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Proteolysis during Development and Senescence of Effective and Plant Gene-Controlled Ineffective Alfalfa Nodules[1]

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Plant-controlled ineffective root nodules, conditioned by the *in*$_1$ gene in *Medicago sativa* L. cv Saranac, undergo premature senescence and have reduced levels of many late nodulins. To ascertain which factors contribute to premature senescence, we have evaluated proteolysis as it occurs throughout the development of ineffective Saranac (*in*$_1$Sa) and effective Saranac nodules. Cysteine protease activities with acidic pH optimum and enzyme proteins were present in both genotypes. We found that acidic protease activity was low in effective Saranac nodules throughout their development. In contrast, by 2 weeks after inoculation, acid protease activity of *in*$_1$Sa nodules was severalfold higher than that of Saranac nodules and remained high until the experiment was terminated 8 weeks later. This increase in protease enzyme activity correlated with an increase in protease protein amounts. Increased protease activity and amount in *in*$_1$Sa nodules was correlated with a decrease in nodule soluble protein. The time at which *in*$_1$Sa nodules initially showed increased protease activity corresponded to when symbiosis deteriorated. High levels of phospho*enol*pyruvate carboxylase (PEPC) protein were expressed in effective nodules by 12 d after inoculation and expression was associated with low proteolytic enzyme activity. In contrast, although PEPC was expressed in *in*$_1$Sa nodules, PEPC protein was not found 12 d after inoculation and thereafter. Acidic protease from *in*$_1$Sa nodules could also degrade purified leghemoglobin. These data indicate that premature senescence and low levels of late nodulins in *in*$_1$Sa nodules can be correlated in part with increased proteolysis.

Effective root nodules formed as the result of the symbiotic association between alfalfa (*Medicago sativa* L.) and *Rhizobium meliloti* require the successful interaction of both the host plant and bacterial genomes (Vance et al., 1988; Brewin, 1991). Alfalfa nodules are classified as indeterminate in growth and are characterized by a persistent meristem (Vance et al., 1979). Along the nodule axis, surrounded by a cortical cell layer, are four different developmental zones: the apical meristem (zone I); the prefixing or infection zone (zone II); the nitrogen-fixing area (zone III); and a senescent zone (zone IV), the latter appearing as nodules age (Truchet et al., 1989; Vasse et al., 1990). In effective alfalfa nodules, a senescent zone is not evident until several weeks after inoculation (Vance et al., 1979; Vasse et al., 1990).

By comparison, ineffective nodules frequently display rapid premature senescence characterized by a strikingly reduced nitrogen-fixing zone and an increased senescent zone (Werner et al., 1985; Egli et al., 1991a). Ineffective alfalfa nodules controlled by the *in*$_1$ gene (ineffective Saranac, *in*$_1$Sa) begin to display features characteristic of senescence within the first week of development (Egli et al., 1991a, 1991b). Nodules formed on *in*$_1$Sa plants have extensive transition and senescent zones as compared to effective nodules of the same age (Vance and Johnson, 1983). Rupture of the peribacteroid membranes, lysis of bacteroids, and disorganization of host-cell cytosol are evidence of the lytic processes occurring in *in*$_1$Sa-infected cells (Vance and Johnson, 1983; Egli et al., 1991a).

The involvement of host-plant proteolytic enzymes in age-related senescence processes of effective nodules has been demonstrated in alfalfa (*Medicago sativa*) (Vance et al., 1979), soybean (Pfeiffer et al., 1983), and French bean (Pladys and Rigaud, 1985, 1988). The first targets of these proteases are nodule cytosolic proteins, especially leghemoglobin, whose disappearance limits O_2 supply to bacteroids. Such proteolytic enzymes are also able to digest bacteroids after rupture of the peribacteroid membrane (Pladys et al., 1986). The fact that proteolytic enzyme activity and amount are strictly localized to the infected cells, as recently demonstrated after protoplast isolation of French bean nodule cells (Pladys et al., 1991), confirms their key role in the lytic process leading to elimination of the bacterial partner. In this way, the precocious lytic process leading to the digestion of bacteroids evidenced in ineffective nodules may also implicate proteolytic enzymes, but this has not been described previously.

The genetics, morphology, and biochemistry of alfalfa lines that form ineffective nodules have been extensively characterized. Ineffective nodules formed by plants homozygous

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Abbreviations: E64, transepoxysuccinyl-L-leucylamido (4-guanido)-butane; *in*$_1$Sa, ineffective saranac; pAPMSF, (*p*-amidinophenyl) methylsulphonyl fluoride; PCMB, *p*-chloromercuribenzoate; PEPC, phospho*enol*pyruvate carboxylase; STI, soybean trypsin inhibitor.

recessive for the in_1 gene contain *Rhizobium*-infected cells, but are reduced 95% to 99% in nitrogenase activity. They also contain less leghemoglobin and less Gln synthetase, glutamate synthase, aspartate aminotransferase, and PEPC activity and protein than do effective nodules (Peterson and Barnes, 1981; Vance and Johnson, 1983; Egli et al., 1989, 1991a, 1991b). Moreover, the early senescence seen in in_1 nodules is accompanied by lysis of both host plant cytosol and bacteroids (Vance and Johnson, 1983; Egli et al., 1989, 1991b). Cytological changes observed for in_1 nodules resemble those that occur in incompatible plant-pathogen reactions (Baird and Webster, 1982; Caetano-Anollés and Gresshoff, 1991; Egli et al., 1991b). With a view to understanding the mechanisms involved in incompatibility mediated by the in_1 gene in alfalfa, proteolytic enzyme activity has been studied in in_1Sa nodules and compared with proteolysis occurring in effective Saranac nodules. Expression of an acidic protease was characterized using anti-protease immunoglobulin G prepared against purified protease from French bean (Pladys et al., 1991).

MATERIALS AND METHODS

Plant Material

Effective alfalfa (*Medicago sativa* L.) cv Saranac and the single gene, recessive and ineffective plant genotype in_1Sa (Peterson and Barnes, 1981) were planted in glasshouse sandbenches amended with P, K, and micronutrients (Vance et al., 1979) and immediately inoculated with *Rhizobium meliloti* strain 102F51 (Liphatech Co., Milwaukee, WI). The planting date was designated as d 0. Plants were harvested at 8:00 AM and nodules were hand collected onto ice from 1 to 7 weeks.

Preparation of Nodule Crude Extracts

Nodules were ground in extraction buffer containing 100 mM Mes-NaOH, pH 6.8, 100 mM Suc, 2% (v/v) 2-mercaptoethanol, and then centrifuged for 15 min at 15,500*g* to obtain the soluble protein fraction. All procedures were carried out at 0 to 4°C. For proteolytic enzyme assays using leghemoglobin as a substrate, nodules were extracted with 100 mM K-phosphate buffer, pH 6.8, containing 5% PVP.

Proteolytic Activity Determination

Protease assays were conducted with azocasein (Peterson and Huffaker, 1975; Pladys and Rigaud, 1985) and leghemoglobin purified from French bean nodules (Pladys and Rigaud, 1982) as substrates. For time course experiments, the substrate was solubilized in citrate-phosphate buffer (pH 5, 100 mM). pH effects on proteolytic activity were studied with azocasein and leghemoglobin in solutions in 100 mM citrate-phosphate buffer (pH 4–7) and Tris-HCl (pH 8). Protein content of extracts was measured by the method of Lowry et al. (1951). Triplicate enzyme assays were run for each extract, and the entire experiment from the planting step was repeated on three separate occasions with similar results.

Inhibition of Protease Activity

Crude extracts of 6-week-old nodules were dialyzed for 3 h against water to eliminate low mol wt effectors and then preincubated for 30 min with the following inhibitors: iodoacetic acid, E64, PCMB, PMSF, pAPMSF, and STI. The assay conditions with azocasein are the same as described above.

SDS-PAGE and Western Blotting

Soluble proteins in cell-free extracts were subjected to electrophoresis in 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. Rabbit polyclonal antibodies to *Phaseolus* nodule acidic protease (Pladys et al., 1991) were used to detect the proteolytic enzyme protein. Blots were treated with anti-protease immunoglobulin G fraction (20 μg/mL) purified by affinity chromatography on protein A Sepharose. PEPC was detected using rabbit polyclonal antibodies to alfalfa PEPC as previously described (Miller et al., 1987) after 8% SDS-PAGE. Proteins of interest were visualized by the goat anti-rabbit horseradish peroxidase development method (Hawkes et al., 1982).

RESULTS

Developmental Changes in in Vitro Proteolytic Activities

Nodules of increasing age from both effective Saranac and in_1Sa were harvested from 1 to 7 weeks after inoculation and assayed immediately. Acidic pH proteolytic activity was measured with both azocasein and bean leghemoglobin as substrates and was expressed per mg of protein digested per hour. Proteolysis was low in effective nodules throughout the experiment but increased slowly after 3 weeks, coinciding with the development of a senescent zone (Fig. 1). In comparison, with the exception of the first two assay times (d 7 and d 11), proteolytic activity of in_1Sa nodules was much greater than that of effective nodules (Fig. 1). By d 11 after inoculation, some 4 to 5 d after nodules were initially visible, there was a linear increase in proteolytic activity of in_1Sa nodules until 5 weeks, and then a burst (5–6 weeks) was seen, especially when leghemoglobin was used as substrate.

Nodule Protein

Soluble protein of effective Saranac nodules increased 2-fold from 5 mg/g fresh weight to approximately 10 mg/g fresh weight between weeks 1 and 2 (Fig. 2) and thereafter remained relatively constant at about 10 mg/g fresh weight. Initially, the amount of soluble protein in in_1Sa nodules was comparable to that in effective Saranac. However, the approximately 2-fold increase seen for Saranac soluble protein did not occur in in_1Sa nodules. Additionally, soluble protein content of in_1Sa nodules decreased 50% between 2 and 4 weeks after inoculation and thereafter remained constant at about 2.5 mg/g fresh weight.

Enzymic Characteristics

The pH optimum for proteolysis of azocasein and leghemoglobin was determined with cell-free extracts obtained from 5-week-old nodules of effective Saranac and in_1Sa (Fig. 3). Nodules from both genotypes exhibited identical pH optima with maximum activity at pH 5 and a range of activity from pH 4 to pH 6 for azocasein and from pH 4 to pH 7 for leghemoglobin.

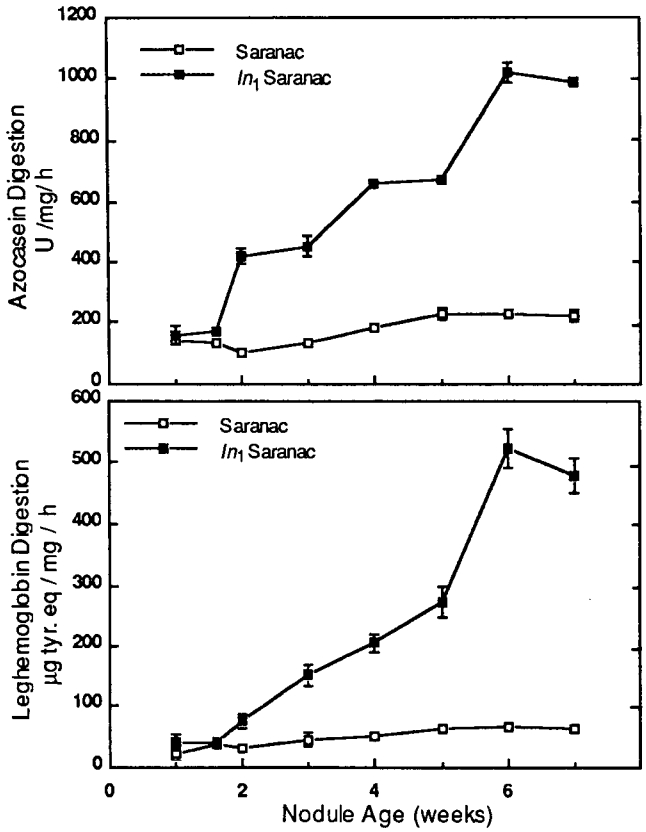

Figure 1. Acidic protease enzyme activities in bacteroid-free supernatants of developing and senescent nodules of effective Saranac and in_1Sa genotypes. Azocasein degradation was measured at pH 5 (0.1 M citrate-phosphate buffer) and activity expressed per mg of protein. One unit of activity was defined as the increase of absorbancy at 340 nm of 0.01. Leghemoglobin degradation was measured by determination of free amino acids and TCA-soluble peptides released after a 60-min incubation. Activity is expressed as µg Tyr equivalents released per mg of protein digested per hour. Each point is the average of three replicates from one representative experiment with SE indicated by the error bars.

Protease inhibitors were used to determine whether protease activity in Saranac and in_1Sa was due to Cys or Ser proteolytic enzymes (Table I). Cys protease inhibitors included PCMB, iodoacetic acid, and E64, whereas Ser protease inhibitors included PMSF, pAPMSF, and STI. Proteolysis by extracts of nodules of both genotypes was strongly inhibited by Cys protease inhibitors. In contrast, there was no inhibition of azocasein digestion by Ser protease inhibitors except for the in_1Sa extract treated with PMSF, a nonspecific inhibitor also known to inhibit Cys proteases. These results clearly show that in Saranac and in_1Sa nodules, crude extract proteolysis was due to similar Cys proteases with acidic pH optima.

Immunoreaction of Protease and PEPC in Nodule Crude Extract

Relative amounts of protease in soluble protein extracts from nodules of different ages were estimated from western

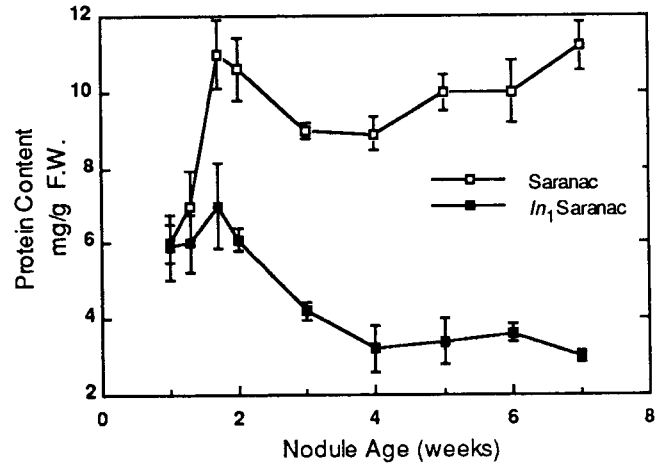

Figure 2. Soluble protein concentration of effective Saranac and in_1Sa nodule extracts obtained at different stages of development. Protein concentration was estimated by the method of Lowry et al. (1951) with BSA as a standard reference. Each point is the average of three replicates with SE indicated by the error bars.

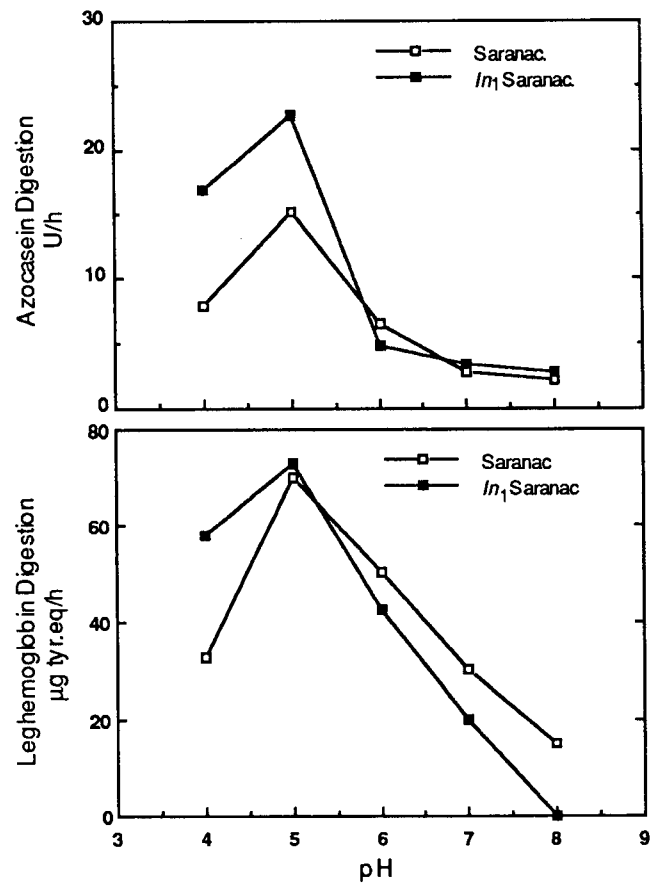

Figure 3. pH dependence of cytosolic proteolytic activities of Saranac and in_1Sa 5-week-old nodules. Incubation mixture (0.5 mL) contained azocasein (2.5 mg) or leghemoglobin (0.3 mM) and 75 µg (Saranac) or 35 µg (in_1Sa) of protein in 0.1 M citrate-phosphate (pH 4–7) and Tris-HCl (pH 8) buffer. Assays were carried out at 40°C for 2 h with shaking (50 revolutions/min).

Table I. *Inhibition of alfalfa nodule proteolytic activity by Cys and Ser protease inhibitors*

Enzymic extracts were perincubated for 30 min at 20°C. Assays were conducted at pH 5 for 1 h at 40°C with azocasein as substrate.

	Inhibitor	Final Concentration	Saranac	in_1Sa
			% *inhibition*	
Cys protease inhibitor	PCMB	0.1 mM	71	80
		1 mM	100	100
	Iodoacetic acid	5 mM	75	70
	E 64	28 μM	50	48
Ser protease inhibitor	PMSF[a]	5 mM	0	28
	pAPMSF	20 μM	0	0
	STI	50 μM	0	0

[a] Known also to be able to inhibit Cys protease.

blots (Fig. 4). Major cross-reactive bands at 58 (arrow) and 40 kD were always present in extracts (40 μg of protein per lane) of in_1Sa nodules, and the density of these bands increased with the age of the nodules. Minor cross-reacting polypeptides of higher molecular mass were also observed. The bean protease antibodies used for western blots recognize a comparable 58-kD polypeptide in purified preparations of bean Cys protease (Pladys et al., 1991). With effective Saranac nodules, polypeptides cross-reacting to the bean protease antibodies were detected only slightly in the youngest nodule extracts (from 1- to 2-week-old nodules). In the lanes containing extracts from 3- to 7-week-old nodules, the 40-kD cross-reacting polypeptide became more obvious with time, whereas the higher molecular mass polypeptides, including the 58-kD one, did not change much in intensity.

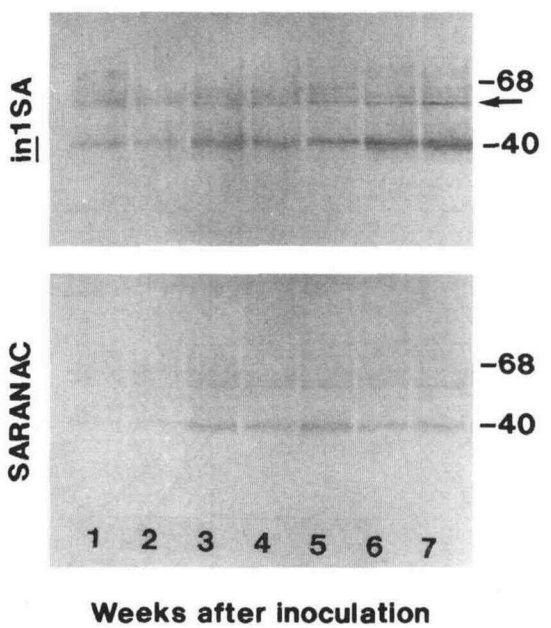

Figure 4. Immunoblots of protease in soluble protein extracts from effective Saranac and in_1Sa roots (1 week) and nodules (2–7 weeks) after inoculation. SDS gels (10%) were loaded with 40 μg of protein per lane. Proteins were blotted onto nitrocellulose and probed with French bean anti-protease immunoglobulin G (20 μg/mL). Forty-kilodalton and 68-kD molecular mass markers were used and the 58-kD band in the in_1Sa nodule extract is indicated by an arrow. A faint band is present in a comparable position in the Saranac lanes.

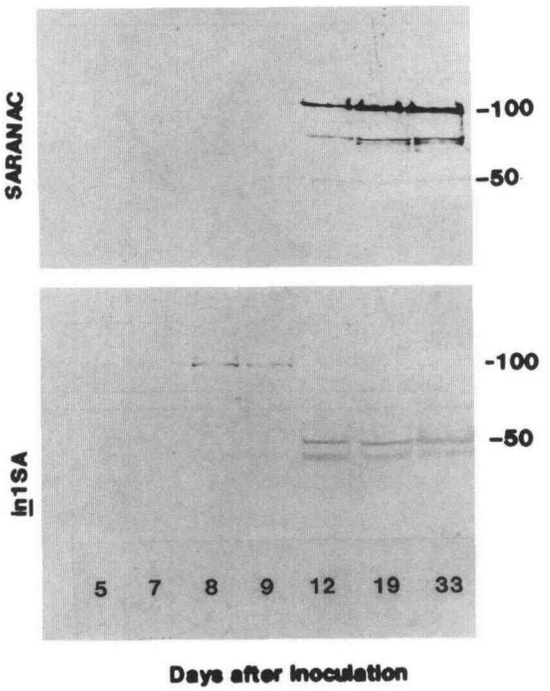

Figure 5. Immunoblots of PEPC in soluble protein extracts from effective Saranac and in_1Sa roots (1 week) and nodules (2–7 weeks). Eight percent SDS gels were loaded with 25 μg of protein per lane. Protein was blotted onto nitrocellulose and probed with anti-alfalfa nodule PEPC polyclonal antibody at a 1:2000 dilution.

Early enhanced proteolytic enzyme activity in in_1Sa nodules clearly altered the amount of nodule PEPC enzyme protein (Fig. 5). The 100-kD protein corresponding to PEPC was first detectable in in_1Sa nodules 8 d after inoculation. On d 9, the 100-kD PEPC enzyme protein was still visible. However, two bands at approximately 50 kD were also slightly visible at this time. On d 12, 19, and 33, only these two bands were visible in soluble protein extracts from ineffective in_1Sa nodules. By comparison, on d 12, 19, and 33, effective Saranac nodules contained a very strong 100-kD PEPC band with some cross-reacting proteins at lower molecular masses, one of which corresponds to the 50-kD product in in_1Sa nodules and the other at 75 to 80 kD, which appeared only in Saranac nodules. The immunoreaction of the 100-kD PEPC band from effective nodules appeared to be much stronger than the apparently diminished reactivity of the 50-kD protein, which could be a breakdown product of the 100-kD PEPC.

DISCUSSION

Early nodule senescence (Vance and Johnson, 1983; Egli et al., 1991a) and reduced ammonia-assimilating enzyme activities (Egli et al., 1989) correlated with the presence of the in_1 gene in alfalfa are accompanied by enhanced activity of

acidic proteolytic enzymes. The increase in acidic proteolytic enzyme activity was accompanied by an increase in protein bands, which cross-react with bean anti-protease immunoglobulin G at 58 and 40 kD. The first visible signs of senescence in in_1Sa nodules are apparent at 12 to 14 d after inoculation (Egli et al., 1991a, 1991b; D.L. Robinson and C.P. Vance, unpublished data). This time coincides with a 2- to 4-fold greater proteolytic enzyme activity in in_1Sa nodules as compared with effective Saranac. The previously reported low-soluble protein concentration in in_1Sa nodules as compared with Saranac (Egli et al., 1989), further verified here, correlates well with the large difference in proteolytic enzyme activity between the two nodule types.

Further evidence of a role for proteolysis in affecting in_1Sa nodule biochemistry and physiology is shown by degradation of endogenous PEPC enzyme protein in in_1Sa nodules. By 12 d after inoculation, little to no intact PEPC protein can be detected in in_1Sa nodules. This event is again coincident with the enhanced activity of the acid proteolytic enzyme. Although we had previously demonstrated reduced PEPC activity and protein in in_1Sa nodules (Egli et al., 1989; Pathirana et al., 1992), we were uncertain as to what factors contributed to these reductions. Furthermore, a recent study from our laboratory (Pathirana et al., 1992) showed that reduced PEPC in in_1Sa nodules could not be explained solely on the basis of decreased synthesis of PEPC mRNA. Although PEPC activity and protein were reduced more than 90% in in_1Sa nodules, PEPC mRNA levels were reduced by only 65%. Thus, posttranslational factors must be invoked in regulation of PEPC in in_1Sa nodules. The high proteolytic enzyme activity occurring concomitant with the degradation of PEPC enzyme protein in in_1Sa nodules strongly suggests that protein degradation contributes significantly to loss of PEPC activity and protein. We have seen similar degradation patterns for glutamine synthetase and leghemoglobin (Egli et al., 1991a and 1991b) in in_1Sa nodules. Additionally, the fact that protease activity in alfalfa nodules can degrade purified bean leghemoglobin provides added evidence for a role for proteolysis in senescence of ineffective in_1Sa nodules.

Premature enhanced proteolysis in in_1Sa nodules and age-related proteolysis in effective Saranac appear to be catalyzed by Cys proteases with acidic pH optima. Immunoreactivity with antibodies against an acidic 58-kD Cys protease from French bean, pH optima, and inhibitor studies support this interpretation. Such acidic proteases have been implicated as factors affecting peribacteroid membrane stability (Brewin et al., 1985; Pladys and Rigaud, 1986) and in eliminating the microbial partner during incompatible plant-microbe associations (Manen et al., 1991; Pladys et al., 1991).

The fact that in_1 requires the nullisomic recessive (Peterson and Barnes, 1981) condition for induction of ineffective nodules suggests that in_1Sa may lack some factor(s) required for effective symbiosis. For example, effective winged bean (*Psophocarpus tetragonolobus* Dc) nodules contain a Kunitz-like protease inhibitor (Manen et al., 1991) that may be important in maintaining symbiosis. If such a factor occurs in alfalfa, it may be missing in in_1Sa nodules, thereby leading to premature senescence. Likewise, the strikingly reduced capacity of in_1Sa nodules to produce NH_3 may trigger enhanced synthesis of acid proteases. Lack of NH_3 production has been implicated in premature senescence and proteolysis in other symbiosis (Pfeiffer et al., 1983; Atkins et al., 1984). Alternatively, enhanced protease synthesis in in_1Sa may be a pleiotropic effect resulting from the alteration of the primary target of in_1.

Although unequivocal evidence that acidic Cys proteases can degrade isolated symbiosomes and bacteroids in alfalfa remains to be established, in_1Sa nodules may be a model system for evaluating the role of proteolysis in senescence and host-microbe interactions during symbiosis.

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LITERATURE CITED

Atkins CA, Shelp BJ, Kuo J, Peoples MB, Pate JS (1984) Nitrogen nutrition and the development and senescence of nodules on cowpea seedlings. Planta **162:** 316–326

Baird LM, Webster BD (1982) Morphogenesis of effective and ineffective root nodules in *Phaseolus vulgaris* L. Bot Gaz **143:** 41–51

Brewin NJ (1991) Development of the legume root nodule. Annu Rev Cell Biol **7:** 191–226

Brewin NJ, Robertson JG, Wood EA, Wells B, Larkins AP, Galfre G, Butcher GW (1985) Monoclonal antibodies to antigens in the peribacteroid membrane from *Rhizobium*-induced root nodules of pea crossreact with plasma membranes and Golgi bodies. EMBO J **4:** 605–611

Caetano-Anollés G, Gresshoff PM (1991) Plant genetic control of nodulation. Annu Rev Microbiol **45:** 345–382

Egli MA, Griffith SM, Miller SS, Anderson MP, Vance CP (1989) Nitrogen assimilating enzymes activities and enzyme protein during development and senescence of effective and plant gene controlled ineffective alfalfa nodules. Plant Physiol **91:** 898–904

Egli MA, Larson RJ, Hruschka WR, Vance CP (1991a) Synthesis of nodulins and nodule-enhanced polypeptides by gene-controlled ineffective alfalfa nodules. J Exp Bot **42:** 969–977

Egli MA, Vance CP, Larson RJ (1991b) Regulation of nodule gene expression in plant-controlled ineffective alfalfa. *In* S Patil, S Ouchi, D Mills, C Vance, eds, Molecular Strategies of Pathogens and Host-Plants. Springer Verlag, Berlin, pp 215–225

Gantt JS, Larson RJ, Farnham MW, Pathirana SM, Miller SS, Vance CP (1992) Aspartate aminotransferase in effective and ineffective alfalfa nodules. Cloning of cDNA and determination of enzyme activity, protein and mRNA levels. Plant Physiol **98:** 868–878

Hawkes R, Niday E, Gordon J (1982) A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem **119:** 142–147

Lowry DH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275

Manen J-F, Simon P, Van Slooten J-C, Osteras M, Frutiger S, Hughes GJ (1991) A nodulin specifically expressed in senescent nodules of winged bean is a protease inhibitor. Plant Cell **3:** 259–270

Miller S, Boylan KLM, Vance CP (1987) Alfalfa root nodule carbon dioxide fixation. III. Immunological studies of nodule phospho*enol*pyruvate carboxylase. Plant Physiol **84:** 501–508

Pathirana SM, Vance CP, Miller SS, Gantt JS (1992) Alfalfa root nodule phospho*enol*pyruvate carboxylase: characterization of the cDNA and expression in effective and plant-controlled ineffective nodules. Plant Mol Biol **20:** 437–450

Peterson LW, Huffaker RC (1975) Loss of ribulose-1,5-diphosphate carboxylase and increase in proteolytic activity during senescence of detached primary barley leaves. Plant Physiol **55:** 1009–1015

Peterson MA, Barnes DK (1981) Inheritance of ineffective nodulation and non-nodulation traits in alfalfa. Crop Sci **21:** 611–616

Pfeiffer NE, Torres CM, Wagner FW (1983) Proteolytic activity in soybean root nodules. Plant Physiol **71:** 797–802

Pladys D, Dimitrijevic L, Rigaud J (1991) Localization of a protease in protoplast preparations in infected cells of French bean nodules. Plant Physiol **97:** 1174–1180

Pladys D, Rigaud J (1982) Acid protease and leghemoglobin digestion in French-bean nodules. Z Pflazenphysiol **108:** 163–169

Pladys D, Rigaud J (1985) Senescence in French bean nodules: occurrence of different proteolytic activities. Physiol Plant **63:** 43–48

Pladys D, Rigaud J (1988) Lysis of bacteroids *in vitro* and during the senescence in *Phaseolus vulgaris* nodules. Plant Physiol Biochem **26:** 179–186

Pladys D, Trinchant JC, Rigaud J (1986) Protease from French-bean nodules host-cells: *in vitro* effects on bacteroids. Physiol Veg **24:** 697–705

Truchet G, Camut S, De Billy F, Odorico R, Vasse J (1989) The *Rhizobium*-legume symbiosis: two methods to discriminate between nodules and other root-derived structures. Protoplasma **149:** 82–88

Vance CP, Egli MA, Griffith SM, Miller SS (1988) Plant regulated aspects of nodulation of N_2 fixation. Plant Cell Environ **11:** 413–427

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

Vance CP, Johnson LEB (1983) Plant determined ineffective nodules in alfalfa (*Medicago sativa*): structural and biochemical comparisons. Can J Bot **61:** 93–106

Vasse J, De Billy F, Camut S, Truchet G (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J Bacteriol **172:** 4295–4306

Werner D, Mellor RB, Hahn MG, Grisebach H (1985) Soybean root response to symbiotic infection: glyceollin I accumulations in an ineffective type of soybean nodules with an early loss of the peribacteroid membrane. Z Naturforsh **40:** 179–181