Carbon Metabolism in Developing Soybean Root Nodules: The Role of Carbonic Anhydrase

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A full-length cDNA clone encoding carbonic anhydrase (CA) was isolated from a soybean nodule cDNA library. In situ hybridization and immunolocalization were performed in order to assess the location of CA transcripts and protein in developing soybean nodules. CA transcripts and protein were present at high levels in all cell types of young nodules, whereas in mature nodules they were absent from the central tissue and were concentrated in cortical cells. The results suggested that, in the earlier stages of nodule development, CA might facilitate the recycling of CO₂ while at later stages it may facilitate the diffusion of CO₂ out of the nodule system. In parallel, sucrose metabolism was investigated by examination of the temporal and spatial transcript accumulation of sucrose synthase (SS) and phosphoenolpyruvate carboxylase (PEPC) genes, with in situ hybridization. In young nodules, high levels of SS gene transcripts were found in the central tissue as well as in the parenchymatous cells and the vascular bundles, while in mature nodules the levels of SS gene transcripts were much lower, with the majority of the transcripts located in the parenchyma and the pericycle cells of the vascular bundles. High levels of expression of PEPC gene transcripts were found in mature nodules, in almost all cell types, while in young nodules lower levels of transcripts were detected, with the majority of them located in parenchymatous cells as well as in the vascular bundles. These data suggest that breakdown of sucrose may take place in different sites during nodule development.

During legume root nodule development and functioning, photosynthate, in the form of sucrose, is the primary source of energy and carbon skeletons provided to both the bacteroids and the plant tissues. Sucrose arrives at the developing nodule through the nodular vascular system and is translocated apoplastically and/or symplastically into the cells, where it is hydrolyzed predominantly by sucrose synthase (Day and Copeland 1991). Immunological and RNA blot analyses of several legumes indicated that sucrose synthase (SS) gene expression is enhanced during effective nodule development while it remains low in ineffective nodule de-

velopment (Anthon and Emmerich 1990; Vance et al. 1997). Accumulation of SS gene transcripts was detected in infected and uninfected cells of the symbiotic zone in indeterminate nodules (de la Pena et al. 1997) and the central tissues as well as the vascular system of determinate nodules (van Ghelue et al. 1996). An exception are the alder nodules, where expression of the SS gene appears to be restricted to the infected cells and to the pericycle of the vascular bundles (van Ghelue et al. 1996).

At the sites of SS activity in nodules, the hydrolyzed products of sucrose (fructose and UDP-glucose) are metabolized by the glycolytic enzymes and/or are used as substrates for cellulose or starch biosynthesis. In plants, a major branch point in glycolysis proceeds from phosphoenolpyruvate to oxaloacetate via the phosphoenolpyruvate carboxylase (PEPC) reaction (Lance and Rustin 1984). It has been demonstrated that expression of PEPC mRNA and protein levels are several-fold elevated during alfalfa (Egli et al. 1989; Pathirana et al. 1992), pea (Suganuma et al. 1997), and soybean (Hata et al. 1998) nodule development. Furthermore, it was recently demonstrated that there are at least three PEPC genes in soybean coding for the respective isoforms (Hata et al. 1998). It was also shown by in situ hybridization and reverse transcription-polymerase chain reaction (RT-PCR) that one of the soybean isoforms is nodule enhanced while another is constitutively expressed (Hata et al. 1998). One of the substrates of the PEPC-catalyzed reaction, bicarbonate, is derived from the solubilization of CO₂ and/or the hydration of CO₂ by carbonic anhydrase (CA). High levels of CA gene transcripts and enzyme activity have been detected during early stages of alfalfa nodule development. However, in mature nodules, the CA gene transcript levels were low and only detected in the inner parenchyma (de la Pena et al. 1997).

The oxaloacetate produced through the action of PEPC could be used either as a substrate for the synthesis of malate or as a source of carbon skeletons for the synthesis of amino acids, amides, or ureides (Chollet et al. 1996). Moreover, the activity of a malate dehydrogenase (MDH) isoform was recently reported to be significantly enhanced in alfalfa developing nodules (Miller et al. 1998). Biochemical studies have suggested that malate and/or succinate, rather than sucrose, are the major, if not the only, carbon compounds transported through the symbiosome membrane, thus providing energy for nitrogen assimilation and carbon skeleton for bacteroid respiration or for biosynthetic processes (Finan et al. 1983; Ronson et al. 1981; Vance and Heichel 1991).

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In this report, we have identified a cDNA clone coding for CA, which is expressed in soybean nodules. The level of accumulation of CA gene transcripts and CA protein was also investigated during soybean nodule development. Our data support the view that the role of CA in CO₂ metabolism may differ between young and mature nodules. We have also examined the spatial and temporal accumulation of gene transcripts coding for enzymes involved in sucrose breakdown (SS) and oxaloacetate production (PEPC). The data obtained suggest that the sites of sucrose breakdown may vary according to the stages of nodule development.

RESULTS

Isolation of cDNA clones coding for CA and PEPC.

To study the expression of genes coding for enzymes involved in CO₂ metabolism, we were concerned with the isolation of cDNA clones coding for CA and PEPC. To this end, a soybean λ gt11 cDNA library prepared from 21-day old nodules was screened under low stringency conditions with a heterologous cDNA (MsCA1)—coding for an alfalfa CA—as a probe. Seven positive clones were identified and purified through successive rounds of screening. The cDNA inserts were cloned into pBluescript KS⁺ and their nucleotide sequence was then determined. The largest insert was 0.85 kbp long (designated as GmCA for *Glycine max* carbonic anhydrase) and contained an open reading frame of 702 bp encoding a polypeptide of 234 amino acids. The sequencing data revealed that none of the cDNA clones has a putative translation start. Re-screening of the cDNA library failed to detect a larger cDNA clone. To obtain a full-length cDNA clone we applied a PCR 5' primer extension technique (Breda et al. 1996). Briefly, PCR amplification was carried out with an oligonucleotide pair designed from a region of GmCA (P1 oligonucleotides) and the pBluescript T3 primer as a primer and an excised λ ZAP cDNA library from soybean nodules as a template. A 300-bp amplification product was sequenced and turned out to be the missing 5' fragment. The total length of the overlapping cDNA clones was 1,082 bp and the largest open reading frame was 783 bp coding for 261 amino acids (Fig. 1). The predicted molecular mass of the translated protein was 29,498 Da, with a calculated pI of 6.25. The deduced amino acid sequence was compared with the amino acid sequences of other CA proteins, from both photosynthetic and nonphotosynthetic tissues (Fig. 1). Phylogenetic analysis inferred from amino acid sequence data of published plant CA genes revealed that soybean CA is closest to that of alfalfa (data not shown). The soybean CA shows 72.3% similarity to the alfalfa nodular CA (de la Pena et al. 1997). The putative soybean protein contains four residues at the positions C 87, E 131, H 147, and C 150 that have been proposed as essential for CA activity and may be occupied with the binding of zinc to the protein molecule (Provart et al. 1993).

Southern blot analysis revealed the presence of two *Eco*RI hybridizing bands (data not shown), suggesting that in soybean there may be two genes or two alleles coding for CA. Northern (RNA) blot analysis indicated that CA transcripts are present in 15 days post infection (d.p.i.) nodules, leaves, roots, and siliques. However, in nodules the transcript level decreases during nodule maturation (data not shown). In situ hybridization studies revealed that the GmCA riboprobe strongly

hybridized with RNAs present in certain cell types in photosynthetic tissues and nodules (Figs. 2 A–C and 3A–D).

Several PEPC cDNA clones were isolated from a λ gt11 cDNA library constructed from mRNA extracted from 21-day-old soybean nodules, with the 5' end fragment of a *Medicago sativa* PEPC cDNA clone as the heterologous probe. These clones were analyzed and all contained partial cDNA fragments that were highly homologous (99% identity) to the soybean PEPC7 cDNA sequence (Hata et al. 1998). The largest cDNA clone (1,000 bp) was designated as GmPEPC.

Spatial and temporal accumulation of PEPC and CA transcript.

The histological localization of the CA and PEPC gene transcripts during soybean nodule development was studied with digoxigenin (DIG)-labeled riboprobes and in situ hybridization. In young nodules (10 d.p.i.), low levels of PEPC transcripts were detectable mainly in the cortical cells and vascular bundles (Fig. 3E). At later stages of nodule development (15 d.p.i.) a relatively stronger signal was observed, particularly in the pericycle cells (Fig. 3F). In mature nodules (28 d.p.i.), the accumulation of PEPC transcripts was significantly enhanced in the same peripheral cells. High levels of PEPC transcript accumulation were also detected in both infected and uninfected cells (Fig. 3G).

The accumulation and distribution of CA transcripts were different from those observed for the PEPC transcripts: in young nodules (10 d.p.i.), high levels of CA transcripts were localized in the cortical cells, including the vascular bundles. An easily detectable signal was also observed in both infected and uninfected cells of the central tissue (Fig. 3A). A similar spatial pattern was observed in older nodules (15 d.p.i.), but lower signal intensity was observed in the central tissue and the vascular bundles (Fig. 3B). The decreased signal intensity was particularly evident in the vascular bundles (Fig. 3B).

At later stages (21 and 28 d.p.i.), the hybridization signal was detected in some cell layers (the inner cortex) surrounding the nodule central tissue, suggesting that the CA gene is mainly transcriptionally active in these cells (Fig. 3C and D). Low transcript levels were found in other external peripheral tissues while the expression of this gene was barely (or not) detectable in the central tissue.

Localization of CA in developing soybean nodules.

The distribution and concentration of CA gene transcripts raised questions concerning the localization of CA protein in these tissues; thus, it was of interest to study the abundance of CA protein in various cell types of developing soybean nodules. To this purpose, GmCA was expressed in *Escherichia coli* and the overexpressed CA protein was purified. Polyclonal antibodies were raised against the recombinant CA protein. A significant degree of cross-reactivity was observed between the recombinant CA and the anti-CA serum (Fig. 4C, lane 2). When extracted root nodule protein was incubated with the immune sera against the recombinant CA, two bands of approximately 29 and 27 kDa were detected (Fig. 4C, lane 1). These immune sera were used to localize the relative accumulation of CA protein in various tissues of young and mature nodules with a tissue print approach. The results obtained clearly suggested that in mature nodules CA protein accumulates in the periphery of the nodular tissue, while no

	10	20	30	40	50	60
GMCA1	-----	-----	-----	-----	-----	-----
MSCA1	-----	-----	-----	-----	-----	-----
ATCAH	MSTAPLSGFFLTSLSPSQSSLQKLSLRTSSSTVACLPPASSSSSSSSSSSSSRVPTLIRNE					
PSCAMRA	MSTSSINGFSLSSLSPAKTSTKRTTLR-----PFVSASLNTSSSSSSSTFPSLIQDK					
SOCCA	MST--ING-CLTSISPS-----RTQLKNTSTLR--PTFIANSRVNPSSSVPPSLIFTRNQ					
ATCAN	-----	-----	-----	-----	-----	-----
	70	80	90	100	110	120
GMCA1	-----	MAK-SSELAIEELKKLLRDKEEFDAVAAAKVEELIAELQ-GCG				
MSCA1	-----	MANQSSELAIEQLKKLLREKEELNEVATTKIEELIVELQ-GCH				
ATCAH	PVFAAPAPIIAPYWEEMGTEAYDEAIEALKLLIEKEELKTVAALKVEQITAALQTGTS					
PSCAMRA	PVFASSSPIITPVLREEMGK-GYDEAIEELQKLLREKTELKATAAEKVEQITAQLGTTSS					
SOCCA	PVFAAPAPIITPTLKEDM---AYEEAIAALKLLSEKGELENEAASKVAQITSELADGGF					
ATCAN	-----	MGNESYEDAIEALKLLIEKDDLKDVAALKVKITADVQAASS				
	-----	Mg--syeeAIEaLKKLLrEKeELk-vAAaKVeqitaeLq-g-s				
	130	140	150	160	170	180
GMCA1	PRPY--EPAVQRIVDGFTHFKINNFDKNSDLYSQLANGQRPKYLVFSCSDSRVSATTILN					
MSCA1	PQPFTIDPAEQRIIDGFTYFKLNNFNKNPELYDRLAKGQSPKFMVFACSDSRVSPSIILN					
ATCAH	SDKKA-FDPVETIKQGFIFKKKEKYETNPALYGELAKGQSPKYMVFACSDSRVCPSHVLD					
PSCAMRA	SDGIPKSEASERIKTGFLHFKKEKYDKNPALYGELAKGQSPPFMVFACSDSRVCPSHVLD					
SOCCA	TTPSASY-PVQRIKEGFIKFKKEKYEKNPALYGELSKGQAPKFMVFACSDSRVCPSHVLD					
ATCAN	SDSKS-FDPVERIKEGFVTFKKEKYETNPALYGELAKGQSPKYMVFACSDSRVCPSHVLD					
	sdp-----	aveRIk-GF--FKkekyekNPALYgeLAKGQsPKfMVFACSDSRVcPShvLd				
	190	200	210	220	230	240
GMCA1	FQPG--EAFMVRNIANMVPPFNQLRYSGVGAAIEYAITALKVPNILVIGHSRCGGIQRIM					
MSCA1	FQPGFTEAFKVRNIANMVPPFNQLRYSGVGATFEYAITALKVESILVIGHSRCGGISRIM					
ATCAH	F--QPGDAFVVRNIANMVPPFDVKYGGVGAAIEYAVLHLKVENIVVIGHSAACGGIKGLM					
PSCAMRA	F--QPGEAFVVRNVANLVPPYDQAKYAGTGAAIEYAVLHLKVSNIIVVIGHSAACGGIKGLL					
SOCCA	FFTQPGEAFMVRNIANMVPPFDKDKYAGVGAAIEYAVLHLKVENIVVIGHSAACGGIKGLM					
ATCAN	F--HPGDAFVVRNIANMVPPFDVKYAGVGAAIEYAVLHLKVENIVVIGHSAACGGIKGLM					
	F--qpgeAFvVRNIANMVPPFDk-kYaGVGAAIEYAvlhLKVeNIvVIGHSAcGGIkgLM					
	250	260	270	280	290	300
GMCA1	SHPED--GSAPFDFIDDWVKIGLPAKLKVLKEYEGYDFKEQCKFCEKESVNNSLVNLKTY					
MSCA1	SHPEDFTGSAPYDFIDDWVKIGLSSSKVKVLKGHECNDFKEQCKFCEMDSVNNSLVNLKTY					
ATCAH	SF--PLDGNNSTDFIEDWVKICLPAKSKVISELGDSAFEDQCGRCEREAVNVSLANLLTY					
PSCAMRA	SF--PFDGTYSTDFIEEWVKIGL-AKAKVKAQHGDAPFAELCTHCEKEAVNASLGNLLTY					
SOCCA	SFFTPDAGPTTTDFIEDWVKICLPAKHKVLAEHGNATFAEQCTHCEKEAVNVSLGNLLTY					
ATCAN	SF--PLDGNNSTDFIEDWVKICLPAKSKVLAESSESAFEDQCGRCEREAVNVSLANRCTY					
	Sf--p-dG--stDFIEDWVKICLpAK-KVlaehe---F-eQC--CEkEaVNVSL-NLLTY					
	310	320	330	340	350	
GMCA1	PYVEKG--IRKNIALLGYYDFVIGEFKLWKYESHITEPISIPLCTH----					
MSCA1	PYVDSEFTIRNENLALLGGYYDFVNGEFKLWKYKTHVTKSVTIPLNGFDMTK					
ATCAH	PFV--REGLVKGTLALKGYYDFVKGAFELWGLEFGLSETSSV-----					
PSCAMRA	PFV--REGLVNKTLALKGYYDFVKGSEFELWGLEFGLSSTFSV-----					
SOCCA	PFVFTRDGLVKKTLALQGGYYDFVNGSFELWGLEYGLSPSQSV-----					
ATCAN	PFV--REGVVKGTLALKGYYAFVNGSFELWELQFGISPVHSI-----					
	PfV--reglvkktLALkGGYYDFVnGsFeLWglefgls---Si-----					

Fig. 1. Alignment of the deduced amino acid sequences of soybean carbonic anhydrase (CA) with homologous sequences from other plants. Dashes indicate gaps introduced to maximize homology. Residues indicated by capital letters on the seventh line are identical in all sequences. Residues indicated by lowercase letters on the seventh line are identical in at least three to six sequences. Accession numbers: *Medicago sativa* X93312 (MSCA1); *Arabidopsis thaliana* P42737 (THCAH); pea P17067 (PSCAMRA); spinach P16016 (SOCCA); *Arabidopsis thaliana* P27140 (ATCAN).

signal was detected in the central tissues (Fig. 4B). On the other hand, a visible signal was observed in all cell types of young nodules (Fig. 4A).

Expression patterns of the SS gene.

Since sucrose is the main carbon source for nodule maintenance and function, the nodular sites of SS gene expression in conjunction with those of the PEPC gene are expected to provide information on the distribution of activities of the SS and PEPC enzymes in developing nodules as well as on the fate of the breakdown products (UDP-fructose and glucose). In young nodules (10 d.p.i.), high levels of SS transcripts were found in the vascular bundles (Fig. 5A) as well as in the part of the vascular bundle connecting the nodule lobe to the root bearing the nodule (Fig. 5A) and the cells of the central tissue. High levels of expression were also observed in parenchymatous cells, particularly in those around the central tissue and the vascular bundles as well as in the boundary cell layers of the distal part in the developing nodules (Fig. 5A). A similar pattern of expression was obtained in sections taken from 15 d.p.i. nodules (Fig. 5B).

In mature nodules (28 d.p.i.), high levels of SS transcripts were detected in vascular bundles, mainly in the pericycle cells, while the signal intensity in all other nodular cell types was significantly reduced (Fig. 5C). High magnification revealed the presence of a low but visible hybridization signal in uninfected cells, while it is not clear whether signal can also be seen in infected cells (Fig. 5G). It should also be pointed out that roots bearing mature (28 d.p.i.) nodules exhibited high levels of SS transcripts (Fig. 5F), whereas no visible signal was observed in roots bearing young (10 d.p.i.) nodules (Fig. 5E).

DISCUSSION

Nodule CA plays differing roles in young and mature soybean nodules.

CA plays an important role in photosynthetic CO₂ fixation in the leaves of C₃ plants by facilitating the diffusion of CO₂ in the stroma (cytoplasmic isoform) and maintaining the supply of CO₂ for Rubisco by speeding up dehydration of bicarbonate (chloroplastic isoform) (Chollet et al. 1996; Fett and Coleman 1994). In the leaves of C₄ plants, CA activity is confined to the cytoplasm of mesophyll cells, where it converts

CO₂ to bicarbonate, the substrate for PEPC (Badger and Price 1994). The role of CA in dark CO₂ fixation is also expected to be significant since it provides the substrate for the carboxylation of oxaloacetate by PEPC (Chollet et al. 1996). In symbiotic nitrogen fixation, dark CO₂ fixation may also play a significant role. It has been suggested that dark CO₂ fixation may provide a large fraction (30%) of the carbon skeletons for amide synthesis or bacteroid metabolism (Rosendal et al. 1990).

In this report we provide evidence that a CA gene is expressed in nonphotosynthetic tissues such as soybean determinate nodules. Its expression patterns suggested that CA may play different physiological roles in young and mature nodules. The spatial and temporal patterns of expression revealed that in young (10 d.p.i.) nodules CA gene transcripts and CA protein were found in all cell types. In young nodules (10 to 15 d.p.i.), the resources of CO₂ are limited while the demand for bicarbonate is great. Because new cell wall synthesis and extensive membrane proliferation are taking place in the newly infected cells, it is expected that the enhanced levels of CA transcripts and consequently CA activity are necessary to convert most of the available CO₂ to bicarbonate. Since in young nodules PEPC gene transcript levels are lower than those seen in mature nodules, it is reasonable to assume that at this developmental stage a large fraction of the bicarbonate is channeled to other biosynthetic processes (e.g., fatty acids or purine biosynthesis) in which CO₂ is recycled, rather than to the production of oxaloacetate, which provides carbon skeletons for amino acid biosynthesis.

In mature nodules (28 d.p.i.), CA gene transcripts and CA protein are exclusively detected in the peripheral tissues (Fig. 3D and 4B). This distribution could be explained on the basis that production of CO₂ in the central tissue is ample, compared with that in earlier stages, due to the large numbers of respiring bacteroids (Vance et al. 1997). Thus, the solubilized CO₂ provides sufficient bicarbonate for biosynthetic processes. The high level of PEPC gene transcripts in mature nodules is in line with the view that a large fraction of bicarbonate is utilized in oxaloacetate production, which is subsequently used in malate and/or amino acid production. Since root respiration is inhibited by the elevated concentration of CO₂ (Nobel and Palta 1989), the localized accumulation of CA protein in mature nodule inner cortical cells possibly reflects the need of a facilitated diffusion of the excess nodular CO₂ to

Fig. 2. In situ localization of carbonic anhydrase (CA) mRNA transcripts in leaves, meristems, and pulvinus. Sections were hybridized with digoxigenin (DIG)-rUTP-labeled antisense RNA, in vitro transcribed from GmCA. Signal is detected as a blue-purple color precipitate. Bars represent 100 μ m. **A**, Cross-section of leaf base. Hybridization signal is detected in phloem (PH) and palisade parenchyma (PP). **B**, Longitudinal section of apical meristem (AP) and leaf primordium (LP). Hybridization signal is detected in all meristematic tissue. **C**, Cross-section of pulvinus. Hybridization signal is detected in the phloem.

Fig. 3. In situ localization of carbonic anhydrase (CA) and phosphoenolpyruvate carboxylase (PEPC) mRNA transcripts during soybean nodule development. Sections of (A and E) 10, (B and F) 15, (C, H, and I) 21, and (D and G) 28 days post infection (d.p.i.) nodules were hybridized to digoxigenin (DIG)-11-UTP-labeled RNA, in vitro transcribed from (A–D and H) GmCA or (E–G and I) GmPEPC. Sense probes were used in sections presented in H and I; in all other panels antisense were used. Signal is detected as a blue-purple color precipitate. Bars represent 100 μ m. A, Hybridization signal is visible in all cell types except those in the outer cortex (OC). B, Hybridization signal is visible in all cell types as in A, but a lower signal intensity is observed in the central tissue and the pericycle of the vascular bundles. C and D, The hybridization signal gradually decreases from the central tissue but is strongly detected in inner cortical cells (IC). E, A low level of signal is detected in parenchymatous cells (PC) and the vascular bundles (VB). F, Hybridization signal is clearly visible in parenchymatous cells (PC). A strong hybridization is found in the pericycle (P) of the vascular bundles. G, High levels of hybridization signal are observed in the peripheral cells including the vascular bundles and the central tissue (CT). H and I, Section from 21 d.p.i. nodules was hybridized with sense probes. No visible hybridization signal is detected.

the rhizosphere, in a way similar to that seen in the facilitated diffusion of atmospheric CO₂ toward the chloroplasts in leaves. This model is also corroborated by the observation that high levels of tobacco CA activity in the cytoplasm of the bundle sheath of transgenic C4-dicot *Flaveria bidentis* resulted in increased permeability of bicarbonate in mesophyll cells (Ludwing et al. 1998).

The presence of two bands cross-reacting with the recombinant CA antisera suggested the presence of two CA isoenzymes. Similar observations have also been reported for the CA present in photosynthetic tissues of various plants (Fett and Coleman 1994; Rumeau et al. 1997). One of these isoenzymes was located in the chloroplast and the other in the cytoplasm. The subcellular location of soybean nodule CA is under investigation.

Sucrose breakdown may take place at different sites in developing nodules.

In legume nodules, carbon skeletons derived from the breakdown of sucrose are used in numerous metabolic processes including amino acid production, plant and bacterial respiration, and the biosynthesis of starch and cellulose. Thus, the sites of sucrose breakdown and the fate of the breakdown products are expected to be dictated, in each developmental stage, by the levels of various enzymes and this is expected to be a reflection of the corresponding mRNA levels, including SS and PEPC mRNAs. Our data demonstrate that the overall level of SS transcripts is high in young nodules while it is declining in mature nodules, with this decline being more prominent in the infected cells of the central tissue, where no visible signal was observed. These data corroborated earlier work that demonstrated that SS activity increased in developing nodules and then decreased during the course of nodule maturation (Anthon and Emmerich 1990; Thummler and Verma 1987). However, in situ hybridization studies on the localization of SS gene transcripts in *Phaseolus vulgaris* nodules have shown that the level of transcripts is much higher in mature than in young nodules (van Ghelue et al. 1996), suggesting that sucrose metabolism in soybean and common bean might differ in the patterns of SS expression, as they have already been shown to differ in glutamine synthetase gene expression (Forde et al. 1989; Miao et al. 1991).

Our data demonstrate that in soybean nodules the distribution and concentration of the SS transcripts in various cell types and at various developmental stages differ significantly. In young nodules, the distribution of SS transcripts indicated that sucrose breakdown takes place in the central tissue as well as in the parenchyma and the vascular system. Since in young nodules the levels of PEPC gene transcripts are low, it is likely that the sucrose breakdown products are directed to a greater extent toward starch and/or cellulose biosynthesis and to a lesser extent are metabolized to oxaloacetate. Interestingly, in mature nodules, although the demand for carbon skeletons and energy is high, the level of SS transcripts is not only very low compared with that of young nodules, but also is much lower in the central tissue than in the parenchyma. Similar results have been obtained in immunocytochemical studies on the localization of the SS protein in mature soybean nodules, where a greater amount of the protein was found in the uninfected and parenchyma cells than in infected cells of the central tissue (Zammit and Copeland 1993). In addition, immunological studies have shown that, in mature soybean nodules, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase is present in a greater amount in the uninfected and parenchymatic cells than in infected cells (Zammit et al. 1992). These indications, along with our observation that high levels of PEPC transcripts are detected in the parenchyma and in all cell types of the central tissue, are further support for the idea that, in mature nodules, sucrose breakdown is taking place mainly in cells other than those located in the central tissue and that carbon compounds needed for malate production or malate itself are mobilized symplastically from other nodular cell types than the infected cells. The root system could be another source of sucrose breakdown products, since a very high level of SS transcripts is detected in the roots bearing the mature nodules (Fig. 5F), compared with that seen in the roots bearing the young nodules (Fig. 5E).

The pericycle cells of the nodule vascular system play an important role in sucrose metabolism.

The nodule vascular system and in particular the pericycle cells appear to be the site of high expression of genes coding for enzymes involved in carbon and nitrogen metabolism (van Ghelue et al. 1996; Charrier et al. 1998) as well as expression

Fig. 4. Immunoblot analysis and immunolocalization of carbonic anhydrase (CA). Western blots (immunoblots) and tissue prints were probed with the CA antibodies and signal was visualized with alkaline-phosphatase (purple-pink color). **A**, A blot containing tissue prints from 10 days post infection (d.p.i.) nodules. **B**, A blot containing tissue prints from 28 d.p.i. nodules. **C**, A blot of a 15% sodium dodecyl sulfate-polyacrylamide gel containing proteins (20 µg) from the cytoplasmic fraction (1) and the purified recombinant CA (0.5 µg) that is expressed in *Escherichia coli* (2).

Fig. 5. In situ localization of sucrose synthase (SS) mRNA transcripts during soybean nodule development. Sections of (A) 10, (B) 15, and (C, D, and G) 28 days post infection (d.p.i.) nodules as well as sections of roots bearing (E) 10 and (F) 28 d.p.i. nodules were hybridized with digoxigenin (DIG)-rUTP labeled RNA, in vitro transcribed from GmSS. An antisense probe was used in sections in A–C and E–G while in the section presented in D a sense probe was used. Bars represent 100 μ m. A and B, High levels of hybridization signal are detected in the parenchymatous cells (PC) and the vascular bundles (VB) as well as in the central tissue (CT). C, A clear hybridization signal was observed in the vascular tissues especially in the pericycle cells (P). D, Section from 21 d.p.i. nodules was hybridized with sense probe. No visible signal of hybridization is detected. E and F, Cross-sections from roots bearing (E) 10 and (F) 28 d.p.i. nodules. A high level of hybridization signal was detected in xylem parenchyma (XP) and sclerenchyma (S) in roots bearing the 28 d.p.i. nodules; no visible signal is detected in roots bearing 15 d.p.i. nodules. G, Enlargement of a region of C reveals presence of a weak hybridization signal in the uninfected cells (UC) of the central tissue.

of other genes, including ENOD40 (Yang et al. 1993; Papadopoulou et al. 1996) and NOD3 (Roussis et al. 1997). In this study we have demonstrated, using the in situ hybridization approach, that irrespective of the developmental stage the level of SS and PEPC mRNA transcripts in the vascular bundles of developing soybean nodules is very high. Similar observations have also been reported for *P. vulgaris* and *Alnus gluticosa* (van Ghelue et al. 1996). It has been postulated that pericycle cells in soybean nodules are metabolically active and that they are involved in an active transport of compounds to and from the vascular bundle they surround (Walsh et al. 1989). Such an active transport is likely to be carried out by a translocator-ATPase activity and requires the consumption of energy that is expected to be derived from the breakdown of the incoming sucrose. The strong expression of SS and PEPC genes in the vascular system of mature nodules (Figs. 3G and 5C) indicates that in nitrogen-fixing nodules the pericycle cells of the nodular vascular system are likely to be very active in metabolizing sucrose to oxaloacetate and thus providing substrates for energy production. This energy demand is expected to be higher in the vascular bundles of mature nodules since they have to simultaneously import sucrose and export ureides and/or amides.

MATERIALS AND METHODS

Growth conditions for plants.

Soybean plants (*Glycine max* cv. Williams) were cultured in gravel as previously described (Bisseling et al. 1978). The plants were inoculated on the day of sowing (day 0) with *Bradyrhizobium japonicum* spc 110.

Isolation, cloning, and sequence analysis of the cDNAs.

A λ gt11 cDNA library of poly (A)⁺ RNA from 21-day-old root nodules of *Glycine max* (cv. Williams) was screened with ³²P-labeled inserts of the MsCA1 and MsPEPC cDNA clones under low stringency conditions. Phage purification and subcloning of the inserts of the positive clones into the vector pBluescript KS⁺ (Stratagene, La Jolla, CA) were performed according to standard methods (Sambrook et al. 1989). Both strands of all inserts were sequenced by the dideoxynucleotide chain termination method (Sanger et al. 1977). To obtain the 5' end region of the CA cDNA, PCR was applied to the excised λ ZapII soybean nodule cDNA library with an 18-mer antisense oligonucleotide (5' ATTCTTTGCACAGCAAGG; P1) derived from the longest open reading frame of GmCA, and the pBluescript T3 primer. The PCR product was purified and cloned into the pGEM-T vector (Promega, Madison, WI) and then both strands were sequenced. An *EcoRI-HindIII* fragment of the Nodulin-100 (nodule specific sucrose synthase) cDNA clone (Thummler and Verma 1987) was subcloned into pBluescript KS⁺ vector according to standard methods (Sambrook et al. 1989).

In situ hybridization.

Nodules 10, 15, 21, and 28 d.p.i. with *B. japonicum* spc 110 as well as several other tissues were picked and then fixed in 4% paraformaldehyde/0.25% glutaraldehyde in 10 mM phosphate buffer at 4°C overnight. Embedding and sectioning were done according to already described methods (van de Wiel et al. 1990). Nodules were block-stained with 0.5% safranin.

Antisense and sense riboprobes were obtained from linearized plasmids by in vitro transcription with T3 or T7 RNA polymerase (Promega). The RNA was labeled with DIG-11UTP according to the Boehringer manual. The probes were partially degraded to an average length of 150 nucleotides. Sections (7 to 10 μ m) were prepared for hybridization according to Scheres et al. (1988) and were put to hybridize overnight at 42°C in 50% formamide, 300 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.025% bovine serum albumin (BSA), 10% dextran sulfate, 60 mM dithiothreitol (DTT), 500 μ g of poly (A) RNA per ml, and 150 μ g of yeast tRNA per ml. The signal detection was performed as described by Papadopoulou et al. 1996.

Expression of GmCA1 in *E. coli* and purification of the recombinant protein.

The GmCA cDNA clone was inserted into the pQE-32 (Qiagen, Hilden, Germany) vector as an *SalI* and *PstI* fragment and its expression in *E. coli* M15 (Qiagen) was induced with 2 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 5 h. Induced cells were pelleted and extracted by shaking in extraction buffer (6 M GuHCl, 0.1 M Na-phosphate, 0.01 M Tris/ HCl). The recombinant 6 $\times$ histag-fusion protein was purified according to the QIAexpress Type IV Kit (Qiagen).

Immunoblotting.

The cytoplasmic and peribacteroid-enveloped bacteroid fractions of the nodules were obtained as described by Katinakis et al. (1988). Cytoplasmic fraction samples (20 μ g of protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15% polyacrylamide) and then electrophoretically transferred to a nylon membrane (Hybond-C extra; Amersham, Athens). Tissue printing of hand sectioned nodules was carried out as described by Cho and Cende (1997). Polyclonal antibodies were raised in rabbit against the overexpressed recombinant GmCA protein. The blots were probed with the polyclonal antibodies and bound antibodies were visualized with the ProtoBlot Western Blot AP System (Promega). All antibodies were used at a 1/2,500 dilution.

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LITERATURE CITED

- Anthon, G. E., and Emmerich, D. W. 1990. Developmental regulation of enzymes of sucrose and hexose metabolism in effective and ineffective soybean nodules. *Plant Physiol.* 92:346-351.
- Badger, M. R., and Price, G. D. 1994. The role of carbonic anhydrase in photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 45:369-392.
- Bisseling, T., van de Bos, R. C., and van Kammen, A. 1978. The effect of ammonium nitrate on the synthesis of nitrogenase and the concentration of leghemoglobin in pea root nodules induced by *Rhizobium leguminosarum*. *Biochim. Biophys. Acta* 539:1-11.

- Breda, C., Sallaud, C., El-Turk, J., Buffard D., de Kozak, I., Esnault, R. and Kondorosi, A. 1996. Defense reaction in *Medicago sativa*: A gene encoding a class 10 PR protein is expressed in vascular bundles. *Mol. Plant-Microbe Interact.* 9:713-719.
- Charrier, B., Trinh, H., Poirier, S., Kondorosi, A., and Ratet, P. 1998. *Flavanone 3-hydroxylase* (F3H) expression and flavonoid localization in nodules of three legume plants reveal distinct tissue specificities. *Mol. Plant-Microbe Interact.* 11:924-932.
- Cho, H. T., and Cende, H. 1997. Expansions and internodal growth of deepwater rice. *Plant. Physiol.* 113:1145-1151.
- Chollet, R., Vidal, J., and O'Leary, M. H. 1996. Phosphoenolpyruvate carboxylase: A ubiquitous highly regulated enzyme in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47:273-298.
- Day, D. A., and Copeland, L. 1991. Carbon metabolism and compartmentation in nitrogen fixing legumes nodules. *Plant Physiol. Biochem.* 29:185-201.
- de la Pena, T. C., Frugier, F., McKhann, H. I., Bauer, P., Brown, S., Kondorosi, A., and Crespi, M. 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.
- Egli, M. A., Griffith, S., Miller, S. S., Aderson, 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.
- Fett, J. P., and Coleman, J. R. 1994. Characterization and expression of two cDNAs encoding carbon anhydrase in *Arabidopsis thaliana*. *Plant. Physiol.* 105:707-713.
- Finan, T. M., Wood, J. M., and Jordon, D. C. 1983. Symbiotic properties of C₄-dicarboxylic acid transport mutants of *Rhizobium leguminosarum*. *J. Bacteriol.* 154:1403-1413.
- Forde, B. G., Day, H. M., Turton, J. F., Shen, W.-C., 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.
- Hata, S., Izui, K., and Kouchi, H. 1998. Expression of a soybean nodule-enhanced phosphoenolpyruvate carboxylase gene that shows striking similarity to another gene for house-keeping isoform. *Plant J.* 13: 267-273.
- Katinakis, P., Klein Lankhous, R. M., Louwerse, J., van Kammen, A., and van den Bos, P. 1988. Bacteroid-encoded proteins are secreted into the peribacteroid space by *Rhizobium leguminosarum*. *Plant Mol. Biol.* 11:183-190.
- Lance, C., and Rustin, P. 1984. The central role of malate in plant metabolism. *Physiol. Veg.* 22:625-641.
- Ludwing, M., von Caemmerer, S., Price, D., Barger, M. R., and Furbank, R. T. 1998. Expression of tobacco carbonic anhydrase in the C₄ dicot *Flaveria bidentis* leads to increased leakiness of the bundle sheath and a defective CO₂-concentrating mechanism. *Plant Physiol.* 117:1071-1081.
- Miao, G. H., Hirel, B., Marsolier, M. C., Ridge, R. W., and Verma, D. P. S. 1991. Ammonia-regulated expression of a soybean gene encoding cytosolic glutamine synthetase in transgenic *Lotus corniculatus*. *Plant Cell* 3:11-22.
- Miller, S. S., Driscoll, B. T., Gregerson, R.G., Gantt, J. S., and Vance, C. P. 1998. Alfalfa malate dehydrogenase (MDH): Molecular cloning and characterization of five different forms reveals a unique nodule-enhanced MDH. *Plant J.* 15:173-184.
- Nobel, P. S., and Palta, J. A. 1989. Soil O₂ and CO₂ effects on root respiration of cacti. *Plant Soil* 120:263-271.
- Papadopoulou, K., Roussis, A., and Katinakis, P. 1996. *Phaseolus* ENOD40 is involved in symbiotic and non-symbiotic organogenetic processes: Expression during nodule and lateral root development. *Plant Mol. Biol.* 30:403-417.
- Pathirana, S. M., Vance, C. P., Miller, S. S., and Gantt, 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.
- Provart, N. J., Majeau, N., and Coleman, J. R. 1993. Characterization of pea chloroplastic carbonic anhydrase. Expression in *Escherichia coli* and site-directed mutagenesis. *Plant Mol. Biol.* 22:937-947.
- Ronson, C. W., Lyttleton, P., and Robertson, J. G. 1981. C₄-dicarboxylate transport mutant of *Rhizobium trifolii* form ineffective nodules. *Proc. Natl. Acad. Sci. USA* 78:4284-4288.
- Rosendal, L., Vance, C. P., and Pedersen, W. B. 1990. Products of dark CO₂ fixation in pea root nodules support bacteroid metabolism. *Plant. Physiol.* 93:12-19.
- Roussis, A., Papadopoulou, K., and Katinakis, P. 1997. NOD3, a novel late nodulin gene from soybean is expressed in the infected cells and the nodule parenchyma. *J. Exp. Bot.* 48:1011-1017.
- Rumeau, D., Cuine, S., Fina, L., Gault, N., Nicole, M., and Peltier, G. 1996. Subcellular distribution of carbonic anhydrase in *Solanum tuberosum* L. leaves: Characterization of two compartments-specific isoforms. *Planta* 199:79-88.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
- Scheres, B., van de Wiel, C., Zalsensky, A., Horvarth, B., Spalink, H. P., van Eck, H., Zwartkruis, F., Wolters, A., Gloudemans, T., van Kammen, A., and Bisseling, T. 1988. The ENOD12 gene product is involved in the infection process during the pea-*Rhizobium* interaction. *Cell* 60:281-294.
- Suganuma, N., Okada, Y., and Kanayama, Y. 1997. Isolation of a cDNA for nodule-enhanced phosphoenolpyruvate carboxylase from pea and its expression in effective and plant-determined ineffective pea nodules. *J. Exp. Bot.* 48:1165-1173.
- Thummler, F., and Verma, D. P. S. 1987. Nodulin-100 of soybean is the subunit of sucrose synthase regulated by the availability of free heme in nodules. *J. Biol. Chem.* 262:14730-14736.
- van de Wiel, C., Scheres, B., Franssen, H., van Lierop, M. J., van Lammeren, A., van Kammen, A., and Bisseling, T. 1990. The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules. *EMBO J.* 9:1-7.
- van Ghelue, M., Ribeiro, A., Solheim, B., Akkermans, A. D., Bisseling, T., and Pawlowski, K. 1996. Sucrose synthase and enolase expression in actinorhizal nodules of *Alnus gluticosa*: Comparison with legume nodules. *Mol. Gen. Genet.* 250:437-446.
- Vance, C. P., and Heichel, G. H. 1991. Carbon in N₂ fixation: Limitation or exquisite adaptation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42:373-392.
- Vance, C. P., Miller, S. S., Driscoll, B. T., Robinson, D. L., Trepp, G., Gantt, J. S., and Samas, D. A. 1997. Nodule carbon metabolism: organic acids for N₂ fixation. Pages 443-448 in: *Biological Nitrogen Fixation for the 21st Century*. C. E. Elmerich, A. Kondorosi, and W. E. Newton, eds. Kluwer Academic, Dordrecht, The Netherlands.
- 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.
- Yang, W. S., Katinakis, P., Hendriks, P., Smolder, A., de Vries, F., Spee, J., van Kammen, A., Bisseling, T., and Frassen, H. 1993. Characterization of GmENOD40, a gene showing novel patterns of cell-specific expression during soybean nodule development. *Plant J.* 3:573-585.
- Zammit, A., and Copeland, L. 1993. Immunocytochemical localization of nodule-specific sucrose synthase in soybean nodules. *Aust. J. Plant Physiol.* 20:25-32.
- Zammit, A., Copeland, L., Miller, C., and Craig, S. 1992. Immunocytological localization of NAD-dependent glyceraldehyde-3-phosphate dehydrogenase in soybean nodules. *Physiol. Plant.* 84:549-594.