Expression Map for Genes Involved in Nitrogen and Carbon Metabolism in Alfalfa Root Nodules

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During root nodule development several key genes involved in nitrogen fixation and assimilation exhibit enhanced levels of expression. However, little is known about the temporal and spatial distribution patterns of these transcripts. In a systematic study the transcripts for 13 of the essential enzymes involved in alfalfa (*Medicago sativa*) root nodule nitrogen and carbon metabolism were localized by in situ hybridization. A serial section approach allowed the construction of a map that reflects the relative distribution of these transcripts. In 33-day-old root nodules, the expression of *nifH*, NADH-dependent glutamate synthase (NADH-GOGAT; EC 1.4.1.14) and a cytosolic isoform of glutamine synthetase (GS13; GS; EC 6.3.1.2) were localized predominantly in a 5- to 15-cell-wide region in the distal part of the nitrogen-fixing zone. This zone was also the region of high expression for leghemoglobin, a second cytosolic glutamine synthetase isoform (GS100), aspartate aminotransferase-2 (AAT-2; EC 2.6.1.1), asparagine synthetase (AS; 6.3.5.4), phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31), and sucrose synthase (SuSy; EC 2.4.1.13). This suggests that, in 33-day-old alfalfa root nodules, nitrogen fixation is restricted to this 5- to 15-cell-wide area. The continued significant expression of the GS100 subclass of GS and AS in the proximal part of the nitrogen-fixing zone implicates these gene products in nitrogen remobilization. A low constitutive expression of NADH-dependent glutamate dehydrogenase (NADH-GDH; EC 1.4.1.2) was observed throughout the nodule. The transcript distribution map will be used as a navigational tool to assist in developing strategies for the genetic engineering of alfalfa root nodules for enhanced nitrogen assimilation.

Additional keywords: amino acids, nitrogenase, oxygen, organic acids, *Sinorhizobium meliloti*.

Alfalfa root nodule initiation and development is the result of a complex interaction between the plant and the soil bacterium *Sinorhizobium meliloti*. During root nodule formation an array of genes are specifically induced in both the plant and the bacterium (Dénarié et al. 1996). In *S. meliloti*, some of these genes belong to the group of nodulation (*nod*, *nol*, *noe*), *fix*, and *nif* genes (Fischer 1994). In the plant nodule, specific genes called nodulins (van Kammen 1984) are divided into two groups, those expressed before the onset of N₂ fixation (early nodulins) and those expressed after the onset of N₂ fixation (late nodulins). While the original definition of nodulins stated that these genes were expressed solely in nodules, we now know that most genes originally identified as nodulins are also expressed in other tissues and even in non-N₂-fixing species (Brewin 1991; Franssen et al. 1992). Several of these nodule genes encode for the enzymes involved in nitrogen and carbon metabolism associated with N₂ fixation (Vance et al. 1994).

After release from the infection thread, *S. meliloti* cells differentiate into bacteroids, and begin to reduce N₂ to ammonia with the nitrogenase enzyme (Nif; EC 1.18.6.1; Mylona et al. 1995). Because oxygen irreversibly denatures nitrogenase (Shaw and Brill 1977), a low oxygen environment is required within the root nodule tissue in order for nitrogen fixation to occur. The maintenance of this microaerobic environment involves an oxygen diffusion barrier (Witty et al. 1986). The oxygen needed for bacterial respiration is supplied by the hemoprotein leghemoglobin (lb), which is expressed in the infected cells of the root nodule (Mylona et al. 1995). In this way it is possible to provide sufficient oxygen for bacteroid respiration in a microaerobic environment (Appleby 1984).

The ammonia derived from N₂ fixation is transported into the plant cytosol (Kaiser et al. 1998) where it is assimilated into glutamine and glutamate by the combined action of glutamine synthetase (GS; EC 6.3.1.2) and the NADH-dependent glutamate synthase (NADH-GOGAT; EC 1.4.1.14; Temple et al. 1998b). In alfalfa root nodules, three different classes of GS are present, two isoforms of cytosolic GS₁ (GS13 and GS100) and a plastid localized GS₂ (Temple et al. 1998a). Evidence suggests that GS₁ accounts for most of the nodule GS activity (Shelp and Atkins 1984; Temple et al. 1998a), indicating that ammonia derived from N₂ fixation is assimilated predominantly in the plant cytosol. Of the two GS₁ isoforms

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occurring in root nodules, GS13 is considered the nodule-enhanced form of GS₁ due to the enhanced abundance of transcripts in this tissue (Temple et al. 1995). Two different forms of GOGAT are found in plant tissues, a ferredoxin-dependent GOGAT (Fd-GOGAT; EC 1.4.7.1) and an NADH-dependent form. In green tissues of alfalfa, NADH-GOGAT enzyme activity and the abundance of its mRNA are low to nondetectable (Gregerson et al. 1993). However, NADH-GOGAT activity increases several-fold during alfalfa root nodule development (Gregerson et al. 1993) and is the predominant form of GOGAT in this tissue (Vance et al. 1995). The glutamine and glutamate synthesized by GS and NADH-GOGAT are further assimilated into aspartate and asparagine via the actions of aspartate aminotransferase (AAT; EC 2.6.1.1) and asparagine synthetase (AS; EC 6.3.5.4). In amide-transporting legumes such as alfalfa, asparagine is the predominant amino acid transported from the nodule to the shoot (Lea et al. 1990). The reaction catalyzed by the NADH-dependent glutamate dehydrogenase (NADH-GDH; EC 1.4.1.2) offers another potential route for ammonia assimilation into glutamate (Oaks 1994). However, the plant enzyme's high k_m for ammonia (>1 mM) has led to an uncertainty over its role in plant metabolism (Lea et al. 1990; Oaks 1994; Temple et al. 1998b). Some authors believe that GDH is involved in glutamate synthesis (Oaks 1994), whereas others argue that the main reaction catalyzed by GDH is the deamination of glutamate yielding 2-oxoglutarate and ammonia (Lea et al. 1990).

The carbon skeletons required for N₂ reduction are provided by the plant, mainly in the form of malate (Rosendahl et al. 1990). A significant portion of malate produced in root nodules passes through the reaction catalyzed by phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31; Maxwell et al. 1984; Rosendahl et al. 1990). PEPC provides oxaloacetate, which can be reduced to malate by malate dehydrogenase (MDH; EC 1.1.1.37). Although organic acids are the energy source for bacteroid respiration, photosynthate in the form of sucrose is the ultimate carbon source required for both N₂ fixation and N assimilation (Reibach and Streeter 1983). An important enzyme in sucrose metabolism of root nodules is sucrose synthase (SuSy; EC 2.4.1.13; Gordon et al. 1995). SuSy catalyzes the hydrolysis of sucrose into fructose and UDP-glucose; these products can be used for both glycolysis and starch biosynthesis (Martin et al. 1993).

Previous studies have demonstrated that the transcripts for enzymes of NH₄⁺ and C assimilation increase dramatically during root nodule development (Dunn et al. 1988; Gantt et al. 1992; Pathirana et al. 1992; Gregerson et al. 1993; Roche et al. 1993; Vance et al. 1994; Shi et al. 1997; Robinson et al., *in press*). However, little is known about the temporal and spatial distribution patterns of these transcripts. In an attempt to increase our understanding of root nodule development, N₂ fixation, and nodule metabolism, we in situ localized 13 plant and bacterial encoded transcripts in alfalfa root nodules by a serial section approach. The following transcripts were localized: Mslbc3, GS100, GS13, NADH-GOGAT, AAT-2, AS, GS2, GDH, PEPC, SuSy, alcohol dehydrogenase (ADH; EC 1.1.1.1), and the bacterial transcripts encoding *nifH* and *dme* (NADH-dependent malic enzyme). The results were compiled and used to create a map that reflects the expression pattern of these genes in 19- and 33-day-old root nodules.

RESULTS

Serial sections made from individual 19- and 33-day-old nodules were placed in consecutive order on three glass slides. Two of the three slides were hybridized either with leghemoglobin (Mslbc3) or *nifH*. The third slide was hybridized with the probe of choice. This approach allowed direct comparisons to be made on selected genes with reference to both Mslbc3 and *nifH* and thus allowed an indirect comparison of the expression pattern of 13 of the essential enzymes involved in nitrogen and carbon metabolism in alfalfa root nodules.

The nodule ultrastructure classification used was based on the nomenclature of Vasse et al. (1990). Five distinct zones were defined along the axis in a longitudinal section through an alfalfa nodule: the nodule meristem (zone I); the invasion zone (zone II); the amyloplast-rich interzone (zone II-III (*)); the nitrogen-fixing zone and proximal inefficient zone (zone III); and the senescent zone (zone IV) (see bright field pictures in Figures 1, 2, and 3).

In situ localization of leghemoglobin and *nifH* in 19- and 33-day-old nodules.

In both 19- and 33-day-old nodules, Mslbc3 transcripts were detectable throughout the central part of a root nodule, including the invasion zone (zone II), interzone (zone II-III), and nitrogen-fixing zone (zone III) (Figs. 1A-D, 2A-D, and 3A-D). The signal reflecting the location of the transcripts is seen in the dark field as white silver grains. Although Mslbc3 transcripts were found throughout the infected cell zones of 33-day-old nodules, expression was highest in the interzone (zone II-III) and in a 5- to 15-cell-wide area of the distal nitrogen-fixing zone (zone III) (Figs. 1C,D, 2C,D, and 3C,D). The *nifH* transcript was localized in the interzone (zone II-III) and nitrogen-fixing zone (zone III) of 19-day-old alfalfa root nodules with the highest accumulation of transcripts being observed in the distal part of the nitrogen-fixing zone (Figs. 1E,F, 2E,F, and 3E,F). In 33-day-old alfalfa root nodules, *nifH* mRNA was localized in the interzone (zone II-III) and in a 5- to 15-cell-wide area of the distal nitrogen-fixing zone (zone III) (Figs. 1G,H, 2G,H, and 3G,H). This 5- to 15-cell-wide zone corresponds to the same region where maximum accumulation of Mslbc3 transcripts occurs. None of the positive-sense riboprobe controls hybridized to the nodule sections (data not shown).

In situ localization of transcripts encoding proteins involved in primary nitrogen metabolism.

In the first set of experiments, the genes involved in nitrogen metabolism were localized in 19- and 33-day-old alfalfa root nodules. The genes used were GS₁ (GS100 and GS13 isoforms), NADH-GOGAT, AAT-2, and AS. The distribution of Mslbc3 and *nifH* transcripts was identical to that described in the previous section (Fig. 1A-H). The transcript encoding the cytosolic GS100 was localized throughout all nodule zones and cortex, irrespective of whether the nodule was harvested 19 or 33 days after planting and inoculation (Fig. 1I-L). In 33-day-old nodules, GS100 transcripts accumulated to the highest level in a 5- to 15-cell-wide area in the distal part of the nitrogen-fixing zone (Fig. 1K and L). In 19-day-old root nodules, nodule-enhanced GS (GS13) expression was localized in the interzone (zone II-III) and the nitrogen-fixing zone, but not in the meristem and invasion zone (Fig. 1M and N). At 33 days,

GS13 transcripts were mostly limited to the interzone and in a 5- to 15-cell-wide area of the distal nitrogen-fixing zone (Fig. 1O and P). GS100 and GS13 transcripts accumulate predominantly in the infected cells of the interzone and nitrogen-fixing zone, which is in agreement with a previous report (Temple et al. 1995). NADH-GOGAT mRNA was confined to the interzone and the nitrogen-fixing zone at day 19 (Fig. 1Q and R). At day 33, the NADH-GOGAT transcript was limited to the interzone and in a 5- to 15-cell-wide area of the distal nitrogen-fixing zone similar to transcript patterns observed for GS13 and *nifH* (Fig. 1S and T). NADH-GOGAT transcripts accumulate predominantly in the infected cells of the interzone and nitrogen-fixing zone (data not shown). In 19-day-old

root nodules, the expression pattern of AAT-2 is similar to that of GS13 and NADH-GOGAT, with transcripts accumulating in the interzone and nitrogen-fixing zone, but not in the meristem, invasion zone, or the root nodule cortex (Fig. 1U and V). In 33-day-old root nodules, the AAT-2 transcript was most abundant in the interzone and the nitrogen-fixing zone, forming a gradient from high to low expression in cells of the interzone to the proximal part of zone III (Fig. 1W and X). AAT-2 transcripts accumulate predominantly in the infected cells of the interzone and nitrogen-fixing zone (data not shown). The AS transcripts were dispersed throughout the nitrogen-fixing zone (zone III) in both 19- and 33-day-old root nodules (Fig. 1Y-BB), but not in the meristem, invasion zone,

Fig. 1. Longitudinal sections through (A, B, E, F, I, J, M, N, Q, R, U, V, Y, Z) 19-day-old and (C, D, G, H, K, L, O, P, S, T, W, X, AA, BB) 33-day-old alfalfa root nodules. A, C, E, G, I, K, M, O, Q, S, U, W, Y, and AA, Corresponding bright-field pictures of the dark-field pictures in B, D, F, H, J, L, N, P, R, T, V, X, Z, and BB. A–D were hybridized with an *Mslb3* antisense RNA probe; E–H with an *nifH* antisense RNA probe; I–L with a GS100 class specific antisense RNA probe; M–P with a GS13 class specific antisense RNA probe; Q–T with an NADH-GOGAT antisense RNA probe; U–X with an AAT-2 antisense RNA probe; Y–BB with an AS antisense RNA probe. Signal reflecting localization of the transcripts can be seen in dark field as white silver grains. Nodule zones are indicated: (I) zone I, meristem; (II) zone II, invasion zone; (*) zone II-III, interzone; (III) zone III, nitrogen-fixing zone; (IV) zone IV, senescence zone. Bar = 400 μ m.

or interzone. It appears that AS transcript accumulation is highest in the distal area of the nitrogen-fixing zone in 33-day-old root nodules (Fig. 1AA and BB). AS transcripts predominantly accumulate in the infected and uninfected cells of the nitrogen-fixing zone and the root nodule cortex, which is in agreement with a previous report (Shi et al. 1997)

In a second set of experiments, GS₂ and NADH-GDH were localized in 19- and 33-day-old root nodules. The distribution of Mslbc3 and *nifH* transcripts was identical to that described above (Fig. 2A–H). In 19-day-old alfalfa root nodules the transcript encoding GS₂ was localized in the interzone and the nitrogen-fixing zone (Fig. 2I and J). No significant signal was detected in the meristem, invasion zone, or root nodule cortex. This pattern was similar for 33 days; the transcript encoding GS₂ accumulated highest in the interzone of the nodule and the transcripts were also detected in the nitrogen-fixing zone (Fig. 2K and L). The transcripts encoding NADH-GDH were expressed at relatively low levels and dispersed equally throughout the nodules, including all zones and the root nodule cortex in both 19- and 33-day-old root nodules (Fig. 2M–P). RNA blot analysis confirmed the low abundance of NADH-GDH transcripts in alfalfa root nodules (Fig. 4). The abundance of the NADH-GDH transcript was highest in leaves and roots.

In situ localization of transcripts encoding proteins involved in carbon metabolism in alfalfa root nodules.

In the third set of experiments, we localized the transcripts of genes encoding key enzymes involved in root nodule carbon metabolism in 19- and 33-day-old root nodules. The genes used encode for PEPC, SuSy, alcohol dehydrogenase (ADH), and the bacterial *dme* (NADH-ME). The accumulation of Mslbc3 and *nifH* transcripts was identical to that de-

scribed above (Fig. 3A–H). In both 19- and 33-day-old root nodules, PEPC transcripts were localized in the invasion zone (Fig. 3I–L). In addition, at both time points, a high accumulation of PEPC transcripts was observed in the interzone. While the PEPC transcript was readily detectable throughout the nitrogen-fixing zone of 19-day-old root nodules, at day 33 the transcripts were localized predominantly within a few cell layers behind the interzone (Fig. 3K and L). PEPC transcripts accumulated in the infected and uninfected cells of the interzone and nitrogen-fixing zone as well as in the root nodule inner cortex, as shown in a previous report (Pathirana et al. 1997). In 19-day-old root nodules, SuSy mRNA was detected in the root nodule meristem, the interzone, the nitrogen-fixing zone, and the root nodule inner cortex (Fig. 3M and N). At 33 days, the transcripts encoding SuSy were found throughout the nodule, except the outer cortex, similar to 19-day-old nodules (Fig. 3O and P). At a higher magnification, it is apparent that in the interzone, SuSy transcripts accumulate highest in the uninfected cells (data not shown). SuSy transcripts accumulate in both the infected and uninfected cells of the nitrogen-fixing zone and in cells of the root nodule inner cortex, as shown in a previous report (Robinson et al., *in press*)

The O₂ concentration occurring in root nodules ([O₂] < 25 nM; Bergensen 1997) is well below that which induces an anoxic response in plant tissue ([O₂] < 0.8 μM; Come et al. 1991). Therefore, we localized ADH transcripts, whose presence is generally associated with anoxia in plants (Walker et al. 1987a; Sachs 1991; Sachs et al. 1996). ADH catalyzes the reduction of acetaldehyde to ethanol. Under fermentative conditions the enzyme is crucial for the regeneration of NAD, necessary for continued glycolysis (Walker et al. 1987b). Enhanced accumulation of ADH transcripts was detected in the interzone and the nitro-

Fig. 2. Longitudinal section through (A, B, E, F, I, J, M, N) 19-day-old and (C, D, G, H, K, L, O, P) 33-day-old alfalfa root nodules. A, C, E, G, I, K, M, and O, Corresponding bright-field pictures of the dark-field pictures in B, D, F, H, J, L, N, and P. A–D were hybridized with an Mslbc3 antisense RNA probe; E–H with an *nifH* antisense RNA probe; I–L with a GS₂ antisense RNA probe; M–P with a GDH antisense RNA probe. Signal reflecting localization of the transcripts can be seen in dark field as white silver grains. Nodule zones are as indicated in Figure 1. Bar = 400 μm.

gen-fixing zone, but not in the meristem, the invasion zone, or the root nodule cortex of 19-day-old root nodules (Fig. 3Q and R). In 33-day-old root nodules, the ADH mRNA was largely restricted to the interzone and the distal nitrogen-fixing zone (Fig. 3S and T). The distribution pattern of ADH mRNA in the nitrogen-fixing zone was confined to the 5- to 15-cell-wide zone similar to that observed for *nifH*, GS13, and NADH-GOGAT.

The bacterial *dme* gene is essential for nitrogen fixation. Nodules formed with a *S. meliloti dme* mutant are *fix*⁻, suggesting that the *dme* gene product, NADH-dependent malic enzyme, is essential for N₂ fixation (Driscoll and Finan 1993). Therefore, examining the *dme* expression pattern provides an ideal tool to determine whether the *nifH* expression pattern is unique or reflects a more general expression pattern of bacterial genes essential for N₂ fixation. In both 19- and 33-day-old root nodules, the *dme* transcript accumulated in all nodule zones (Fig. 3U–X). Transcripts for *dme* accumulated highest in the interzone, particularly in 19-day-old root nodules (Fig. 3U and V).

DISCUSSION

In this study we report the localization of 13 different transcripts encoding key enzymes involved in nitrogen and carbon metabolism in alfalfa root nodules. The serial section approach chosen allowed a comparison of the expression pattern for differ-

Fig. 4. Analysis of NADH-GDH mRNA in various alfalfa tissues. Poly A⁺-RNA (2 µg) was isolated from roots (lane R), effective nodules (lane N), cotyledons (lane C), stems (lane S), and leaves (lane L) of alfalfa cultivar Saranac, electrophoresed in a 1.5% formaldehyde-agarose gel, transferred and probed with ³²P-labeled NADH-GDH cDNA fragment. The size of the GDH transcript is indicated to the left.

Fig. 3. Longitudinal section through (A, B, E, F, I, J, M, N, Q, R, U, V) 19-day-old and (C, D, G, H, K, L, O, P, S, T, W, X) 33-day-old alfalfa root nodules. A, C, E, G, I, K, M, O, Q, S, U, and W, Corresponding bright-field pictures of the dark-field pictures in B, D, F, H, J, L, N, P, R, T, V, and X. A–D were hybridized with an *Mslbc3* antisense RNA probe; E–H with an *nifH* antisense RNA probe; I–L with a phosphoenolpyruvate carboxylase (PEPC) antisense RNA probe; M–P with a sucrose synthase (SuSy) antisense RNA probe; Q–T with an alcohol dehydrogenase (ADH) antisense RNA probe; U–X with an NADH-malic enzyme (*dme*) antisense RNA probe. Signal reflecting localization of the transcripts can be seen in dark field as white silver grains. Nodule zones are as indicated in Figure 1. Bar = 400 µm.

ent genes and facilitated the construction of a map that reflects the relative distribution of the 13 transcripts throughout the central root nodule tissue (Fig. 5). The signal intensity for a given probe was used as an indicator determining the relative amount of

transcript for a given probe. It should be noted that no absolute quantifications are possible with in situ hybridization data. Therefore, the map reflects the qualitative distribution pattern for the various transcripts localized in different root nodule zones.

Fig. 5. Transcript distribution map reflects qualitative distribution pattern of 13 different mRNAs localized in different nodule zones. A thick bar indicates a high abundance of transcripts; a drawn line indicates the transcripts were moderately abundant; a dashed line indicates occasional expression. Nodule zones are indicated: (I) zone I, meristem; (II) zone II, invasion zone; (*) zone II-III, interzone; (III) zone III, nitrogen-fixing zone; (IV) zone IV, senescence zone.

The nodule nomenclature used as defined by Vasse et al. (1990) describes five consecutive developmental zones in alfalfa root nodules: the meristem (zone I); the invasion zone (zone II); the interzone (zone II-III (*)); the nitrogen-fixing zone (zone III); and the senescence zone (zone IV). Figure 5 shows the overall distribution pattern of the 13 different transcripts localized in alfalfa root nodules. This study reports the first systematic localization of transcripts for essential enzymes involved in nitrogen and carbon metabolism. The map will be used to assist our efforts in metabolic engineering of the alfalfa root nodule N and C metabolism with the goal of the enhancement of N assimilation. Three different transcripts were detected in the nodule meristem (zone I): GS100, GDH, and SuSy. In the invasion zone (zone II) GS100, GDH, PEPC, SuSy, Mslbc3, and the bacterial encoded *dme* transcripts were detected. In the interzone (zone II-III) all transcripts with the exception of AS were detected. The abundance of the rhizobial *dme* transcripts was highest in the infected cells of the interzone. Of all the transcripts, only AS is initially triggered in and localized to zone III. In 33-day-old alfalfa root nodules, the localization of GS13, NADH-GOGAT, *nifH*, and ADH transcripts was almost completely restricted to a 5- to 15-cell-wide area in the distal part of the nitrogen-fixing zone. The GS100, AAT-2, AS, and Mslbc3 transcripts were all most abundant in that same 5- to 15-cell-wide zone. SuSy, AAT-2, AS, GS100, Mslbc3, and the *dme* transcripts were also found throughout the nitrogen-fixing zone.

In root nodules a microaerobic environment allows proper functioning of the oxygen-sensitive nitrogenase enzyme (Mylona et al. 1995). The infected plant cells express large quantities of leghemoglobin (lb), which transfer sufficient oxygen to the bacteroid (Appleby 1984). Earlier in situ localization studies indicated that lb expression is triggered in a single cell layer behind the invasion zone (de Billy et al. 1991). Our findings indicate that lb expression is triggered in the invasion zone and that the abundance of lb mRNA is highest in the interzone and the distal part of the nitrogen-fixing zone. The difference between previously reported lb expression patterns and those presented here could be attributed to small experimental differences such as exposure duration and the amount of radiolabeled probe used per hybridization. In *Vicia faba* roots, lb gene expression is induced 1 h after Nod-factor application (Heidstra et al. 1997), making lb the earliest gene responding to applied Nod factor reported to date. Taken together, these findings would suggest that the lb transcripts are present very early and occur in the invasion zone of alfalfa root nodules.

Because O₂ concentrations are very low in the nodule interior (Bergensen 1997), we determined the expression pattern of ADH, a gene generally associated with anoxia in plants (Walker et al. 1987b). Under low oxygen conditions, ADH genes are rapidly induced (Walker et al. 1987a; Sachs 1991; Sachs et al. 1996). ADH expression patterns could reflect anoxic regions in root nodule tissue. ADH transcripts were detected in the interzone zone and the distal part of the nitrogen-fixing zone. The appearance of ADH transcripts in the interzone coincides with the expression of the oxygen-sensitive bacterial regulator pair *fixL* and *fixJ* (Soupene et al. 1995). This finding supports the suggestion that the O₂ concentration undergoes a dramatic decrease in the interzone II-III boundary (Soupene et al. 1995), which results in the induction of *nif* and *fix* genes (Franssen et al. 1992; Labes et al. 1993; de Maagd et

al. 1994). Two-dimensional nodule oximetry studies performed on alfalfa root nodules indicate a uniform distribution of oxygenated lb throughout the root nodule tissue (Shimada et al. 1997), which would suggest a similar distribution of free O₂ in this tissue (Becana and Klucas 1992). In vitro experimental evidence suggests that pH greatly influences the O₂ affinity of lb (Appleby et al. 1983), although no in vivo data are available. Therefore, it is not clear whether the distribution of oxygenated lb reflects the distribution of free oxygen in this organ. The ADH expression pattern may indicate that there are significant differences in the distribution of free O₂ along the longitudinal axis of an indeterminate root nodule.

In root nodules a large amount of oxygen is needed to fuel the process performed by the bacterial nitrogenase protein. Nitrogenase consists of two components, an Fe protein encoded by *nifH* and a molybdenum-iron (MoFe) protein encoded by *nifD* and *nifK* (Howard and Rees 1996). In an attempt to identify the areas in which nitrogenase is expressed, we localized *nifH* in 19- and 33-day-old root nodules. While *nifH* is expressed in the interzone and throughout the nitrogen-fixing zone of 19-day-old root nodules, in 33-day-old alfalfa root nodules *nifH* transcripts accumulate exclusively in the interzone and a 5- to 15-cell-wide area of the distal nitrogen-fixing zone. In order to test whether the *nifH* expression pattern is unique or reflects a more general pattern of expression for bacterial genes essential for N₂ fixation, malic enzyme (*dme*) transcripts were localized in root nodules. Rhizobial *dme* mutants are incapable of N₂ fixation and therefore indicate that the conversion of malate to pyruvate is essential for this process (Driscoll and Finan 1993). In 19- and 33-day-old alfalfa root nodules, *dme* transcripts are localized throughout the interior of the root nodule. This indicates that the *nifH* expression pattern is the result of specific gene regulation.

Acetylene reduction assays performed on alfalfa root nodule segments indicate that N₂ fixation occurs in the distal part of the nitrogen-fixing zone (Vasse et al. 1990). This distal part corresponds well to the 5- to 15-cell-wide area of *nifH*, GS13, and NADH-GOGAT expression in 33-day-old nodules. The abundance of Mslbc3, GS100, AAT-2, and AS transcripts is also strongest in this region. It has been estimated that the half-life of the nitrogenase enzyme protein is approximately 2 days (Bisseling et al. 1980). Considering this evidence, the restricted expression pattern of *nifH*, GS13, and NADH-GOGAT indicates that there is little to no N₂ fixation and subsequent assimilation of fixed N in the proximal part of the nitrogen-fixing zone.

The reaction catalyzed by GDH offers a potential alternative route for the assimilation of ammonia into glutamate (Oaks 1994). However, the role of GDH in plant metabolism is still unresolved (Lea et al. 1990; Oaks 1994). While some authors believe that a substantial amount of glutamate is formed by GDH (Oaks 1994) others argue that the GDH enzyme functions mainly to deaminate glutamate producing 2-oxoglutarate and ammonia (Lea et al. 1990). RNA blot analysis showed that, in alfalfa, NADH-GDH message is abundant in roots and leaves with low but detectable amounts in stems and nodules. In *Arabidopsis* the NADH-GDH transcripts are also most abundant in leaves and roots (Turano et al. 1997). Our in situ localization of NADH-GDH transcripts in indeterminate alfalfa root nodules shows no differences in the transcript abundance and distribution between day 19 and day 33. NADH-GDH transcript accumulation is low and rather

uniform throughout all root nodule zones and the nodule inner cortex regardless of nodule age. Because there is no enhanced accumulation of NADH-GDH transcripts in the nitrogen-fixing zone, the results suggest that NADH-GDH probably plays only a minor role in the initial assimilation of fixed nitrogen in an alfalfa root nodule. This finding provides further evidence to support the suggestion that the main route of ammonia assimilation into amino acids in N_2 -fixing root nodules occurs through the GS/GOGAT pathway.

The observation that significant levels of GS100 and AS transcripts were found in the proximal part of the nitrogen-fixing zone while N_2 fixation appears to be confined to the distal area leads to the question of what role these enzymes might play in nonfixing tissues. When root nodules become older, protease activity increases (Pladys and Vance 1993), and a senescent zone (zone IV) is formed (Brewin 1991). Senescence is a complex and highly regulated developmental process that results in the remobilization of various compounds to other parts of the plant (Buchanan-Wollaston 1997). In leaf tissue it has been observed that organelle and membrane integrity is maintained until the last phase of senescence, allowing the proper cooperation of the compartments during remobilization (Feller and Fischer 1994). A similar situation may be occurring in the proximal part of the nitrogen-fixing zone of indeterminate root nodules. Three lines of evidence support this. First, there is an abundance of proteases and protease inhibitors in the proximal part of the root nodule tissue (Manen et al. 1991; Kardailsky and Brewin 1996). Second, ultrastructural studies indicate that the bacteroid morphology in the proximal part of the nitrogen-fixing zone is different from that in the distal part (Vance et al. 1979; Paau et al. 1980; Vasse et al. 1990). Third, N_2 fixation appears to be restricted to the distal part of the nitrogen-fixing zone, indicating a fundamental difference in nitrogen metabolism between the distal and proximal regions of the nodule. Several studies indicate an important role for cytosolic GS and AS during senescence (Feller and Fischer 1994; Buchanan-Wollaston 1997). Significant quantities of nitrogen in senescing tissue are converted to transportable amino acids—in particular, glutamine and asparagine (Feller and Fischer 1994). In senescing rice leaves, a cytosolic GS is thought to be important in the remobilization of nitrogenous compounds to N-sink tissues (Kamachi et al. 1992). The cytosolic GS induced during leaf senescence in *Arabidopsis thaliana* is the homologue of GS100 (Bernhard and Matile 1994). The expression of AS has been found to be induced in senescing spears and leaves of asparagus (Davies and King 1993). Considering this evidence, the expression pattern of GS100 and AS in 33-day-old alfalfa root nodules suggests that the corresponding enzymes are involved not only in primary nitrogen assimilation but also in the remobilization of nitrogenous compounds from the proximal part of the nitrogen-fixing zone. Elevated levels of GDH activity have been reported during plant senescence and following a variety of stress applications (Oaks 1994), although the nature of the metabolic function of this enzyme during these processes is still controversial (Feller and Fischer 1994). The role of GDH in the remobilization of nitrogen in the proximal part of the nitrogen-fixing zone is also unclear and no increase in transcript abundance was observed.

In this study, we reported the localization of 13 different *S. meliloti* and alfalfa transcripts in 19- and 33-day-old root nod-

ules. The serial section approach chosen allowed for the construction of a map that reflects the relative distribution of these transcripts in the root nodule tissue. This map revealed the restricted and enhanced accumulation of several transcripts to the distal part of the nitrogen-fixing zone, indicating that this area is metabolically very active in N_2 fixation and primary N assimilation. The expression of the *nifH* gene is restricted to this zone, suggesting that the expression of *nifH* may be used as a molecular marker determining the area of N_2 fixation in an alfalfa root nodule. In a 33-day-old root nodule, N_2 fixation is confined in a 5- to 15-cell-wide zone in the distal nitrogen-fixing zone. In the proximal nitrogen-fixing zone, events associated with senescence occur and the nitrogen may be remobilized by GS and AS. The ADH expression pattern may reflect very low concentrations of oxygen in the root nodule tissue. The transcript distribution map will be used as a navigational tool in developing strategies for genetic engineering of alfalfa root nodule N metabolism and will assist our efforts to enhance plant nitrogen assimilation.

MATERIALS AND METHODS

Plant material and bacterial strains.

Medicago sativa L. 'Saranac' seeds were obtained from JoAnn Lamb (U.S. Department of Agriculture-Agricultural Research Service, St. Paul, MN). Plants were maintained in glasshouse sand benches and were inoculated with effective *Sinorhizobium meliloti* 102F51, as described by Egli et al. (1989). For all studies, the planting date was designated as day 0. At 19 and 33 days after planting and inoculation the plants were harvested, washed free of sand, and placed on ice. The first two nodules from the main root were immediately fixed and embedded for use in subsequent in situ hybridization experiments. Nodules for in situ hybridization studies were harvested between 15:00 and 16:00 to minimize possible diurnal rhythms.

Probes used for in situ hybridization.

The following probes were used: pGS13G and pGS304, which contain the 3' untranslated regions of pGS13 and pGS100, respectively. These represent specific mRNAs for two classes of previously described cytosolic forms of GS from alfalfa root nodules (Temple et al. 1995). Additional probes were a 1.8-kbp (*Bam*HI-*Not*I) NADH-GOGAT cDNA insert (Gregerson et al. 1993), a 1.4-kbp AAT-2 cDNA fragment (Gantt et al. 1992), a 0.6-kbp AS cDNA fragment (Shi et al. 1997), a 0.7-kbp ADH-1 cDNA fragment, a 1.4-kbp (*Bgl*II-*Bgl*II) SuSy cDNA fragment (Robinson et al., *in press*), and a 1.4-kbp *dme* gene fragment. The ADH-1 and *dme* genes were generous gifts of Bob Gregerson (Lyon College, Batesville, AR) and Turlough Finan (McMaster University, Hamilton, Ontario, Canada). GDH and GS₂ were isolated from a alfalfa nodule cDNA library made in the Lambda-UniZapXR vector (Stratagene, La Jolla, CA) with heterologous probes: GDH (Turano et al. 1997) and GS₂ (Sukanya et al. 1994) as described by Shi et al. (1997). The isolated clones were partially sequenced to confirm their identity. For in situ hybridizations, a 1.8-kbp AS cDNA fragment, a 1.6-kbp GDH cDNA fragment, and a 1.7-kbp GS₂ cDNA fragment were used. As controls, a 1.1-kbp *nifH* gene fragment and a 0.9-kbp leghemoglobin cDNA were used. The *nifH* gene fragment and the leghemoglobin cDNA fragment were generous gifts from

Mike Sadowsky (University of Minnesota, St. Paul) and Ann Hirsch (University of California, Los Angeles), respectively.

Preparation of RNA probes.

All probes used in this study were generated from linearized plasmids. Each construct was linearized with the appropriate enzyme and transcribed in the sense and antisense directions with SP6, T3, or T7 RNA polymerases and ^{35}S -UTP as described by the manufacturer (Stratagene, La Jolla, CA). With the exception of pGS13G and pGS304, the probes were partially degraded to an average length of 150 nucleotides by heating at 60°C in 0.06 M Na_2CO_3 /0.04 M NaHCO_3 (Cox and Goldberg 1988).

In situ hybridization.

The tissue for all in situ hybridizations was collected as follows: The planting date was considered to be day 0; at harvest dates (19 and 33 days) the two top root nodules on the main root were used for subsequent studies. In situ hybridization was carried out essentially as described by Fleming et al. (1993). Tissues were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2). The tissues were rinsed twice in the same buffer and twice in deionized water, and dehydrated in a graded ethanol series. Subsequently the absolute ethanol was replaced with xylene and the tissue was embedded in Paraplast (Oxford Labware, St. Louis, MO). The embedded tissues were sectioned to a thickness of 7 μm and affixed to poly-L-lysine coated slides. Hybridizations were performed in mineral oil as described by Heintzen et al. (1994), and the final washing conditions were $0.1\times$ SSC ($1\times$ SSC is 0.15 M NaCl, 15 mM sodium citrate) including 1 mM DTT (dithiothreitol) at 50°C for 20 min. The slides were coated with a liquid photoemulsion (NBT-2; Eastman Kodak, Rochester, NY) and exposed in complete darkness at 4°C. The length of exposure for different probes was adjusted to obtain the optimal signal intensity, but was typically between 1 and 4 weeks. After development, the sections were stained with 0.05% toluidine blue O, dehydrated in ethanol and xylene, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Sections were viewed and photographed with a Labophot microscope (Nikon, Garden City, NY) equipped with dark- and bright-field optics.

RNA blot analysis.

Total RNA was isolated from alfalfa roots, nodules, stems, leaves, and cotyledons as described by Gregerson et al. (1993). Poly A⁺ RNA was obtained by a single pass over an oligo(dT) column (Gibco BRL, Gaithersburg, MD). RNA gel blotting was performed essentially as described by Gantt et al. (1992). Two micrograms of poly A⁺ RNA was separated on denaturing agarose gels, transferred to ZetaProbe (Bio-Rad, Hercules CA) membranes, and hybridized with ^{32}P -labeled GDH cDNA fragment. Probing with ^{32}P -labeled polyuridine showed that RNA loading in each lane was comparable.

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