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Analyses of phosphoenolpyruvate carboxylase gene structure and expression in alfalfa nodules

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Summary

Phosphoenolpyruvate carboxylase (PEPC) plays a crucial role in the assimilation of CO_2 during symbiotic N_2 fixation in legume root nodules. In this study, an alfalfa PEPC gene (*PEPC-7*), whose transcripts are found at elevated levels in nodules relative to either leaves or roots, has been isolated and characterized. The intron/exon structure of this gene is identical to that of most other plant PEPC genes except for the presence of an additional intron in the 5′ untranslated region. *In situ* RNA hybridization studies showed that PEPC transcripts were present in the nodule meristem, the infection zone, the nitrogen-fixing zone, and the parenchyma. PEPC transcripts were also found in vascular tissue of roots and nodules and in the pulvinus of petioles. In transgenic alfalfa, a chimeric reporter gene was expressed in these same regions except that little expression was found in the nodule meristem. Analyses of promoter deletions suggest that the region between −634 and −536 is of particular importance in directing transcriptional activity to the infected zone of nodules. Within this region is a mirror repeat sequence that is potentially capable of forming an H-DNA structure. These results indicate that *PEPC-7* has a central role in nitrogen-fixing nodules and that regulation of transcription is an important determinant of its activity.

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Introduction

Phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31), a ubiquitous enzyme in plants and bacteria, catalyzes the conversion of phosphoenolpyruvate and bicarbonate to oxaloacetate and inorganic phosphate (Utter and Kolenbrander, 1972). PEPC plays a key role in photosynthetic carbon assimilation in CAM and C4 plants by catalyzing the initial incorporation of CO_2 into an organic acid (Bowes, 1993; Hatch, 1992; O'Leary, 1982; Ting, 1985). PEPC activity also appears to play a key role in a variety of non-photosynthetic processes, perhaps the most well recognized of which occurs in nitrogen-fixing root nodules of legumes. Enhanced levels of PEPC appear to occur in effective nodules of all legumes as well as in the non-legume *Alnus glutinosa*, which fixes N_2 in symbiosis with *Frankia* (Deroche and Carrayol, 1988). The amount (1–2% of total protein) and specific activity of PEPC in nodules is comparable to that found in the leaves of C4 and CAM species (Deroche and Carrayol, 1988; Egli *et al.*, 1989; Miller *et al.*, 1987).

In addition to supplying all the carbon needed to generate energy for N_2 reduction and for the growth of the bacterial symbiont, the host plant also generates large quantities of keto acids that provide the carbon skeletons for the assimilation of ammonia. In perennial legumes such as alfalfa, it has been estimated that reduction of N_2 and assimilation of ammonia consume about onethird of total photosynthate (Maxwell *et al.*, 1984). PEPC reassimilates a substantial amount of the CO_2 respired in nodules. Oxaloacetate derived from the PEPC-catalyzed reaction has several metabolic fates. Included among these are its import into mitochondria for use in the tricarboxylic acid (TCA) cycle, its conversion to malate and succinate, which are imported into bacteroids and used as a carbon and energy source, and its transamination by aspartate aminotransferase to produce aspartate. In amide-transporting species such as alfalfa, much of the aspartate is converted to asparagine by asparagine synthetase (Ta *et al.*, 1986).

Many plants possess multiple isoforms of PEPCs, perhaps each being associated with a different metabolic pathway. Ting and Osmond (1973a,b) analyzed PEPCs from photosynthetic and non-photosynthetic tissues of a number of C3, C4 and CAM species and proposed that at least four distinct isoforms exist based on kinetics and

ion-exchange chromatography. These include the C4 photosynthetic PEPC present in the leaves of C4 plants, C3 PEPC in the leaves of C3, C4 and CAM plants, CAM PEPC in the leaves of CAM plants, and a non-autotrophic PEPC present in the roots of all plants. The functionally active form of all these PEPCs consists of a homotetramer with a monomeric molecular mass of about 100 kDa. Our understanding of PEPC isoforms and their expression has been enhanced by the isolation of PEPC cDNAs and genes from a wide variety of plants. Sequence analysis of PEPC cDNAs and genes suggests that most plant PEPCs can be loosely placed into three related groups (Toh *et al.*, 1994). These groups consist of PEPCs that are expressed at relatively low levels throughout dicot or monocot plants and PEPCs that are expressed at high levels in mesophyll cells of C4 plants. The inducible ice plant CAM PEPC isoform appears to be most closely related to monocot C3 isoforms (Toh *et al.*, 1994). In spite of their high level of expression, the characterized alfalfa and soybean nodule PEPCs are most related by their sequences to PEPCs of dicots that are constitutively expressed at low levels.

Although PEPC clearly plays a central role in symbiotic nitrogen fixation, little is known about the mechanisms underlying PEPC gene expression during the establishment and maintenance of symbiosis. As a first step toward understanding the mechanisms by which PEPC gene expression is controlled in symbiotic root nodules, we isolated a PEPC cDNA clone from an alfalfa root nodule cDNA library and studied the levels of PEPC activity, protein and transcripts during nodule development (Pathirana *et al.*, 1992). In developing nodules, PEPC activity increases to a level that is 10- to 15-fold greater than that found in roots and leaves. This increase in activity is correlated with comparable increases in PEPC protein and transcript. Our results comparing PEPC gene expression in nitrogen-fixing (effective) and non-nitrogen-fixing (ineffective) alfalfa nodules suggest that full gene expression is associated first with nodule initiation and development and then with the initiation and maintenance of effective symbiosis (Pathirana *et al.*, 1992).

To investigate further the regulatory mechanisms that control PEPC gene expression in root nodules, we have cloned a PEPC gene from alfalfa, determined its structure and its expression in nodule, root and leaf tissues, and examined the role of its putative promoter in directing reporter gene expression in transgenic alfalfa plants.

Results

Structure of the PEPC-7 *gene*

The nucleotide sequence of an alfalfa PEPC gene designated *PEPC-7* was determined along with 1614 bp of 5′ and 318 bp of 3′ flanking regions. The amino acid sequence deduced from the coding region of *PEPC-7* is identical to the amino acid sequence deduced from the previously sequenced pPEPC-61 cDNA (Pathirana *et al.*, 1992), and the nucleotide sequence of the *PEPC-7* coding region is 99% similar to that of pPEPC-61 cDNA. Primer extension (Pathirana *et al.*, 1992) and RNase protection assays (described below) defined the 5′ end of the mRNA, which presumably corresponds to the site of transcription initiation, and showed that the transcribed region of *PEPC-7* is 5557 bp long. The positions of the exons and introns within *PEPC-7* were determined by alignment of the pPEPC-61 cDNA and the genomic sequences. The transcribed region of *PEPC-7* consists of eleven exons. Each intron contains the nearly invariant 5′-GT...AG-3′ intron boundary sequences and generally conforms to the plant consensus intron junction sequences (Hanley and Schuler, 1988). The exon/intron organization of *PEPC-7* is compared with that of the *Mesembryanthemum crystallinum* (ice plant) gene encoding the C3 PEPC isoform (Cushman and Bohnert, 1989a) in Figure 1. Nine of the ten introns found in *PEPC-7* are located in the same position relative to the encoded amino acid sequence as are the introns found in nearly all plant PEPC genes sequenced to date. However, *PEPC-7* contains an additional intron (407 nucleotides) in its 5′ leader sequence.

Sequence analysis of 128 bp in the 3′-untranslated region of 13 PEPC cDNA clones isolated from an alfalfa root nodule cDNA library revealed the presence of two expressed PEPC genes that differ in their nucleotide sequences by one substitution and one 2 bp insertion. Five of the 13 cDNA clone sequences were identical to the *PEPC-7* gene sequence, with the remaining eight sequences identical to the pPEPC-61 cDNA. These results suggest that *PEPC-7* is expressed in nodules, and that, in alfalfa, the nodule-enhanced form of PEPC is encoded by a multi-allelic gene or by recently duplicated genes.

Mapping of the 5′ ends of PEPC-7 *mRNA expressed in alfalfa nodules, roots and leaves*

Previously, we determined the sequence of the 5′ end of nodule PEPC transcripts by cloning and sequencing primer extension products. Three cDNA clones of identical length, each derived from independent amplification reactions, were sequenced (Pathirana *et al.*, 1992). The sequences of these cDNAs are identical to the corresponding sequences in *PEPC-7*. To examine the expression of *PEPC-7* in nodule, root and leaf tissues and to confirm the location of the 5′ end of *PEPC-7* transcripts, RNase protection assays were conducted. Figure 2 shows that, in all three tissue types, the major RNA fragment protected was the same size (estimated to be 78 ± 2 nucleotides). These results placed the 5′ end of *PEPC-7* mRNA at nucleotides +4 to +8, very near the site mapped by primer extension analyses. These

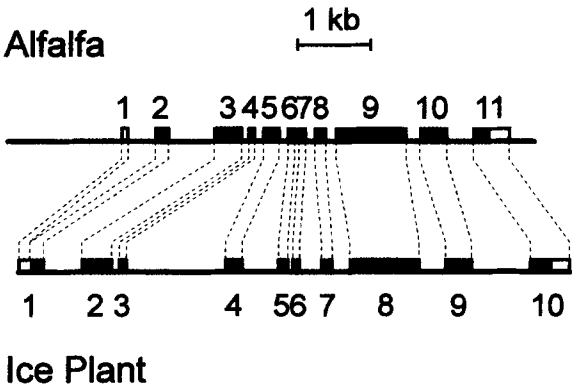


Figure 1. Comparison of the exon/intron organization of alfalfa *PEPC-7* with that of the ice plant *Ppc2* gene encoding the C3 isoform of PEPC.
Exons are represented by boxes. Filled boxes represent the translated regions of the genes. The extent of DNA sequencing in the 5′ and 3′ flanking regions of *PEPC-7* is indicated by the lines flanking exons 1 and 11, respectively.

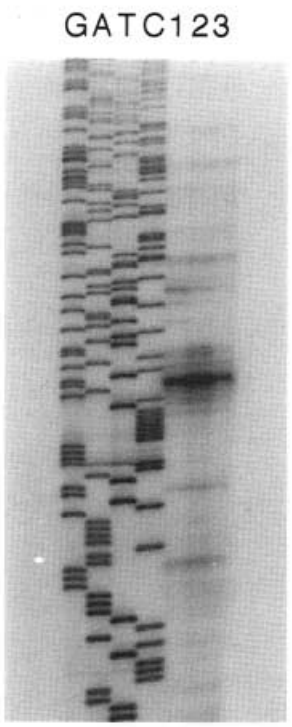


Figure 2. Analysis of *PEPC-7* transcripts in alfalfa roots, nodules and leaves by RNase protection assays.
Alfalfa total RNA from roots (100 μg, lane 1), nodules (35 μg, lane 2) and leaves (100 μg, lane 3) were hybridized to a [^{32}P]-labeled *PEPC-7* antisense RNA probe and digested with RNase A and RNase T1. pBluescript KS$^-$ was sequenced and used as a DNA size marker. Products of the reactions were fractionated through a 6% polyacrylamide DNA sequencing gel.

RNase protection assays also demonstrated that *PEPC-7* expression is not restricted to nodules and its expression is therefore not nodule-specific. However, the amount of *PEPC-7* transcript in nodules is substantially higher than that detected in roots and leaves, indicating that expression of this gene is highly enhanced in nodules.

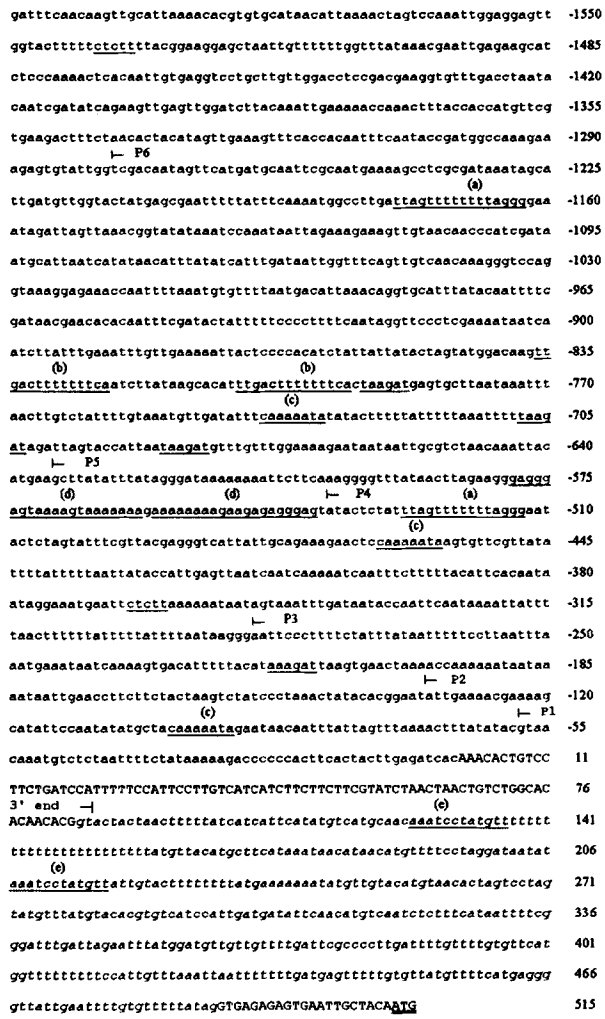


Figure 3. The nucleotide sequence of the region upstream of the *PEPC-7* translation initiation codon.
Position 1 corresponds to the 5′ end of mRNA as mapped by primer extension. The exon sequences are in upper case lettering, the gene's first intron is in italicized lower-case lettering, and the gene's 5′ flanking sequence is in lower-case lettering. Sequences previously identified as being important in late-nodulin gene expression (CTCTT and (T/A)AAGAT) are underlined as are repeated sequences that are identified by letters above the repeat (repeat d is a mirror repeat). The 5′ ends of the promoterregion deletions (P6, P5, etc.) used in reporter gene studies are marked, as is their common 3′ end. The translation initiation codon is marked by a double underline.

Analysis of the 5′ flanking sequence of the PEPC-7 *gene*

Figure 3 shows the nucleotide sequence upstream of the *PEPC-7* ATG translational start codon. *PEPC-7* contains potential TATA and CCAAT boxes located 36 bp and 113 bp upstream from the proposed transcription start site, respectively. We previously observed that maximum levels of PEPC transcripts and enzyme protein occur in mature nodules soon after the onset of nitrogenase activity (Pathirana *et al.*, 1992). This expression pattern is typical of late nodulins, such as leghemoglobin (Marcker *et al.*,

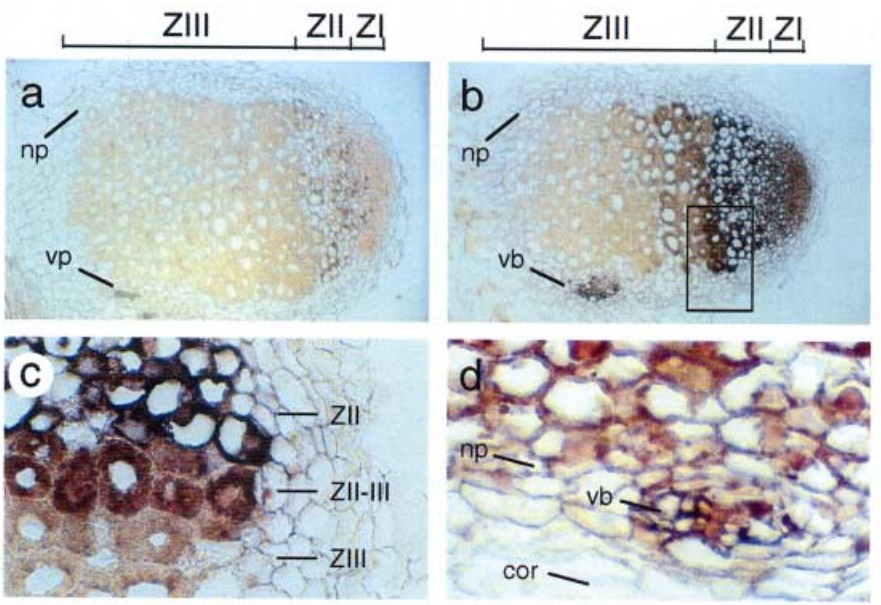


Figure 4. Localization of PEPC transcripts in alfalfa nodules by *in situ* hybridization. Alkaline phosphatase activity used to visualize digoxigeninlabeled RNA probe is detected as a maroon color. (a and b) Day 19 nodules hybridized to digoxigeninlabeled PEPC sense (a) or antisense (b) RNA probes. (c) Enlargement of boxed region in panel b showing interzone II–III. (d) Close-up of day 19 nodule hybridized to digoxigenin-labeled PEPC antisense probes highlighting a vascular bundle and the nodule parenchyma. Abbreviations: ZI, zone I (meristematic region); ZII, zone II (infection zone); ZIII, zone III (nitrogen-fixing zone); ZII–III (interzone II–III); vb, vascular bundle; np, nodule parenchyma; cor, nodule cortex.

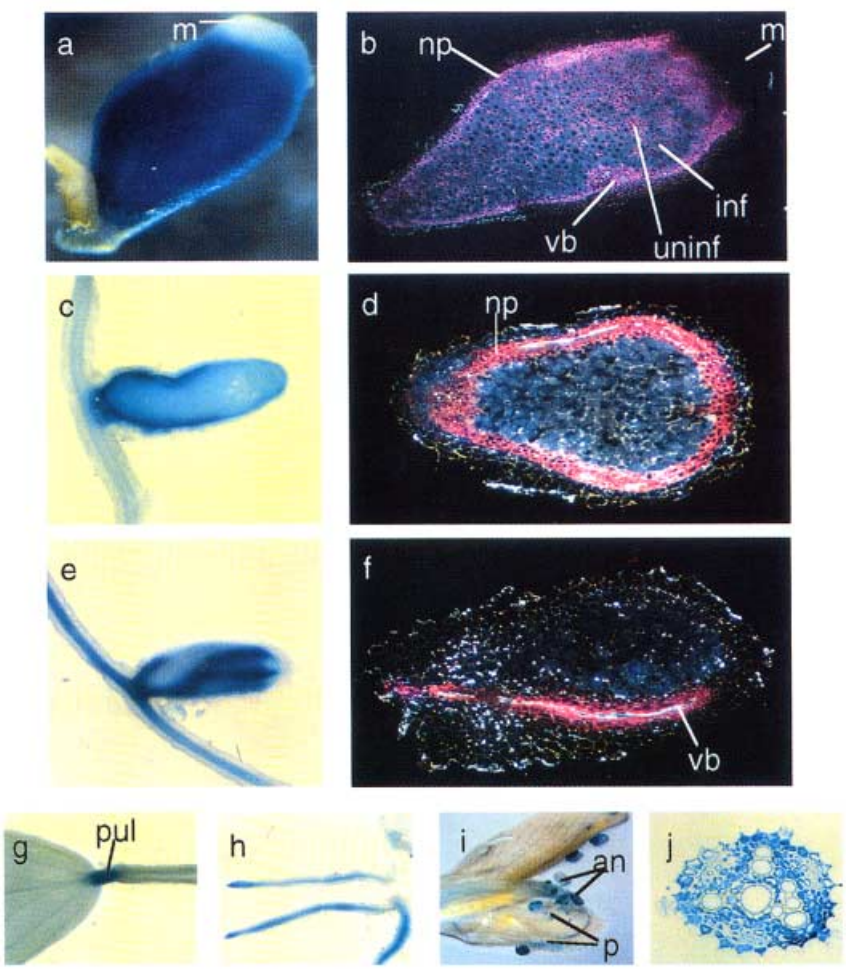


Figure 5. See facing page for legend.

1984), N23 (Jorgensen *et al.*, 1988), glutamine synthetase (Forde *et al.*, 1989), and plastid-localized aspartate aminotransferase (Gantt *et al.*, 1992). Two sequence motifs found in other late nodulin genes, including genes encoding leghemoglobin (Metz *et al.*, 1988; Stougaard *et al.*, 1987) and the N23 gene family (reviewed by de Bruijn and Schell, 1993), are present in *PEPC-7*: 5′-(A/T)AAGAT-3′ at −218, −686, −708 and −792, and 5′-CTCTT-3′ at −365 and −1539 (Figure 3). However, the conserved spacing of these motifs observed in the leghemoglobin genes (Szczyglowski *et al.*, 1994) is not found in *PEPC-7*.

Several other sequences that may be important *cis*-regulatory elements are present in the 5′ flanking region of *PEPC-7*. A 42 bp homopurine sequence, that includes a mirror repeat (a sequence that is the same whether read 5′ to 3′ or 3′ to 5′), centered at position −558, begins at position −579. Several repeat sequences are also found in the 5′ flanking region. 5′-TTAGTTTTTTT(T)AGGG-3′ (repeat a in Figure 3) is repeated at positions −524 and −1173; 5′-TTGACTTTTTTTCA-3′ (repeat b) is repeated at positions −807 and −836; and 5′-CAAAAATA-3′ (repeat c) is repeated at positions −100, −465 and −739. No significant similarities were identified when the 5′ flanking region of *PEPC-7* was compared with the 5′ flanking regions of PEPC genes encoding maize C4 (Hudspeth and Grula, 1989), ice plant and *Flavaria* C3 (Cushman and Bohnert, 1989b; Hermans and Westhoff, 1992) or ice plant CAM (Cushman and Bohnert, 1989a) specific isoforms.

One repeat sequence (repeat e) was found in the first intron of *PEPC-7*. This intron also contains an unusual string of 24 thymidines.

Localization of PEPC transcripts in developing nodules

In situ hybridization assays were used to examine the cellular distribution of PEPC transcripts. Figure 4 compares results obtained from control sense (Figure 4a) and antisense (Figure 4b) digoxigenin-labeled probes when hybridized to sections taken from day 19 nodules. Sense and antisense RNA probes correspond to 798 nucleotides of coding sequence. Both digoxigenin- and [^{35}S]-labeled probes were used in these experiments with very similar results. PEPC transcripts, detected as maroon staining, are clearly present in nodule vascular tissue and in zones I, II and III, which correspond to the meristematic zone, the infection zone and the nitrogen-fixing zone, respectively. In Figure 4(b), staining is also faintly visible in the nodule parenchyma (or inner cortex as defined by Hirsch, 1992). Figure 4(c) is a magnified view of interzone II–III (Vasse *et al.*, 1990) where the transition from the infection zone to the nitrogen-fixing zone occurs. Nitrogen fixation is initiated in bacteroids found in the distal (apical) regions of zone III (Vasse *et al.*, 1990). Figure 4(b) and (c) show that PEPC transcripts are clearly present in cells that do not contain active nitrogenase. Figure 4(d) shows the presence of PEPC transcripts in vascular tissues and in the nodule parenchyma but the transcripts are undetectable in the nodule cortex. PEPC transcripts were also detected in root vascular tissue and in pulvini (data not shown), but, surprisingly, not in leaf guard cells, where PEPC is thought to play an important role in regulating cellular pH and charge balance (data not shown).

Expression of phosphoenolpyruvate carboxylase (PEPC-7) promoter-GUS gene fusions in transgenic alfalfa

Our previous data demonstrated that, during the development of alfalfa nodules, PEPC transcripts increase to a level that is 10- to 15-fold higher than that found in root and leaf tissues (Pathirana *et al.*, 1992). These data together with the *in situ* hybridization results described above led us to investigate the transcriptional regulation of the *PEPC-7* gene. We predicted that the 5′ flanking region of the gene contains promoter elements that direct and limit expression to cells shown to contain PEPC transcripts. To test this hypothesis and increase our understanding of PEPC gene expression in alfalfa, we constructed chimeric genes containing sequences of varying length upstream of the presumed transcriptional start site fused to the β-glucuronidase (*GUS*) reporter gene. The 5′ end points of the promoter

Figure 5. *PEPC-7* promoter-β-glucuronidase (*GUS*) reporter gene activity in transgenic alfalfa.
GUS activity is seen as a blue color in whole organs and as a pink–red color in thin sections. All nodules were taken from day 17 plants.
(a) GUS reporter gene activity in a nodule taken from a transgenic plant containing the P6 promoter fragment.
(b) Dark-field micrograph of a nodule thin section taken from transgenic plants containing the P6 promoter fragment. GUS activity is evident in both infected and uninfected cells in the nitrogenfixing zone. Staining is also observed in the parenchyma cell layer while little or no staining is observed in the senescent zone or in the cortex.
(c) GUS reporter gene activity in a nodule taken from a transgenic plant containing the P5 promoter fragment.
(d) Dark-field micrograph of a nodule thin section taken from a transgenic plant containing the P5 promoter fragment. Little or no GUS activity is found in the nitrogen-fixing zone or in the cortex. However, intense staining is observed in the nodule parenchyma. Note that the section does not include the meristematic region.
(e) GUS reporter gene activity in a nodule taken from a transgenic plant containing the P4 promoter fragment.
(f) Dark-field micrograph of a nodule thin section taken from a transgenic plant containing the P4 promoter fragment. The only tissue that appears to stain for GUS activity is the vascular bundle.
(g to i) GUS reporter gene activity in a pulvinus (g), young roots (h) and a flower (i) of plants transformed with the P6–GUS reporter gene.
(j) GUS reporter gene activity in the root vascular bundle of a transgenic plant containing the P4–GUS reporter gene.
Abbreviations: inf, infected cell; uninf, uninfected cell; pul, pulvinus; an, anther; p, pollen.

sequences are shown in Figure 3. The putative promoter fragments contain the entire first exon and two nucleotides of the first *PEPC-7* intron at their 3′ end. These chimeric genes were then introduced into alfalfa and reporter gene activity was examined in regenerated plants. The number of independently transformed plants analyzed in these studies ranged from 8 (P6) to 20 (P2). Most plants transformed with the same construct displayed similar patterns of GUS staining.

GUS gene expression driven by the longest promoter fragment, P6 (–1277 to 86 relative to the mapped 5′ end of the transcript), was the most thoroughly examined. Figure 5(a) shows that the P6 promoter is active in the nodule, with little *GUS* expression detected in the nodule meristem. Darkfield microscopy of nodule thin sections (Figure 5b) revealed that P6-driven GUS expression is present in the nodule parenchyma, in both infected and uninfected cells of the infection (II) and nitrogen-fixing (III) zones, and in the nodule vascular tissue; however, little or no staining was observed in the nodule meristem. The P6 promoter fragment also confers *GUS* expression to pulvini and leaf vascular tissue (Figure 5g), root tips (Figure 5h) and pollen (Figure 5i). Consistent with the *in situ* hybridization results, no GUS expression was detected in leaf guard cells (data not shown).

The activity of the P6 promoter was also examined in nodules of different ages (data not shown). Very young nodules (day 7) showed promoter activity in root vascular tissue and the nodule parenchyma with less activity in the symbiotic zone. Overall activity increased in day 9 nodules with similar promoter activities in root vascular tissue, the nodule parenchyma and the symbiotic zone. The most intense staining was observed in 12-, 19-, and 33-day-old nodules with increased and approximately equal activities in the parenchyma and symbiotic zone. Promoter activity in root vascular tissue did not significantly change in day 9 to day 33 nodules.

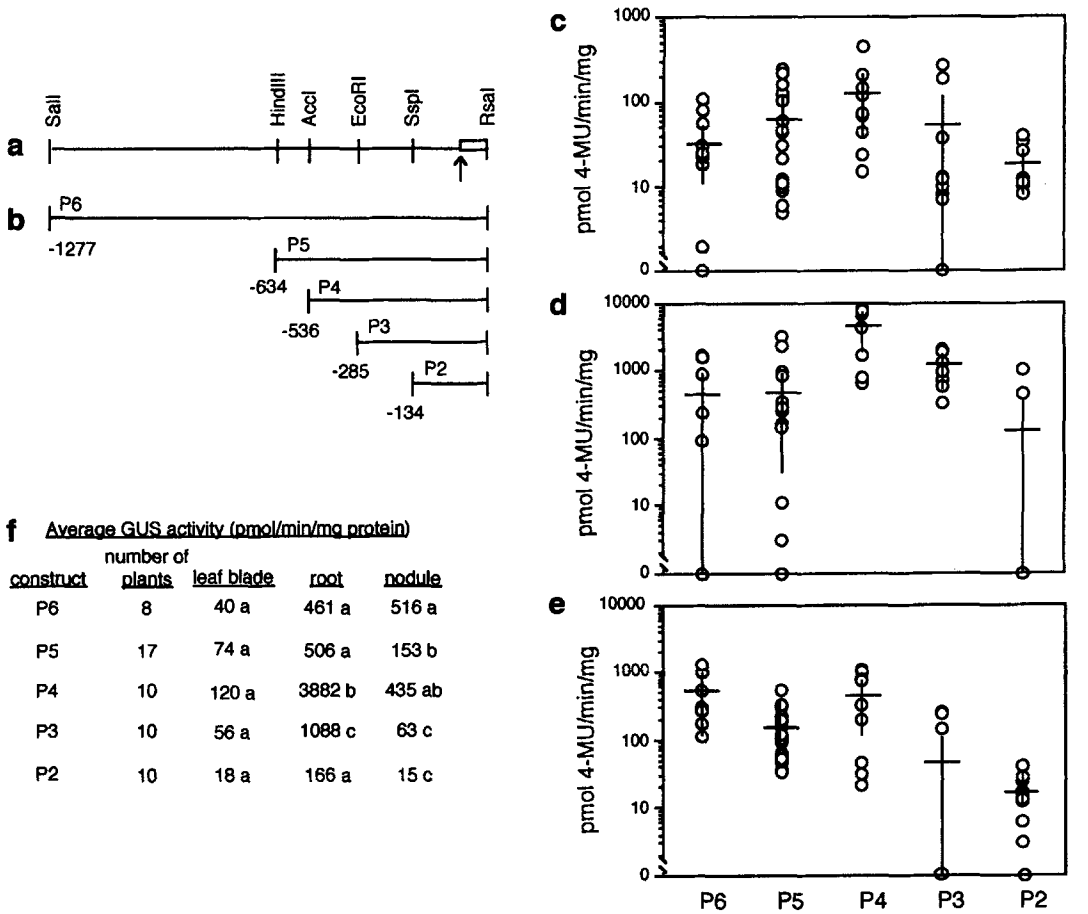
When the P6 promoter was shortened, significantly different patterns of *GUS* expression were observed. The P5 promoter fragment (–634 to 86) was much less active in the symbiotic zone than was the P6 promoter (compare Figure 5c and d with 5a and b). However, reporter gene activity remained high in the nodule parenchyma (Figure 5c and d). When the distal 98 bp of the P5 promoter were deleted, forming the P4 promoter (–536 to 86), virtually all reporter gene expression was lost except that observed in nodule and root vascular tissue (Figure 5e, f and j). Furthermore, the P4 promoter directed very high levels of GUS activity to vascular tissue throughout the plant (data not shown). Low levels of GUS expression were observed in plants transformed with the P3 promoter (–285 to 86) and the expression pattern was essentially the same as for the P4 promoter (data not shown). Very little or no GUS activity was observed in transgenic plants containing the P2 (–134 to 86) or P1 (–58 to 86) promoter fragments (data not shown). Verification that plants were in fact transformed with the P3–GUS, P2–GUS or P1–GUS constructs was obtained by PCR analyses of DNA extracted from the regenerated plants.

GUS activity in transgenic plants was quantified by a fluorometric assay (Jefferson *et al.*, 1987) and the results are shown in Figure 6. None of the promoter deletions used in our experiments significantly altered *GUS* expression in leaves (Figure 6c and f). However, removal of the *SalI*/*Hin*dIII fragment, creating the P5 promoter (Figure 6a and b), resulted in a significant decrease in total GUS activity in the nodule but appeared to have little effect on activity in roots (Figure 6d–f). Deletion of nucleotides –634 to –536 resulted in a dramatic increase in the GUS activity found in roots (Figure 6d–f) and a large increase (over that seen in P5–GUS transgenic plants) in whole-nodule GUS activity (Figure 6e and f). These increases appear to be due to activation of the transgene in vascular tissues (see Figure 5). P3–GUS transgenic plants had similar staining pattern as P4–GUS transgenic plants but with lower overall GUS activity. Deletion of the 5′ region to –134 (P2) decreased reporter gene expression to near background levels in leaves and nodules.

Discussion

Although many PEPC genes have been isolated and partially characterized, neither detailed analysis of the putative promoter region nor *in situ* hybridization studies have been reported for legumes, in which PEPC functions in an important and specialized manner. Here we report the isolation and analysis of the *PEPC-7* gene from alfalfa. RNase protection experiments (Figure 2) showed that *PEPC-7* encodes a message that is highly expressed in nodules, which is consistent with our previous studies (Pathirana *et al.*, 1992) showing that PEPC activity, protein and mRNA are significantly higher in nodules than in other plant organs. However, these experiments also demonstrate that, unlike the C4 PEPC isoform, which is expressed only in specific cells of C4 plants, *PEPC-7* is expressed throughout much of the plant.

The structure of the alfalfa *PEPC-7* gene is noticeably different than published PEPC genes in that it contains an intron in the 5′ leader sequence of the transcribed region. Because the number and positions of nearly all other introns in the gene (Yanai *et al.*, 1994) are conserved among the characterized plant genes encoding monocot and dicot C3, C4 and CAM PEPC isotypes, this intron appears to be a relatively recent addition to the gene's structure. However, it has recently been shown that the PEPC genes in *Flavaria* also contain an intron in their 5′ untranslated region (K. Ernst, Universität Düsseldorf, personal communication). In plants, an intron in the 5′ untranslated region has



f Average GUS activity (pmol/min/mg protein)

construct	number of plants	leaf blade	root	nodule
P6	8	40 a	461 a	516 a
P5	17	74 a	506 a	153 b
P4	10	120 a	3882 b	435 ab
P3	10	56 a	1088 c	63 c
P2	10	18 a	166 a	15 c

Figure 6. Diagram of *PEPC-7* 5′ flanking regions used in *PEPC–GUS* chimeric gene constructs and quantification of GUS activity in nodules, roots and leaves.
(a) The arrow shows the transcription initiation site and the open box is the first *PEPC-7* exon. *Sal*I, *Hin*dIII, *Acc*I, *Eco*RI and *Ssp*I are the restriction enzyme sites used to define the 5′ ends of the P6, P5, P4, P3 and P2 promoter fragments, respectively. The *Rsa*I restriction enzyme site determined the 3′ end of each construct.
(b) 5′ Flanking regions used to construct GUS chimeric gene fusions.
(c to e) GUS activity was determined by a fluorometric assay in leaves (c), roots (d) and nodules (e). Each circle represents the activity from an individual, independently transformed, plant. Horizontal bars are average activities and vertical bars are 95% confidence intervals. Note the different scale in (c).
(f) Mean GUS activities were calculated from the number of independently transformed plants indicated. For each tissue, activity values followed by different letters are significantly different at $P \leq 0.05$.

been reported in several genes including those encoding ascorbate peroxidase (Mittler and Zilinskas, 1992), sucrose synthase (Werr *et al.*, 1985), actin (Pearson and Meagher, 1990), phytochrome (Christensen *et al.*, 1992) and ubiquitin (Christensen and Quail, 1989). Sequences contained within the leader introns of some of these genes have been identified as regulatory elements. The first intron of alfalfa *PEPC-7* contains a direct repeat of 12 bases (5′-AAAT-CCTATGTT-3′) at positions +125 and +207, and 24 consecutive T residues beginning at position +135 (Figure 3). Whether these sequences or any other sequences present in the 5′ leader intron play a significant role in *PEPC-7* expression is unknown, although our reporter gene studies suggest that most of the sequences regulating *PEPC-7* gene expression are found in the gene's 5′ flanking region.

Transgenic alfalfa plants containing the chimeric P6–GUS reporter gene construct showed GUS to be strongly expressed in zones II and III, nodule parenchyma, and nodule vascular tissue, with little or no expression in the nodule meristem. However, upon longer periods of staining, a low level of GUS activity could sometimes be detected in the meristem. With the exception of detectable PEPC transcripts in the meristem, our *in situ* hybridization data are consistent with the reporter gene data. The general concordance of *in situ* hybridization and reporter gene studies both within the nodule and in other organs (discussed below) suggests that PEPC gene expression is regulated primarily by transcription and that *PEPC-7* transcription is largely controlled by elements found within a region 1.3 kb upstream of the transcription start site. However, several possibilities may explain the presence of PEPC transcripts in the meristem and our inability to

consistently detect reporter gene activity. For example, some *PEPC-7* promoter elements may lie outside the region used as the P6 promoter; another PEPC gene, closely related to *PEPC-7*, may be expressed in the meristem; the PEPC and GUS transcripts may be differentially stabile in the meristem; or the stability of the GUS protein may vary in different tissues.

PEPC immunolocalization studies have been conducted in soybean and alfalfa nodules. Suganuma *et al.* (1987) reported that soybean PEPC activity was highest in uninfected cells in the symbiotic zone, lowest in infected cells, and intermediate in parenchyma cells. Kouchi *et al.* (1988) agreed with Suganuma *et al.* (1987) that infected cells had the lowest PEPC activity, but found the nodule parenchyma cells to have the highest activity. In alfalfa, we have studied the intracellular location of PEPC by immunogold electron microscopy (Robinson *et al.*, 1996) and these results are consistent with our reporter gene and *in situ* hybridization studies. PEPC protein was found in the cytoplasm of both infected and uninfected cells of the symbiotic zone and at a somewhat reduced level in the nodule parenchyma cells (meristematic cells were not examined). Furthermore, the pericycle cells of the nodule vascular system also appeared to contain a high amount of PEPC. The pericycle cells contain dense cytoplasm with numerous mitochondria, suggesting that they have a high rate of metabolism, and have cell wall in-growths that are thought to facilitate the transport of assimilated nitrogen into nodule xylem (Walsh *et al.*, 1989). Anaplerotic assimilation of CO_2 catalyzed by PEPC has been proposed to occur in metabolically active tissues (Latzko and Kelly, 1983). Therefore, PEPC gene activity in pericycle cells of nodule vascular bundles is consistent with the proposed anaplerotic function of the gene product.

High levels of reporter gene expression were also detected in young roots of some plants containing the P6–GUS transgene. This observation agrees with that of Smith (1985) who reported high levels of PEPC activity in pea root tips. As in the case with pericycle cells, PEPC activity in actively growing roots may help replenish TCA cycle intermediates.

PEPC enzyme protein and activity has also been associated with the accumulation of malate in guard cells during stomatal opening (Allaway, 1973; Michalke and Schnabl, 1987; Raschke and Schnabl, 1978). It is believed that malic acid acts to lower the increase in cytosolic pH caused by proton extrusion during guard cell opening. Malate ions may also be important in maintaining the charge balance during the uptake of potassium. However, we could not detect *PEPC-7* gene expression in guard cells by either reporter gene assays or *in situ* hybridization studies. While RNase protection assays demonstrated that *PEPC-7* is expressed in leaves, reporter gene and *in situ* hybridization studies leave open the possibility that another gene encodes guard-cell PEPC.

Both *in situ* hybridization and reporter gene expression studies show PEPC gene activity in the pulvini of alfalfa petioles. Pulvini, the motor organs of leaflets located at the base of the leaf blade, mediate diurnal leaf movements and leaf movements in response to external stimuli such as light or touch. Several lines of physiological and biochemical evidence suggest that these leaf movements are brought about by osmotic changes in motor cells of the extensor and flexor regions of the pulvini by a mechanism similar to that operating in guard cells during the opening and closing of stomata (Hosokawa and Kiyosawa, 1983; Mayer *et al.*, 1985). An increase in malate ion concentration has been detected in *Phaseolus* pulvini during the light period, relative to the levels detected in the dark period (Bialczyk and Lechowski, 1986) and PEPC activity has also been found in *Phaseolus* pulvini (Vanhinsberg and Horton, 1990). In contrast to a proposed function for PEPC in guard-cell function, our *in situ* hybridization and reporter gene results provide additional support for the functioning of PEPC during leaf movements.

Expression of the P6–GUS reporter gene was observed in pollen of both buds and dehiscent flowers. Several laboratories have demonstrated that supplementing pollen suspension cultures with increased concentrations of CO_2 stimulates pollen germination (Malik *et al.*, 1983; Roberts *et al.*, 1983). Since pollen contains no functional chloroplasts, it has been proposed that CO_2-stimulated pollen germination may occur through non-photosynthetic fixation of CO_2 by PEPC. The expression of *PEPC-7* in alfalfa pollen is consistent with a functional role for PEPC in pollen development or germination.

We were interested in further characterizing the PEPC promoter region. To do this, we constructed a series of promoter deletion constructs and analyzed their activity in transgenic plants. Our results suggest that the region between –1265 and –705 contains an element(s) that influences the amount of PEPC gene transcription in the symbiotic zone and represses transcription in the meristem (compare Figure 5a, b, c and d). This region of the promoter contains one member of a 16 bp repeat, both members of a 14 bp repeat, and two of the five (A/T)AAGAT sequence motifs that may be important in *PEPC-7* transcriptional control.

Further deletion of the promoter region led to a complex change in the pattern of transcription. In plants containing the P4–GUS construct, GUS activity was nearly absent in the symbiotic zones and was much reduced in the nodule parenchyma and meristem. However, transcription was activated to high levels in vascular tissue throughout the plant (Figure 5e, f and j) Our interpretation of this result is that a negative regulatory element controlling the extent of PEPC gene transcription in vascular tissue was deleted

and a positive element that normally interacts with this element remains in the P4 promoter. The portion of the *PEPC-7* promoter between positions –634 and –536 contains a 42 bp purine-rich sequence beginning at position –579 (Figure 3) which is a candidate for the negative regulatory element. This sequence forms a mirror repeat centered around position –558. Such purine/pyrimidinerich motifs have been observed to form an intramolecular triple helix structure known as H-DNA (Htun and Dahlberg, 1988), which have been suggested to play a role in regulation of gene expression (Lu and Ferl, 1992; Mirkin and Frank-Kamenetskii, 1994; Wells *et al.*, 1988). The H-DNA structure that may form in this region is shown in Figure 7. A similar purine-rich sequence has been identified in the maize *Adh1* promoter within a region required for the induction of this gene under anaerobic stress (Lu and Ferl, 1992). Evidence supporting the formation of H-DNA in the *Adh1* promoter has been obtained from *in vitro* studies (Lu and Ferl, 1992) and it has been proposed that this sequence motif plays an important role in regulating *Adh1* gene transcription (Lu and Ferl, 1992). The presence of a comparable sequence motif in a region of *PEPC-7* promoter that appears to regulate cell-specific expression in nodules opens the possibility that H-DNA may also be an important structure in the regulation of *PEPC-7* transcription. This may be a particularly salient feature since the interior of root nodules contain little free O_2.

The fact that the region between –634 and –536 appears to affect control of PEPC expression in the nodule parenchyma has implications for gas diffusion into nodules. One of the key components associated with regulation of nitrogenase activity and nodule development is nodule O_2 concentration (Soupène *et al.*, 1995). Too much free O_2 in the symbiotic zone irreversibly inactivates nitrogenase while too little O_2 impedes ATP production. Several studies have shown that a variable O_2 diffusion barrier within the parenchyma regulates O_2 supply to nitrogenase (Hunt and Layzell, 1993; Witty and Minchin, 1994; references therein). Changes in nitrogenase activity associated with a wide array of treatments have been attributed to rapid increases or decreases in the variable O_2 diffusion barrier (Vance and Heichel, 1991). The mechanisms contributing to regulation of the O_2 diffusion barrier, although currently unknown, have been postulated to involve modifications in hydroxyprolinerich glycoproteins; shifts in influx and efflux of calcium; modulation in carbohydrate metabolism; and malate-induced changes in pH and proton extrusion (similar to that occurring in guard cells and pulvini). The presence of a specific promoter element involved in directing PEPC gene expression to the nodule parenchyma plus immunogold localization of PEPC protein to this area (Robinson *et al.*, 1996) suggest that malate derived from oxaloacetate synthesized via PEPC may be a factor in regulation of the variable O_2 diffusion barrier. Recent

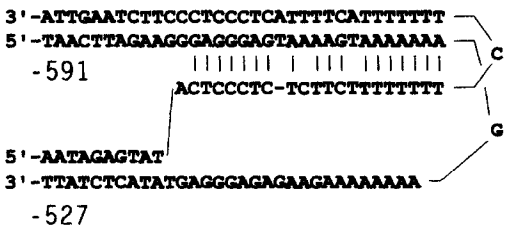


Figure 7. Proposed structure of H-DNA formed in the *PEPC-7* promoter.

results from de la Pena *et al.* (1996) showing localized expression of a carbonic anhydrase gene to the nodule parenchyma further strengthen our hypothesis.

In contrast to the changing pattern of *GUS* expression in nodules, the pattern of reporter gene expression in roots and in cells associated with leaf, root and nodule vascular bundles was similar in plants transformed with the P6–GUS, P5–GUS or P4–GUS constructs, although a large quantitative change was observed in vascular expression. This observation suggests that expression of *PEPC-7* in nodules is regulated by mechanisms different from those regulating *PEPC-7* expression in roots and vascular bundles.

The importance of PEPC in CO_2 fixation in C4 and CAM plants and in nitrogen assimilation in legumes is well documented. This enzyme may play an additional adaptive role in stress since it is induced by phosphate starvation in lupine proteiod roots (Johnson *et al.*, 1994) and by high concentrations of salt in tomato roots (Cramer and Lips, 1995). Regulation of PEPC activity is known to be controlled both transcriptionally and post-translationally. Further investigation of the mechanisms by which PEPC activity responds to multiple developmental and physiological signals may provide insight into how primary metabolic pathways can be adapted to specialized functions.

Experimental procedures

Isolation and characterization of PEPC genomic and cDNA clones

A λDASH II (Stratagene) alfalfa (*Medicago sativa* L.) cv Saranac genomic DNA library (Gregerson *et al.*, 1994) was screened by hybridization with a [^{32}P]-labeled probe derived from the alfalfa PEPC cDNA clone pPEPC-61 (Pathirana *et al.*, 1992). Several clones were found to hybridize to probes corresponding to the 3′ and 5′ ends of the cDNA clone. One genomic clone, λPEPC7, was chosen for further study.

Several overlapping restriction fragments of λPEPC-7 were subcloned into the plasmid vector pBluescript KS$^-$ (Stratagene) for sequencing. Overlapping deletions of these subclones were generated as described by Henikoff (1987), and, from these individual fragments, 7490 base pairs of sequence, containing the entire transcribed region of the *PEPC-7* gene, were determined. Double-stranded DNA was sequenced by the chain termination method using Sequenase 2.0 (US Biochemicals).

To obtain the sequence in the 3′ untranslated region of several

PEPC cDNA clones, a γgt22A (Gibco/BRL) alfalfa cv. Saranac cDNA library (Gregerson *et al.*, 1993) was screened with a [^{32}P]-labeled probe corresponding to nucleotides 1 to 507 of alfalfa PEPC cDNA (Pathirana *et al.*, 1992). Two oligonucleotide primers, 5′-CCGTACGACTTGTGACCAAT-3′ (PEPC-3L) and 5′-GCGTATAGT-AGCAGCAGTCA-3′ (PEPC-3R), complementary to nucleotides 2986–3005 and 3258–3239 of pPEPC-61 cDNA, respectively, were synthesized and used in a 1:50 ratio PEPC-3L:PEPC-3R to amplify single-stranded DNA asymmetrically from 13 PEPC-positive recombinant phage (McCabe, 1990). The amplified DNA was sequenced directly.

RNase protection analysis

RNase protection experiments were performed with slight modifications of the procedure described by Firestein *et al.* (1987). A radiolabeled antisense RNA probe was synthesized using [α-^{32}P]UTP and T3 RNA polymerase (Stratagene). This RNA probe corresponds to the nucleotides +113 to −72 of *PEPC-7* (Figure 3). Total RNA extracted from alfalfa nodules, roots or leaves was mixed with 5×10^5 cpm of probe in 15 μl of 5 M guanidine thiocyanate and incubated at 27°C for 16 h. Reactions were treated with 4 units of RNase A (US Biochemicals) and 160 units of RNase T1 (US Biochemicals) in 450 μl RNase digestion buffer (30 mM Tris–HCl, pH 7.2; 300 mM NaCl; 1 mM EDTA) for 60 min at 37°C. Digestion products were treated with 200 μg ml^{-1} proteinase K for 30 min at 37°C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated in ethanol. The protected RNA fragments were fractionated by electrophoresis in a 6% polyacrylamide sequencing gel.

In situ *hybridization*

To generate a suitable template for *in vitro* transcription of *in situ* hybridization probes, a 798 bp *Pst*I/*Eco*RI fragment of the pPEPC-61 plasmid (Pathirana *et al.*, 1992) was isolated and cloned into pBluescript KS$^-$. This plasmid (pPEPC-IS) was then digested with *Eco*RI prior to the synthesis of an antisense RNA probe by *in vitro* transcription with T7 RNA polymerase. Control probe was transcribed by T3 RNA polymerase after digestion of pPEPC-IS with *Xba*I. RNA probes were synthesized using digoxigenin-11-UTP (Boehringer Mannheim), and, prior to alkaline hydrolysis, the amount of labeled transcript was estimated as suggested by the manufacturer.

In situ hybridizations were performed essentially as described by Bochenek and Hirsch (1990). Plant tissues were fixed overnight at 4°C in a glutaraldehyde/paraformaldehyde solution and embedded in paraffin. The fixed tissues were sectioned (9 μm) and attached to Vectabond-coated slides. Digoxigeninlabeled probe was detected using alkaline phosphataseconjugated antidigoxigenin antibodies as described by the manufacturer (Boehringer Mannheim). At least 75 nodules were evaluated during these studies.

Construction of chimeric genes

Six overlapping deletion fragments of the 5′ flanking region of *PEPC-7* were generated by cleavage at restriction endonuclease sites. The 5′ ends of these promoter fragments, designated as P1, P2, P3, P4, P5 and P6, were 58, 134, 285, 536, 634 or 1277 nucleotides upstream from the 5′ end of the mature *PEPC-7* transcript (+1), respectively (Figure 3). The 3′ end of each of these fragments was at position +86. DNA fragments were initially subcloned into pBluescript KS$^-$ (P1, P2, P3, P4) or SK+ (P5, P6) (Stratagene) vectors and subsequently removed by cleavage at the *Sal*I and *Bam*HI restriction sites (in the case of P6), the *Hin*dIII and *Bam*HI restriction sites (in the case of P5, P3, P2 and P1), or the *Xba*I and *Sma*I restriction sites (in the case of P4), and inserted into the corresponding sites in the plant transformation vector pBI101 (Jefferson, 1987), thereby producing P6–GUS, P5–GUS, P4–GUS, P3–GUS, P2–GUS and P1–GUS chimeric reporter genes.

Alfalfa transformation

The promoter–*GUS* constructs were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. Leaf disks of a highly regenerable clone selected from Regen SY (Bingham, 1991) were inoculated and cultured essentially by the method of Austin *et al.* (1995). Briefly, surface-disinfected leaf explants were dipped into an overnight bacterial culture, blotted briefly to remove excess liquid, and then plated on B5h medium (Brown and Atanassov, 1985). Explants were cocultured for 8 days, then rinsed three times in sterile water, and placed on B5h containing 25 mg l^{-1} kanamycin and 500 mg l^{-1} ticarcillin. After 21 days, explants and calli were transferred to B5h medium with antibiotics but lacking plant growth regulators. Embryos were removed from callus tissue after three weeks and placed on MS medium (Murashige and Skoog, 1962) with antibiotics for plant development to occur. Only one transformed plant was assessed from each explant to ensure that each plant was the result of an independent transformation event. DNA was extracted from transgenic plants and subjected to PCR analysis to verify the integrity of the transgenes.

Analysis of β-glucuronidase activity

Histochemical detection of GUS activity in nodules, roots, leaves or flowers was performed by a modification of the protocol described by Jefferson (1987). Tissues, which were sliced for efficient uptake of the staining solution, were incubated in 1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc), 0.1 M sodium phosphate buffer (pH 7.5), 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 10 mM EDTA (pH 7.5) and 0.1% Triton X-100 *in vacuo* for 15 min followed by incubation at 37°C for 12 to 16 h. After staining, the tissues were rinsed in water and stored in 70% ethanol until they were photographed.

In order to observe cellular localization of GUS activity, stained nodules were resin-embedded and sectioned. The stained tissues were fixed in 3% glutaraldehyde and 100 mM sodium phosphate, pH 7, in vacuo for 3 h. After rinsing the tissues with distilled water, they were dehydrated by incubation in 25, 50, 75 and 95% ethanol for 15 min in each solution, followed by 100% ethanol for 2 h. Dehydrated tissues were then infiltrated and embedded in LR White resin and sectioned using a glass knife. Sections (4 μm) were mounted on slides and observed using darkfield conditions.

GUS activity was determined by fluorometry essentially as described by Jefferson (1987). Samples of nodules (20 mg), roots (50 mg) and leaves (50 mg) were removed from plants and homogenized in extraction buffer (10 μl mg^{-1}), pelleted, and the supernatant used to quantify GUS activity with 4methylumbelliferyl β-D-glucuronic acid (MUG) as the substrate. Tissues from regenerated nontransformed plants were used to quantify background activity. Enzyme activity in each tissue, less background, was compared by single-factor analysis of variance and means separated by the Tukey–Kramer multiple comparisons test.

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References

Allaway, W.G. (1973) Accumulation of malate in guard cells of *Vicia faba* during stomatal opening. *Planta,* **110,** 63–70.

Austin, S., Bingham, E.T., Mathews, D.E., Shahan, M.N., Will, J. and Burgess, R.R. (1995) Production and field performance of transgenic alfalfa (*Medicago sativa* L.) expressing α-amylase and manganese-dependent lignin peroxidase. *Euphytica,* **85,** 381–393.

Bialczyk, J. and Lechowski, Z. (1986) Diurnal changes in the malic acid concentration in *Phaseolus coccineus* L. pulvini. *Plant Cell Physiol.* **27,** 981–987.

Bingham, E.T. (1991) Registration of alfalfa hybrid Regen-SY germplasm for tissue culture and transformation research. *Crop Sci.* **31,** *1098.*

Bochenek, B. and Hirsch, A.M. (1990) *In-situ* hybridization of nodulin mRNAs in root nodules using non-radioactive probes. *Plant Mol. Biol. Rep.* **8,** 237–248.

Bowes, G. (1993) Facing the inevitable: Plants and increasing atmospheric CO_2. *Annu. Rev. Plant Physiol. Mol. Biol.* **44,** 309–327.

Brown, D.C.W. and Atanassov, A. (1985) Role of genetic background in somatic embryogenesis in *Medicago. Plant Cell Tissue Org. Cult.* **4,** 111–122.

de Bruijn, F.J. and Schell, J. (1993) Regulation of plant genes specifically induced in developing and mature nitrogen-fixing nodules: *cis*-acting elements and *trans*-acting factors. In *Control of Plant Gene Expression* (Verma, D.P.S., ed.). Boca Raton: CRC Press, pp. 241–258.

Christensen, A.H. and Quail, P.H. (1989) Sequence analysis and transcriptional regulation by heat shock of polyubiquitin transcripts from maize. *Plant Mol. Biol.* **12,** 619–632.

Christensen, A.H., Sharrock, R.A. and Quail, P.H. (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Mol. Biol.* **18,** 675–689.

Cramer, M.D. and Lips, S.H. (1995) Enriched rhizosphere CO_2 concentrations can ameliorate the influence of salinity on hydroponically grown tomato plants. *Plant Physiol.* **94,** 425–432.

Cushman, J.C. and Bohnert, H.J. (1989a) Nucleotide sequence of the Ppc2 gene encoding a housekeeping isoform of phosphoenolpyruvate carboxylase from *Mesembryanthemum crystallinum. Nucl. Acids Res.* **17,** 6743–6744.

Cushman, J.C. and Bohnert, H.J. (1989b) Nucleotide sequence of the gene encoding a CAM specific isoform of phosphoenolpyruvate carboxylase from *Mesembryanthemum crystallinum. Nucl. Acids Res.* **17,** 6745–6746.

Deroche, M.E. and Carrayol, E. (1988) Nodule phosphoenolpyruvate carboxylase: a review. *Physiol. Plant.* **74,** 775–782.

Egli, M.A., Griffith, S.M., Miller, S.S., Anderson, M.P. and Vance, C.P. (1989) Nitrogen assimilating enzyme activity and enzyme protein during development and senescence of effective and plant-controlled ineffective alfalfa nodules. *Plant Physiol.* **91,** 898–904.

Firestein, G.S., Gardner, S.M. and Roeder, W.D. (1987) Quantitative molecular hybridization with unfractionated solubilized cells using RNA probes and polyacrylamide gel electrophoresis. *Anal. Biochem.* **167,** 381–386.

Forde, B.G., Day, H.M., Turton, J.F., Shen, W.-J., Cullimore, J.V. and Oliver, J.E. (1989) Two glutamine synthetase genes from *Phaseolus vulgaris* L. display contrasting developmental and spatial patterns of expression in transgenic *Lotus corniculatus* plants. *Plant Cell,* **1,** 391–401.

Gantt, J.S., Larson, R.J., Farnham, M.W., Pathirana, M.S., Miller, S.S. and Vance, C.P. (1992) Aspartate aminotransferase in effective and ineffective alfalfa nodules: cloning of a cDNA and determination of enzyme activity, protein and mRNA levels. *Plant Physiol.* **98,** 868–878.

Gregerson, B.G., Miller, S.S., Twary, S.N., Gantt, J.S. and Vance, C.P. (1993) Molecular characterization of NADH-dependent glutamate synthase from alfalfa nodules. *Plant Cell,* **5,** 215–226.

Gregerson, B.G., Miller, S.S., Petrowski, M., Gantt, J.S. and Vance, C.P. (1994) Genomic structure, expression and evolution of the alfalfa aspartate aminotransferase genes. *Plant Mol. Biol.* **25,** 387–399.

Hanley, B.A. and Schuler, M.A. (1988) Plant intron sequences: evidence for distinct groups of introns. *Nucl. Acids Res.* **16,** 7159–7176.

Hatch, M.D. (1992) C4 Photosynthesis: an unlikely process full of surprises. *Plant Cell Physiol.* **33,** 333–342.

Henikoff, S. (1987) Unidirectional digestion with exonuclease III in DNA sequence analysis. *Methods Enzymol.* **155,** 156–165.

Hermans, J. and Westhoff, P. (1992) Homologous genes for the C4 isoform of phosphoenolpyruvate carboxylase in a C3 and a C4 *Flavaria* species. *Mol. Gen. Genet.* **234,** 275–284.

Hirsch, A.M. (1992) Developmental biology of legume nodulation. *New Phytol.* **122,** 211–237.

Hosokawa, Y. and Kiyosawa, K. (1983) Diurnal K^+ and anion transport in *Phaseolus pulvini. Plant Cell Physiol.* **24,** 1065–1072.

Htun, H. and Dahlberg, J.E. (1988) Single strands, triple strands, and kinks in H-DNA. *Science,* **241,** 1791–1796.

Hudspeth, R.L. and Grula, J.W. (1989) Structure and expression of the maize gene encoding the phosphoenolpyruvate carboxylase isozyme involved in C4 photosynthesis. *Plant Mol. Biol.* **12,** 579–589.

Hunt, S. and Layzell, D.B. (1993) Gas exchange of legume nodules and the regulation of nitrogenase activity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44,** 483–511.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* **5,** 387–405.

Johnson, J.E., Allan, D.L. and Vance, C.P. (1994) Phosphorous stressed-induced proteoid roots show altered metabolism in *Lupinus albus. Plant Physiol.* **104,** 657–665.

Jorgensen, J.E., Stougaard, J., Marcker, A. and Marcker, K.A. (1988) Root nodule-specific gene regulation: analysis of the soybean N23 gene promoter in heterologous symbiotic systems. *Nucl. Acids Res.* **16,** 39–50.

Kouchi, H., Fukai, K., Katagiri, H., Minamisawa, K. and Tajima, S. (1988) Isolation and enzymological characterization of infected and uninfected cell protoplasts from root nodules of *Glycine max. Physiol. Plant.* **73,** 327–334.

Latzko, E. and Kelly, G.J. (1983) The many-faceted function of phosphoenolpyruvate carboxylase in C3 plants. *Physiol. Veg.* **21,** 805–815.

Lu, G. and Ferl, R.J. (1992) Site-specific oligodeoxynucleotide binding to maize Adh1 gene promoter represses Adh1–GUS gene expression *in vivo. Plant Mol. Biol.* **19,** 715–723.

McCabe, P.C. (1990) Production of single stranded DNA by asymmetric PCR. In *PCR Protocols. A Guide to Methods and Applications* (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., eds). San Diego: Academic Press, pp. 76–83.

Malik, C.P., Basra, A.S. and Gill, R.K. (1983) Non-photosynthetic

fixation of carbon dioxide in pollen tube growth and metabolism. *J. Palinol.* **19,** 207–221.

Marcker, A., Lund, M., Jensen, E.O. and Marcker, K.A. (1984) Transcription of the soybean leghemoglobin genes during nodule development. *EMBO J.* **3,** 1691–1695.

Maxwell, C.A., Vance, C.P., Heichel, G.H. and Stade, S. (1984) CO_2 fixation in alfalfa and birdsfoot trefoil root nodules and partitioning of ^{14}C to the plant. *Crop Sci.* **24,** 257–264.

Mayer, W.E., Flach, D., Raju, M.V.S., Starrach, N. and Wiech, E. (1985) Mechanics of circadian pulvini movements in *Phaseolus coccineus* L. *Planta,* **163,** 381–390.

Metz, B.A., Welters, P., Hoffman, H.-J., Jensen, E.O., Schell, J. and de Bruijn, F.J. (1988) Primary structure and promoter analysis of leghemoglobin genes of the stem-nodulated tropical legume *Sesbania rostrata*: conserved coding sequences, *cis*-elements and *trans*-acting factors. *Mol. Gen. Genet.* **214,** 181–191.

Michalke, B. and Schnabl, H. (1987) The status of adenine nucleotides and malate in chloroplast mitochondria and supernatant of guard cell protoplasts from *Vicia faba*. *J. Plant Physiol.* **130,** 243–253.

Miller, S.S., Boylan, K.L.M. and Vance, C.P. (1987) Alfalfa root nodule carbon dioxide fixation III. Immunological studies of nodule phosphoenolpyruvate carboxylase. *Plant Physiol.* **84,** 501–508.

Mirkin, S.M. and Frank-Kamenetskii, M.D. (1994) HDNA and related structures. *Annu. Rev. Biophys. Biomol. Struct.* **23,** 541–576.

Mittler, R. and Zilinskas, B.A. (1992) Molecular cloning and characterization of a gene encoding pea cytosolic ascorbate peroxidase. *J. Biol. Chem.* **267,** 21 802–21 807.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15,** 473–497.

O'Leary, M.H. (1982) Phosphoenolpyruvate carboxylase: an enzymologist's view. *Annu. Rev. Plant Physiol.* **33,** 297–315.

Pathirana, S.M., Vance, C.P., Miller, S.S. and Gantt, J.S. (1992) Alfalfa root nodule phosphoenolpyruvate carboxylase: characterization of the cDNA and expression in effective and plant-controlled ineffective nodules. *Plant Mol. Biol.* **20,** 437–450.

Pearson, L. and Meagher, R.B. (1990) Diverse soybean actin transcripts contain a large intron in the 5′ untranslated leader: structural similarity to vertebrate muscle actin genes. *Plant Mol. Biol.* **14,** 513–526.

de la Pena, T.C., Frugier, F., McKhann, H.I., Bauer, P., Brown, S., Kondorosi, A. and Crespi, M.D. (1997) A carbonic anhydrase gene is induced in the nodule primordium and its cell-specific expression is controlled by the presence of *Rhizobium* during development. *Plant J.* **11,** 407–420.

Raschke, K. and Schnabl, H. (1978) Availability of chloride affects balance between potassium chloride and potassium malate in guard cells of *Vicia faba* L. *Plant Physiol.* **62,** 84–87.

Roberts, I.N., Guade, T.C., Harrod, G. and Dikinson, H.G. (1983) Pollen-stigma interactions in *Brassica oleracea*: a new pollen germination medium and its use in elucidating the mechanism of self incompatibility. *Theor. Appl. Genet.* **65,** 231–238.

Robinson, D.L., Pathirana, S.M., Gantt, J.S. and Vance, C.P. (1996) Immunogold localization of nodule-enhanced phospho-enolpyruvate carboxylase in alfalfa. *Plant Cell Environ.* **19,** 602–608.

Smith, A.M. (1985) Capacity for fermentation in roots and *Rhizobium* nodules of *Pisum sativum* L. *Planta,* **166,** 264–270.

Soupène, E., Foussard, M., Boistard, P., Truchet, G. and Batut, J. (1995) Oxygen as a key developmental regulator of *Rhizobium meliloti* N_2-fixation gene expression within the alfalfa root nodule. *Proc. Natl Acad. Sci. USA,* **92,** 3759–3763.

Stougaard, J., Sandal, N.N., Gron, A., Kuhle, A. and Marcker, K.A. (1987) 5′ analysis of the soybean leghaemoglobin lbc3 gene: regulatory elements required for promoter activity and organ specificity. *EMBO J.* **6,** 3565–3569.

Suganuma, N., Kitou, M. and Yamamoto, Y. (1987) Carbon metabolism in relation to cellular organization of soybean root nodules and respiration of mitochondria aided by leghemoglobin. *Plant Cell Physiol.* **28,** 113–122.

Szczyglowski, K., Szabados, L., Fujimoto, S.Y., Silver, D. and de Bruijn, F.J. (1994) Site-specific mutagenesis of the nodule-infected cell expression (NICE) element and the ATrich element ATRE-BS2* of the *Sesbania rostrata* leghemoglobin *glb3* promoter. *Plant Cell,* **6,** 317–332.

Ta, C.T., Faris, M.A. and MacDowall, F.D.H. (1986) Pathways of nitrogen metabolism in nodules of alfalfa (*Medicago sativa* L.). *Plant Physiol.* **80,** 1002–1005.

Ting, I.P. (1985) Crassulacean acid metabolism. *Annu. Rev. Plant. Physiol.* **36,** 595–622.

Ting, I.P. and Osmond, C.P. (1973a) Photosynthetic phosphoenolpyruvate carboxylases. Characteristics of alloenzymes from leaves of C3 and C4 plants. *Plant Physiol.* **51,** 439–447.

Ting, I.P. and Osmond, C.P. (1973b) Multiple forms of plant phosphoenolpyruvate carboxylase associated with different metabolic pathways. *Plant Physiol.* **51,** 448–453.

Toh, H., Kawamura, T. and Izui, K. (1994) Molecular evolution of phosphoenolpyruvate carboxylase. *Plant Cell Environ.* **17,** 31–43.

Utter, M.F. and Kolenbrander, H.M. (1972) Formation of oxaloacetate by CO_2 fixation on phosphoenolpyruvate. In *The Enzymes* (Boyer, P.D., ed.). New York: Academic Press, pp. 117–136.

Vance, C.P. and Heichel, G.H. (1991) Carbon in N_2 fixation: limitation or exquisite adaptation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42,** 373–392.

Vanhinsberg, N. and Horton, R.F. (1990) Carbon dioxide assimilation in pulvini of *Phaseolus vulgaris* L. *Biochem. Physiol. Pflanzen,* **186,** 37–42.

Vasse, J., de Billy, F., Camut, S. and Truchet, G. (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *J. Bacteriol.* **172,** 4295–4302.

Walsh, K.B., McCully, M.E. and Canny, M.J. (1989) Vascular transport and soybean nodule function: nodule xylem is a blind alley, not a throughway. *Plant Cell Environ.* **12,** 395–405.

Wells, R.D., Collier, D.A., Jeffery, C.H., Shimizu, M. and Wohlrab, F. (1988) The chemistry and biology of unusual DNA structures adopted by oligopurine·oligopyrimidine sequences. *FASEB J.* **2,** 2939–2949.

Werr, W., Frommer, W.B., Mass, C. and Starlinger, P. (1985) The structure of the sucrose synthase gene on chromosome 9 of *Zea mays* L. *EMBO J.* **4,** 1373–1380.

Witty, J.F. and Minchin, F.R. (1994) A new method to detect the presence of continuous gas-filled pathways for oxygen diffusion in legume nodules. *J. Exp. Bot.* **45,** 967–978.

Yanai, Y., Okumura, S. and Shimada, H. (1994) Structure of *Brassica napus* phosphoenolpyruvate carboxylase genes: missing introns causing polymorphisms among gene family members. *Biosci. Biotech. Biochem.* **58,** 950–953.

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