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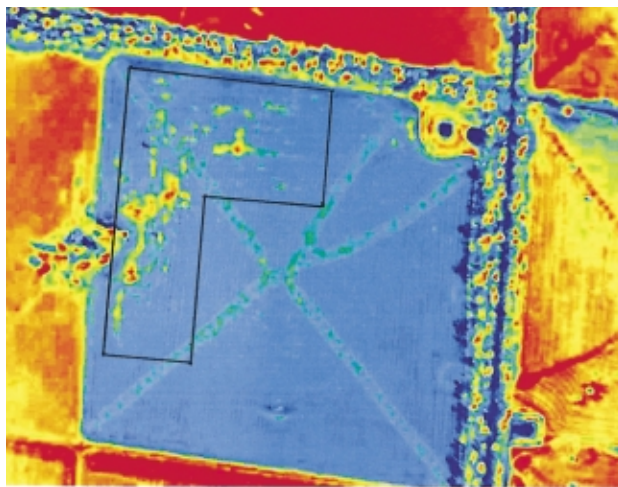
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Application of biochemical studies to improving nitrogen fixation

C. P. Vance[AB] *and J. F. S. Lamb*[A]

[A]US Department of Agriculture, Agricultural Research Service, Plant Science Research Unit, and the Department of Agronomy and Plant Genetics, University of Minnesota, 411 Borlaug Hall, 1991 Upper Buford Circle, St Paul, MN 55108, United States.
[B]Author for correspondence; e-mail: vance004@tc.umn.edu

Abstract. Improvement of symbiotic nitrogen fixation requires a multidisciplinary approach with a comprehensive program ranging from microbial ecology to plant breeding and genomics. Achievement of symbiotic nitrogen fixation requires at least 100 genes from each partner interacting in a favorable environment. The more information that we obtain from applied and fundamental studies of *Rhizobium*–legume and *Frankia*–non-legume symbioses, the greater are our chances to extend nitrogen fixation to non-fixing species. Studies with alfalfa (*Medicago sativa* L.) aimed at improving symbiotic nitrogen fixation have resulted in significant advances in germplasm development, plant biochemistry, microbial ecology and the understanding of plant genes involved in nodule nitrogen and carbon metabolism. However, translation to field improvement of symbiotic nitrogen fixation has proven elusive.

Additional keywords: root nodules, nitrogen assimilation, carbon assimilation, molecular, plant breeding.

Introduction

Symbiotic nitrogen (N_2) fixation is one of the most important biochemical processes on earth. Annually about 90×10^9 kg of N_2 is fixed symbiotically, principally by *Rhizobium/Bradyrhizobium/Sinorhizobium* bacteria in root nodules of legume crops (Vance 1998). In agricultural systems symbiotically fixed N can: (i) be an immediate source of N to the fixing species for dry matter and seed production; (ii) be released from the fixing species to companion crops to supplement their N needs; and (iii) be useful as a green manure providing N to crops grown in rotations (Heichel *et al.* 1989; Peoples *et al.* 1995). The inherent capacity for N_2 fixation by the legume–rhizobia symbiosis is a mainstay in cost-effective, ecologically sound approaches to sustainable agricultural practices.

Formation of a successful symbiotic association requires the coordinated expression of both bacterial and plant genes in the appropriate ecological niche (Schultze and Kondorosi 1998; Perret *et al.* 2000). Roots of the legume plants release flavonoids and isoflavonoids that act as signals for the induction of nodulation (*nod*) genes in the rhizobial partner (Laeremans and Vanderleyden 1998). Bacterial *nod* genes synthesise complex lipo-chitooligosaccharides (LCO, nod factors) that signal root cortical cell division and nodule initiation. The bacteria then penetrate the susceptible root hairs and migrate through infection threads to the nodule initials. Thereupon, plant cells become infected, nitrogenase expression is induced and plant cells assimilate fixed N and transport the products of N assimilation (Vance 1997). Because of these unique features, nodules are made up of several cell types (meristematic cells, infected and uninfected cells, vascular tissue, oxygen diffusion barrier cell layers, parenchyma and schlerenchyma cells). To date, at least 100 genes, 50 from the bacterial partner and 50 from the host plant, have been implicated in the establishment of effective N_2-fixing symbioses (Hirsch and LaRue 1997; Szczyglowski *et al.* 1998; Suganuma 1999). Undoubtedly more genes affecting symbiosis will be identified. Thus, it becomes obvious that genetic improvement of N_2 fixation is not a trivial task.

Numerous strategies have been advanced to improve N_2 fixation including, but not limited to: (i) selection of highly effective and competitive rhizobial strains; (ii) development of legume cultivars that have restricted nodulation to highly effective rhizobia; (iii) increasing the capacity for rhizobia to synthesise nod factors; (iv) enhancing the carbon uptake capacity of rhizobia; (v) selection for increased root nodule nitrogenase activity; and (vi) using mutagenesis approaches to develop super-nodulating legume cultivars (Ladha *et al.* 1992; Bliss and Hardarson 1993). A review of each of these approaches is beyond the scope and intent of this contribution. Rather, here we present an overview of studies from our laboratories over the last several years to apply biochemical studies to improving symbiotic N_2 fixation in alfalfa (*Medicago sativa* L.). The strategies that have been employed involve: (i) defining the metabolic pathway for the assimilation of fixed nitrogen; (ii) conducting bidirectional selection for biochemical traits thought to limit N_2 fixation; (iii) developing plants incapable of N_2 fixation (ineffective) for comparative biochemical and molecular studies and for

Table 1. Predominant amino acids in root nodules of species that transport symbiotically fixed nitrogen as amides

Data derived from Sprent (1980), Ta *et al.* (1986), Vance *et al.* (1987), Rosendahl *et al.* (1990),
Baker *et al.* (1997), and C. P. Vance unpublished data
Values are µmol/g fresh weight

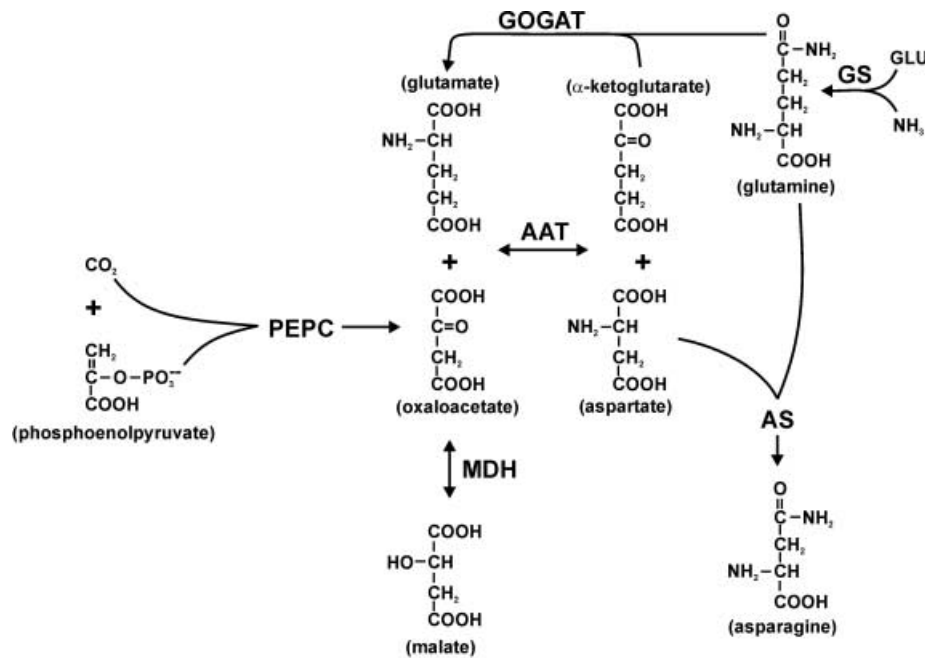| Amino acid	Alfalfa	Trefoil	Pea	Myrica	Mean
Asparagine	44.70	17.20	9.50	21.20	23.15
Aspartate	0.75	1.20	0.20	0.28	0.61
Glutamine	4.08	1.00	0.43	1.23	1.68
Glutamate	4.20	1.40	1.77	2.02	2.35
Alanine	2.25	1.31	1.10	1.00	1.42
γ-aminobutyric	2.70	1.50	0.67	0.48	1.34
Serine	0.69	2.00	0.30	0.28	0.81

Figure 1. Metabolic scheme for the initial assimilation of ammonia derived from symbiotic nitrogen fixation into amino acids. Scheme also includes the synthesis of oxaloacetate and malate with carbon derived from root nodule CO_2 fixation and phosphoenolpyruvate. Enzymes involved include: glutamine synthetase (GS), glutamate synthase (GOGAT), aspartate aminotransferase (AAT), asparagine synthetase (AS), phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH).

controls in field measurements of N_2 fixation; (iv) isolation, characterisation, and modification of the expression of genes thought to limit the assimilation of fixed N.

Define the metabolic pathway for assimilation of fixed nitrogen

In the legume–rhizobia symbiosis, ammonia, synthesised from the reduction of N_2 by nitrogenase, is released from the bacteroid and transported via an ammonium transporter (Kaiser *et al.* 1998) across the symbiosome membrane into the plant cytosol where initial assimilation into amino acids occurs (Lea and Ireland 1999). The predominant amino acids in most temperate legumes [e.g. alfalfa, pea (*Pisum sativum* L.) and trefoil (*Lotus pedunculatus* L.)], as well as some non legumes (e.g. *Myrica*), are the amides asparagine (Asn) and glutamine (Gln) along with aspartate (Asp), glutamate (Glu), alanine (Ala), γ-aminobutyric acid (Gaba) and serine (Ser) (Table 1). Large quantities of these amino acids, particularly asparagine, are also transported from the nodule in the xylem stream to distal organs of temperate legume species (Sprent

1980; Ta *et al.* 1986; Vance *et al.* 1987; Rosendahl *et al.* 1990; Baker *et al.* 1997). We, along with others, have used $^{14}CO_2$, inhibitors, and $^{15}N_2$ to show that primary N assimilation in nodules occurs first into Gln and Glu with subsequent rapid incorporation into Asp and Asn (Fig. 1) (Ta *et al.* 1986; Rosendahl *et al.* 1990). Thus, the initial assimilation of ammonia derived from symbiotic N_2 fixation into amino acids in a large number of interactions occurs through the combined action of 4 enzymes (Fig. 1). Glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.1.14), frequently designated as the GS/GOGAT cycle, catalyse the synthesis of Gln and Glu, respectively. Further incorporation of N into Asp and Asn occurs through the action of aspartate aminotransferase (AAT, EC 2.6.1.1) and asparagine synthetase (AS, EC 6.3.5.4) (Ireland and Lea 1999). The carbon (C) skeletons for initial assimilation of N are derived from TCA intermediates α-ketoglutarate and oxaloacetate (Vance 1998). Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) and malic dehydrogenase (MDH, EC 1.1.1.82) provide a substantial amount of C to bacteroids to fuel the nitrogenase reaction and to replenish the organic acid pool for the synthesis of Asp (Vance and Heichel 1991; Day and Copeland 1991). Thus, the fixation and assimilation of N is inextricably linked to C metabolism.

Although we were not the first group to report that effective root nodules had significant activities for GS, GOGAT, AAT, AS, PEPC and MDH, we were the first to conclusively demonstrate that the activity of these enzymes was tightly linked to nitrogenase activity and nodule function. Groat and Vance (1981, 1982) demonstrated that alfalfa nodule GS, NADH-GOGAT and AS activities rapidly declined with treatments that reduced nitrogenase activity but activities recovered as nitrogenase recovered. Moreover, they showed that ineffective nodules, both plant- and bacteria-controlled, had reduced enzyme activities. In addition, Groat *et al.* (1984) demonstrated that alfalfa germplasm selected for increased nitrogenase activity via acetylene reduction had genetic variation for PEPC and NADH-GOGAT and that these enzymes were highly correlated with nitrogenase activity. Other studies by Vance and Johnson (1983), Maxwell *et al.* (1984), Snapp and Vance (1986) and Egli *et al.* (1989) showed a similar tight link between effective nodulation and root nodule NADH-GOGAT, PEPC, AAT and MDH activities. The foundation studies initiated by Groat in the late 1970s and early 1980s laid the framework for subsequent efforts that led to the purification, characterisation, and production of antibodies for nodule enzymes of C and N assimilation (described elsewhere in this paper).

Bidirectional selection for biochemical traits related to nitrogen fixation

Since nitrogenase activity expressed in effective root nodules is the focal point for N_2 fixation, initial efforts in
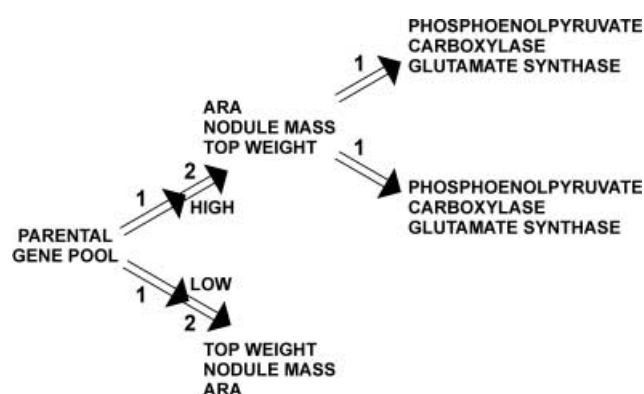

Figure 2. Schematic diagram for bidirectional selection for morphological and biochemical traits related to nitrogen fixation. Initially 2 cycles of selection were completed for acetylene reduction activity (ARA), nodule mass, shoot dry weight and root mass. Plants selected initially for high ARA, nodule mass, shoot dry weight and root mass were then subjected to another cycle of selection for phosphoenolpyruvate carboxylase (PEPC) and glutamate synthase (GOGAT).

breeding for a biochemical component involved bidirectional selection for nitrogenase (Fig. 2). Using acetylene reduction activity (ARA) as a measure of nitrogenase, Seetin and Barnes (1977) demonstrated significant (4-fold) variation in ARA among individual alfalfa plants. They also showed that high × high ARA crosses gave progeny with twice the ARA of low × low ARA crosses. Similar results have been reported for clover (Smith *et al.* 1982) and bean (Bliss and Hardarson 1993).

To verify that nitrogenase activity as measured by ARA could be a useful component in a breeding program and to establish which plant traits related to ARA, an extensive greenhouse experiment was established to evaluate the response to bidirectional selection for individual plant ARA, shoot dry weight, nodule mass and fibrous root mass (Viands *et al.* 1981). Two broad-based gene pools differing in winter hardiness were used as base populations. Both gene pools responded to selection for the 4 traits, and highly significant differences were observed between the high and low subpopulations for each characteristic in each germplasm source (Barnes *et al.* 1984). Nodule mass, ARA, fibrous root mass and shoot dry weight were highly intercorrelated (Table 2). Nodule mass explained 31–42% of the variation in nitrogenase activity. However, 40–60% of that variation

Table 2.   Selection for traits related to biological nitrogen fixation

Nodule mass explained 31–42% of the variation in nitrogenase
Data are derived from Viands *et al.* (1981) and Barnes *et al.* (1984)

Trait	Response
Nitrogenase (ARA)	***
Nodule mass	***
Shoot dry weight	***
Fibrous root mass	***

remained unexplained. As mentioned in the previous section, Groat *et al.* (1984) found significant differences in subpopulations for nodule GS, GDH, NADH-GOGAT and PEPC activities but only NADH-GOGAT and PEPC were highly correlated with selection for nitrogenase.

Field studies using isotope dilution procedures and antibiotic-resistant mutants of *Rhizobium* were conducted with alfalfa subpopulations from the glasshouse selection experiments (Heichel *et al.* 1981; Hardarson *et al.* 1982). Rankings for N_2 fixation measured in the glasshouse by ARA generally were maintained in the field over years according to the ^{15}N evaluations. However, both high and low subpopulations failed to yield more than the unselected parental germplasm pools (Barnes *et al.* 1984), suggesting that inbreeding depression had occurred while selecting for physiological traits. In contrast, host selection for physiological and morphological traits associated with N_2 fixation had modified host–*Rhizobium* compatibility. Alfalfa subpopulations selected for increased nitrogenase activity exhibited increased preference for effective *Rhizobium* strains and decreased preference for less effective indigenous strains.

The lack of response to selection noted in field trials of greenhouse-selected material could be the result of numerous factors (Heichel *et al.* 1989). The primary reason is probably because ARA is a single indirect assessment of nitrogenase activity, while field performance is an integrated event over an entire season. Moreover, later experiments by Minchin *et al.* (1983) showed that the ARA assay contains inherent errors that can interfere with accuracy and reproducibility of data. In addition, the field environment can be vastly different to the greenhouse, resulting in a significant impact on gene expression. Barnes *et al.* (1984) also noted that inbreeding depression was observed during selection for physiological traits and may have reduced the performance of elite lines. In any event, the problems encountered when transferring greenhouse selection results to the field remain large, and breeding programs must recognise these difficulties.

Efforts to improve N_2 fixation by selection for nitrogenase or other related traits may have been compromised if the plant's potential to assimilate N was limited due to inadequate amounts of plant enzymes involved in nodule N metabolism. Since we had established the route of N assimilation in alfalfa and had shown that nodule NADH-GOGAT and PEPC activities were highly correlated with nitrogenase activity, we thought it important to evaluate the genetic variation and response to selection for several plant enzymes involved in nodule N assimilation.

Jessen *et al.* (1987) evaluated 6 diverse alfalfa germplasm sources, including subpopulations developed by Viands *et al.* (1981) that varied for traits associated with N_2 fixation, for *in vitro* activities of NADH-GOGAT and PEPC. Variability existed for activities of nodule NADH-GOGAT and PEPC and those relative differences among alfalfa genotypes were

reproducible. They concluded that NADH-GOGAT and PEPC activities should be investigated as selection criteria in an alfalfa breeding program to enhance N_2 fixation. One cycle of bidirectional selection for NADH-GOGAT and PEPC activities was conducted on subpopulations previously selected for seedling vigor, large shoot mass, large nodule mass, large root mass and high ARA (Jessen *et al.* 1988). Both enzymes responded to this selection procedure. The high subpopulations had nodule NADH-GOGAT and PEPC activities that averaged 20 and 18% greater, respectively, than the low subpopulations. This was the first time that the activities of 2 enzymes in a single organ were altered by selection. Although selection for high enzyme activities did not enhance plant performance, selection for reduced nodule NADH-GOGAT and PEPC resulted in decreased shoot dry weight and N_2 fixation. These results suggested that threshold levels of these nodule enzyme activities must be maintained to achieve adequate crop growth. Furthermore, the lack of a positive growth response in the high selections implied that either these enzymes are not limiting or enzymes further downstream involved in aspartate and asparagine formation are insufficient.

To evaluate steps downstream from PEPC and NADH-GOGAT, Degenhart *et al.* (1992) investigated the potential of nodule AS and AAT activities to increase N_2 fixation and N assimilation in alfalfa. One cycle of bidirectional selection for AAT and AS activities was conducted in 3 unrelated alfalfa germplasm sources including 2 sources developed by Jessen *et al.* (1988) for high nodule enzyme activity (GOGAT and PEPC). Asparagine synthetase activity exhibited low heritability and a poor association with ARA, plant N concentration and plant dry weight and was not recommended as a selection criterion for improving N_2 fixation in alfalfa. Heritability estimates of ATT activity were moderately high, but associations with plant dry weight, N concentration and ARA were not strong enough to warrant its use for improving N_2 fixation. In subsequent studies Farnham *et al.* (1992) showed that selection for total nodule AAT activity specifically affected a particular isozyme of AAT, namely AAT-2, while AAT-1 was not altered. Selection altered AAT-2 specific activity and concentration of AAT-2 protein but the total production of AAT-2 polypeptide was unchanged, because nodule mass varied inversely with nodule AAT-2. For example, selections having high nodule AAT-2 activity were reduced in nodule mass. Although similar compensation has been observed previously in that selection for improved seed weight and protein leads to plants with reduced total yield, Farnham *et al.* (1992) were the first to demonstrate compensation at the molecular level.

To further assess whether selection for root nodule N assimilation enzyme activities and other traits related to N_2 fixation affected field performance of alfalfa, several subpopulations selected under N_2-dependent conditions in the greenhouse were established in yield trials at 2 locations. Ineffective alfalfa was grown as the non-fixing control and

Table 3. Designation, genotype, and phenotype of ineffective and non-nodulating alfalfas

IN, ineffective nodules; NN, no nodules
Data are derived from Peterson and Barnes (1981) and Vance *et al.* (1984)

Designation	Genotype	Phenotype
*in₁*Saranac	in_1	nod⁺fix⁻: normal pale
*in₁*Agate	in_1	nod⁺fix⁻¹: normal pale
MN-3226 (IN)	in_2	nod⁺fix⁻: tumor-like, empty
MN-3811 (IN)	in_3	nod⁺fix⁻: tumor-like, empty
MNPL-480 (IN)	$in_4 in_5$	nod⁺fix⁻: tumor-like, empty
MNNC-1008 (NN)	$nn_1 nn_2$	nod⁻myc⁻

N_2 fixation was assessed by the difference method. No differences in total herbage yield or percentage N derived from symbiosis were found between selected and unselected populations (J. F. S. Lamb, M. P. Russelle and C. P. Vance unpublished data). The selected populations appeared to have significant inbreeding depression resulting from recurrent selection.

While yield trials from our breeding experiments have been disappointing, these studies have provided valuable information for future reference. Foremost is that selection for a single biochemical or physiological trait will not be sufficient to improve a complex trait like N_2 fixation. Progress in improving N_2 fixation will require methods that allow stacking of several traits during a breeding program. Secondly, because of the difficulty of transferring results from greenhouse studies to the field, parallel field selection must accompany greenhouse experiments. Moreover, plants capable of N_2 fixation should not only be selected under low N conditions but also high N environments to ensure that leaf N assimilation is improved in synchrony with symbiotic N_2 fixation. Because of inbreeding depression, selection pressure in the base populations must be adjusted and all populations outcrossed to achieve maximum gain. Lastly, we have discovered that selection for several individual biochemical traits in a single organ is possible but improved N_2 fixation and yield are functions of numerous parameters requiring integration over the totality of plant growth and development.

Develop non-nitrogen-fixing alfalfa genotypes for use in fundamental and applied studies

Early in the course of evaluating N metabolic pathways and assessing field rates of N_2 fixation it became apparent that the development of ineffective lines of alfalfa would be a necessity. During several experiments in which thousands of plants were grown in the greenhouse under symbiotic conditions, we would occasionally find chlorotic, stunted plants. These plants could be rescued by growth on nitrate. Invariably these chlorotic, stunted plants turned out to be either ineffectively nodulated or non-nodulated.

At least 5 genes in alfalfa designated $in_1 - in_5$ control ineffective nodulation (Table 3) (Peterson and Barnes 1981). All genes controlling ineffectiveness are recessive with tetrasomic inheritance. These plants are ineffective with all strains of *S. meliloti*. A non-nodulation trait is conditioned by 2 tetrasomically inherited genes, nn_1 and nn_2. The in_1 genotype in both the Saranac and Agate backgrounds has numerous nodules relatively normal in external morphology but bacteroids exhibit early senescence (Vance and Johnson 1983; Vance *et al.* 1984). By contrast, the $in_2 - in_5$ genotypes have normal invasion but few bacteria are released. Nodule growth proliferates but cells are devoid of bacteroids (empty). These nodules are cream colored and appear tumor-like. Although all genotypes have been utilised, the most thoroughly studied has been *in₁*Saranac, because it is the most normal in nodule morphology, and displays root and herbage characteristics similar to those of effective genotypes. Several physiological and biochemical comparisons of *in₁*Saranac and effective Saranac are shown in Table 4.

Table 4. Biochemical and physiological characteristics of ineffective *in₁*Saranac and effective Saranac

Data derived from Maxwell *et al.* (1984), Vance *et al.* (1984), Egli *et al.* (1989), and C. P. Vance unpublished data

Parameter	*in₁*Saranac	Saranac	Reduction (%)
Acetylene reduction (nmol/g FW)	0–2	172	99
Asparagine + aspartate in xylem sap (nmol/cm³)	150	7000	98
Nodule CO_2 fixation (μg/kg.s)	96	400	76
Nodule respiration (mg/kg.s)	0.5	5	90
Label in xylem sap amino acids (% total ¹⁴C)	3	85	96
Soluble protein (mg/g FW)	4	17	76
Enzyme activity (nmol/min.mg protein)			
GOGAT	5	105	95
PEPC	100	750	87
Nodule malate (μmol/g FW)	0.5	9	95
Nodule amino acids (nmol/g FW)	600	15 000	96
Herbage N yield (mg/plant)	6	56	91

The ineffective and non-nodulating germplasms have been released to the public (Barnes *et al.* 1988, 1990) and small amounts of seed are available upon request. Most recently, the *in₁*-ineffective gene has been moved into several diverse germplasms showing a range of dormancy traits (J. F. S. Lamb and D. A. Samac unpublished data).

Numerous discoveries related to nodule growth and function have been facilitated by the development of ineffective and non-nodulating genotypes (Table 4): (i) accurate field measurements of symbiotic N_2 fixation in alfalfa are now easily accomplished (Heichel *et al.* 1984); (ii) genes controlling non-nodulation and mycorrhizal infection are closely linked (Duc *et al.* 1989); (iii) the role of GS, NADH-GOGAT, AAT, AS, PEPC and MDH in nodule N and C metabolism was conclusively defined (Egli *et al.* 1989; Vance *et al.* 1994); (iv) maximum expression of genes involved in nodule N and C metabolism require effective N_2 fixation (Maxwell *et al.* 1984; Snapp and Vance 1986); (v) control of plant gene expression involves both transcriptional and post-translational mechanisms (Pathirana *et al.* 1992; Shi *et al.* 1997; Vance *et al.* 1997); (vi) proteolysis during nodule senescence can be selective (Pladys and Vance 1993); (vii) nodule CO_2 fixation provides a major source of C for N_2 fixation and N assimilation (Maxwell *et al.* 1984); (viii) the components of respiration resulting from nodule maintenance and N_2 fixation can be readily separated (Maxwell *et al.* 1984; Snapp and Vance 1986); (ix) ineffective genotypes appear to be more efficient at utilising soil N than effective genotypes (M. P. Russelle unpublished data); and (x) N_2 fixation can continue at significant levels even in the presence of very high amounts of N fertiliser (Lamb *et al.* 1995).

Isolate, characterise, and modify the expression of genes involved in alfalfa nodule nitrogen and carbon metabolism

Because large quantities of symbiotically fixed N are assimilated into amino acids in root nodules (Vance 1997) and because amides and other N-containing products appear to play a role in feedback control of nitrogenase activity (Parsons *et al.* 1993; Hartwig 1998), root nodules are exquisite models for biochemical and molecular studies to improve N assimilation. Additionally, the fact that root nodules are not only strong sinks for C (Vance and Heichel 1991) but they can also fix CO_2 into organic acids (Maxwell *et al.* 1984) at quite high rates make them ideal for determining how C metabolism and N assimilation are intertwined. Our efforts toward developing the biochemical and molecular foundation to improve N_2 fixation as well as N and C assimilation have involved isolation and characterisation of many of the major enzymes involved in the initial reactions catalysing C and N assimilation in alfalfa nodules. Using reverse genetics and mutant complementation strategies the corresponding cDNAs were isolated, characterised, and used to determine tissue

mRNA expression patterns and whether root nodule expression was dependent upon nitrogenase activity. The importance of NADH-GOGAT, MDH, AS, AAT, GS, PEPC and sucrose synthase (SS) to nodule N and C metabolism is evidenced by their striking enhancement in effective root nodules (Fig. 3 and Table 5). Summarised below is an overview of strategies and approaches we have used to define the central role of NADH-GOGAT and MDH in alfalfa nodule N and C metabolism. Although we have used similar strategies to study other enzymes (listed in Table 5 and Figs 1 and 3), space restraints preclude their incorporation here. Much of the information presented on NADH-GOGAT and MDH is applicable to other N_2-fixing crops.

NADH-glutamate synthase

Glutamate-2-oxyglutarate aminotransferase (glutamate synthase, GOGAT) catalyses the transfer of the amide group from glutamine to α-ketoglutarate to yield 2 molecules of Glu (Fig. 1). This enzyme in conjunction with GS is collectively referred to as the GS/GOGAT cycle (Temple *et al.* 1998; Ireland and Lea 1999). The synthesised Glu can be used either to replenish the pool of Glu for subsequent Gln synthesis via GS or to provide amino groups for other amino acids and N-containing compounds like Asp and Asn. In higher plants GOGAT occurs as 2 distinct forms, ferredoxin-dependent, Fd-GOGAT (EC 1.2.7.1), and NADH-GOGAT (EC 1.4.1.14). These forms differ in M_r, subunit composition, enzyme kinetics, antigenic specificity, tissue localisation, and metabolic function (Suzuki and Gadal 1984; Temple *et al.* 1998). Fd-GOGAT is an iron–sulfur flavoprotein with a subunit molecular mass of 130–180 kDa that is generally considered to function as a monomer. The enzyme has a pH optimum of 6.9–7.5 and apparent K_m values for ferredoxin, Gln, and α-ketoglutarate of 2–6, 100–1000, and 7–70 µmol/L, respectively. Studies with *Arabidopsis*, barley and pea mutants either lacking Fd-GOGAT or having reduced enzyme activity show that this form of the enzyme functions in assimilation of ammonia derived from reduction of NO_3^- and ammonia generated during photorespiration (Ireland and Lea 1999 and references therein). Expression of antisense orientation Fd-GOGAT in tobacco substantiates the above roles for Fd-GOGAT.

Maize roots contain a Fd-GOGAT isoform that is immunologically distinct from the enzyme found in leaves, suggesting that the 2 forms are encoded by distinct genes. The root isoform has been implicated in the assimilation of ammonia derived from soil NO_3^-. Recent studies indicate that *Arabidopsis* also contains 2 distinct and apparently functional Fd-GOGAT genes (*GLU1* and *GLU2*) (Lam *et al.* 1996). The *GLU1* gene is expressed predominantly in leaves, and *GLU2* expression is more abundant in roots. Legume root nodules are also reported to contain low Fd-GOGAT
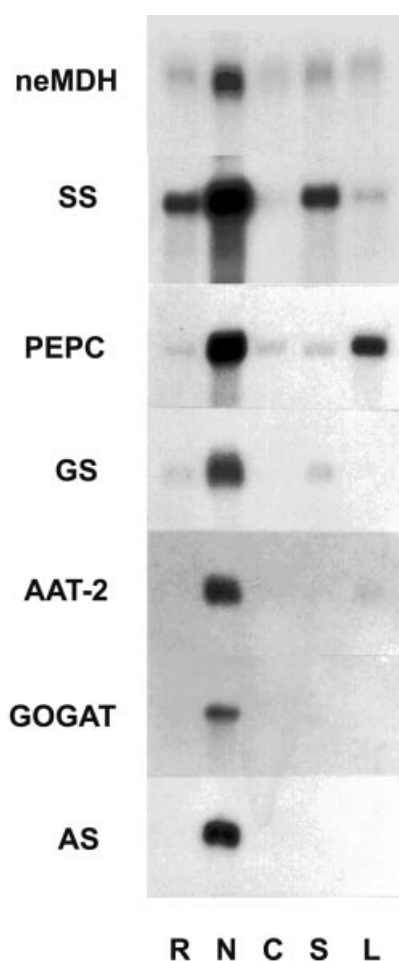
Figure 3. Abundance of transcripts encoding nodule enhanced forms of MDH, SS (sucrose synthase), PEPC, GS, AAT-2, NADH-GOGAT, and AS. PolyA$^+$RNA source was roots (R), nodules (N), cotyledons (C), stems (S) and leaves (L). Each lane contains 2 μg of polyA$^+$RNA. Data derived from Vance *et al.* (1994), Shi *et al.* (1997), Miller *et al.* (1998) and Robinson *et al.* (1999). See text for abbreviations.

activity and mRNA; however, its role is not understood (Suzuki *et al.* 1988).

NADH-GOGAT, like Fd-GOGAT, is also an iron–sulfur flavoprotein, but this enzyme is found primarily in non-green tissues. NADH-GOGAT has been purified and characterised from legume root nodules and rice cell cultures (Chen and Cullimore 1988; Anderson *et al.* 1989; Goto *et al.* 1999). In nitrogen-fixing legume nodules, NADH-GOGAT activity has been found to increase markedly during nodule development, and this activity is associated with a single form of the enzyme. In higher plants, NADH-GOGAT exists as monomers with a native subunit mass of about 225–230 kDa; has a pH-optimum range from 7.5 to 8.5; and apparent K_m values for NADH, Gln, and α-ketoglutarate of 4–13, 400–1000, and 39–960 μmol/L, respectively. There is negligible NADH-GOGAT mRNA, enzyme activity, or enzyme protein in alfalfa leaves and roots (Fig. 3). However,

alfalfa nodules contain a single NADH-GOGAT isozyme that increase in activity, protein, and mRNA during effective nodule development (Gregerson *et al.* 1993; Trepp *et al.* 1999*b*). By comparison, bean nodule NADH-GOGAT appears to occur as 2 isoforms (I and II) (Chen and Cullimore 1988), with the observed increase in GOGAT activity during nodule development resulting primarily from an increase in activity of isozyme II. Two distinct cDNAs for NADH-GOGAT have been isolated from a bean root nodule cDNA library and both are expressed in effective bean nodules (M. Lara, L. Blanco and C. Vance unpublished data).

Since much of our early data pointed to root nodule NADH-GOGAT as a key enzyme in the assimilation of symbiotically fixed N (Groat and Vance 1981; Groat *et al.* 1984) and breeding studies suggested that the enzyme may be a target for modification to improve N_2 fixation (Jessen *et al.* 1987, 1988), we extensively characterised this protein at the biochemical and molecular level. Alfalfa NADH-GOGAT appears to be encoded by a single 14 kbp gene comprised of 22 exons interrupted by 21 introns (Vance *et al.* 1995; Trepp *et al.* 1999*b*). A 2.7 kbp region 5' from the translation initiation site confers nodule specific reporter gene activity used in a chimeric β-glucuronidase (GUS) construct and transformed into alfalfa and birdsfoot trefoil (*Lotus corniculatus* L.). Both infected and uninfected nodule cells display GUS activity but activity appears greater in the infected cells. Promoter deletion studies show that nodule specificity in the N_2-fixing zone is controlled by an element(s) between –1064 and –655 bp (Vance *et al.* 1995; Trepp *et al.* 1999*b*). This 409 kbp region contains an 88 bp AT rich direct repeat that could be involved in nodule specificity (Fig. 4).

The alfalfa *NADH-GOGAT* gene encodes a 7.2 kbp mRNA which translates into a 240 kDa protein (Gregerson *et al.* 1993). The deduced amino acid sequence of NADH-GOGAT is similar to both Fd-GOGAT from maize and NADPH-GOGAT from *Escherichia coli*. Amino acid sequence determination of the native protein showed that the pre-protein is processed at position 101 resulting in a mature protein of 229 kDa (2093 amino acids). Similar to Fd-GOGAT, the deduced amino acid sequence of the presequence resembles a plastid targeting polypeptide. Recent immunogold localisation studies have shown that the enzyme is primarily localised in amyloplasts of infected root nodule cells (Trepp *et al.* 1999*a*). Amyloplasts of infected cells contained 3-fold more NADH-GOGAT protein than amyloplasts of uninfected cells (Table 6). Likewise, *in situ* hybridisation studies of NADH-GOGAT mRNA showed that infected cells contained striking amounts of message while the signal in uninfected cells was much lower (Trepp *et al.* 1999*b*). Protein immunoblots and mRNA blots also showed that expression of NADH-GOGAT activity, protein, and mRNA were very high in effective nodules but low to nondetectable in ineffective nodules (Fig. 5). In comparison, GS, AAT, AS,

Table 5. Activity and relative abundance of alfalfa nodule enzymes involved in nitrogen and carbon assimilation

Data derived from Egli *et al.* (1989), Gantt *et al.* (1992), Pathirana *et al.* (1992), Gregerson *et al.* (1993), Shi *et al.* (1997), Miller *et al.* (1998), Robinson *et al.* (1999) and unpublished data

Enzyme	Specific activity (nmol/min.mg protein)	Relative abundance (% soluble protein)	Nodule mRNA expression (dpm) fix[+]	fix[−]
NADH-glutamate synthase	90	0.1–0.4	1500	200
Glutamine synthetase[A]	120	1.0–2.0	2000	400
Aspartate aminotransferase (AAT-2)	800	1.0–2.0	4750	1040
Asparagine synthetase	50	0.2–0.6	10 000	1500
Sucrose synthase	600	0.5–1.0	4000	400
Phosphoenolpyruvate carboxylase	730	2.0–3.0	12 000	3000
Malate dehydrogenase (neMDH)	10 000	0.1–0.7	850	270

[A]Value comparisons of radioactivity can be made between fix[+] and fix[−] nodules, but those between enzymes are not valid because they are from different experiments.

PEPC, MDH, SS, mRNAs and protein are found at substantial levels in ineffective nodules.

Although Fd-GOGAT has been reported in root nodules and suggested to be important in nodule N metabolism (Suzuki *et al.* 1988), we have been unable to detect significant amounts of either Fd-GOGAT mRNA or enzyme activity (Vance *et al.* 1995). We isolated a partial Fd-GOGAT cDNA from alfalfa and evaluated expression of the message in various tissues (Fig. 6). Cotyledons and leaves had abundant Fd-GOGAT message while roots, nodules, and stems contained little to no Fd-GOGAT mRNA.

Most recently we have transformed alfalfa with an antisense construct in efforts to down regulate and interfere with NADH-GOGAT expression (Schoenbeck *et al.* 2000). An antisense construct driven by the promoter for nodule enhanced AAT was transformed into alfalfa and several plants were obtained that had reduced nodule NADH-

GOGAT mRNA, protein, and activity (Fig. 7). These plants were impaired in assimilation of symbiotically fixed N. Growth was impaired when plants were grown symbiotically but could be rescued by addition of nitrate. Interestingly, the antisense plants were not reduced in N_2 fixation. Such an effect would be expected based upon the feedback control hypothesis for regulation of nitrogenase. This hypothesis states that a downstream product of fixation such as amides or ureides feeds back to inhibit nitrogenase under conditions of adequate N availability (Hartwig 1998). However, when N sinks are present, N from symbiosis is transported to these sinks and not available for feedback inhibition. The antisense NADH-GOGAT plants reduced capacity to assimilate fixed N functions essentially as an unlimited sink.

Our postulate that NADH-GOGAT occupies perhaps the key step in assimilation of symbiotically fixed N in alfalfa is based upon several findings: (i) NADH-GOGAT in alfalfa

Figure 4. Partial nucleotide sequence of alfalfa NADH-GOGAT. Underlined is the 88 bp direct repeat that may control nodule infected cell zone specific expression of NADH-GOGAT transcription. Arrow indicates transcription initiation start site. Data derived from Vance *et al.* (1995).

Saranac

A

in1 Saranac

B

5 7 8 9 12 19 33

Figure 5. Expression pattern of NADH-GOGAT during development of (*A*) effective and (*B*) ineffective 'Saranac' root nodule. Total RNA was isolated from roots (days 5 and 7), roots + nodules (day 8) and nodules (days 9–33) after planting and inoculation. Each lane contains 10 µg of total RNA. Data derived from Gregerson *et al.* (1993).

occurs as a single gene encoding a single enzyme while other enzymes in nodule N and C assimilation are encoded by multiple genes encoding multiple isozymes; (ii) the protein comprises only a small, 0.1–0.4%, of nodule soluble protein while the other enzymes are more abundant; (iii) the enzyme is the junction for channeling amino acid synthesis to Asp in amyloplasts and Glu in the cytosol (Fig. 8); (iv) selection for reduced NADH-GOGAT enzyme activity and expression of antisense NADH-GOGAT transcripts have a negative effect on plant growth but can be rescued by nitrate fertilisation; and (v) all other genes involved in primary N and C metabolism are expressed at fairly high levels in ineffective nodules, while NADH-GOGAT expression has a requirement for effective nodules.

Malate dehydrogenase

Malate dehydrogenase catalyses the reversible reduction of oxaloacetate to malate (Gietl 1992). This enzyme is important in several metabolic pathways and higher plants contain
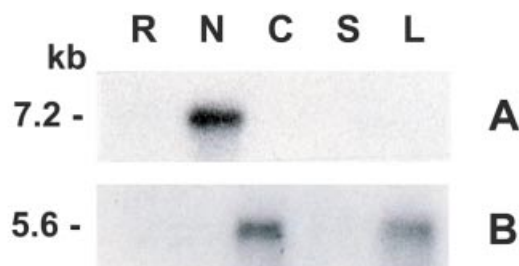
Table 6. Density of gold particles/0.25 µm^2 in various cell types and subcellular organelles of alfalfa nodules upon immunolabelling with NADH-GOGAT antibodies

Data are the average of 30 profiles from 3 nodules and are derived from Trepp *et al.* (1999*a*)

Cell type/organelle	Labelling density
Cytosol	0.13
Intercellular space	0.29
Cell wall	0.36
Mitochondria	0.13
Bacteroids	0.14
Amyloplasts of infected cell	3.5
Amyloplasts of uninfected cell	1.2

multiple isozymes that differ in co-enzyme specificity and subcellular location. While the reaction favors malate production, whether oxaloacetate or malate forms depends upon physiological conditions and enzyme location. Chloroplasts contain an NADP-dependent MDH (plMDH) that plays a critical role in balancing reducing equivalents between the cytosol and stroma. Plants also contain at least 4 NAD-dependent MDHs which are found in: (i) the cytosol (cMDH) and peroxisomes (pMDH) involved in malate-aspartate shuttles; (ii) the mitochondria (mMDH) involved in the TCA cycle; and (iii) the glyoxysomes (gMDH) functioning in β-oxidation (Gietl 1992). The enzyme has been purified from several plant sources and antibodies produced against plMDH, gMDH, and pMDH. The p and gMDHs are serologically indistinguishable, while the plMDH is antigenically unique. The M_r of MDHs varies from 66 to 80 kDa in its native state. The enzyme is a dimer and subunit relative molecular weight (M_r) varies from 33 to 42 kDa. Gietl's extensive review (1992) lists kinetic constants for the various forms of MDH. Several MDH cDNAs have been isolated and sequences compared from both plant and animal sources (see Gietl 1992; Miller *et al.* 1998 and references
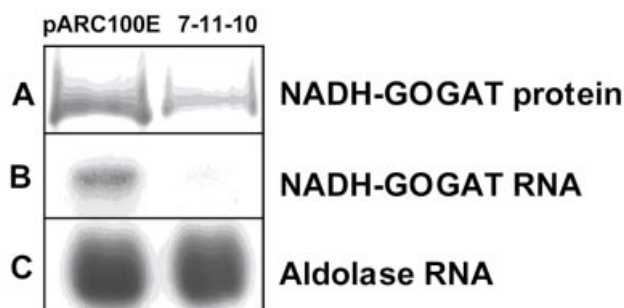

R N C S L
kb
7.2 -   A
5.6 -   B

Figure 6. Transcript abundance for (*A*) NADH-GOGAT and (*B*) Fd-GOGAT in roots (R), nodules (N), cotyledons (C), stems (S) and leaves (L) of alfalfa. Each lane contains 2 µg polyA$^+$RNA. Data derived from Vance *et al.* (1995).

pARC100E 7-11-10

A   NADH-GOGAT protein

B   NADH-GOGAT RNA

C   Aldolase RNA

Figure 7. Comparison of NADH-GOGAT protein and mRNA in nodules from the antisense transformant, 7-11-10, and the control pARC100E. (*A*) Protein immunoblot, (*B*) RNA blot, and (*C*) control RNA blot, aldolase. Each lane on protein immunoblot contains 50 µg nodule soluble protein. Each lane on RNA blots contain 10 µg total RNA. Data derived from Schoenbeck *et al.* (2000).
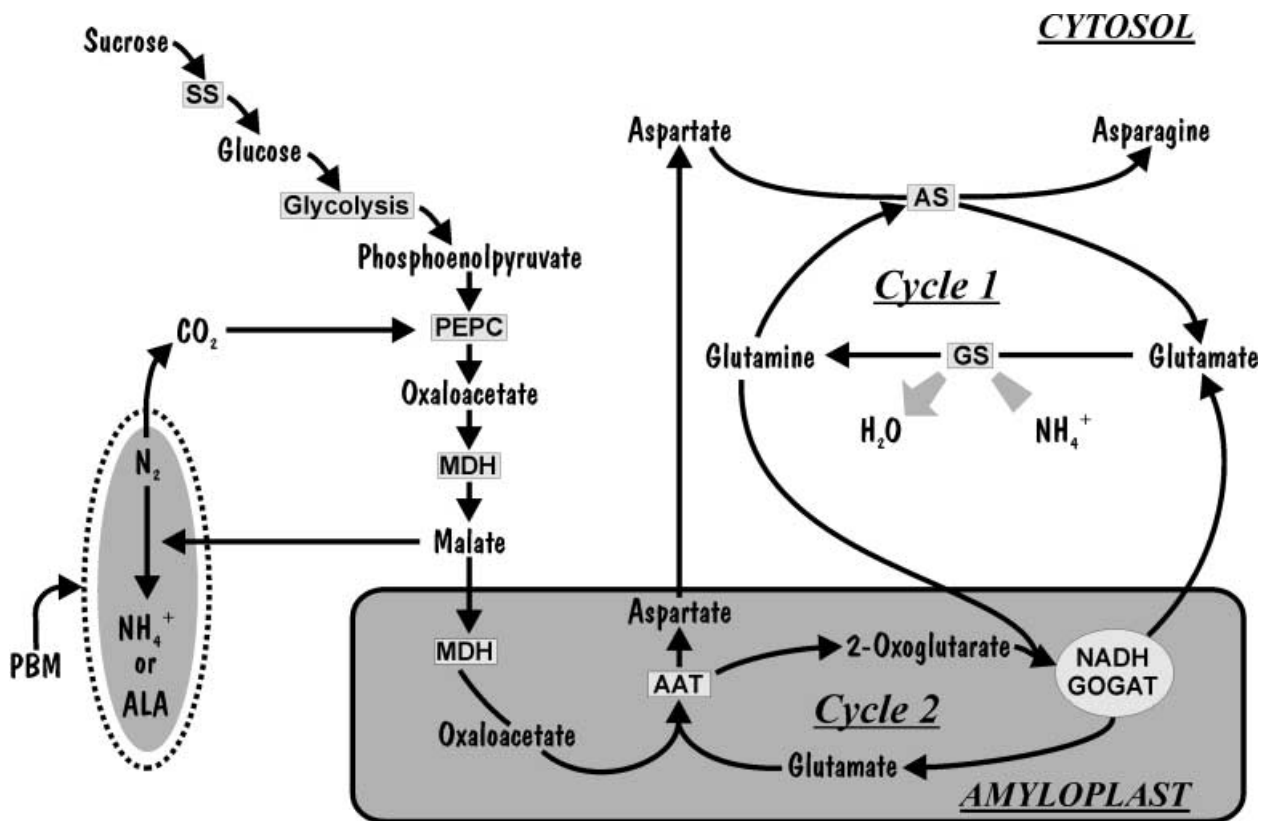
Figure 8. Nitrogen and carbon assimilation in alfalfa nodules and the cellular localisation of the enzymes involved. Two linked glutamate cycles are proposed to be involved in asparagine biosynthesis. Cycle 1 is cytosolic, while cycle 2 is in amyloplasts. The cycles are connected through plastid localised NADH-GOGAT. There are also amyloplast forms of malate dehydrogenase (MDH) and aspartate aminotransferase (AAT). In contrast sucrose synthase (SS), phosphoenolpyruvate carboxylase (PEPC), glutamine synthetase (GS), and asparagine synthetase (AS) are located in the cytosol. It should be noted that nodules also contain modest amounts of a plastid form of GS and a cytosolic form of MDH. Figure after Trepp *et al.* (1999*a*).

therein). MDH genes have also been isolated from several bacterial sources.

Nitrogen-fixing legumes have yet another use for MDH. In root nodules, malate serves as the primary carbon source to support bacteroid respiration and N_2 fixation (Day and Copeland 1991), therefore a nodule-enhanced (neMDH) may be critical for nodule function. Immunological and biochemical studies suggest that nodules of lupin (Ratajczak *et al.* 1989) and pea have unique nodule enhanced forms of the enzyme. Pea nodules contain at least 4 different forms of MDH and the root nodule form catalysed high *in vitro* rates of malate production (Appels and Haaker 1988).

Using complementation of an *E. coli* MDH⁻ mutant, RT-PCR, and screening with heterologous probes, we recently isolated 5 distinct alfalfa MDH cDNAs (Miller *et al.* 1998). Chloroplast MDH was obtained by RT-PCR amplification of leaf mRNA using degenerate primers based on known plMDH proteins. Mitochondrial MDH and gMDH were isolated by screening at low stringency a nodule cDNA library with heterologous probes from watermelon. Two

previously unreported MDHs, the neMDH and cMDH, were isolated by complementation of an *E. coli* MDH⁻ mutant via electroporation with an alfalfa nodule plasmid cDNA library and were characterised in detail.

Messenger RNA blots showed the mMDH, gMDH, and cMDH genes to be fairly uniformly expressed in all tissues evaluated. In comparison, plMDH was expressed in green tissues of cotyledons, stems, and leaves with neMDH showing striking expression in effective root nodules but little mRNA in other organs. Nodule developmental studies showed that neMDH mRNA expression was correlated with effective nodule formation and was reduced in ineffective nodules (Fig. 9). Cytosolic MDH was uniformly expressed throughout development of both effective and ineffective nodules. A similar cDNA with comparable expression patterns has been characterised in pea (Fedorova *et al.* 1999).

The alfalfa neMDH cDNA is 1045 bp containing a single ORF of 1224 bp that encodes a 408 amino acid protein with a deduced M_r of 43 kDa. This protein contains an 82 amino acid N-terminal extension relative to the processing site of
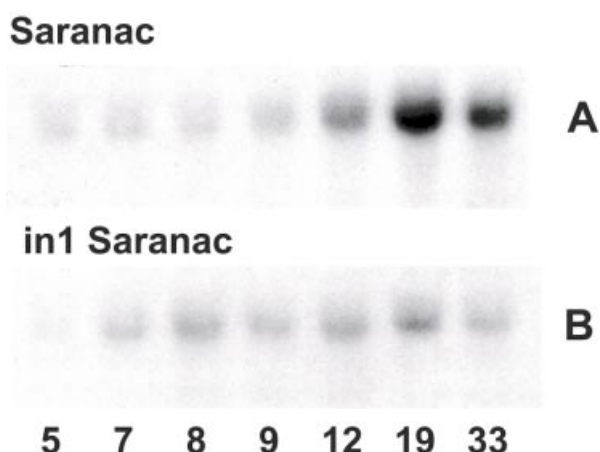
Figure 9. Expression of nodule enhanced (ne) MDH during development of (*A*) effective and (*B*) ineffective 'Saranac' root nodules. Total RNA was isolated from roots (days 5 and 7), roots + nodules (day 8), and nodules (days 9–33) after planting and inoculation. Each lane contains 10 µg of total RNA. Data derived from Miller *et al.* (1998).
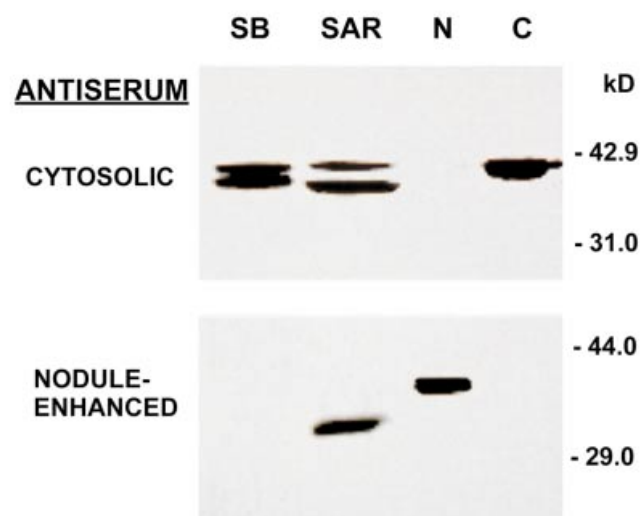

Figure 10. Immunological specificity of cytosolic (upper panel) and nodule enhanced (lower panel) malate dehydrogenase antiserum. Lanes correspond to effective soybean nodule protein (SB), effective Saranac nodule protein (SAR), recombinant nodule enhanced MDH protein (N) and recombinant cytosolic (C) protein. Molecular mass is indicated in kDa. Antiserum to cytosolic MDH does not recognise nodule enhanced MDH protein (upper panel), nor does nodule enhanced MDH antiserum recognise cytosolic MDH protein (lower panel). Note antibodies to nodule enhanced MDH recognise a recombinant nodule enhanced MDH of about 40 kDa while the polypeptide recognised *in planta* has been processed to about 34 kDa.

m- and gMDHs. The estimated M_r of the mature (processed) protein is 34 kDa. Antibodies to neMDH recognise a 33 kDa polypeptide from nodules (Fig. 10). Furthermore, when neMDH is expressed in *E. coli*, the enzyme is cleaved to give a 35 kDa protein. Thus, neMDH is processed both *in planta* and in *E. coli*. The putative presequence is predicted to target the enzyme to either plastids or mitochondria with near equal probability. Current immunogold localisation studies have tentatively shown that neMDH is located in amyloplasts of infected cells and cMDH is in uninfected cells (G. Trepp, R. Litjens and C. Vance unpublished data).

Antibodies that were produced to recombinant expressed neMDH and cMDH were antigenically distinct (non-crossreactive). The antibodies to neMDH did not recognise cMDH protein nor did cMDH antibodies recognise neMDH protein (Fig. 10). Protein immunoblots and immuno-precipitation of enzyme activity demonstrated that neMDH was the major form of MDH protein and enzyme activity in nodules, comprising at least 50–60% of the total nodule MDH activity.

Kinetic analysis of the neMDH enzyme showed very novel characteristics (Table 7). The enzyme has extremely high affinity for both oxaloacetate and malate. The neMDH enzyme turnover number for malate oxidation and oxaloacetate reduction were 4-fold and 30-fold greater, respectively, than those for cMDH. The specificity constants of neMDH show high specificity for oxaloacetate and NADH over malate and NAD^+. These data suggest that neMDH dramatically favors malate production.

Several lines of evidence indicate that neMDH may be responsible for the strikingly high concentrations of malate seen in nodules. First, the K_M values for oxaloacetate and NADH are substantially lower than for malate and NAD^+ and are similar to those reported for an neMDH from pea (Appels

and Haaker, 1988). Second, neMDH transcripts and protein are more highly expressed in nodules than any other tissue. Third, immunoprecipitations show neMDH comprises the largest proportion of total nodule MDH (at least 50%). Fourth, the active site turnover rate (k_{cat}) and substrate specificity constants of neMDH highly favor malate synthesis, which in essence pulls the reaction in the direction of malate. Lastly, neMDH upon initial analysis appears to be localised primarily to the amyloplasts of infected cells in effective nodules.

Synopsis

Improvement of symbiotic N_2 fixation involves selection for a very complex trait encompassing large numbers of genes from both the microsymbiont and host plant. Selection of a single biochemical or morphological trait is unlikely to lead to a significant improvement in N_2 fixation. Failure to make parallel selection in both the greenhouse and field can seriously compromise practical advancement in a breeding program. Attempts to improve N_2 fixation by selection for root nodule enzymes showed that several enzymes can be selected for in a single plant organ. But, to achieve improved plant performance these enzymes need to be stacked in the absence of inbreeding depression. Selection for nodule biochemical and physiological traits laid the foundation for isolating, characterising and elucidating the role of NADH-GOGAT, MDH, PEPC, AS, AAT and GS in symbiotic N_2 fixation. Isolation of the genes encoding these enzymes offers new

Table 7. **Kinetic parameters of the nodule-enhanced and cytosolic forms of alfalfa MDH
produced in *Escherichia coli***

Data are derived from Miller *et al.* (1998)

Substrate	Nodule-enhanced MDH		Cytosolic MDH	
	K_M (mmol/L)	Specificity constant $(mmol/L.min)^{-1}$	K_M (mmol/L)	Specificity constant $(mmol/L.min)^{-1}$
Malate	2.22 ± 0.21	1391	3.71 ± 0.30	235
NAD^+	0.328 ± 0.027	3090	0.363 ± 0.017	2410
Oxaloacetate	0.076 ± 0.014	206 723	0.421 ± 0.007	1330
NADH	0.078 ± 0.009	201 423	0.037 ± 0.008	15 135

tools that may prove useful in altering plant N nutrition and C metabolism. Expression of antisense transcripts has been used to down regulate AAT, PEPC and NADH-GOGAT (Mett *et al.* 1996; Schulze *et al.* 1998; Schoenbeck *et al.* 2000) resulting in each case with decreased plant growth. The remaining practical question is whether these and other genes can be overexpressed via transgenic technology and improve N_2 fixation and N assimilation.

The development of ineffective lines incapable of N_2 fixation and/or nodulation is requisite for biochemical and genetic studies of N_2 fixation. They may be selected as variants among normal populations or generated through mutagenesis. Although we have found ineffective alfalfa lines enormously beneficial for fundamental and applied studies, a novel practical application has recently been demonstrated for these genotypes. Since ^{15}N studies showed that the *in₁* genotypes had 25–30% greater efficiency in nitrate uptake and grew normally in the presence of applied N, they are currently being used to remediate excess N in fertiliser spill areas and feed pastures over porous soils (M. Russelle, J. Lamb and C. Vance unpublished data). These ineffective genotypes not only help remediate the excess N but they also act as indicators by becoming chlorotic as N levels are reduced to acceptable standards. The ineffective genotypes would also be useful in application of riparian strips between wetlands and crop production fields.

The localisation of nodule enhanced NADH-GOGAT and MDH in amyloplasts of infected cells suggests that this organelle must play a pivotal role in cellular partitioning of N and C metabolism. Nodule amyloplasts are the major site for Glu, Asp, α-ketoglutarate, and oxaloacetate biosynthesis for N assimilation. In contrast, Gln and Asn biosynthesis occur almost completely in the cytosol. We have proposed that 2 cycles linked through NADH-GOGAT could explain how synthesis of amino acids of primary N assimilation in alfalfa is regulated (Fig. 8). A cytosolic cycle (cycle 1) incorporates Glu derived from NADH-GOGAT into Gln with subsequent synthesis of Asn. An amyloplast cycle (cycle 2) catalyses synthesis of Asp from Glu. The Asp is released into the cytosol to be used in Asn synthesis and the α-ketoglutarate is regenerated for use by GOGAT. A neMDH coupled to

amyloplasts could channel C for both amino acid biosynthesis and bacteroid respiration. Isotopic labelling and enzyme localisation support this hypothesis.

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