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Alfalfa root nodule phosphoenolpyruvate carboxylase: characterization of the cDNA and expression in effective and plant-controlled ineffective nodules

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Abstract

Phosphoenolpyruvate carboxylase (PEPC) plays a key role in N_2 fixation and ammonia assimilation in legume root nodules. The enzyme can comprise up to 2% of the soluble protein in root nodules. We report here the isolation and characterization of a cDNA encoding the nodule-enhanced form of PEPC. Initially, a 2945 bp partial-length cDNA was selected by screening an effective alfalfa nodule cDNA library with antibodies prepared against root nodule PEPC. The nucleotide sequence encoding the N-terminal region of the protein was obtained by primer-extension cDNA synthesis and PCR amplification. The complete amino acid sequence of alfalfa PEPC was deduced from these cDNA sequences and shown to bear striking similarity to other plant PEPCs. Southern blots of alfalfa genomic DNA indicate that nodule PEPC is a member of a small gene family. During the development of effective root nodules, nodule PEPC activity increases to a level that is 10- to 15-fold greater than that in root and leaf tissue. This increase appears to be the result of increases in amount of enzyme protein and PEPC mRNA. Ineffective nodules have substantially less PEPC mRNA, enzyme protein and activity than do effective nodules. Maximum expression of root nodule PEPC appears to be related to two signals. The first signal is associated with nodule initiation while the second signal is associated with nodule effectiveness. Regulation of root nodule PEPC activity may also involve post-translational processes affecting enzyme activity and/or degradation.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number M83086.

Introduction

Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) catalyzes the irreversible carboxylation of phosphoenolpyruvate to oxaloacetate [22]. The enzyme is widely distributed in plants and microbes, playing a crucial role in carbon metabolism. In plants, most studies have focused on the role of PEPC in C_4 and CAM (crassulacean acid metabolism) species where the enzyme provides an effective mechanism of concentrating CO_2 within leaves [22, 30, 43]. Numerous studies have documented physical and biochemical properties of PEPC in C_4 and CAM species [1, 2, 17, 18, 21, 29, 43] and more recently cDNAs and genomic clones which encode the enzyme in these species have been isolated [4, 6, 12, 15, 16, 19, 33]. The enzyme is a tetramer comprised of subunits of 100 to 110 kDa and several isoforms of PEPC have been detected in both C_4 and CAM species [4, 6, 12, 16, 19]. Regulation of the enzyme is achieved through both transcriptional and post-translational events. Light induces an increase in the steady-state level of PEPC mRNA and enzyme protein in maize and sorghum [4, 12, 16] while salt stress increases the steady-state levels of mRNA and enzyme protein in ice plant (*Mesembryanthemum crystallinum*) [6, 30, 33]. Post-translational regulation of PEPC is achieved through phosphorylation of the protein [1, 2, 17, 21], oligomerization as affected by phosphoenolpyruvate, malate and glucose-6-phosphate [29, 43, 44] and protein turnover [18]. By comparison, much less is known about the C_3 and nonautotrophic (root) forms of PEPC.

Although Latzko and Kelly [22] hypothesized several functions for PEPC in C_3 plants, a clearly defined function for the enzyme has been demonstrated only in legume root nodules. Carbon dioxide fixation in root nodules is integrally associated with dinitrogen fixation [20, 25, 34, 40]. The enzyme functions in concert with glutamine synthetase (EC 6.3.1.2), glutamate synthase (EC 1.4.1.14), aspartate aminotransferase (EC 2.6.1.1) and asparagine synthetase (EC 6.3.5.4) to synthesize the amino acids aspartate and asparagine, the major nitrogen transport compounds in alfalfa and other amide-transporting species [20, 25, 34]. Moreover, root nodule malate and succinate, which provide the energy for bacteroid metabolism, are synthesized in part through the action of PEPC [20, 25, 34]. We have shown that up to 25% of the carbon required for nitrogenase activity and ammonia assimilation can be supplied by PEPC [25].

Increases in PEPC activity occur early in effective nodule development and activity remains high until nodule senescence occurs, during which time enzyme activity decreases 50 to 90% [7, 8, 24, 25]. Additionally, PEPC activity in plant-controlled ineffective nodules is strikingly reduced as compared to the activity in effective nodules [8, 25, 34]. Using highly specific polyclonal antibodies prepared to alfalfa PEPC, we have previously shown: (1) that nodule PEPC activity in effective and ineffective nodules was directly related to the amount of enzyme protein; (2) that the subunit molecular mass of nodule PEPC was 100 ± 5 kDa; (3) that nodule PEPC was antigenically related to the PEPC of C_4 leaves [28]. However, little is known of the mechanisms that regulate root nodule PEPC enzyme activity or synthesis.

Since legume root nodule PEPC plays such a significant role in biological nitrogen fixation, it is important to gain a thorough understanding of the mechanisms that regulate its activity. To begin these studies, we have deduced from isolated cDNA sequences the primary structure of the root nodule enhanced form of PEPC. We have also examined the amounts of PEPC mRNA during development of effective and ineffective alfalfa nodules and compared these mRNA levels to the amounts of enzyme activity and enzyme protein.

Materials and methods

Plant material

Alfalfa (*Medicago sativa* L.) cv. Saranac and the single-gene, recessive ineffective plant genotype Ineffective Saranac (in_1Sa) seeds were obtained from Dr D. K. Barnes (USDA-ARS, St. Paul, MN; [32]). Over 90% of the in_1Sa genotype is

from the Saranac background. The fact that alfalfa is an outcrossing tetraploid species precludes formation of isogenic lines. Nodule size and bacteroid development of the *in*1 genotype closely approximates the effective wild-type nodules. In the Saranac background, in_1 nodules are comparable to effective nodules in size and initial development [8], but bacteroids quickly deteriorate, leghemoglobin is reduced, and nodules senesce early. Plants were grown in glasshouse sandbenches inoculated with effective *Rhizobium meliloti* as previously described [8]. The date that seeds were planted in the sandbench was designated day 0. For developmental analyses, plant material was collected on days 5, 7, 8, 9, 12, 19 and 33. Plants were harvested at 0800 and roots (day 5), nodules on 2 mm root sections (days 7, 8) or nodules (day 9 and older) were hand-collected onto ice and used immediately for measurement of PEPC activity and for protein extraction and RNA isolation.

Protein extraction and enzyme assays

Triplicate samples of roots or nodules were ground in extraction buffer (100 mM 2-(*N*-morpholino)ethane sulfonic acid (MES), pH 6.8, 100 mM sucrose, 2% 2-mercaptoethanol, 15% ethylene glycol, 2 mM phenylmethylsulfonyl fluoride, 0.2 mM antipain) and centrifuged 15 min at $15\,500 \times g$ to obtain the soluble protein fraction [8]. PEPC activity was assayed *in vitro* as described by Miller *et al.* [28]. Protein content of extracts and nodule nitrogenase activities were determined as previously described [8].

SDS-polyacrylamide gel electrophoresis and western blotting

Soluble proteins in cell-free extracts were separated by electrophoresis in 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose as described previously [28]. Each lane was loaded with 30 μg of protein. Rabbit polyclonal antibodies to alfalfa nodule PEPC were used to detect and ascertain relative PEPC enzyme protein on western blots [28].

Isolation and characterization of PEPC cDNA

A cDNA synthesis kit (Pharmacia) was used to construct an oligo-dT primed alfalfa nodule cDNA library in the vector λgt11 [11]. The library was screened with monospecific antisera prepared against alfalfa PEPC [28] using horseradish peroxidase-conjugated goat anti-rabbit antibodies as a detection system. Approximately 2×10^5 recombinant clones were screened. From this screening, ten recombinant antigen-producing bacteriophage were purified and the sizes of the cDNA inserts were determined. The recombinant containing the largest cDNA was subcloned (pPEPC-61) into pBluescript KS^- (Stratagene) and its nucleotide sequence was determined from nested deletion [14] fragments by the dideoxy termination method using Sequenase (US Biochemical).

The RACE procedure [10], as modified below, was used to obtain cDNA clones that encode the N-terminal amino acids of PEPC. One μg of nodule poly(A)$^+$ RNA was reverse transcribed in *Taq* DNA polymerase buffer (50 mM KCl, 10 mM Tris pH 8.8 (at 42 °C), 0.1% Triton X-100, 1.5 mM $MgCl_2$) supplemented with $MgCl_2$ to a final concentration of 3.75 mM and containing 1.5 units/μl RNasin (Promega Biotech) 1 mM of each dNTP, 10 units of AMV reverse transcriptase (Promega Biotech) and 10 pmol of reverse transcriptase primer (5′-TGGTTCT-TCAATGCATCA-3′) that is complementary to nucleotides 590 to 573 (Fig. 1). Following incubation at 42 °C for 1 h and at 52 °C for 30 min, unextended primers were removed using a Centricon 100 spin filter (Amicon) and the cDNA was tailed with dA. The solution was then diluted to 0.5 ml in preparation for amplification. Amplification of the single-stranded tailed-cDNA was carried out in a 50 μl reaction mixture that contained *Taq* DNA polymerase buffer supplemented with $MgCl_2$ to a final concentration of 2.5 mM, 200 μM of each dNTP, 25 pmol PEPC-5AMP

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AAACACTGTCCTTCTGATCCATTTTTCCATTCCTTGTCATCATCTTCTTCTTCGTATCTAACTAACTGTCTGGCACACAACACGGTGAGAGAGTGAATTGCTACA 105
ATG GCA AAC AAG ATG GAA AAA ATG GCA TCA ATT GAT GCA CAG CTT AGA CAA TTG GTT CCT GCA AAA GTG AGT GAA GAT GAT 186
 M   A   N   K   M   E   K   M   A   S   I   D   A   Q   L   R   Q   L   V   P   A   K   V   S   E   D   D  27
AAA CTT ATT GAG TAT GAT GCT TTG TTG TTG GAT CGG TTT CTT GAT ATT CTT CAA GAT TTA CAT GGA GAG GAT CTG AAG GAT 267
 K   L   I   E   Y   D   A   L   L   L   D   R   F   L   D   I   L   Q   D   L   H   G   E   D   L   K   D  54
TCT GTT CAA GAA GTG TAT GAA CTG TCT GCT GAA TAT GAA AGA AAG CAT GAT CCT AAG AAA CTT GAA GAG CTT GGA AAT TTG 348
 S   V   Q   E   V   Y   E   L   S   A   E   Y   E   R   K   H   D   P   K   K   L   E   E   L   G   N   L  81
ATC ACA AGT TTC GAT GCA GGT GAC TCA ATT GTT GTT GCC AAG TCC TTT TCA CAC ATG CTT AAC TTG GCC AAC TTA GCT GAA 429
 I   T   S   F   D   A   G   D   S   I   V   V   A   K   S   F   S   H   M   L   N   L   A   N   L   A   E  108
GAG GTT CAA ATT GCG CAC CGC CGA AGG AAC AAG TTG AAG AAA GGT GAT TTT AGG GAT GAG AGC AAT GCA ACC ACT GAA TCT 510
 E   V   Q   I   A   H   R   R   R   N   K   L   K   K   G   D   F   R   D   E   S   N   A   T   T   E   S  135
GAC ATT GAG GAA ACT CTC AAG AAA CTT GTG TTT GAC ATG AAG AAA TCT CCT CAA GAG GTT TTT GAT GCA TTG AAG AAC CAG 591
 D   I   E   E   T   L   K   K   L   V   F   D   M   K   K   S   P   Q   E   V   F   D   A   L   K   N   Q  162
ACT GTT GAT CTT GTT CTT ACT GCT CAT CCT ACT CAG TCG GTT CGT CGA TCT TTG CTT CAA AAG CAC GGA AGG GTA AGG AAC 672
 T   V   D   L   V   L   T   A   H   P   T   Q   S   V   R   R   S   L   L   Q   K   H   G   R   V   R   N  189
TGT TTA TCT CAA TTG TAT GCT AAA GAC ATC ACT CCT GAT GAT AAG CAG GAG CTT GAT GAA GCT CTC CAG AGG GAG ATT CAA 753
 C   L   S   Q   L   Y   A   K   D   I   T   P   D   D   K   Q   E   L   D   E   A   L   Q   R   E   I   Q  216
GCT GCA TTC CGT ACT GAC GAA ATC AAG AGG ACT CCA CCA ACT CCC CAA GAT GAA ATG AGA GCT GGG ATG AGT TAC TTC CAT 834
 A   A   F   R   T   D   E   I   K   R   T   P   P   T   P   Q   D   E   M   R   A   G   M   S   Y   F   H  243
GAA ACA ATT TGG AAG GGT GTC CCT AAA TTT CTT CGC CGT GTT GAT ACG GCA TTG AAG AAC ATA GGG ATT AAC GAA CGT GTT 915
 E   T   I   W   K   G   V   P   K   F   L   R   R   V   D   T   A   L   K   N   I   G   I   N   E   R   V  270
CCC TAT AAT GCT CCT CTT ATT CAA TTT TCT TCT TGG ATG GGT GGT GAT CGT GAC GGT AAT CCA AGA GTG ACT CCT GAA GTG 996
 P   Y   N   A   P   L   I   Q   F   S   S   W   M   G   G   D   R   D   G   N   P   R   V   T   P   E   V  297
ACA AGG GAT GTT TGC TTA CTA GCT AGA ATG ATG GCT GCT AAC TTG TAT TAT TCA CAG ATA GAA GAT CTT ATG TTT GAA CTT 1077
 T   R   D   V   C   L   L   A   R   M   M   A   A   N   L   Y   Y   S   Q   I   E   D   L   M   F   E   L  324
TCT ATG TGG CGT TGC AAT GAC GAG CTA CGT GTT CGC GCA GAA GAA CTT CAC AGG AAT TCC AAG AAA GAT GAA GTT GCA AAA 1158
 S   M   W   R   C   N   D   E   L   R   V   R   A   E   E   L   H   R   N   S   K   K   D   E   V   A   K  351
CAC TAT ATA GAG TTT TGG AAA AAA ATT CCT TTG AAT GAA CCA TAC CGT GTT GTA CTC GGG GAG GTA AGG GAC AAG CTC TAT 1239
 H   Y   I   E   F   W   K   K   I   P   L   N   E   P   Y   R   V   V   L   G   E   V   R   D   K   L   Y  378
CGC ACT CGT GAG CGT TCT CGT TAT CTC CTA GCT CAT GGC TAC TGT GAA ATT CCT GAA GAA GCC ACA TTC ACC AAT GTC GAT 1320
 R   T   R   E   R   S   R   Y   L   L   A   H   G   Y   C   E   I   P   E   E   A   T   F   T   N   V   D  405
GAG TTT CTG GAA CCT CTT GAA CTC TGC TAC AGA TCA CTC TGT GCT TGT GGT GAT CGT GCA ATT GCT GAT GGA AGC CTT CTT 1401
 E   F   L   E   P   L   E   L   C   Y   R   S   L   C   A   C   G   D   R   A   I   A   D   G   S   L   L  432
GAT TTC TTG AGG CAA GTT TCC ACT TTT GGA CTG TCA CTT GTA AGG CTT GAT ATA CGG CAA GAG TCT GAT CGT CAC ACT GAC 1482
 D   F   L   R   Q   V   S   T   F   G   L   S   L   V   R   L   D   I   R   Q   E   S   D   R   H   T   D  459
GTG ATG GAT GCC ATT ACC AAA CAT TTG GAA ATT GGA TCC TAC CAA GAA TGG TCT GAA GAA AAA AGA CAG GAA TGG CTT TTG 1563
 V   M   D   A   I   T   K   H   L   E   I   G   S   Y   Q   E   W   S   E   E   K   R   Q   E   W   L   L  486
TCC GAG TTG ATT GGC AAA AGG CCA CTC TTT GGA CCT GAC CTA CCC CAA ACC GAT GAA ATT AGA GAT GTT TTA GAC ACG TTC 1644
 S   E   L   I   G   K   R   P   L   F   G   P   D   L   P   Q   T   D   E   I   R   D   V   L   D   T   F  513
CGT GTC ATA GCA GAA CTT CCA TCT GAC AAC TTT GGA GCC TAC ATC ATT TCG ATG GCA ACT GCA CCG TCT GAT GTG CTG GCA 1725
 R   V   I   A   E   L   P   S   D   N   F   G   A   Y   I   I   S   M   A   T   A   P   S   D   V   L   A  540
GTT GAG CTT CTT CAA CGT GAA TGC AAA GTC AGG AAT CCA TTA AGA GTC GTT CCG TTG TTT GAA AAG CTT GAT GAT CTT GAG 1806
 V   E   L   L   Q   R   E   C   K   V   R   N   P   L   R   V   V   P   L   F   E   K   L   D   D   L   E  567
TCT GCT CCT GCT GCA TTG GCT CGG TTG TTC TCC ATA GAC TGG TAC ATT AAC CGG ATC GAT GGG AAG CAA GAA GTT ATG ATT 1887
 S   A   P   A   A   L   A   R   L   F   S   I   D   W   Y   I   N   R   I   D   G   K   Q   E   V   M   I  594
GGA TAT TCT GAT TCA GGA AAA GAT GCT GGA AGG TTT TCT GCA GCA TGG CAG CTA TAT AAG GCT CAG GAG GAC CTC ATC AAA 1968
 G   Y   S   D   S   G   K   D   A   G   R   F   S   A   A   W   Q   L   Y   K   A   Q   E   D   L   I   K  621
GTC GCA CAG AAA TTT GGT GTT AAG CTA ACC ATG TTC CAC GGT CGT GGT GGA ACT GTT GGA AGA GGA GGT GGA CCT ACC CAT 2049
 V   A   Q   K   F   G   V   K   L   T   M   F   H   G   R   G   G   T   V   G   R   G   G   G   P   T   H  648
CTT GCT ATC TTG TCT CAA CCA CCA GAA ACA ATT CAC GGA TCT CTT CGT GTG ACA GTT CAA GGT GAA GTT ATT GAA CAG TCG 2130
 L   A   I   L   S   Q   P   P   E   T   I   H   G   S   L   R   V   T   V   Q   G   E   V   I   E   Q   S  675
TTC GGT GAG GAA CAC TTG TGC TTT AGG ACA CTG CAA CGT TTC ACT GCT GCT ACT CTA GAA CAT GGA ATG CGT CCC CCA AGC 2211
 F   G   E   E   H   L   C   F   R   T   L   Q   R   F   T   A   A   T   L   E   H   G   M   R   P   P   S  702
TCT CCA AAA CCA GAA TGG CGC GCC TTG ATG GAT CAG ATG GCT GTC ATT GCA ACT GAG GAA TAC CGT TCA ATT GTG TTC AAG 2292
 S   P   K   P   E   W   R   A   L   M   D   Q   M   A   V   I   A   T   E   E   Y   R   S   I   V   F   K  729
GAA CCA CGT TTT GTT GAG TAT TTC CGT CTG GCT ACA CCA GAG ATG GAG TAT GGT AGG ATG AAC ATT GGA AGT CGA CCG GCA 2373
 E   P   R   F   V   E   Y   F   R   L   A   T   P   E   M   E   Y   G   R   M   N   I   G   S   R   P   A  756
AAG AGA AGG CCT AGT GGA GGC ATT GAA ACA CTG CGT GCG ATA CCA TGG ATC TTT GCC TGG ACA CAG ACA AGG TTT CAT CTT 2454
 K   R   R   P   S   G   G   I   E   T   L   R   A   I   P   W   I   F   A   W   T   Q   T   R   F   H   L  783
CCA GTA TGG CTG GGC TTT GGA GCA GCA TTT AGA CAA GTT GTT CAG AAG GAT GTT AAG AAT CTC CAT ATG CTG CAA GAG ATG 2535
 P   V   W   L   G   F   G   A   A   F   R   Q   V   V   Q   K   D   V   K   N   L   H   M   L   Q   E   M  810
TAC AAT CAA TGG CCT TTC TTT AGG GTT ACA ATT GAT TTA GTT GAA ATG GTG TTT GCC AAG GGT GAC CCT GGT ATT GCA GCA 2616
 Y   N   Q   W   P   F   F   R   V   T   I   D   L   V   E   M   V   F   A   K   G   D   P   G   I   A   A  837
CTG AAT GAT AGG CTC CTA GTT TCA AAG GAT CTG TGG CCA TTT GGG GAA CAA TTG AGA AGC AAA TAT GAA GAA ACT AAG AAA 2697
 L   N   D   R   L   L   V   S   K   D   L   W   P   F   G   E   Q   L   R   S   K   Y   E   E   T   K   K  864
CTC CTA CTT CAG GTG GCT GCA CAC AAG GAA GTT CTT GAA GGT GAC CCC TAC TTG AAG CAA AGA CTC AGA CTC CGT GAT TCG 2778
 L   L   L   Q   V   A   A   H   K   E   V   L   E   G   D   P   Y   L   K   Q   R   L   R   L   R   D   S  891
TAC ATT ACA ACC CTT AAT GTT TTC CAA GCC TAC ACA TTG AAA CGG ATC CGC GAT CCA AAC TAC AAG GTG GAG GTG CGC CCC 2859
 Y   I   T   T   L   N   V   F   Q   A   Y   T   L   K   R   I   R   D   P   N   Y   K   V   E   V   R   P  918
CCA ATA TCG AAA GAG TCT GCT GAA ACA AGT AAA CCA GCT GAT GAA CTT GTA ACA TTG AAT CCA ACA AGT GAA TAT GCT CCT 2940
 P   I   S   K   E   S   A   E   T   S   K   P   A   D   E   L   V   T   L   N   P   T   S   E   Y   A   P  945
GGT TTG GAA GAC ACA CTC ATT CTT ACC ATG AAG GGT ATT GCT GCT GGC ATG CAG AAC ACT GGT TAA ATTTTGGTTACATTTTTCA 3025
 G   L   E   D   T   L   I   L   T   M   K   G   I   A   A   G   M   Q   N   T   G   *                      966
CTTGTATTTGTTTCTTTATGTTAAGTGTGTACTAAGATTTCATAAATACTAGATGAATCTAGTTGCAAACAAGCACTTCAAGTGAGTGCTTTTTTCTTTTTCTTTTT 3132
CTTTTCATAAGAATTTCACATCAGGTTTTGTTGGTGTGCTTCCTTACTTTGCTGCCACACAAATGAGTTATGCAATTGATGTTATGTTTCAAGGCATAAATTTTGTT 3239
GAGTGCTGCTACTATACGCTTTCTTGTTCAATTTAATATGAGACTGAAAAACATAGAAAATAGGAACAATTATAATAA                              3317
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primer (5′-TTCAGTGGTTGCATTGCT-3′) complementary to nucleotides 507 to 490 (Fig. 1), 10 pmol dT-adapter primer (5′-GACTCGAG GATCCAAGCTTTTTTTTTTTTTTTT-3′), 25 pmol adapter primer (5′-GACTCGAGG ATCCAAGCTTT-3′), 10 μl of the cDNA and 2.5 units of *Taq* DNA polymerase (Promega Biotech). The mixture was cycled 25 times in a Perkin-Elmer Cetus DNA Thermal Cycler as follows: 94 °C for 30 s; 47 °C for 30 s; and 72 °C for 30 s. Amplification products from three independent syntheses were subcloned into the vector pCR1000 (Invitrogen) and sequenced as described above.

RNA isolation and northern blots

Total RNA was isolated from freshly collected root and nodule samples of effective Saranac and ineffective in_1Sa according to procedures detailed by Thompson et al. [38]. Poly(A)$^+$ RNA was obtained by one cycle of oligo(dT)-cellulose chromatography.

Total RNA (10 μg/lane) and poly(A)$^+$ RNA (0.5 μg/lane) were electrophoretically separated on 1.5% agarose gels containing formaldehyde and transferred to Zetaprobe (Bio-Rad) in 10 × SSC (1 × SSC is 150 mM NaCl, 15 mM trisodium citrate) overnight. Northern blots were probed with denatured ^{32}P-labeled pPEPC-61 cDNA. Northern blots were hybridized overnight in 7% SDS, 50% formamide, 0.25 M NaCl, 0.25 M NaH_2PO_4, pH 7.4 at 43 °C as described by the manufacturer. After hybridization, blots were washed sequentially with: 2 × SSC, 0.1% SDS at room temperature for 15 min; 0.5 × SSC, 0.1% SDS at room temperature for 15 min; and 0.1% × SSC, 0.1% SDS at 65 °C for 15 min. Radioactivity on blots was quantitated with an AMBIS Radioanalytic Imaging System (San Diego, CA). The amount of radioactivity that hybridized to PEPC mRNA was determined with similar results from three separate blots. After quantitation of radioactivity, filters were exposed to X-ray film. The amount of poly(A)$^+$ RNA in each lane of the northern blots was estimated by hybridization to ^{32}P-labeled poly(U).

Plant DNA extraction and Southern blots

Genomic DNA was isolated from shoots of 12-day-old Saranac alfalfa seedlings as described by Shure *et al.* [35] and modified by S. Dellaporta and J. Chen (pers. commun.). Approximately equimolar amounts of alfalfa (10 μg/lane) and *R. meliloti* (0.01 μg/lane) DNA were digested with restriction endonucleases, electrophoresed through agarose and transferred to Magna nylon membrane (Micron Separations). A ^{32}P-labeled, 971 bp *Ava* I/*Xba* I fragment (extending from position 1214 to position 2185 shown in Fig. 1) from pPEPC-61 cDNA was used as a hybridization probe. Hybridization was carried out overnight in 50% formamide, 6 × SSPE (1 × SSPE is 150 mM NaCl, 10 mM NaH_2PO_4 pH 7.4, 1 mM EDTA), and 0.2% SDS at 42 °C as recommanded by the manufacturer. The filter was washed sequentially with 2 × SSC, 0.1% SDS at room temperature for 30 min, 0.5 × SSC, 0.1% SDS at room temperature for 30 min and 0.1 × SSC, 0.1% SDS at 65 °C for 30 min.

Results

Characterization of PEPC primary sequence

A λgt11 cDNA library was constructed from poly(A)$^+$ RNA extracted from 20-day-old root nodules and screened with antibodies prepared against purified PEPC protein. A recombinant bacteriophage containing a 2945 bp insert was

Fig. 1. Nucleotide sequence of PEPC cDNA and the deduced amino acid sequence of alfalfa nodule PEPC. The 5′ end of pPEPC-61 cDNA corresponded to position 373. Amino acid residues are in single letter code. The asterisk denotes the end of the protein coding region. Underlined residues 8–16, 595–604, and 635–646 correspond to those regions conserved for phosphorylation, active site, and substrate-binding sites, respectively.

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 Ms PEPC       MANKMEKMASIDAQLRQLVPAKVSEDDKLIEYDALLLDRFLDILQDLHGEDLKDSVQEVYELSAEYERKHDPK  73
Mc PEPC2             .........L............V.....................IRET...C..R.....G.....
Mc PEPC1      MSTVKLDRLT.......L.A.K.....................N.....I.ET...L..Q......T....
 Zm PECP  MASTKAPGPG..HH..........G.............V....N.......PS.REF...C..V..D..G.G.TT

 Ms PEPC  KLEELGNLITSFDAGDSIVVAKSFSHMLNLANLAEEVQIAHRRR-NKLKKGDFRDESNATTESDIEETLKKLVFDMKK  150
Mc PEPC2  ..D...SVL..L.........................Y...IK......LS..NS.........F.R..AELG.
Mc PEPC1  ......SMV..L.....................SR.K.VK.V.....M..NT.M....M....RR.IV.L..
 Zm PEPC  ..G...AKL.GLAPA.A.L..S.IL.................NS.....G.A..GS.........R..SEVG.

 Ms PEPC  SPQEVFDALKNQTVDLVLTAHPTQSVRRSLLQKHGRVRNCLSQLYAKDITPDDKQELDEALQREIQAAFRTDEIKRTP  228
Mc PEPC2  ..E.I...........F.................I....A.............................R...
Mc PEPC1  ....I.ET.........F.................I.D..A.............................R..Q
 Zm PEPC  ..E...E.........F.......A.......NA.I....T..N.....D.....................R.AQ

 Ms PEPC  PTPQDEMRAGMSYFHETIWKGVPKFLRRVDTALKNIGINERVPYNAPLIQFSSWMGGDRDGNPRVTPEVTRDVCLLAR  306
Mc PEPC2  ..........................................................................
Mc PEPC1  ..................N........L.........T.....................................
 Zm PEPC  ........Y....I...V.....................L...VS..R.........................

 Ms PEPC  MMAANLYYSQIEDLMFELSMWRCNDELRVRAEELHRNSKKDEVAKHYIEFWKKIPLNEPYRVVLGEVRDKLYRTRERS  384
Mc PEPC2  .......F.............T....A..D...SS.RR.--.........H..P......I..D......N.....
Mc PEPC1  .....M.F...DE.........T....E......KY....--S........Q..SS.....I.AD......Y.....
 Zm PEPC  .......ID...E.....................SS.GSK-.TKY......Q..P......I..H......N....A

 Ms PEPC  RYLLAHGYCEIPEEATFTNVDEFLEPLELCYRSLCACGDRAIADGSLLDFLRQVSTFGLSLVRLDIRQESDRHTDVMD  462
Mc PEPC2  .QM.S..VSD...D..Y.SLEQ.........G........P....T........F.....................
Mc PEPC1  .Q...SEVS...V.....EI.Q.....................PV........M...A....C..K.......E.....
 Zm PEPC  .H...S.VS..SA.SS..SIE..........K...D...K.........L....F.......K.......E.....I.

 Ms PEPC  AITKHLEIGSYQEWSEEKRQEWLLSELIGKRPLFGPDLPQTDEIRDVLDTFRVIAELPSDNFGAYIISMATAPSDVLA  540
Mc PEPC2  ...Q.......K..............S.......S.....E..A.....LH.L......C..........S......
Mc PEPC1  ...T..G....RD.T.....D......R..........R....A.....IN........S....V.............
 Zm PEPC  ...T..G....R..P.D..........R.....LP.........A..IGA.H.L....P.S..P..............

 Ms PEPC  VELLQRECKVRNPLRVVPLFEKLDDLESAPAALARLFSIDWYINRIDGKQEVMIGYSDSGKDAGRFSAAWQLYKAQED  618
Mc PEPC2  ........R.KQ.............A....V.........K...N..................L.....A......E
Mc PEPC1  ..........KK...........A...A...SMT....V...K.....................L.....M..V..E
 Zm PEPC  ........G..Q..P......R.A..Q....SVE....V...MD..K...Q..V...........L.......R...E

 Ms PEPC  LIKVAQKFGVKLTMFHGRGGTVGRGGGPTHLAILSQPPETIHGSLRVTVQGEVIEQSFGEEHLCFRTLQRFTAATLEH  696
Mc PEPC2  .VQ..KEY.................................VN.....................................
Mc PEPC1  .L..SK.............................A..A...G......I...........Q.........Y.......
 Zm PEPC  MAQ..KRY.....L.......................D..N..I..........FC.........Q.............

 Ms PEPC  GMRPPSSPKPEWRALMDQMAVIATEEYRSIVFKEPRFVEYFRLATPEMEYGRMNIGSRPAKRRPSGGIETLRAIPWIF  774
Mc PEPC2  ..H..I.........L.EI..V...K.....................L...........S..K......S........
Mc PEPC1  ..N..K.........L.....V.........................T...........S..K......S........
 Zm PEPC  ..H..V.......K...E...V.......V.V..A.......S....T.................G...T.........

 Ms PEPC  AWTQTRFHLPVWLGFGAAFRQVVQKDVKNLHMLQEMYNQWPFFRVTIDLVEMVFAKGDPGIAALNDRLLVSKDLWPFG  852
Mc PEPC2  ...................GYAI.......SV..K...E.........................Y.K....EE.....
Mc PEPC1  ..............V.G.LKH.LE..IR.HN..RD...NF.........L..........E....Y.K....EE.QS..
 Zm PEPC  S.............V....KFAID...R.FQV.K....E.......L..L............G.Y.E...AEE.K...

 Ms PEPC  EQLRSKYEETKKLLLQVAAHKEVLEGDPYLKQRLRLRDSYITTLNVFQAYTLKRIRDPNYKVEVRPPISKESAE-TS-  928
Mc PEPC2  .R..ADF....G.I.KI.G..DL.......R..............AL.............N.QL.PH....YMDS..N
Mc PEPC1  .R..AN..D..RR..E..G..DL..............P.......C...........DF..TE..HL...IMDA--H
 Zm PEPC  K...D..V..QQ....I.G..DI.....F...G.V..NP.................F..TPQ..L...F.DE--N

 Ms PEPC  KPADELVTLNPTSEYAPGLEDTLILTMKGIAAGMQNTG                                      966
Mc PEPC2  ...A...K...S.........................
Mc PEPC1  .A.A...K.....................V...L....
 Zm PEPC  ...G-..K...A...P.....................
```

Fig. 2. Comparison of the deduced alfalfa nodule PEPC amino acid sequence (Ms PEPC) to those of ice plant nonautotrophic (Mc PEPC2 [5]), ice plant stress-inducible (Mc PEPC1 [33]) and maize leaf (Zm PEPC [16]) PEPCs. Identical residues are identified by dots (·). Dashed lines (–) indicate gaps introduced into the sequences to maximize identity. Residue numbering is from the N-terminal amino acid of alfalfa PEPC.

subcloned and sequenced. The cDNA in this recombinant plasmid (pPEPC-61) lacks a poly(A) tract, as do most of the clones in the library, and contains a single long open reading frame of 877 codons. The 5′ end of this partial-length cDNA corresponds to position 373 in Fig. 1.

To obtain the entire protein coding sequence of the PEPC gene, we amplified the 5′ region of PEPC mRNA by the RACE procedure [10]. Three cDNA clones, each derived from independent RACE reactions, were isolated and sequenced. The sequence of the cDNA contained in pPEPC-61 exactly matched the sequences of the 3′ portion of the PCR-derived clones from its 5′ end up to the 3′ end of the pPEPC-5AMP primer (position 490). This demonstrated that the RACE procedure amplified the 5′ end of PEPC mRNA. The three RACE-derived cDNA clones are identical in length (489 bp) and extended the pPEPC-61 cDNA sequence by 372 nucleotides. In the combined sequence, the first ATG triplet is found at position 106 and is followed by a 965 codon open reading frame which encodes a 110 896 Da protein. The nucleotides flanking this methionine codon, ACAATGGC, match the plant consensus translation initiation sequence, ACAATGGC [13]. Additionally, an in-frame stop codon is present 12 nucleotides upstream of the proposed site of translational initiation. These data show that we have deduced the complete amino acid sequence of the protein.

When the deduced amino acid sequence was used to search a protein sequence database, significant similarities were found with ice plant, maize and bacterial PEPCs. Alignment of the alfalfa PEPC amino acid sequence with the nonautotrophic and stress-induced PEPCs of ice plant (a CAM plant) as well as the autotrophic PEPC from maize (a C_4 plant) leaf showed a high degree of similarity throughout the entire protein (Fig. 2). The alfalfa sequence is 85 and 82% identical to the uninduced and salt-induced form of ice plant PEPC, respectively, and is 78% identical to maize leaf C_4 PEPC from amino acid position 8 to the C-terminus. Comparison of the alfalfa nodule PEPC-deduced amino acid sequence to other PEPCs showed that alfalfa PEPC contains several conserved sequences [4, 6] which are proposed to be involved in: (1) phosphorylation (MASIDAQLR, residues 8 to 16); (2) the active site of the enzyme (GYSDSGKDAG, residues 595 to 604; and (3) glycine-rich substrate-binding site (GRGGTVGRGGGP, residues 635 to 646).

Northern blot analysis of PEPC mRNA in roots, nodules and leaves

PEPC mRNA was initially characterized by northern blot analysis of poly(A)$^+$ RNA extracted from uninfected roots, effective nodules and leaves (Fig. 3). The size of the mRNA is approximately 3.6 to 3.7 kb, independent of the tissue from which it was extracted. While root and leaf poly(A)$^+$ RNA had only a very faint band corresponding to PEPC, direct determination of hybridized radioactivity by AMBIS analysis showed that nodules contain about 15-fold more PEPC mRNA than do uninfected roots and leaves.

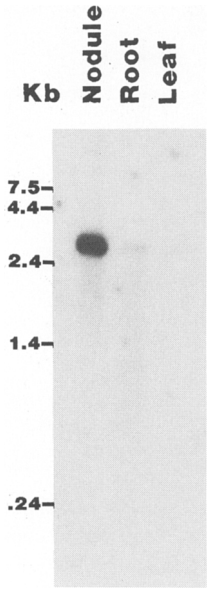

Fig. 3. Northern blot analysis of PEPC mRNA in poly(A)$^+$ RNA isolated from effective nodules, uninfected roots, and leaves of effective Saranac alfalfa. A total of 0.5 μg poly(A)$^+$ RNA was applied in each lane. Size markers are indicated in kb. The data shown are representative of four separate blots.

Southern blot analysis of PEPC

To estimate the number of alfalfa PEPC genes, ^{32}P-labeled pPEPC-61 cDNA was hybridized to Saranac alfalfa DNA digested with a variety of restriction enzymes (Fig. 4). All restriction digests contained multiple fragments that hybridized to the probe, indicating that PEPC is encoded by a small multigene family or that the PEPC gene contains several introns. Alternatively, the hybridization pattern could reflect allelic variation at a single genetic locus in this tetraploid species. The presence of additional, significantly divergent PEPC genes cannot be ruled out since hybridization was performed at a moderately high stringency.

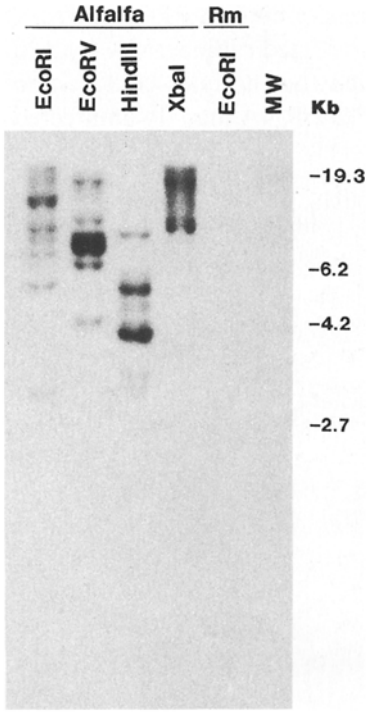

Fig. 4. Southern blot analysis of alfalfa genomic DNA. Alfalfa genomic DNA was digested to completion with *Eco* RI, *Eco* RV, *Hin*d III, or *Xba* I, size-fractionated on an 0.8% agarose gel, transferred to a nylon membrane and hybridized with a ^{32}P-labeled, 971 bp *Ava* I/*Xba* I fragment (extending from position 1214 to position 2185 shown in Fig. 1) of pPEPC-61 cDNA. DNA size markers are indicated in kb. The lane designated Rm is *Rhizobium meliloti* total DNA digested to completion with *Eco* RI.

Nitrogenase activity, PEPC enzyme activity and PEPC protein

Although we have previously published a study of PEPC activity and PEPC enzyme protein in developing alfalfa nodules [8], we thought it important to repeat that experiment sampling nodules more frequently at early time points for this study. Nodule initiation and visible emergence of nodules from roots occur between days 7 and 10, a period during which nitrogenase activity increases from zero to maximum specific activity. Since in our previously published study we sampled on days 7 and 10, we did not know the sequence of events during the important intervening days. We also wanted to extend our understanding of the relationships between nitrogenase and PEPC activity by comparing the synthesis of PEPC enzyme protein with that of PEPC mRNA during this period.

Nitrogenase activity (Fig. 5) in Saranac nodules was not detectable until day 9 even though small white nodule outgrowths begin to emerge from roots by 7 days after planting and inoculation [11]. Nitrogenase specific activity in effective Saranac nodules continued to increase through day 12, remained constant to day 19 and then decreased slightly by day 33. By comparison, ni-

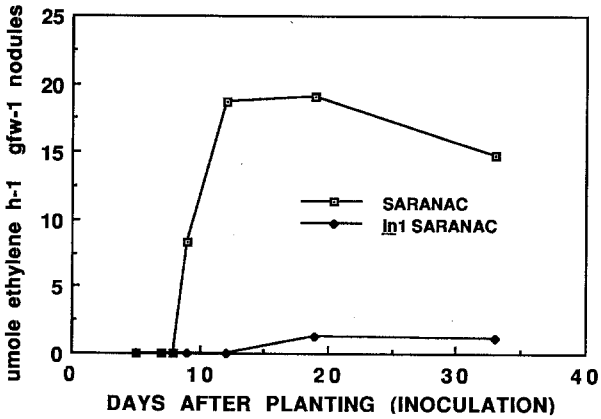

Fig. 5. Nitrogenase activity (acetylene reduction assay) of effective Saranac and ineffective *in*$_1$Sa plants throughout nodule development. On day 5, no nodules were present on roots. Small nodules were emerging from roots on days 7 and 8. By day 9, nodules had merged from roots. Each data point represents the average of three separate determinations.

trogenase specific activity of in_1Sa nodules was not detectable through day 12 and was only 5% that of effective nodules on days 19 and 33.

The pattern of PEPC activity late in nodule development was strikingly different between effective Saranac and ineffective in_1Sa (Fig. 6). In both genotypes, there were small but significant increases in PEPC activity by days 7 and 8. PEPC specific activity in Saranac nodules increased from 0.17 to 0.90 μmol/min per g fresh weight between days 5 and 8 while that in in_1Sa nodules increased from 0.16 to 0.67 μmol/min per g fresh weight over the same period. These early increases in nodule PEPC activity occurred prior to detectable nitrogenase activity and were coincident with nodule formation and emergence. From days 9 to 12 differences in nodule PEPC between Saranac and in_1Sa became obvious. By day 12, PEPC activity in effective Saranac was approximately 3-fold higher than that in ineffective in_1Sa nodules. PEPC specific activity in Saranac continued to increase from day 12 to day 33, while that in in_1Sa declined gradually throughout the same period. On day 33, Saranac nodule PEPC specific activity was 16-fold greater than that in in_1Sa nodules. Bacteroids contain no detectable PEPC activity [28].

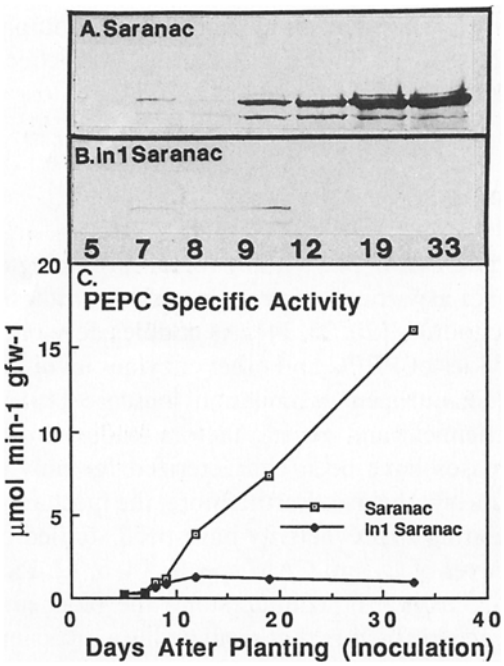

Fig. 6. PEPC enzyme activity and protein during development of effective Saranac and ineffective in_1Sa nodules. Panel A: PEPC enzyme protein in 30 μg of soluble protein from effective Saranac. Panel B: PEPC enzyme protein in 30 μg of soluble protein from ineffective in_1Sa. Panel C: Total *in vitro* PEPC specific enzyme activity for effective Saranac and ineffective in_1Sa. On day 5, no nodules were present on roots. Small root sections having emerging nodules were harvested on days 7 and 8. Nodules, free of root tissue, were harvested on the remaining days. The data shown reflect three separate experiments.

Western blots probed with monospecific PEPC antibodies showed that increased PEPC activity throughout the development of effective Saranac nodules correlated well with the increase in PEPC enzyme protein (Fig. 6). A slowly increasing amount of PEPC enzyme protein is detectable in root tissue as nodules form and emerge (days 5 through 8). Then as nitrogenase becomes active, the amount of nodule PEPC increases substantially. A prominent increase in PEPC enzyme protein occurs by day 9, with further increases evident on days 12, 19 and 33. With protein extracted from ineffective in_1Sa, the amount of PEPC enzyme protein appears to be similar to that found in Saranac tissue through day 9. However, on days 12, 19 and 33, PEPC enzyme protein was essentially undetectable even when 30 μg of protein was applied on the gel. Bacteroids contain no immunologically reactive PEPC [28]. These data not only confirm those of Egli *et al.* [8], but also extend their findings by showing that the initial increase in PEPC activity and protein appears to be related to nodule development and not nitrogenase activity.

PEPC mRNA levels during nodule development

We next examined the amount of PEPC mRNA in effective and ineffective nodules. Total RNA was extracted from root and nodule tissue as described in Materials and methods. Due to the small amount of tissue from which RNA was extracted, northern blots of total RNA were used to estimate PEPC mRNA levels. RNA samples from tissue collected after day 8 have significant

amounts of rhizobial RNA in addition to plant RNA. Thus, estimates of the increase in PEPC mRNA during nodule development probably underestimate the actual increase. Hybridization of the northern blots to ^{32}P-labeled poly(U) showed that the amount of poly(A)$^+$ RNA in each lane of effective Saranac total RNA was comparable to or slightly less than that in each lane of ineffective *in*$_1$Sa total RNA (data not shown). The PEPC mRNA in effective nodules increases from a relatively low, basal level on day 5 to a relatively high level on day 8 (Fig. 7). The abundance of PEPC mRNA in effective nodules remains high or increases slightly from day 8 to day 33. Direct assessment of radioactivity hybridized to PEPC mRNAs in effective nodules shows a 4-fold increase between days 5 and 8, with a further 2.5-fold increase by day 9, followed by a further 1.5-fold increase to day 33 leading to a total increase of about 13-fold between day 5 and 33. The increase in PEPC mRNA as nodules formed is similar to the increase in PEPC activity and PEPC enzyme protein.

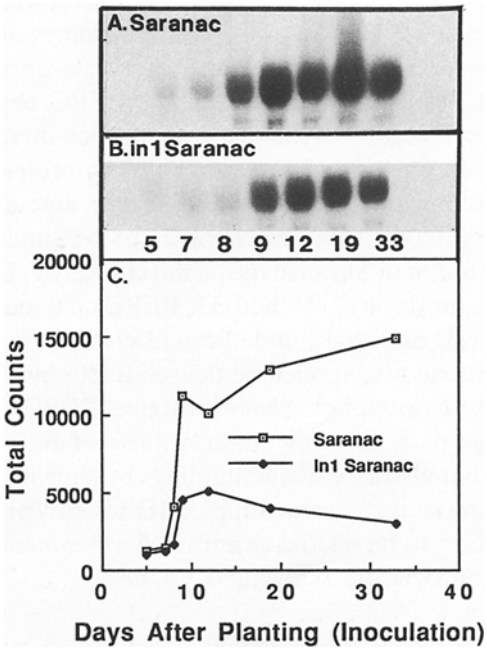

Fig. 7. Northern blots of PEPC mRNA expression throughout nodule development of effective Saranac and ineffective *in*$_1$Sa alfalfa. Panel A: effective Saranac. Panel B: ineffective *in*$_1$Sa. Panel C: radioactivity hybridized to PEPC mRNA of effective Saranac (□) and ineffective *in*$_1$Sa (●) on each day. Total RNA was isolated at various days after planting. On day 5, no nodules were present on roots. Small root sections having emerging nodules were harvested on days 7 and 8. Nodules, free of root tissue, were harvested on the remaining days. Each lane contains 10 μg total RNA. Data are representative of three separate blots and counts hybridized represent the average of three separate blots.

The overall developmental pattern for PEPC mRNAs in *in*$_1$Sa was similar to that of effective Saranac with three exceptions: (1) the total amount of radioactive PEPC cDNA probe hybridized to RNA extracted from *in*$_1$Sa was generally about one-third to one-fourth of that hybridized to Saranac RNA after day 7; (2) the increase in PEPC mRNA that occurred after nitrogenase activity is detected on day 9 in effective Saranac nodules did not occur in *in*$_1$Sa nodules; (3) on day 33 the amount of PEPC mRNA present in *in*$_1$Sa tissue decreased about 20% as compared to day 19, which did not occur in the effective nodules.

Discussion

Nodule PEPC plays a key role in providing carbon for aspartate and organic acid biosynthesis in root nodules [20, 25, 34]. As nodules develop, the activities of PEPC and other enzymes involved in nodule nitrogen assimilation increase [8]. The biochemical and genetic factors leading to such increases have been characterized for only glutamine synthetase. Furthermore, the mechanisms regulating PEPC activity have been studied only in leaves of C_4 and CAM species [4, 6, 12, 15, 16, 33]. Our goal is to understand the biochemical and genetic factors that regulate the expression of the key enzymes involved in ammonia assimilation in root nodules. As an initial step in achieving this goal, we have isolated and characterized PEPC cDNA clones and deduced the complete primary structure of the nodule-enhanced form of PEPC. Utilizing antibodies to alfalfa nodule PEPC, we screened an expression cDNA library. We first isolated a partial-length 2.9 kb PEPC cDNA clone that contained an open reading frame of 877 codons but lacked the N-terminal

coding region of the cDNA. cDNAs encoding this N-terminal region of the protein were obtained by the RACE protocol [10].

The deduced amino acid sequence of alfalfa nodule PEPC is about 80% identical to the uninduced and salt-induced forms of ice plant PEPC and to maize autotrophic PEPC. The deduced sequence of alfalfa PEPC also contains all of the conserved residues [4, 6, 16, 33] proposed to be associated with substrate binding, protein phosphorylation and the active site in plant PEPCs, suggesting that alfalfa nodule PEPC enzyme activity may be regulated like these other PEPC enzymes. The deduced molecular mass of alfalfa nodule PEPC protein is about 111 kDa, which is very similar to the estimated masses of the *in vivo* protein [28, 41] and *in vitro* synthesized polypeptide (S. Miller and C. Vance, unpublished). Two isoforms of nodule PEPC have been reported for alfalfa, lupine and soybean [7, 24, 28, 31] and could be the products of separate genes. Alternatively, they could arise from the same gene through post-translational modification. Although our data do not resolve the issue, it does appear that alfalfa nodule PEPC is either a member of a small gene family or that considerable sequence polymorphisms exist near a single PEPC locus in the tetraploid genome of alfalfa. In maize [12, 15, 16], sorghum [4] and ice plant [6], PEPC occurs as isozymes that are encoded by distinct genes.

It is interesting to note that increased PEPC enzyme protein and activity in effective nodules appears to occur in two phases. The first phase occurs by days 7 and 8, and coincides with nodule emergence from roots. The second phase, commencing at day 9 and continuing through day 33, occurs after nitrogenase activity is detectable (Fig. 6). By contrast, only the first-phase increase appears to occur in ineffective in_1Sa nodules and this increase is delayed about 24 h (Figs. 6 and 7). Thus, in_1Sa nodules apparently lack the factor(s) required for maximal PEPC expression. We have seen similar patterns of development for several other nodule enzymes including glutamine synthetase and aspartate aminotransferase [8, 11].

The striking increase in PEPC mRNA abundance during nodule development (Fig. 7) mirrors the apparent biphasic increase in PEPC activity. This suggests that the amount of PEPC enzyme protein and activity is determined, at least in part, by changes in the amount of PEPC mRNA. We propose that the observed increase in PEPC mRNA occurs in two independent phases. The initial increase is due to an event involved with organ (nodule) development since (1) it occurs prior to detectable nitrogen fixation and (2) it coincides with nodule initiation and emergence from the root in both Saranac and in_1Sa nodules. The second stage of PEPC mRNA accumulation apparently requires a signal that is associated with effective nodules and/or nitrogen fixation. This second signal, absent in in_1Sa nodules, is probably unrelated to bacterial release from infection threads into host plant cytosol of infected cells, since release occurs in both Saranac and in_1Sa [8, 32].

Although light induction of PEPC activity in maize and sorghum [4, 12, 15, 16] and salt induction of PEPC activity in ice plant [6, 30, 33] are known to involve increases in the amounts of PEPC mRNA and protein, other factors occurring post-translationally have also been implicated in regulating PEPC enzyme activity. These factors include: (1) the phosphorylation state of the enzyme protein [1, 17, 18, 21]; (2) the oligomerization and allosteric behavior of the enzyme protein [1, 17, 29, 43, 44]; (3) the rate of PEPC protein turnover [18]. Regulation of PEPC activity in alfalfa nodules may also involve post-translational events. Support for this suggestion is drawn from a comparison of the amount of PEPC enzyme protein and the amount of PEPC mRNAs in ineffective in_1Sa nodules. On day 9 and thereafter, PEPC mRNAs are relatively abundant in in_1Sa nodules while PEPC activity remains low and enzyme protein is virtually undetectable (Figs. 6 and 7). These observations suggest that PEPC mRNAs either are not being translated efficiently or, alternatively, that PEPC enzyme protein is being rapidly degraded. Early senescence, which occurs in ineffective in_1Sa nodules and is apparent by light microscopy by day 13 [8], may play a role in such events. It is currently unknown how senescence affects either

the translation of PEPC mRNAs or degradation of PEPC enzyme protein. Additional evidence supporting post-translational control of nodule PEPC activity is drawn from the observation that alfalfa nodule PEPC can be phosphorylated *in vitro* (S. Miller and C. Vance, unpublished).

While events that occur very early in nodule differentiation seem to be controlled by rhizobial signals which function at very low concentrations [23, 27], additional signals are required to yield functionally effective nodules [27]. These additional signals may be the result of metabolic alterations resulting from bacteroid metabolism and/or nitrogen fixation. Since effective bacteroids release ammonia into the plant cytosol and the interior of effective nodules has a very low O_2 concentration, both ammonia and oxygen have been suggested as signals involved in regulation of plant genes during the latter stages of symbiosis. However, it has been difficult to demonstrate unequivocally that either of these are primary signals for the regulation of late nodulin gene expression. Experiments with common bean [3] and pea [42] have shown that ammonia production in nodules is not required for maximal accumulation of glutamine synthetase and leghemoglobin. By contrast, glutamine synthetase and leghemoglobin expression in soybean nodules was directly related to the availability of ammonia [26]. Likewise, low O_2 concentrations in nodules have been implicated, without direct evidence, in the regulation of nodule uricase [45] and sucrose synthase [39, 45].

Irrespective of the nature of signals involved in regulating nodule gene expression, some appear to be conserved between species. Regulation of the soybean Lbc3 and N-23 gene expression [36], as well as glutamine synthetase from common bean [9] and leghemoglobin glb3 from *Sesbania* [37], have been evaluated in transgenic *Lotus corniculatus* carrying chimeric gene fusions containing the 5′-upstream region of these genes fused to reporter genes. In all instances organ specific expression was displayed in a heterologous host, indicating that diverse legumes contain functionally similar nodulin transcriptional activation factors and *cis*-acting elements. With the isolation of the alfalfa nodule-enhanced PEPC and aspartate aminotransferase [11] cDNAs, we have initiated experiments aimed at understanding the expression of genes encoding enzymes important in the assimilation of carbon and nitrogen with the objective of defining the mechanisms by which these genes are regulated.

Acknowledgements

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