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Nitrogen Assimilating Enzyme Activities and Enzyme Protein during Development and Senescence of Effective and Plant Gene-Controlled Ineffective Alfalfa Nodules[1]

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ABSTRACT

Effective (N_2-fixing) alfalfa (*Medicago sativa* L.) and plant-controlled ineffective (non-N_2-fixing) alfalfa recessive for the *in*$_1$ gene were compared to determine the effects of the *in*$_1$ gene on nodule development, acetylene reduction activity (ARA), and nodule enzymes associated with N assimilation and disease resistance. Effective nodule ARA reached a maximum before activities of glutamine synthetase (GS), glutamate synthase (GOGAT), aspartate aminotransferase (AAT), asparagine synthetase (AS), and phospho*enol*pyruvate carboxylase (PEPC) peaked. Ineffective nodule ARA was only 5% of effective nodule ARA. Developmental profiles of GS, GOGAT, AAT, and PEPC activities were similar for effective and ineffective nodules, but activities in ineffective nodules were lower and declined earlier. Little AS activity was detected in developing ineffective nodules. Changes in GS, GOGAT, AAT, and PEPC activities in developing and senescent effective and ineffective nodules generally paralleled amounts of immunologically detectable enzyme polypeptides. Effective nodule GS, GOGAT, AAT, AS, and PEPC activities declined after defoliation. Activities of glutamate dehydrogenase, malate dehydrogenase, phenylalanine ammonia lyase, and caffeic acid-*o*-methyltransferase were unrelated to nodule effectiveness. Maximum expression of nodule N-assimilating enzymes appeared to require the continued presence of a product associated with effective bacteroids that was lacking in *in*$_1$ effective nodules.

Legume root nodules are morphologically and biochemically unique organs. Their formation involves differentiation of root cortical cells to give rise to a nodule meristem, synthesis of new vascular elements, and development of infected and uninfected nodule cells, both of which undergo alterations in ultrastructure and metabolism (21, 28). These processes result in the formation of organs that provide an ecological niche for N_2-fixing *Rhizobium* symbionts, and that can assimilate NH_4^+ derived from N_2 fixation into amides or ureides that are ultimately exported to other organs.

Both plant and bacterial genes are required for nodule development and N_2 fixation. Evidence for the importance of plant genes to N_2 fixation has come from identification of plant-synthesized, nodule-specific proteins (nodulins) and from isolation of plant mutants altered in nodulation and N_2 fixation capacity (28).

From 9 to 30 nodulin polypeptides or mRNAs have been identified in mature nodules from various legumes including soybean, pea, and alfalfa (6–8, 26). Some nodulins are subunits of enzymes required for N assimilation or nodule C metabolism, such as GS[2] (EC 6.3.1.2), uricase, and sucrose synthase (reviewed in ref. 28). While proteins and activities of some other enzymes required for N and C assimilation, such as GOGAT (EC 1.4.1.13), AAT (EC 2.6.1.1), and PEPC (EC 4.1.1.31), are strikingly increased in nodules (16, 27), it is unknown if these enzyme activities are correlated with amounts of enzyme protein, or if these enzymes are comprised of nodule-specific polypeptides.

Studies of the development of effective and bacterially induced ineffective nodules have shown that N assimilation capacity and expression of most nodulins begins at or slightly before the onset of N_2 fixation (4, 7, 8, 13, 14, 19). Nodulin expression appears to be induced in response to specific steps in nodule morphogenesis (8). However, reduced nodulin expression in bacterially induced ineffective nodules has led several authors to suggest that other factors, perhaps products of N_2 fixation, may also regulate nodulin expression (6, 12).

To date, some 45 plant genotypes with altered nodulation and N_2 fixation capacities have been identified in eight legume species (28), but little is known of the developmental, biochemical, and molecular manifestations of these plant genes. To examine the effect of genetically reduced N_2 fixation on nodule development, and to determine how N assimilation capacity is regulated, we have compared the development of normal, N_2-fixing alfalfa cv 'Saranac' nodules to that of ineffective nodules formed by plants which are nulliplex for

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[2] Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; AAT, aspartate aminotransferase; PEPC, phospho*enol*pyruvate carboxylase; AS, asparagine synthetase; PAL, phenylalanine ammonia lyase; C-OMT, caffeic acid *o*-methyltransferase; GDH, glutamate dehydrogenase; MDH, malate dehydrogenase; ARA, acetylene reduction activity.

the in_1 gene in the 'Saranac' background (18). In alfalfa, fixed N is converted to asparagine by the enzymes GS, GOGAT, AAT, and AS (21), with a significant portion of exported carbon derived from dark CO_2 fixation by PEPC (32). Mature in_1Sa nodules fix little N_2, undergo early senescence, and have reduced activities of GS, GOGAT, and PEPC (30). We have (a) measured the activities of plant enzymes involved in nodule N and C metabolism and (b) estimated the amounts of GS, GOGAT, AAT, and PEPC polypeptides, relative to N_2 fixation capacity, throughout nodule initiation, development, and senescence. Because early senescence of in_1Sa nodules resembles an incompatible plant-pathogen reaction (33), we have also compared activities of PAL (EC 4.3.1.5) and C-OMT, enzymes required for synthesis of phytoalexins and lignin precursors, throughout development. A preliminary report on this study has been published (27).

MATERIALS AND METHODS

Plant Material

Alfalfa (*Medicago sativa* L.) cv 'Saranac' and in_1Sa (18) seeds were planted in sand benches amended with P, K, and micronutrients (29) and immediately inoculated with *Rhizobium meliloti* strain 102F51 (Nitragin Co., Milwaukee, WI). The planting date was designated as d0. Glasshouse conditions were as previously described (29). No other nutrients were supplied to plants. Plants were harvested at 0800 h and roots (d3,5), nodules on 5-mm root sections (d7), or nodules (d10 and older) were hand-collected onto ice for use in enzyme assays as previously described (29). Plants used for acetylene reduction assays were harvested at 1400 h. After assays were performed on d38, plants were defoliated to induce nodule senescence.

Microscopy

Unfixed roots were stained for 15 min with 0.1% w/v methylene blue, rinsed in water, and examined by low power light microscopy to determine the stage of nodule development.

Preparation of Cell-Free Extracts

Triplicate samples of roots or nodules were ground in extraction buffer (100 mM Mes-NaOH [pH 6.8], 100 mM sucrose, 2% v/v 2-mercaptoethanol, 15% v/v ethylene glycol, 2 mm PMSF, 0.2 mM antipain) and centrifuged 15 min at 15,500*g* to obtain the soluble protein fraction (11).

In Vitro Enzyme Assays

Radiochemicals were obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). GS and AS were assayed by determining the rate of conversion of L-[U-^{14}C]glutamate (0.02 μCi μmol^{-1}) or L-[U-^{14}C]aspartate (0.033 μCi μmol^{-1}) into [^{14}C] glutamine and [^{14}C]asparagine (11). For PAL assays, 0.4 mL of extract was incubated with 1 mL of 100 mM Tris (pH 8.5), containing 4.2 μmol L-[U-^{14}C]phenylalanine (0.023 μCi μmol^{-1}). For C-OMT assays, 0.4 mL of extract was incubated with 0.85 mL of 100 mM K phosphate (pH 7.5), containing 3.6 μmol of Na ascorbate, 0.87 μmol of $MgCl_2$, 1 μmol of [methyl-^{14}C]S-adenosylmethionine (0.05 μCi μmol^{-1}, freshly diluted from stock), and 2.5 μmol of caffeic acid (freshly made), for 3 h at 30°C. Reactions were stopped with 0.1 mL of 6 N HCl. [^{14}C]Ferulic acid and [^{14}C]*trans*-cinnamic acid were recovered by extracting twice with 2 mL of ethyl acetate and were quantitated by liquid scintillation counting of anhydrous extracts. Control PAL and C-OMT assays containing no plant extracts were performed to determine the background partitioning of [^{14}C] into ethyl acetate. GOGAT, GDH (11), AAT, MDH (25), and PEPC (32) were assayed spectrophotometrically by monitoring the disappearance of NADH at 340 nm in direct or coupled assays. Protein content of extracts was measured by the Lowry assay (15). Enzyme activities ($\times$ mg protein^{-1}) are expressed as means $\pm$ 1 SE of assays of triplicate extracts from one time course experiment. The entire experiment was repeated on three separate occasions with similar results.

SDS-PAGE and Western blotting

Soluble proteins in cell-free extracts were electrophoresed in 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose as previously described (26). Protein loads per lane varied, as described in Figure 3. Rabbit polyclonal antibodies to alfalfa nodule GOGAT (1), AAT (9), and PEPC (16) and mouse polyclonal antibodies to alfalfa nodule GS (10) were used to detect the corresponding antigens on Western blots (26). Representative blots are shown for each antigen.

Acetylene Reduction Assays

Eight intact plants were placed in each of three 25-mL serum vials at 100% humidity. Acetylene (10% final concentration) was added 10 min after enclosing plants. Samples (0.1 mL) were withdrawn from the tubes approximately 15, 25, and 35 min after addition of acetylene, and ethylene concentration was determined by gas chromatography (29). Results are the mean ARA $\pm$ 1 SE of triplicate assays.

RESULTS

Nodule Development

Alfalfa seeds inoculated with *R. meliloti* had germinated by d3, when roots were first collected. The first signs of infection (infection threads and microscopic nodules) were observed on d6. From d7 to d10, the diameter of the largest nodules increased from approximately 0.5 to 1.0 mm. On d10, the largest Saranac nodules were faint pink, and the first trifoliate leaves of both Saranac and in_1Sa plants were partially expanded. By d17, Saranac nodules were pink and foliage was dark green, while in_1Sa nodules were white or faint pink, and foliage was pale green and appeared N-deficient. On d38, the fresh weights of Saranac and in_1Sa plants were 4.31 and 1.53 g, respectively. Proximal ends of in_1Sa nodules were greenish and senescent. Defoliation caused extensive senescence of the proximal ends of Saranac nodules, as previously reported (31). Regrowth of Saranac foliage began by d47.

Acetylene Reduction Activity in Developing and Senescent Nodules

Saranac nodule ARA (g nodule fresh weight^{-1}) increased from almost nil on d7 to a maximum on d10 and remained constant through d38 (Fig. 1). Similar to Saranac, *in*$_1$Sa nodule ARA increased from d7 to d10, but the maximum ARA of *in*$_1$Sa nodules was only 5% of Saranac nodule ARA. Four d after defoliation (d42), ARA of both Saranac and *in*$_1$Sa nodules had decreased approximately 80%, relative to the rates on d38. Saranac nodule ARA had partially recovered by d47.

Nodule Protein

Soluble protein (g fresh weight^{-1}) of both Saranac and *in*$_1$Sa nodules increased sharply from d7 to d10 and gradually from d10 to d38 (Fig. 1). Young *in*$_1$Sa nodules (d10,17) contained only 60% as much protein as Saranac nodules. By d38, Saranac and *in*$_1$Sa nodules contained similar amounts of protein. After defoliation, nodule soluble protein declined more in *in*$_1$Sa nodules (60%) than in Saranac nodules (20%), relative to d38.

Developmental Changes in *In Vitro* Activities of GS, GOGAT, AAT, AS, and PEPC

In vitro specific activities of GS, GOGAT, AAT, AS, and PEPC were relatively low in roots (d3,5) and d7 nodules

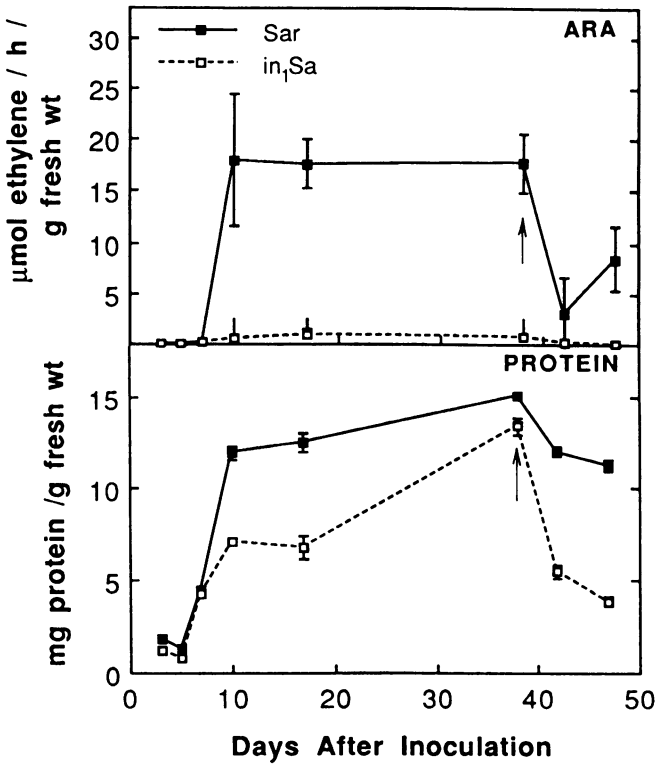

Figure 1. Acetylene reduction activity and soluble protein concentration of effective 'Saranac' and ineffective *in*$_1$Sa roots and nodules at intervals after planting and inoculation with *R. meliloti*. After assays on d38, plants were defoliated (*arrow*) to induce nodule senescence.

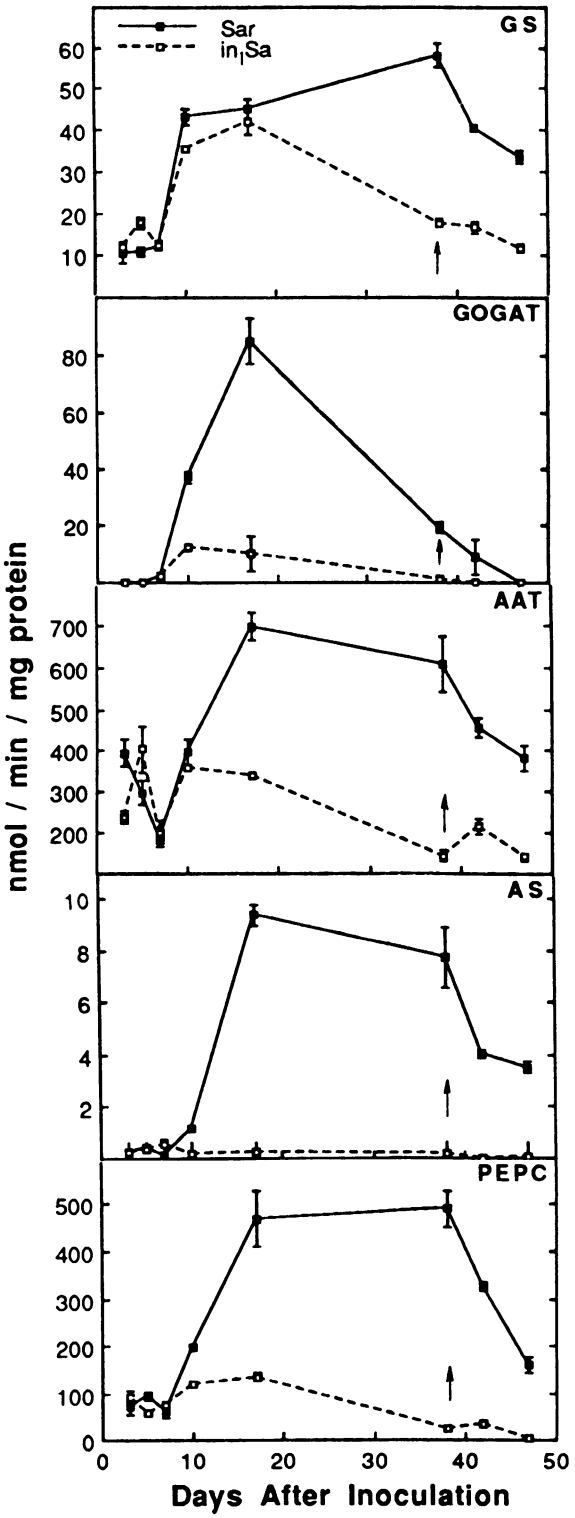

Figure 2. *In vitro* specific activities of GS, GOGAT, AAT, AS, and PEPC in soluble protein extracts from roots (d3,5) and developing and senescent nodules of Saranac and *in*$_1$Sa genotypes. Plants were defoliated on d38 (*arrow*).

(Fig. 2). *In vitro* enzyme activities in effective Saranac nodule extracts increased dramatically from d7 (GS, GOGAT, AAT, PEPC) or d10 (AS) to d17 or d38 (GS). A different pattern was observed for in_1Sa nodule development. Activities of GS, GOGAT, AAT, and PEPC increased from d7 to d10, but the increases in GOGAT and PEPC activities were less than those for Saranac nodules, and there was no increase in AS activity of in_1Sa nodules. Increases in enzyme activities in developing in_1Sa nodules ceased after d10 (GOGAT, AAT, PEPC) or d17 (GS), earlier than in Saranac nodules. By d38, enzyme activities in in_1Sa nodule extracts were approximately 24% (GS), 9% (GOGAT), 9% (AAT), 2% (AS), and 4% (PEPC) of those found in Saranac nodules. Nine d after defoliation, Saranac nodule GS, AAT, and AS activities were reduced about 50% relative to d38; PEPC was reduced by 70% (relative to d38); and GOGAT activity was almost nil. There were variable effects on in_1Sa nodule GS, GOGAT, AAT, and PEPC activities (mg protein^{-1}), although activities × g fresh weight^{-1} all declined after defoliation.

Immunodetection of GS, GOGAT, AAT, and PEPC in Roots and Nodules

Relative amounts of GS, GOGAT, AAT, and PEPC polypeptides in soluble protein extracts from roots and nodules of different ages were estimated from Western blots (Fig. 3). A 37-kD polypeptide corresponding to the alfalfa GS subunit (10) was present in extracts (10 μg protein) from both roots and nodules of Saranac and in_1Sa plants. The density of this band increased greatly from d7 to d10, corresponding to the time of the largest increase in GS specific activity during nodule development (Fig. 2). Saranac nodules on d38 contained the greatest amount of GS polypeptide, which decreased after defoliation. Significant amounts of GS polypeptide were also found in 20 μg of protein from d10 to d38 in_1Sa nodules, but little was present after defoliation.

Much more GOGAT polypeptide was detected in 100 μg of protein from Saranac nodules than from in_1Sa nodules at all ages (Fig. 3). The 200-kD GOGAT polypeptide (1) was faintly present in roots (d3,5), but increased sharply as nodules developed. The amount of GOGAT polypeptide in Saranac nodules greatly increased from d7 to d17, similar to GOGAT specific activity (Fig. 2). In contrast, in_1Sa nodules contained little GOGAT on d7 and d10 and almost no GOGAT after d10 (Fig. 3). Unlike GOGAT activity, GOGAT polypeptide did not decline significantly from d17 to d38.

Amounts of AAT polypeptide (in 12 μg of protein) also varied during nodule development. A 40-kD polypeptide corresponding to AAT-2 (9) was present in roots and nodules of all ages (Fig. 3). The amount of AAT-2 polypeptide increased from d7 to d17 in Saranac nodule extracts and from d7 to d10 in in_1Sa nodule extracts, similar to changes in AAT specific activity (Fig. 2). After defoliation, the amount of AAT-2 in both Saranac and in_1Sa nodules was reduced (Fig. 3). A 36-kD band was also visible on these blots but did not appear on blots developed with purified IgG eluted from the AAT-2 band.

Activity of PEPC during nodule development (Fig. 2) was related to the amount of PEPC protein (Fig. 3). The 100-kD PEPC polypeptide (16) was present in 15 μg of protein from both roots (d3,5) and nodules, and it increased significantly from d7 to d17 in Saranac nodules and from d7 to d10 in in_1Sa nodules. PEPC protein in developing Saranac nodules decreased only after defoliation, but PEPC protein in in_1Sa nodules decreased after d17. Little PEPC polypeptide could be detected in 30 μg of protein from in_1Sa nodules after defoliation.

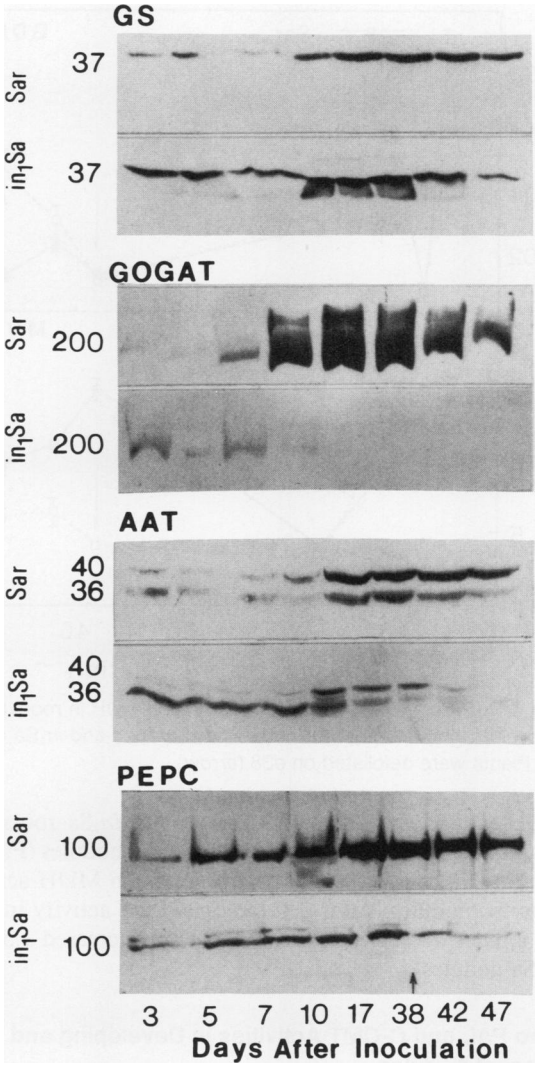

Figure 3. Immunoblots of GS, GOGAT, AAT, and PEPC polypeptides in soluble protein extracts from Saranac and in_1Sa roots (d3,5) and developing and senescent nodules. Plants were defoliated on d38 (*arrow*). SDS gels were loaded with 10 μg (GS; Saranac), 20 μg (GS; in_1Sa), 100 μg (GOGAT), 12 μg (AAT), 15 μg (PEPC; all lanes except in_1Sa d42, 47), or 30 μg (PEPC; in_1Sa d42, 47) of protein per lane.

In Vitro GDH and MDH Activities in Roots and Developing Nodules

Activity of GDH was unrelated to ARA (Fig. 4). Throughout development, GDH activities in Saranac and in_1Sa were similar. High GDH activity was found in young roots (d5) and at the onset of N_2 fixation (d10). Nodule GDH activity increased after defoliation, particularly for the in_1Sa genotype.

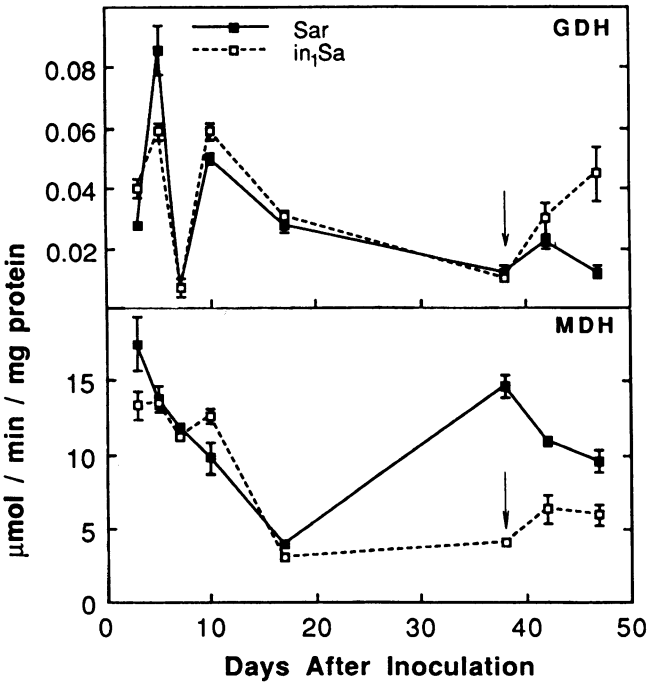

Figure 4. *In vitro* specific activities of GDH and MDH in roots (d3,5) and developing and senescent nodules of Saranac and *in*$_1$Sa genotypes. Plants were defoliated on d38 (*arrow*).

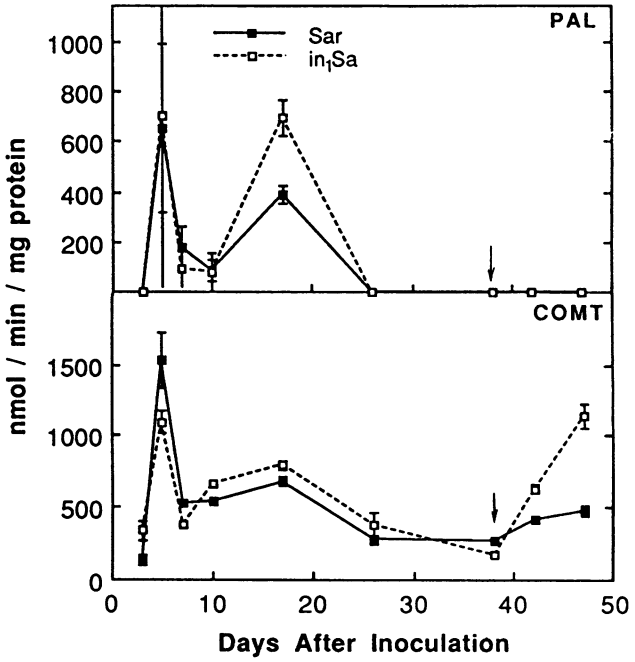

Figure 5. *In vitro* specific activities of PAL and C-OMT in soluble protein extracts of roots (d3,5) and developing and senescent nodules of Saranac and *in*$_1$Sa genotypes. Plants were defoliated on d38 (*arrow*).

MDH activity was high in Saranac and *in*$_1$Sa roots and young nodules and in mature (d38) Saranac nodules (Fig. 4). Mature *in*$_1$Sa nodules had only 29% as much MDH activity as Saranac nodules. After defoliation, MDH activity in Saranac nodules decreased by 73% (d47) but increased 1.5-fold in *in*$_1$Sa nodules.

In Vitro PAL and C-OMT Activities in Developing and Senescent Nodules

Activities of C-OMT and PAL varied throughout nodule development but followed similar patterns in Saranac and *in*$_1$Sa genotypes (Fig. 5). PAL specific activity was highest in d5 roots and in d17 nodules. The higher PAL activity (mg protein^{-1}) of d17 *in*$_1$Sa nodules (compared to Saranac nodules) was not consistently observed. Both genotypes contained similar PAL activity × g fresh weight^{-1}.

In vitro C-OMT activity (mg protein^{-1}) in Saranac and *in*$_1$Sa nodules was similar throughout development, although activity × g fresh weight^{-1} was higher in Saranac than in *in*$_1$Sa nodules (Fig. 5). Young (d5) roots contained the highest C-OMT activity; d17 nodules contained moderate levels of C-OMT activity. Nodule C-OMT activity increased approximately 2.5-fold 4 d after defoliation and continued to increase in *in*$_1$Sa, but not Saranac, nodules.

DISCUSSION

While numerous reports have documented the effect of bacterially induced ineffectiveness on nodule enzyme activities and plant gene expression during nodule development (3, 6–8, 14, 26), ours is the first report of the effect of the plant genome on similar parameters. *In vitro* activities of enzymes directly involved in nodule N assimilation in *in*$_1$ gene-controlled ineffective nodules differed from those of effective Saranac nodules in two ways: (a) there was a smaller initial increase in some enzyme activities from d7 to d10 (around the time when N_2 fixation reached a maximum), and (b) the duration of increased activity was reduced. The final result was that *in vitro* activities of GS, GOGAT, AAT, AS, and PEPC and amounts of immunologically detectable polypeptides of GS, GOGAT, AAT, and PEPC were reduced in mature *in*$_1$Sa nodules, compared to effective Saranac nodules (Figs. 2 and 3). This divergence in nodule biochemistry and gene expression during development of ineffective *in*$_1$Sa and effective Saranac nodules is similar to the effects on enzyme activities, proteins, and mRNAs found in bacterially induced ineffective nodules (5–7, 26) or in nodules grown in an Ar:O_2 atmosphere (2).

Parallel increases in amounts of enzyme polypeptides and *in vitro* activities of GOGAT, AAT, and PEPC during development of effective and *in*$_1$Sa root nodules indicate that their activities may be regulated by transcription or translation, similar to GS (5, 6), uricase (3), and xanthine dehydrogenase (17). Transcription of nodulins and nodule-stimulated genes increases during effective nodule development and is generally reduced in bacterially induced ineffective nodules (6–8). Increased activities of GOGAT, AAT, and PEPC may likewise be due to expression of nodule-specific subunits. One-dimensional SDS-PAGE immunoblot analyses of alfalfa nodule soluble proteins show that GOGAT, AAT, and PEPC are comprised of polypeptides that are more highly expressed in nodules than in roots (Fig. 3). Nodule-specific subunits differing in charge (*e.g.* GS; ref. 5) cannot be separated by SDS-

PAGE, so further investigation of the nodule specificity of alfalfa GOGAT, AAT, and PEPC polypeptides will require two-dimensional gel electrophoresis.

Differences among the profiles of *in vitro* activities of GS, GOGAT, AAT, AS, and PEPC during nodule development and senescence of effective Saranac and ineffective *in*$_1$Sa nodules suggest that these enzymes may be regulated differently. Large increases in GS and AAT activities and polypeptides occurred between d7 and d10 in both Saranac and *in*$_1$Sa nodules, even though ARA of *in*$_1$Sa nodules was severely reduced. Only small increases in GOGAT and PEPC activities and polypeptides occurred in *in*$_1$Sa nodules. These initial increases in nodule GS, AAT, GOGAT, and PEPC activities and polypeptides occurred despite the effects of the *in*$_1$Sa gene, suggesting that release of bacteria or bacteroids into nodule cells, irrespective of effectiveness, may be one signal for increased nodule enzyme activity.

During effective nodule development, N-assimilating enzymes appear to be (a) initially expressed during organogenesis and (b) further expressed at increased levels during the time N_2 fixation rates are high. Only (a) appears to occur in ineffective *in*$_1$Sa nodule development. The lack of time points from d7 to d10 prevents us from assigning a role for fixed N as a signal for induction of N-assimilating enzymes. Dunn *et al.* (6) have shown that a low amount of nodule-specific GS is expressed even in the absence of N_2 fixation. Relative to effective nodules, GS expression in older bacterially induced (6) or plant-controlled ineffective alfalfa nodules (this paper) is reduced, which suggests that availability of fixed N may modulate GS expression.

The developmental profile of AS activity was significantly different from ARA and that of other N-assimilating enzymes. The maximum rate of increase in *in vitro* AS activity of effective Saranac nodules occurred later (d10–d17) than for other N-assimilating enzymes (d7–d10). Also, AS activity was low to undetectable throughout development of *in*$_1$Sa nodules. Studies of lupin and soybean nodule development have shown that *in vitro* activity of AS increases after that of GS (19, 22). These observations suggest that increased alfalfa nodule AS activity requires not only organogenesis but also a factor associated with effective bacteroids, which are reduced or absent in *in*$_1$Sa nodules (30). It is unlikely that GS is directly responsible for increased AS activity, since little AS activity was found in *in*$_1$Sa nodules which had near-normal GS activity. However, *in vivo* AS activity of alfalfa nodules is reduced when nodules are treated for 2 h with inhibitors of GS or GOGAT (23), suggesting that increased AS activity somehow depends on the initial reactions of N assimilation. Differences between Saranac and *in*$_1$Sa nodule AS activities are unlikely to be due to small molecules present in crude extracts, since desalting increased *in vitro* AS activity of Saranac and *in*$_1$Sa nodule extracts to the same extent (MA Egli, unpublished data). Instead, AS activity in effective nodules may be controlled by changes in gene expression. Inhibitor studies have shown that increased *in vitro* AS activity of excised corn root tips requires protein and RNA synthesis and that these effects are apparent within 3 h of the time when AS activity begins to increase (24).

Changes in activities of N- and C-assimilating enzymes during effective alfalfa nodule development were similar to those observed in other legumes (4, 13, 14, 17, 19, 20), in that activities were closely related to N_2 fixation. Exceptions were GDH and MDH. Differences in profiles of GDH activity and ARA during development and after defoliation (this paper; ref. 11) indicate that GDH is not involved in NH_4^+ assimilation in alfalfa nodules. The relatively high *in vitro* GDH activity in young roots and senescent nodules is more consistent with a role in N recycling. Although MDH is likely to supply C skeletons for N assimilation, its *in vitro* activity was at least threefold greater than that of PEPC or N-assimilating enzymes and thus might not be expected to be closely coupled to N assimilation.

Defoliation of alfalfa plants induces senescence of the late symbiotic zone at the proximal end of the nodule (31). Previously, we showed that defoliation of effective alfalfa plants decreased specific activities of PEPC and GOGAT, ARA, and soluble protein in nodules, while GS activity was unchanged. These parameters were nearly constant in unharvested controls (11, 16, 31). In the current study, effective nodule ARA decreased sharply and GS, GOGAT, AAT, AS, and PEPC specific activities and polypeptides decreased moderately after defoliation (Figs. 2 and 3). The decline in GS activity was unexpected, based on previous results (11). Due to the lack of nondefoliated controls in this experiment, we cannot determine if the decrease in enzyme activities (or polypeptides) which occurred after defoliation resulted from defoliation or normal ontogeny. For example, effective nodule GOGAT activity and also enzyme activities in *in*$_1$Sa nodules declined prior to defoliation on d38. The relatively large amounts of Saranac nodule GS, AAT, and PEPC activities (50–70% of the amount on d38) remaining after defoliation, and the low to moderate levels of these enzymes in d38 *in*$_1$Sa nodules, suggest that a fraction of these enzymes is located in cells distal to the late symbiotic zone, in regions which are unaffected by defoliation-induced nodule senescence (31) or *in*$_1$ gene-induced senescence (30).

The early senescence phenotype of ineffective *in*$_1$Sa nodules has been likened to an incompatible interaction in plant disease (30), in which bacteria are recognized as pathogens rather than as symbionts. Phytoalexin synthesis and lignification are commonly observed in incompatible interactions, and some bacterially induced ineffective soybean nodules do contain elevated levels of the soybean phytoalexin, glyceollin I (33). However, no significant differences in the *in vitro* activities of enzymes required for synthesis of phytoalexins (PAL) or lignin precursors (PAL, C-OMT) were observed in developing Saranac and *in*$_1$Sa nodules. Effective and ineffective nodule PAL and C-OMT activities peaked around d17 (Fig. 5), prior to a large increase in nodule mass (data not shown). We suggest that nodule C-OMT may be involved in formation of lignin precursors for vascular elements during nodule growth. Activity of C-OMT increased after defoliation, especially in *in*$_1$Sa nodules which showed little recovery after defoliation; thus C-OMT may also play a role in senescent nodules. Determination of the roles of PAL and C-OMT in developing and senescent nodules will require knowledge of the tissues in which these enzymes are active.

Pronounced differences between development of ineffective

in$_1$Sa nodules and effective alfalfa nodules were found in the activity or expression of enzymes directly involved with N assimilation, similar to other types of nodules which fix little or no N_2 (2, 5, 6, 14). The earliest effects of the *in*$_1$ gene on N-assimilating enzymes were apparent by the time N_2 fixation in effective nodules reached a maximum, and these effects were more severe in older *in*$_1$Sa nodules. High activities of nodule GS, GOGAT, AAT, AS, and PEPC appeared to depend on a bacteroid-derived product that was lacking in ineffective *in*$_1$Sa nodules. As with GS (5, 6), high GOGAT, AAT, and PEPC activities resulted from increased amounts of enzyme protein.

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LITERATURE CITED

1. **Anderson MP, Vance CP, Heichel GH, Miller SS** (1989) Purification and characterization of NADH-glutamate synthase from alfalfa root nodules. Plant Physiol **90:** 351–358
2. **Atkins CA, Shelp BJ, Storer PJ, Pate JS** (1984) Nitrogen nutrition and the development of biochemical functions associated with nitrogen fixation and ammonia assimilation of nodules on cowpea seedlings. Planta **162:** 327–333
3. **Bergmann H, Preddie E, Verma DPS** (1983) Nodulin 35: a subunit of specific uricase (uricase II) induced and localized in the uninfected cells of soybean nodules. EMBO J **2:** 2333–2339
4. **Christeller JT, Laing WA, Sutton WD** (1977) Carbon dioxide fixation by lupin root nodules I. Characterization, association with phosphoenolpyruvate carboxylase, and correlation with nitrogen fixation during nodule development. Plant Physiol **60:** 47–50
5. **Cullimore JV, Bennett MJ** (1988) The molecular biology and biochemistry of plant glutamine synthetase from root nodules of *Phaseolus vulgaris* L. and other legumes. J Plant Physiol **132:** 387–393
6. **Dunn K, Dickstein R, Feinbaum R, Burnett BK, Peterman TK, Thoidis G, Goodman HM, Ausubel FM** (1988) Developmental regulation of nodule-specific genes in alfalfa root nodules. Mol Plant-Microbe Interact **1:** 66–74
7. **Fuller F, Verma DPS** (1984) Appearance and accumulation of nodulin mRNAs and their relationship to the effectiveness of root nodules. Plant Mol Biol **3:** 21–28
8. **Govers F, Nap JP, Van Kammen A, Bisseling T** (1987) Nodulins in the developing root nodule. Plant Physiol Biochem **25:** 309–322
9. **Griffith SM, Vance CP** (1989) Aspartate aminotransferase in alfalfa root nodules. I. Purification and partial characterization. Plant Physiol **90:** 1622–1629
10. **Groat R, Schrader L** (1982) Isolation and immunochemical characterization of plant glutamine synthetase in alfalfa (*Medicago sativa* L.) nodules. Plant Physiol **70:** 1759–1761
11. **Groat RG, Vance CP** (1981) Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.): Developmental patterns and response to applied nitrogen. Plant Physiol **67:** 1198–1203
12. **Hirel B, Bouet C, King B, Layzell D, Jacobs F, Verma DPS** (1987) Glutamine synthetase genes are regulated by ammonia provided externally or by symbiotic nitrogen fixation. EMBO J **6:** 1167–1171
13. **Lara M, Cullimore JV, Lea PJ, Miflin BJ, Johnston AWB, Lamb JW** (1983) Appearance of a novel form of plant glutamine synthetase during nodule development in *Phaseolus vulgaris* L. Planta **157:** 254–258
14. **Larsen K, Jochimsen BU** (1987) Appearance of purine-catabolizing enzymes in fix$^+$ and fix$^-$ root nodules on soybean and effect of oxygen on the expression of the enzymes in callus tissue. Plant Physiol **85:** 452–456
15. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275
16. **Miller S, Boylan KLM, Vance CP** (1987) Alfalfa root nodule carbon dioxide fixation III. Immunological studies of nodule phosphoenolpyruvate carboxylase. Plant Physiol **84:** 501–508
17. **Nguyen J, Machal L, Vidal J, Perrot-Rechenmann C, Gadal P** (1986) Immunochemical studies on xanthine dehydrogenase of soybean root nodules: Ontogenic changes in the level of enzyme and immunocytochemical localization. Planta **167:** 190–195
18. **Peterson MA, Barnes DK** (1981) Inheritance of ineffective nodulation and non-nodulation traits in alfalfa. Crop Sci **21:** 611–616
19. **Reynolds PHS, Boland MJ, Blevins DG, Schubert KR, Randall DD** (1982) Enzymes of amide and ureide biogenesis in developing soybean nodules. Plant Physiol **69:** 1334–1338
20. **Reynolds PHS, Farnden KJF** (1979) The involvement of aspartate aminotransferases in ammonium assimilation in lupin nodules. Phytochemistry **18:** 1625–1630
21. **Schubert KR** (1986) Products of biological nitrogen fixation in higher plants: Synthesis, transport, and metabolism. Annu Rev Plant Physiol **37:** 539–574
22. **Scott DB, Farnden KJF, Robertson JG** (1976) Ammonia assimilation in lupin nodules. Nature **263:** 703–705
23. **Snapp S, Vance CP** (1986) Asparagine biosynthesis in alfalfa (*Medicago sativa* L.) root nodules. Plant Physiol **82:** 390–395
24. **Stulen I, Oaks A** (1977) Asparagine synthetase in corn roots. Plant Physiol **60:** 680–683
25. **Sulebele G, Silverstein E** (1969) Malate dehydrogenase and aspartate aminotransferase of *Phycomyces blackesleeanus.* Arch Biochem Biophys **133:** 425–435
26. **Vance CP, Boylan KLM, Stade S, Somers DA** (1985) Nodule specific proteins in alfalfa (*Medicago sativa* L.). Symbiosis **1:** 69–84
27. **Vance CP, Egli MA, Griffith SM, Miller SS, Anderson MP** (1988) Plant regulated gene expression in alfalfa nodules. *In* R Palacios, DPS Verma, eds, Proceedings of the 4th International Symposium on Molecular Genetics of Plant-Microbe Interactions, American Phytopathological Society Press, St Paul MN, pp. 331–332
28. **Vance CP, Egli MA, Griffith SM, Miller SS** (1988) Plant regulated aspects of nodulation and N_2 fixation. Plant Cell Environ **11:** 413–427
29. **Vance CP, Heichel GH, Barnes DK, Bryan JW, Johnson LE** (1979) Nitrogen fixation, nodule development, and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. Plant Physiol **64:** 1–8
30. **Vance CP, Johnson LEB** (1983) Plant determined ineffective nodules in alfalfa (*Medicago sativa* L.): structural and biochemical comparisons. Can J Bot **61:** 93–106
31. **Vance CP, Johnson LEB, Halvorsen AM, Heichel GH, Barnes DK** (1980) Histological and ultrastructural observations of *Medicago sativa* root nodule senescence after foliage removal. Can J Bot **58:** 295–309
32. **Vance CP, Stade S, Maxwell CA** (1983) Alfalfa root nodule carbon dioxide fixation I. Association with nitrogen fixation and incorporation into amino acids. Plant Physiol **72:** 469–473
33. **Werner D, Mellor RB, Hahn MG, Grisebach H** (1985) Soybean root response to symbiotic infection: Glyceollin I accumulation in an ineffective type of soybean nodules with an early loss of the peribacteroid membrane. Z Naturforsch **40:** 179–181