Proteoid root; N, normal root; L, leaf.

DISCUSSION

In this report, we have advanced the fundamental understanding of plant adaptation to −P stress by: (a) isolating 2,102 ESTs from proteoid roots of P-deficient white lupin; (b) identifying 35 genes that are more highly expressed under −P in different stages of proteoid root development; (c) demonstrating differential patterns of gene expression under −P in proteoid roots, normal roots, and leaves; and (d) identifying genes potentially more highly expressed in roots of P-deficient *M. truncatula* by heterologous hybridization against white lupin nylon filter arrays. Our results revealed a suite of responses in proteoid roots of P-deficient white lupin that may be involved in: inorganic phosphate (P_i) acquisition, recycling, and transport; an adapted C metabolism; enhanced secondary metabolism; root development; and signal transduction. Interestingly, although 35 genes showed enhanced expression under P-deficient conditions, some 2,000 genes showed little change in expression in −P proteoid roots versus +P normal roots.

cDNA libraries and Nylon Filter Array Data

A comparison of expression patterns revealed by nylon filter arrays and contig representation in both proteoid root cDNA libraries displayed good correlation. Contigs comprised of five or more ESTs that showed enhanced expression in more mature proteoid roots (14 DAE) compared with juvenile proteoid roots (10 DAE) were more highly represented in the library of more mature proteoid roots (12 and 14 DAE), namely contigs with homology to cytokinin oxidase (EST nos. 241, 847, 998, 1,032, and 1,036), MATE (EST nos. 131, 449, 471, 584, 685, 729, 1,020, 1,160, 1,281, and 1,297), and a Fru-bis-P aldolase isoform (EST nos. E170, E292, E304, 133, 350, 538, 647, 786, 786, 834, 836, 896, and 1,399). However, the majority of contigs that showed higher representation in one of the cDNA libraries did not display enhanced expression in the corresponding tissue in nylon filter arrays. This might be due in part to the relatively strict selection criteria used for the analysis of nylon filter arrays. More sensitive methods like RNA gel blots and reverse transcription (RT)-PCR would be useful to further explore the expression pattern of these contigs. It should also be acknowledged that a comparison of cDNA representation and nylon filter arrays could be complicated by the potential of cross hybridization of closely related sequences (Wu et al., 2001; Fedorova et al., 2002; Miller et al., 2002).

Although we identified 35 genes with enhanced expression in proteoid roots compared with +P normal roots (Tables II and III), we could only identify one contig (EST nos. 241, 847, 998, 1,032, and 1,036, homology to cytokinin oxidase) that displayed less than 0.5-fold expression in all replicate arrays. One reason for the low representation of ESTs that displayed a significant decrease of expression in proteoid roots compared with +P normal roots, might be the fact that both cDNA libraries were derived from proteoid root tissue. Thus, genes with low expression in proteoid roots might be underrepresented in these libraries.

A complete list of the 2,102 ESTs including BLASTX annotations, nylon filter array results, and GenBank accession numbers are accessible on our supplemental Web site (http://home.earthlink.net/~whitelupinacclimation).

Table IV. *White lupin ESTs that displayed induced expression in M. truncatula −P normal roots, compared with +P normal roots*

EST Identification No.	GenBank Accession No. of EST	Accession No. of GenBank Hit	BLASTX E Value	Annotation	Possible Function	Ratio of Expression[a] (−P Normal Root: +P Normal Root)
183	CA409919	CAC32462.1	3E-83	Suc synthase	Glycolysis	4.9 ± 1.5
1,211	CA409671	TPIS_COPJA	3E-54	Triose-P isomerase	Glycolysis	4.7 ± 1.9
1,141	CA409604	G3PC_MAGLI	2E-72	NAD-dependent glyceraldehyde-3P dehydrogenase	Glycolysis	3.7 ± 0.5
508	CA410244	CAB75428.1	1E-68	Enolase	Glycolysis	3.4 ± 1.4
1,035	CA409498	CAC28225.1	8E-60	Phospho*enol*pyruvate carboxylase	Glycolysis	5.8 ± 2.1
1,033	CA409496	CAA11025.1	2E-72	Aquaporin	Transport	3.9 ± 1
20	CA409945	AF325121_1	6E-44	Brassinosteroid biosynthetic protein	Root elongation	2.2 ± 0.1
912	CA410659	NP_192570.1	1E-60	Nodulin-like protein	Integral membrane protein	6.2 ± 0.2

[a] Values represent averages of two independent nylon filter arrays.

P_i Acquisition and Recycling

Secretion of acid phosphatase from roots of P-deficient plants is a known adaptive mechanism to release organically bound P_i (Duff et al., 1991; Tadano and Sakai, 1991; Gilbert et al., 1999). Previous results from our laboratory revealed induced expression of two acid phosphatase isoforms, a secreted and a membrane-bound form (Gilbert et al., 1999; Miller et al., 2001) in −P proteoid roots. In our selection of 2,102 ESTs, four ESTs (nos. 769, 823, 1,085, and 1,146) correspond to the secreted acid phosphatase and one EST (no. 617) corresponds to the membrane-bound isoform. Consistent with previous results (Miller et al., 2001), the secreted isoform displayed high induction in 14-DAE −P proteoid roots. In addition, we identified an EST (no. 901) with homology to LEPS2, a P starvation-induced novel acid phosphatase from tomato (*Lycopersicon esculentum*; Baldwin et al., 2001). Baldwin et al. (2001) reported rapid induction of LEPS2 under −P in roots, stems, and leaves. Consistent with these findings, nylon filter arrays and RNA gel blots of the corresponding EST number 901 in white lupin revealed enhanced expression under −P in roots (normal and proteoid) and leaves. The enhanced expression in P-deficient leaves suggests involvement of EST number 901 in internal P_i remobilization rather than in P_i acquisition. Due to the low substrate specificity, acid phosphatases are presumed to play a role in the nonspecific hydrolysis of internal P_i to restore the P_i pool. A P_i starvation-inducible extracellular acid phosphatase in aerial tissues of higher plants has been speculated to be involved in scavenging P_i from xylem-derived phosphocholine, a major component of plant xylem sap (Plaxton and Carswell, 1999).

A combination of P_i released by acid phosphatase and enhanced uptake is considered an important means for plants grown under −P to acquire P_i from the rhizosphere (Plaxton and Carswell, 1999; Raghothama, 1999). Previous results from our laboratory (Liu et al., 2001) revealed induced expression of a putative P_i transporter, referred to as LaPT1, in P-deficient proteoid roots of white lupin. Two ESTs (nos. 649 and 657) from our collection proved identical to the LaPT1 gene and displayed enhanced induction in −P proteoid roots.

Induction of intracellular or RNase isozymes has been reported for P_i-starved Arabidopsis, tobacco (*Nicotiana tabacum*), and cultured tomato suspension cells (Jost et al., 1991; Loffler et al., 1993; Bariola et al., 1994; Dodds et al., 1996). We identified an EST (no. 741) with homology to an RNase that showed significant induction in −P proteoid roots and, to a lesser degree, in −P normal roots. Enhanced expression of an RNase isoform is consistent with the report of a drastic decrease of RNA concentration in mature proteoid roots of white lupin (Johnson et al., 1994; Neumann et al., 2000).

Organic Acid Synthesis

The excretion of malate and citrate from proteoid roots of white lupin in response to −P has been well documented (Dinkelaker et al., 1989; Johnson et al., 1996a; Neumann et al., 2000). Excretion of organic acids from roots has also been implicated as an adaptation to Al tolerance (Jones, 1998; Ryan et al., 2001). Organic acids allow for the displacement of P_i from Al^{3+}-, Fe^{3+}-, and Ca^{2+}-phosphates (Dinkelaker et al., 1989; Gerke et al., 1994), thus freeing bound P_i. In addition, plant uptake of P_i hydrolyzed by acid phosphatase is thought to be improved by the presence of citrate, a chelate that binds metals otherwise competing for released P_i (Braum and Helmke, 1995). We have shown previously that genes of most enzymes of the glycolytic pathway are represented in our EST collection from 12- and 14-DAE proteoid roots. These ESTs display significant induction in −P proteoid roots, indicating the involvement of this pathway in organic acid synthesis in proteoid roots

(Uhde-Stone et al., 2003). In addition, enhanced expression of two ESTs with homology to a glyoxysomal malate synthase (nos. 771 and 1,407) support the involvement of this enzyme in the synthesis of organic acids. The 2,102 ESTs presented here include ESTs with homologies to all enzymes of the glycolytic pathway except an ATP-dependent phosphofructokinase and a pyruvate kinase. Interestingly, these two enzymes are thought to be bypassed under −P (Duff et al., 1989; Plaxton and Carswell, 1999). The induction of glycolytic bypass enzymes that do not need ATP or ADP + P_i are thought to facilitate intracellular P_i scavenging (Theodorou et al., 1992; Theodorou and Plaxton, 1996; Plaxton and Carswell, 1999). ESTs with homology to an inorganic pyrophosphate-dependent phosphofructokinase, a phospho*enol*pyruvate carboxylase, and a malate dehydrogenase, enzymes that catalyze the supposed bypass reactions, were represented in our EST collection and displayed enhanced expression in −P proteoid roots compared with +P normal roots. We did not, however, find evidence for the expression of an NADP-dependent glyceraldehyde 3-P dehydrogenase, an enzyme that is thought to catalyze another glycolytic bypass reaction. Although we found no EST with homology to the NADP-dependent form of this enzyme, we did identify 15 highly expressed redundant ESTs corresponding to the regular (non-bypass) NAD-dependent glyceraldehyde 3-P dehydrogenase. These data indicate that the NAD-dependent glyceraldehyde 3-P dehydrogenase is not bypassed by an NADP-dependent glyceraldehyde 3-P dehydrogenase in proteoid roots of P-deficient white lupin. This finding is consistent with that of Penaloza et al. (2002), who reported enhanced expression of a gene with homology to an NAD-dependent glyceraldehyde 3-P dehydrogenase in P-deficient, compared with P-sufficient, proteoid roots. Although we identified only one isoform of glyceraldehyde 3-P dehydrogenase, we found two isoforms of a Fru bis-P aldolase, both forms sharing about 70% sequence identity in the sequenced 5′ coding region. One isoform was primarily represented in the cDNA collection from early proteoid roots (EST nos. E57, E242, E295, E379, E433, E640, E736, 152, 155, and 750), whereas the other isoform was more abundant in the cDNA collection from 12- to 14-DAE proteoid roots (EST nos. E170, E292, E304, 133, 350, 538, 647, 786, 834, 836, 896, and 1,399). Only the latter isoform showed enhanced expression of the corresponding ESTs in −P proteoid roots compared with +P normal roots, indicating that a different isoform of this enzyme might be involved in the increased glycolysis that seems to occur in proteoid roots.

Organic Acid Transport and Proton Excretion

Because malate and citrate are fully dissociated in the cytosol and cell membranes are impermeable to ions, excretion of organic acids from roots probably involves some type of channel protein (Neumann et al., 2000; Ryan et al., 2001). Studies using anion channel antagonists have indicated a possible involvement of anion channel proteins in citrate exudation of proteoid roots (Neumann et al., 2000). Genes encoding a corresponding channel or other protein that facilitates organic acid excretion have not yet been isolated.

An interesting candidate for a putative organic acid transporter is a gene represented by 10 ESTs in our collection of mature proteoid roots (EST nos. 131, 449, 471, 584, 685, 729, 1,020, 1,160, 1,281, and 1,297) that was not present in the EST collection obtained from juvenile stages. The consensus sequence of this contig displayed homology to a putative MATE protein from Arabidopsis. MATE proteins are a large family of putative transporters and are thought to be involved in excretion of a variety of drugs and toxins (Debeaujon et al., 2001; Diener et al., 2001). Nylon filter arrays and RNA gel-blot analysis revealed some induction in P-deficient normal and 12-DAE proteoid roots, but much higher induction in more mature −P proteoid roots at 14 DAE. This timing of induction is consistent with malate and citrate excretion from proteoid roots. Initial findings indicate that the putative MATE gene identified here is also responsive to high Al (+Al) stress (C. Uhde-Stone, C.P. Vance, and D.L. Allan, unpublished data), a condition known to result in organic acid excretion in many plants (Ma et al., 2001). So far, only a few MATE proteins have been analyzed in plants, among them a putative Fe sensor (Rogers et al., 2000; Rogers and Guerinot, 2002) and a protein required for flavonoid sequestration in vacuoles (Debeaujon et al., 2001).

Metabolic Adaptations of Respiration

A gene encoding a putative FDH (EST nos. 218 and 728) displayed high induction in proteoid roots collected at 14 DAE from P-deficient white lupin. FDH catalyzes the oxidation of formate to CO_2 in the presence of NAD^+. In bacteria and unicellular algae, formate is produced in large quantities under anaerobic conditions by the action of pyruvate formate lyase (Kreuzberg, 1984; Ferry, 1990). It is noteworthy that a pyruvate formate lyase-encoding gene showed significantly enhanced expression under P_i starvation in *Chlamydomonas reinhardii* (Dumont et al., 1993). Higher plant mitochondria from nonphotosynthetic plant tissue can also display high FDH expression (Colas des Francs-Small et al., 1993). Correspondingly, the putative FDH gene from proteoid roots of white lupin contains a likely ($P = 0.998$) mitochondrial-targeting sequence (Claros and Vincens, 1996). FDH has been previously reported to show induced expression under Fe deficiency in roots of tomato (Herbik et al., 1996) and barley (*Hordeum vulgare*; Suzuki et al., 1998), probably as a con-

sequence of oxygen deficiency in Fe-deficient tissue. Suzuki et al. (1998) speculated that an impairment of Fe-containing enzymes in oxidative respiration causes an increase in anaerobic metabolism under Fe limitation. Similarly, induction of FDH in −P proteoid roots of white lupin could result from the reduced mitochondrial respiration that has been reported in −P proteoid roots (Johnson et al., 1994; Neumann et al., 1999).

Secondary Metabolism

The accumulation of aromatic secondary metabolites appears to be a common response of plant cells undergoing P_i deprivation (for review, see Plaxton and Carswell, 1999). Plaxton and Carswell (1999) noted that the initial sequence of reactions in the aromatic pathway may serve to recycle P_i from phosphate esters. Enhanced expression of several key enzymes of the aromatic pathway, including 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase, Phe ammonia lyase, and chalcone synthase, has been previously reported in P_i-deficient plant cells (for review, see Plaxton and Carswell, 1999). In addition, isoflavonoids have been shown to be excreted from proteoid roots of white lupin (Neumann et al., 2000).

Table III shows that a number of genes involved in the phenylpropanoid pathway, the pathway that ultimately leads to products like flavonoids and lignin, were more highly expressed in −P proteoid roots of white lupin. These ESTs encode a cytochrome P 450 (EST no. 374), a caffeoyl-CoA 3-*O*-methyltransferase (EST nos. 789, 1,112, and 1,129), and a putative laccase (EST no. 955). Some cytochrome P 450s are postulated to be involved in flavone, flavonoid, and anthocyanin synthesis (Kitada et al., 2001). Caffeoyl-CoA 3-*O*-methyltransferase and laccase encode later steps in phenylpropanoid metabolism and are both implicated in lignin synthesis, a finding that is consistent with the striking proliferation of root growth that occurs during proteoid root development.

Plant Hormones and Proteoid Root Development

The plant hormone auxin has been implicated in the regulation of many aspects of plant growth including proteoid root development (Gilbert et al., 2000). The role of cytokinin, however, is less clear. Although auxins are known to promote lateral root primordia, cytokinins have been reported to have an antagonistic effect on proteoid root development (Neumann et al., 2000). ESTs with homology to cytokinin oxidase (EST nos. 241, 847, 998, 1,032, and 1,036), an enzyme that catalyzes the irreversible degradation of cytokinin (Rinaldi and Comandini, 1999), were induced in 14-DAE proteoid roots but showed reduced expression in 10- and 12-DAE −P proteoid roots compared with +P normal roots. Moreover, ESTs with homology to cytokinin oxidase were found redundantly in the EST collection from 12- and 14-DAE proteoid roots but not in that from 7- and 10-DAE −P proteoid roots. Modified expression of cytokinin oxidase at different stages of proteoid root development may be important in mediating proteoid root induction and/or maturity.

Proteoid root development is known to be accompanied by extensive root hair growth, resulting in a more than 100-fold increased surface area (Dinkelaker et al., 1995; Skene, 2001). The plant hormone ethylene has been proposed to regulate the induction of root hairs (Cao et al., 1999). The production of ethylene in plants is thought to be at least in part controlled by 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase in a gene-dependent manner (Chae et al., 2000). The enhanced expression of a predicted ACC oxidase gene (EST no. 865) in −P proteoid roots is consistent with earlier findings that show an enhanced ethylene formation by −P proteoid roots (Gilbert et al., 2000). It should be noted, however, that the application of ethylene inhibitors had no noticeable effect on proteoid root numbers (Gilbert et al., 2000).

Signal Transduction and Regulation

−P is one of many possible abiotic stresses plants might face in the environment. Plants have developed mechanisms to recognize and respond to a variety of stresses. Frequently, this response involves intracellular Ca^{2+}, a second messenger in signal transduction of eukaryotes (for review, see Knight, 2000). Stress- or plant hormone-induced changes in the cytosolic Ca^{2+} concentration can be transduced via calmodulin, Ca-dependent protein kinases, and other Ca^{2+}-controlled proteins. The enhanced expression of ESTs with homology to calmodulin (EST nos. 342 and 343) and Ca^{2+}-binding protein (EST no. 210) in −P proteoid roots suggests the involvement of Ca^{2+}-regulated processes in the response of white lupin to −P. The transduction of an abiotic stress signal, like −P, via calmodulin can affect a wide array of downstream growth and development responses.

Genes Induced in Roots of P-Deficient *M. truncatula*

To compare our findings of differential gene expression under −P in white lupin with the legume model organism *M. truncatula*, we searched the MtGI (http://www.tigr.org). The MtGI allows the in silico identification of ESTs that are more highly represented (≥60%) in a cDNA library of roots from P-deficient *M. truncatula* compared with other cDNA libraries accessible via MtGI. ESTs that were more highly represented in the P-deficient *M. truncatula* library included TCs with homology to acid phosphatases and cytochrome P450.

Proteoid roots do not form in *M. truncatula* in response to −P; rather, this species is mycorrhizal (Harrison, 1998). As a consequence, analysis of gene expression in P-deficient *M. truncatula* has been performed mainly on plants grown in mycorrhizal association. In the nylon filter arrays we performed, we sought to identify genes that show enhanced expression in roots of P-deficient *M. truncatula* that were grown without mycorrhizal inoculation. As might be expected, the majority of genes did not hybridize strongly with cDNA clones derived from white lupin. Heterologous hybridization of nylon filter arrays does not allow us to distinguish between low expression and low (less than 80%) sequence similarity of *M. truncatula* genes. It is, however, possible to compare the level of expression in +P and −P roots of *M. truncatula* for those genes that show sufficient hybridization. Similar to white lupin, *M. truncatula* displayed significantly induced expression of genes involved in the glycolytic pathway (Table IV). When grown under P-deficient conditions, *M. truncatula* responds by increasing its root-shoot ratio, a well-documented response to −P in many plants (Lynch and Brown, 1998). In this context, it is of interest that a gene with homology to a brassinosteroid biosynthetic protein (EST no. 20), a homolog to the cell elongation protein diminuto, was found to be induced in P-deficient roots of *M. truncatula*. This gene was represented by a white lupin EST (EST no. 20) but did not display significantly enhanced expression in −P proteoid roots. Brassinosteroid biosynthetic protein is thought to control the biosynthesis of campesterol, a compound implicated in growth modulation (Schaeffer et al., 2001).

CONCLUSION

Higher plants vary greatly in their ability to obtain and utilize scarcely available P_i. Taken together, the results presented here indicate that the effective adaptation of proteoid roots from white lupin to −P is a result of a complex coordinated regulation of gene expression that influences P_i acquisition and remobilization, carbon and secondary metabolism, and developmental processes. Enhanced expression of glycolysis-related genes in both proteoid roots of P-deficient white lupin and roots of P-deficient *M. truncatula* indicate some parallels in the response of both plant species to −P. Functional studies in alfalfa (*Medicago sativa*), *M. truncatula*, yeast (*Saccharomyces cerevisiae*), and *Xenopus* sp. oocytes are necessary to identify the proposed functions of proteins encoded by genes. Isolation of genomic clones and identification of promoter elements of P-responsive genes will certainly further our understanding of coordinated gene expression in response to −P.

MATERIALS AND METHODS

Plant Material

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

RNA Isolation

For isolation of total RNA, plant tissue was harvested in liquid nitrogen and stored at −80°C. About 5 g of root tissue or 1 g of leaf tissue, respectively, was ground under liquid nitrogen with mortar and pestle and added to 10 mL of cold extraction buffer (0.2 M Na acetate and 10 mM EDTA [pH 5]) and 10 mL of cold phenol. Samples were ground further in a Polytron at half speed for 30 s, than mixed by inversion for 10 min and centrifuged for 10 min at 10,000*g*. The supernatant was added to 5 mL of phenol and 5 mL of chloroform:isoamylalcohol (24:1 [v/v]), mixed by inversion for 10 min, and centrifuged for 10 min at 10,000*g*. This step was repeated using 10 mL of chloroform:isoamylalcohol (24:1 [v/v]) for extraction. One-third of the supernatant's volume of 8 M LiCl was added for a final concentration of 2 M LiCl and precipitated overnight at 4°C. The RNA was pelleted by centrifugation at 15,000*g* for 15 min. The pellet was washed first with 3 mL of 2 M LiCl and than washed twice with 70% (w/v) ethanol. The pellet was dried and resuspended in about 100 μL of diethyl pyrocarbonate-treated water, dissolved for 30 min at 65°C, and stored at −80°C.

Preparation and Screening of a cDNA Library

Proteoid roots of white lupin grown under P-deficient conditions were harvested 7 10, 12, and 14 DAE, and total RNA was isolated. Poly(A^+) RNA was obtained from total RNA using oligo(dT) cellulose. The poly(A^+) RNA obtained from early developmental stages (7 and 10 DAE) and those from later developmental stages (12 and 14 DAE) were combined into two pools, each in a 1:1 (w/v) ratio. From each pool, 7 μg of RNA was used for construction of two proteoid root cDNA libraries in the phage Uni-ZAP XR vector according to the manufacturer's instructions (Stratagene, La Jolla, CA). The linkage of two restriction site adapters, *Eco*RI and *Xho*I, allowed 5′- and 3′-directional cloning of the cDNA product into the Uni-ZAP XR vector. The cDNA from both libraries were size selected via Sephacryl S-500 spin columns as part of the procedure described by Stratagene.

Generation of ESTs

For conversion of the two cDNA phage libraries (ZAP XR vector) into the plasmid form (pBluescript), mass excision was performed according to the procedure described by Stratagene. Single colonies of *Escherichia coli* strain SOLR carrying the excised phagemid were replicated, and glycerol stocks were stored in microtiter plates at −80°C. DNA was isolated using the QIAprep 96 Turbo Miniprep Kit according to manufacturer's instructions (Qiagen USA, Valencia, CA). A portion of the obtained DNA was used for 5′ single-stranded sequencing at the Advanced Genetic Analysis Center (St. Paul, MN) using standard T_3 sequencing primer. The obtained 5′ single-strand sequences were edited and compared with the nonredundant database at the National Center for Biotechnology using the BLASTX program.

Nylon Filter Arrays

The cDNA portion of each clone was amplified by PCR, using standard T_3 and T_7 primers. Before spotting, the quality of each PCR product was evaluated by gel electrophoresis. For manual spotting, 0.5 μL (about 0.2 μg) of the amplified cDNA was spotted in parallel onto Gene Screen Plus membranes (NEN Life Science Products, Boston) using an eight-channel pipetter. For automated spotting with the Q-bot (Genetix), a 96-pin gravity gridding head with 0.4-mm pin diameter was used to spot the PCR products (about 0.4 μg DNA μL^{-1}) in replicate on Gene Screen Plus membranes that

had been soaked in 6× SSC. A 4 × 4 gridding pattern with an equal spot distance of 2,250 μm was used.

After spotting, nylon filters were positioned face up for 10 min onto Whatman paper (Whatman, Clifton, NJ) soaked with denaturing solution (1.5 M NaCL and 0.5 M NaOH), followed by 5 min of neutralization solution (1.5 M NaCl and 1 M Trizma Base), than dried and exposed at 120 joules cm^{-2} under a UV cross linker.

Total RNA was isolated from −P proteoid roots at different stages of development (10, 12, and 14 DAE), and from +P and −P normal roots and +P and −P leaves at 14 DAE. For hybridization, ^{32}P-labeled first strand cDNA probe was synthesized by RT of 30 μg of total RNA using SuperScriptII reverse transcriptase according to the manufacturer's instructions (Stratagene). The reaction mixture included 30 μg of total RNA, and 0.5 μg of oligo$(dT)_{12\text{-}18}$ primer (5 units μL^{-1}) that was annealed by heating to 70°C for 10 min in a total volume of 7 μL. Added to the mixture were 4 μL of 5× first strand buffer, 2 μL of 0.1 M dithiothreitol, 1 μL of dNTP mix (2.5 mM each of dCTP, dGTP, dTTP, and 0.0625 mM dATP), 5 μL of [α-^{32}P]dATP (10 mCi mL^{-1}), and 1 μL (200 units) of Superscript II reverse transcriptase. After 1 h of labeling at 42°C, 1 μL of 5 mM ATP was added, and the incubation was allowed to proceed for an additional 30 min. Unincorporated [^{32}P] dATP was removed by passing the mixture through Sephadex G50-G150 columns. ^{32}P incorporation was quantified via liquid scintillation. The final concentration of each probe was adjusted to 10^6cpm mL^{-1} hybridization solution.

Hybridizations were performed in 50% (w/v) formamide, 0.5 M Na_2HPO_4, 0.25 M NaCl, 7% (w/v) SDS, and 1 mM EDTA at 42°C. Blots were washed with three subsequent washes (1× SSC, 0.1% [w/v] SDS; 0.5× SSC, 0.1% [w/v] SDS; and 0.1× SSC, 0.1% [w/v] SDS) at 42°C.

Data Analysis of Nylon Filter Arrays

Radioactivity of each spot was quantified using a Phosphor Screen imaging system (Molecular Dynamics, Sunnyvale, CA). For manual spotted filters, the signal intensity for each spot of the parallel hybridizations was quantified using the software ImageQuant (Molecular Dynamics). For automated spotted arrays, the signal intensity of each spot was determined automatically using the software Array-Pro Analyzer (Media Cybernetics, Carlsbad, CA). Both programs allow the normalization of quantified signals against the background. The normalized intensities were reported as an Excel (Microsoft, Redmond, WA) file sheet and linked to the corresponding cDNA clones.

To identify cDNA clones that were differentially expressed under −P, the intensities from the various test hybridizations (−P proteoid root at different stages of development, −P normal root, and −P leaf) were divided by the intensities from the control hybridization (+P normal root and +P leaf). Ratios of greater than or equal to 2 in all replicates were considered an indication of a significant change of expression. ESTs with homology to polyubiquitin (nos. 518, 587, 787, 820, and 1,359) served as controls for uniform expression among the parallel hybridizations. Original signal intensities and transformed data for all experiments are available from our Web site (http://home.earthlink.net/~whitelupinacclimation).

RNA Gel Blots

Total RNA from different tissues (15 μg) was separated electrophoretically on 1.5% (w/v) denaturing agarose gels and transferred to Zeta-Probe Blotting Membranes (Bio-Rad Laboratories, Hercules, CA) following standard capillary blotting procedures (Sambrook et al., 1989). Blots were hybridized to ^{32}P-labeled cDNA of selected ESTs and probes were prepared by random primer labeling. The RNA was isolated from 10-, 12-, and 14-DAE −P proteoid roots and from +P and −P normal roots and +P and −P leaves at 14 DAE. Hybridizations were performed in 0.5 M Na_2HPO_4, 7% (w/v) SDS, and 10 mM EDTA at 65°C. Blots were washed with three subsequent washes (2× SSC, 0.5% [w/v] SDS; 1× SSC, 0.5% [w/v] SDS; and 0.5× SSC, 0.5% [w/v] SDS) at 65°C before autoradiography. Equivalent loading of each lane was assessed by probing blots with lupin polyubiquitin DNA. Loading variability was no greater than 20%.

Reverse RNA Gel Blots

About 0.4 μg (1 μL) of PCR-amplified cDNAs was loaded in parallel on a 1% (w/v) agarose gel in Tris-Acetate-EDTA, containing four parallel rows of loading wells. After electrophoresis, the gel was transferred to Immobilon membrane (Millipore, Bedford, MA) following standard capillary blotting procedures (Sambrook et al., 1989). ^{32}P-labeled first strand cDNA probe was synthesized by RT of 30 μg of total RNA using SuperScriptII reverse transcriptase according to the manufacturer's instructions (Stratagene). The RNA was isolated from mature (14 DAE) −P proteoid roots and +P normal roots. Equal activities of the probes (1 million cpm mL^{-1}) were used for hybridization. Hybridizations were performed in 50% (w/v) formamide, 0.125 M Na_2HPO_4, 0.25 M NaCl, 7% (w/v) SDS, and 1 mM EDTA at 42°C. Blots were washed with three subsequent washes (2× SSC, 0.1% [w/v] SDS; 0.5× SSC, 0.1% [w/v] SDS; and 0.1× SSC, 0.1% [w/v] SDS) at 42°C. Radioactivity of each band was quantified using the Phosphor Screen imaging system described above.

ACKNOWLEDGMENTS

The authors wish to thank Ernest Retzel and staff, especially Rod Staggs, Bob Milius, and Tina Schmidt, from the Center for Computational Genomics and Bioinformatics (University of Minnesota, Minneapolis) for support with the computational analysis. We also gratefully acknowledge Kate Vanden-Bosch and Ariana Lindemann (Department of Plant Biology, University of Minnesota, St. Paul) for providing the facility for automated spotting (Q-bot) and for technical assistance.

Received October 29, 2002; returned for revision November 11, 2002; accepted December 21, 2002.

LITERATURE CITED

Aono T, Kanada N, Ijima A, Oyaizu H (2001) The response of the phosphate uptake system and the organic acid exudation system to phosphate starvation in *Sesbania rostrata*. Plant Cell Physiol **42:** 1253–1264

Baldwin JC, Karthikeyan AS, Raghothama KG (2001) Leps2, a phosphorus starvation-induced novel acid phosphatase from tomato. Plant Physiol **125:** 728–737

Bariola PA, Howard CJ, Taylor CB, Verburg MT, Jaglan VD, Green PJ (1994) The Arabidopsis ribonuclease gene RNS1 is tightly controlled in response to phosphate limitation. Plant J **6:** 673–685

Bieleski RL (1973) Phosphate pools, phosphate transport, and phosphate availability. Ann Rev Plant Physiol **24:** 225–252

Braum S, Helmke PA (1995) White lupin utilizes soil phosphorus that is unavailable to soybean. Plant Soil **176:** 95–100

Cao XF, Linstead P, Berger F, Kieber J, Dolan L (1999) Differential ethylene sensitivity of epidermal cells is involved in the establishment of cell pattern in the Arabidopsis root. Physiol Plant **106:** 311–317

Chae HS, Cho YG, Park MY, Lee MC, Eun MY, Kang BG, Kim WT (2000) Hormonal cross-talk between auxin and ethylene differentially regulates the expression of two members of the 1-aminocyclopropane-1-carboxylate oxidase gene family in rice (*Oryza sativa* L.). Plant Cell Physiol **41:** 354–362

Charlton WA (1983) Patterns and distribution of lateral root primordia. Ann Bot **51:** 417–427

Chiou TJ, Liu H, Harrison MJ (2001) The spatial expression patterns of a phosphate transporter (MtPT1) from *Medicago truncatula* indicate a role in phosphate transport at the root/soil interface. Plant J **25:** 281–293

Claros MG, Vincens P (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem **241:** 779–786

Colas des Francs-Small C, Ambard-Bretteville F, Small ID, Remy R (1993) Identification of a major soluble protein in mitochondria from nonphotosynthetic tissues as NAD-dependent formate dehydrogenase. Plant Physiol **102:** 1171–1177

Debeaujon I, Peeters AJ, Leon-Kloosterziel KM, Koornneef M (2001) The TRANSPARENT TESTA12 gene of Arabidopsis encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. Plant Cell **13:** 853–871

del Pozo JC, Allona I, Rubio V, Leyva A, de la Pena A, Aragoncillo C, Paz-Ares J (1999) A type 5 acid phosphatase gene from *Arabidopsis thaliana* is induced by phosphate starvation and by some other types of phosphate mobilising/oxidative stress conditions. Plant J **19:** 579–589

Diener AC, Gaxiola RA, Fink GR (2001) Arabidopsis ALF5, a multidrug efflux transporter gene family member, confers resistance to toxins. Plant Cell **13:** 1625–1638

Dinkelaker B, Hengeler C, Marschner H (1995) Distribution and function of proteoid roots and other root clusters. Bot Acta **108:** 169–276

Dinkelaker B, Romheld V, Marschner H (1989) Citric acid excretion and precipitation of calcium citrate in the rhizosphere of white lupin (*Lupinus albus* L.). Plant Cell Environ **12:** 285–292

Dodds PN, Clarke AE, Newbigin E (1996) Molecular characterisation of an S-like RNase of *Nicotiana alata* that is induced by phosphate starvation. Plant Mol Biol **31:** 227–238

Duff SM, Plaxton WC, Lefebvre DD (1991) Phosphate-starvation response in plant cells: de novo synthesis and degradation of acid phosphatases. Proc Natl Acad Sci USA **88:** 9538–9542

Duff SMG, Moorhead GBG, Lefebvre DD, Plaxton WC (1989) Phosphate starvation inducible "bypasses" of adenylate and phosphate dependent glycolytic enzymes in *Brassica nigra* suspension cells. Plant Physiol **90:** 1275–1278

Dumont F, Joris B, Gumusboga A, Bruyninx M, Loppes R (1993) Isolation and characterization of cDNA sequences controlled by inorganic phosphate in *Chlamydomonas reinhardii*. Plant Sci. **89:** 55–67

Dunaeva M, Adamska I (2001) Identification of genes expressed in response to light stress in leaves of *Arabidopsis thaliana* using RNA differential display. Eur J Biochem **268:** 5521–5529

Fedorova M, Van De Mortel J, Matsumoto PA, Cho J, Town CD, VandenBosch KA, Gantt JS, Vance CP (2002) Genome-wide identification of nodule-specific transcripts in the model legume *Medicago truncatula*. Plant Physiol **130:** 519–537

Ferry JG (1990) Formate dehydrogenase. FEMS Microbiol Rev **7:** 377–382

Gardner WK, Barber DA, Parbery DG (1983) The acquisition of phosphorus by *Lupinus albus* L.: III. The probable mechanism by which phosphorus movement in the soil/root interface is enhanced. Plant Soil **70:** 107–124

Gardner WK, Parbery DG, Barber DA (1982) The acquisition of phosphorus by *Lupinus albus* L.: I. Some characteristics of the soil/root interface. Plant Soil **68:** 19–32

Gerke J, Roemer W, Jungk A (1994) The excretion of citric and malic acid by proteoid roots of *Lupinus albus* L.: effect on soil solution concentrations of phosphate, iron, and aluminum in the proteoid rhizosphere in samples of an oxisol and a lurizol. Z Pflanzenernaehr Bodenkd **157:** 289–294

Gilbert GA, Knight JD, Vance CP, Allan DL (1999) Acid phosphatase activity in phosphorus-deficient white lupin roots. Plant Cell Environ **22:** 801–810

Gilbert GA, Knight JD, Vance CP, Allan DL (2000) Proteoid root development of phosphorus deficient lupin is mimicked by auxin and phosphonate. Ann Bot **85:** 921–928

Gilroy S, Jones DL (2000) Through form to function: root hair development and nutrient uptake. Trends Plant Sci **5:** 56–60

Girke T, Todd J, Ruuska S, White J, Benning C, Ohlrogge J (2000) Microarray analysis of developing Arabidopsis seeds. Plant Physiol **124:** 1570–1581

Gyorgyey J, Vaubert D, Jimenez-Zurdo JI, Charon C, Troussard L, Kondorosi A, Kondorosi E (2000) Analysis of *Medicago truncatula* nodule expressed sequence tags. Mol Plant-Microbe Interact **13:** 62–71

Harrison MJ (1998) Development of the arbuscular mycorrhizal symbiosis. Curr Opin Plant Biol **1:** 360–365

Herbik A, Giritch A, Horstmann C, Becker R, Balzer HJ, Baumlein H, Stephan UW (1996) Iron and copper nutrition-dependent changes in protein expression in a tomato wild type and the nicotianamine-free mutant chloronerva. Plant Physiol **111:** 533–540

Johnson JF, Allan DL, Vance CP (1994) Phosphorus stress-induced proteoid roots show altered metabolism in *Lupinus albus*. Plant Physiol **104:** 657–665

Johnson JF, Allan DL, Vance CP, Weiblen G (1996a) Root carbon dioxide fixation by phosphorus-deficient *Lupinus albus*: contribution to organic acid exudation by proteoid roots. Plant Physiol **112:** 19–30

Johnson JF, Vance CP, Allan DL (1996b) Phosphorus deficiency in *Lupinus albus*. Altered lateral root development and enhanced expression of phospho*enol*pyruvate carboxylase. Plant Physiol **112:** 31–41

Jones DL (1998) Organic acids in the rhizosphere: a critical review. Plant Soil **205:** 25–44

Jost W, Bak H, Glund K, Terpstra P, Beintema JJ (1991) Amino acid sequence of an extracellular, phosphate-starvation-induced ribonuclease from cultured tomato (*Lycopersicon esculentum*) cells. Eur J Biochem **198:** 1–6

Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D, Bohnert HJ (2001) Gene expression profiles during the initial phase of salt stress in rice. Plant Cell **13:** 889–905

Kitada C, Gong Z, Tanaka Y, Yamazaki M, Saito K (2001) Differential expression of two cytochrome P450s involved in the biosynthesis of flavones and anthocyanins in chemo-varietal forms of *Perilla frutescens*. Plant Cell Physiol **42:** 1338–1344

Knight H (2000) Calcium signaling during abiotic stress in plants. Int Rev Cytol **195:** 269–324

Kreuzberg K (1984) Starch fermentation via formate producing pathway in *Chlamydomonas reinhardii*, *Chlorogonium elongatum*, and *Chlorella fusca*. Physiol Plant **61:** 87–94

Leggewie G, Willmitzer L, Riesmeier JW (1997) Two cDNAs from potato are able to complement a phosphate uptake-deficient yeast mutant: identification of phosphate transporters from higher plants. Plant Cell **9:** 381–392

Liu C, Muchhal US, Uthappa M, Kononowicz AK, Raghothama KG (1998a) Tomato phosphate transporter genes are differentially regulated in plant tissues by phosphorus. Plant Physiol **116:** 91–99

Liu H, Trieu AT, Blaylock LA, Harrison MJ (1998b) Cloning and characterization of two phosphate transporters from *Medicago truncatula* roots: regulation in response to phosphate and to colonization by arbuscular mycorrhizal (AM) fungi. Mol Plant-Microbe Interact **11:** 14–22

Liu J, Uhde-Stone C, Li A, Vance CP, Allan DL (2001) A phosphate transporter with enhanced expression in proteoid roots of white lupin (*Lupinus albus* L.). Plant Soil **237:** 257–266

Loffler A, Glund K, Irie M (1993) Amino acid sequence of an intracellular, phosphate-starvation-induced ribonuclease from cultured tomato (*Lycopersicon esculentum*) cells. Eur J Biochem **214:** 627–633

Lynch JP, Brown KM (1998) Regulation of root architecture by phosphorus availability. *In* Lynch JP, Deikman J, eds. Phosphorus in Plant Biology: Regulatory Roles in Molecular, Cellular, Organismic, and Ecosystem Processes. American Society of Plant Physiology, Rockville, MD, pp 148–156

Ma JF, Ryan PR, Delhaize E (2001) Aluminum tolerance in plants and the complexing role of organic acids. Trends Plant Sci **6:** 273–278

Marschner H (1995) Mineral Nutrition of Higher Plants, Ed 2. Academic Press Inc., San Diego

Marschner H, Dell B (1994) Nutrient uptake in mycorrhizal symbiosis. Plant Soil **159:** 89–102

Marschner H, Romheld V, Cakmak I (1987) Root-induced changes of nutrient availability in the rhizosphere. J Plant Nutr **10:** 1175–1184

Marschner HV, Romheld V, Horst WJ, Martin P (1986) Root-induced changes in the rhizosphere: importance for the mineral nutrition of plants. Z Pflanzenernaehr Bodenkd **149:** 441–456

Massonneau A, Langlade N, Leon S, Smutny J, Vogt E, Neumann G, Martinoia E (2001) Metabolic changes associated with cluster root development in white lupin (*Lupinus albus* L.): relationship between organic acid excretion, sucrose metabolism and energy status. Planta **213:** 534–542

Miller NA, Gong Q, Bryan R, Ruvolo M, Turner LA, LaBrie ST (2002) Cross-hybridization of closely related genes on high-density macroarrays. Biotechniques **32:** 620–625

Miller SS, Liu J, Allan DL, Menzhuber CJ, Fedorova M, Vance CP (2001) Molecular control of acid phosphatase secretion into the rhizosphere of proteoid roots from phosphorus-stressed white lupin. Plant Physiol **127:** 594–606

Neumann G, Massonneau A, Langlade N, Dinkelaker B, Hengeler C, Romheld V, Martioia E (2000) Physiological aspects of cluster root function and development in phosphorus-deficient white lupin (*Lupinus albus* L.). Ann Bot **85:** 909–919

Neumann G, Massonneau A, Martinoia E, Romheld V (1999) Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. Planta **208:** 373–382

Ohlrogge J, Benning C (2000) Unraveling plant metabolism by EST analysis. Curr Opin Plant Biol **3:** 224–228

Penaloza E, Gutierrez A, Martinez J, Munoz G, Bravo LA, Corcuera LJ (2002) Differential gene expression in proteoid root clusters of white lupin (*Lupinus albus*). Physiol Plant **116:** 28–36

Plaxton WC, Carswell MC (1999) Metabolic aspects of the phosphate starvation response in plants. *In* HR Lerner, ed, Plant Responses to Environmental Stress: From Phytohormones to Genome Reorganization. Marcel-Dekker, New York, pp 350–372

Raghothama KG (1999) Phosphate acquisition. Ann Rev Plant Physiol Plant Mol Bio **50:** 665–693

Rinaldi AC, Comandini O (1999) Cytokinin oxidase strikes again. Trends Plant Sci **4:** 300

Rogers EE, Eide DJ, Guerinot ML (2000) Altered selectivity in an Arabidopsis metal transporter. Proc Natl Acad Sci USA **97:** 12356–12360

Rogers EE, Guerinot ML (2002) FRD3, a member of the multidrug and toxin efflux family, controls iron deficiency responses in Arabidopsis. Plant Cell **14:** 1787–1799

Ryan P, Delhaize E, Jones D (2001) Function and mechanism of organic anion exudation from plant roots. Annu Rev Plant Physiol Plant Mol Biol **52:** 527–560

Sambrook J, Fritch E, Maniatis T (1989) Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sas L, Rengel Z, Tang C (2001) Excess cation uptake, and extrusion of protons and organic acid anions by *Lupinus albus* under phosphorus deficiency. Plant Sci **160:** 1191–1198

Sasaki Y, Asamizu E, Shibata D, Nakamura Y, Kaneko T, Awai K, Masuda T, Shimada H, Takamiya K, Tabata S et al. (2000) Genome-wide expression-monitoring of jasmonate-responsive genes of Arabidopsis using cDNA arrays. Biochem Soc Trans **28:** 863–864

Schaeffer A, Bronner R, Benveniste P, Schaller H (2001) The ratio of campesterol to sitosterol that modulates growth in Arabidopsis is controlled by STEROL METHYLTRANSFERASE 2;1. Plant J **25:** 605–615

Skene KR (2001) Cluster roots: model experimental tools for key biological problems. J Exp Bot **52:** 479–485

Smith SE, Gianinazzi-Pearson V, Koide R, Cairney JWG (1994) Nutrient transport in mycorrhizae: structure, physiology, and consequences for efficiency of symbiosis. Plant Soil **159:** 103–113

Suzuki K, Itai R, Nakanishi H, Nishizawa NK, Yoshimura E, Mori S (1998) Formate dehydrogenase, an enzyme of anaerobic metabolism, is induced by iron deficiency in barley roots. Plant Physiol **116:** 725–732

Tadano T, Sakai H (1991) Secretion of acid phosphatase by roots of several crop species under phosphorus-deficient conditions. Soil Sci Plant Nutr **37:** 129–140

Theodorou ME, Cornel FA, Duff SM, Plaxton WC (1992) Phosphate starvation-inducible synthesis of the alpha-subunit of the pyrophosphate-dependent phosphofructokinase in black mustard suspension cells. J Biol Chem **267:** 21901–21905

Theodorou ME, Plaxton WC (1993) Metabolic adaptations of plant respiration to nutritional phosphate deprivation. Plant Physiol **101:** 339–344

Theodorou ME, Plaxton WC (1996) Purification and characterization of pyrophosphate-dependent phosphofructokinase from phosphate-starved *Brassica nigra* suspension cells. Plant Physiol **112:** 343–351

Thimm O, Essigmann B, Kloska S, Altmann T, Buckhout TJ (2001) Response of Arabidopsis to iron deficiency stress as revealed by microarray analysis. Plant Physiol **127:** 1030–1043

Uhde-Stone C, Gilbert G, Johnson JF, Litjens R, Zinn KE, Temple SJ, Vance CP, Allan DL (2003) Acclimation of white lupin to phosphorus deficiency involves enhanced expression of genes related to organic acid metabolism. Plant Soil (in press)

Vance CP (2001) Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. Plant Physiol **127:** 390–397

White JA, Todd J, Newman T, Focks N, Girke T, de Ilarduya OM, Jaworski JG, Ohlrogge JB, Benning C (2000) A new set of Arabidopsis expressed sequence tags from developing seeds. The metabolic pathway from carbohydrates to seed oil. Plant Physiol **124:** 1582–1594

Williamson LC, Ribrioux SP, Fitter AH, Leyser HM (2001) Phosphate availability regulates root system architecture in Arabidopsis. Plant Physiol **126:** 875–882

Wu S-H, Ramonell K, Gollub J, Somerville S (2001) Plant gene expression profiling with DNA microarrays. Plant Physiol Biochem **39:** 917–926