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Sucrose Synthase in Legume Nodules Is Essential for Nitrogen Fixation[1]

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The role of sucrose synthase (SS) in the fixation of N was examined in the *rug4* mutant of pea (*Pisum sativum* L.) plants in which SS activity was severely reduced. When dependent on nodules for their N supply, the mutant plants were not viable and appeared to be incapable of effective N fixation, although nodule formation was essentially normal. In fact, N and C resources invested in nodules were much greater in mutant plants than in the wild-type (WT) plants. Low SS activity in nodules (present at only 10% of WT levels) resulted in lower amounts of total soluble protein and leghemoglobin and lower activities of several enzymes compared with WT nodules. Alkaline invertase activity was not increased to compensate for reduced SS activity. Leghemoglobin was present at less than 20% of WT values, so O_2 flux may have been compromised. The two components of nitrogenase were present at normal levels in mutant nodules. However, only a trace of nitrogenase activity was detected in intact plants and none was found in isolated bacteroids. The results are discussed in relation to the role of SS in the provision of C substrates for N fixation and in the development of functional nodules.

Legume nodules are primarily dependent on the import and metabolism of Suc to provide the energy and C skeletons for biological N fixation, the assimilation of ammonia, and the export of nitrogenous fixation products. Suc is synthesized in the leaves and exported in the phloem to sinks such as the nodules. Once unloaded in the nodule cortex, Suc must diffuse into the infected region of the nodule to be metabolized. The products of Suc catabolism (usually malic acid; Udvardi and Day, 1997) are then used by bacteroids to fuel the fixation of N (Vance and Heichel, 1991; Gordon, 1995). Ammonia is exported from the bacteroids into the infected cell cytosol, where it must be rapidly assimilated. Amino acids and/or ureides are then synthesized for subsequent export from the nodule.

Suc is first metabolized by one of two enzymes, SS (EC 2.4.1.13) or AI (EC 3.2.1.26; in mature nodules there is no acid invertase activity). These reactions produce UDP-Glc and free hexoses, which, after phosphorylation by hexokinases, enter the glycolytic or oxidative pentose phosphate pathways and are metabolized to PEP. PEP is converted to oxaloacetic acid and then to L-malate by PEPC (EC 4.1.1.31) and MDH (EC 1.1.1.37), respectively.

The initial hydrolysis of Suc is a key step in N fixation. The gene encoding SS was discovered to be one of a class of genes termed "nodulins," which are highly or uniquely expressed in nodules (Thummler and Verma, 1987). In addition to its role in the provision of C for nodule N fixation, we have also provided evidence that the control of SS gene expression in nodules may be an important means of regulating C metabolism and N fixation (Gordon et al., 1997). Using experimental perturbations we previously demonstrated in soybean that the nodule SS gene is quickly down-regulated in response to stresses that simultaneously reduce N fixation (Gonzalez et al., 1995; Gordon et al., 1997). Our hypothesis is that the reduction in nodule SS activity by down-regulation of SS gene expression reduces the supply of carbohydrate for N fixation and assimilation. It is also possible that in vivo activity of the enzyme may be adjusted by covalent modification such as phosphorylation/dephosphorylation (Huber et al., 1996; Zhang and Chollet, 1997; Nakai et al., 1998).

Less is known about AI, the other nodule enzyme capable of Suc hydrolysis. AI does not appear to be regulated allosterically, and the first AI gene has only recently been cloned from plants (Gallagher and Pollock, 1998). There is no evidence that AI is affected by stress perturbations that affect N fixation (Gonzalez et al., 1995; Fernandez-Pascual et al., 1996; Gordon et al., 1997). Present evidence suggests that AI has little if any role in the modulation of N fixation. However, the unequivocal function of SS and AI in nodule metabolism remains to be established.

These questions can now be addressed with the availability of *rug4* mutants of pea (*Pisum sativum* L.) in which SS activity is greatly reduced (Craig et al., 1999). These mutants were created by a program that examines seed development and storage products at the John Innes Centre (Norwich, UK) (Wang and Hedley, 1993). In some of the mutants isolated, which were designated *rug4*, the only change in enzyme activities relating to starch metabolism

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Abbreviations: AAT, Asp aminotransferase; ADH, alcohol dehydrogenase; AI, alkaline invertase; ANA, apparent nitrogenase activity; GOGAT, Gln-oxoglutarate aminotransferase; GS, Gln synthetase; Lb, leghemoglobin; MDH, malate dehydrogenase; PDC, pyruvate decarboxylase; PEPC, PEP carboxylase; PFK, phosphofructokinase; SS, Suc synthase; WT, wild type.

in developing embryos was reduced SS activity, which resulted in lower seed starch content and a characteristic wrinkled seed phenotype (Craig et al., 1999). It was also observed that, when grown in low-N soils, these mutant plants appeared to be N deficient. In contrast, WT plants grew normally under the same conditions.

Preliminary analysis has shown that SS activity is also greatly reduced in nodules produced by these mutants (Craig et al., 1999). Furthermore, these authors have demonstrated that a gene encoding SS co-segregates with the *rug4* locus. This SS gene is identical in sequence to a cDNA obtained from pea nodules (accession no. AF079851) and a partial SS cDNA from pea testa (Dejardin et al., 1997). The gene is also 67% identical to another SS gene from pea testa (accession no. AJ001071). In addition, one of the mutant genes has been sequenced to reveal a single point mutation at position 1757 that results in the conversion of Arg-578 to Lys (Craig et al., 1999). This change at position 578 appears to have no connection with Ser-11, which corresponds to the residue capable of phosphorylation in maize (Ser-15) and mung bean (Ser-11) (Huber et al., 1996; Nakai et al., 1998).

Preliminary work on *rug4* mutant nodules by Craig et al. (1999) was based on young plants grown in suboptimum conditions on agar slopes. Their results showed reduced activity of SS in nodules, but apparently no differences in other enzyme activities or in acetylene-reduction-based ANA. However, δN^{15} analyses indicated that mutant plants derived little of their N from N_2 fixation. The objective of the work described here was to carry out much more detailed physiological, biochemical, and growth analyses on mutant and WT plants to test the hypothesis that deficient SS activity in nodules renders them incapable of effective N fixation.

MATERIALS AND METHODS

Plant Growth

Three near isogenic lines of pea (*Pisum sativum* L.), including the WT and two allelic mutants at the *rug4* locus (*rug4-a* and *rug4-b*), described by Wang et al. (1990) and Wang and Hedley (1993), were the focus of work in this study (seeds were from the backcross-6 F_2 generation). Plants were grown from seed and inoculated with *Rhizobium leguminosarum* (RCR 1045) in 0.8-L pots of vermiculite (one plant per pot) in a controlled-environment cabinet (25°C/20°C day/night temperature, 70% RH, 500 μmol m^{-2} s^{-1} PPFD, and 15-h photoperiod). Plants were supplied each day with a mineral nutrient solution lacking N (Ryle et al., 1978).

To produce seeds and compare growth when supplied with nutrients containing mineral N, separate sets of plants were grown four per 4-L pot in a commercial potting compost (Levington Horticulture, Suffolk, UK), and supplied with water each day in a greenhouse with supplementary lighting to provide a 12-h photoperiod. Day temperature was approximately 20°C and night temperature was maintained above 10°C. In the mutant lines, germinating shoots were slower to appear above the soil surface, but thereafter the plants showed no obvious differences in appearance, leaf color, or stature compared with WT plants. All plants produced flowers and set seed normally.

Nodule Harvest and Growth Analysis

Cabinet-grown plants were harvested at 3, 4, 5, and 6 weeks for growth analysis and assay of nodule biochemical parameters. Vermiculite was carefully washed from the roots of three replicate plants of each line, and nodules were quickly placed onto ice, weighed into 100- to 200-mg samples in screwcap vials, and frozen in liquid N. The remaining plants were divided into shoots and roots, and, with one nodule subsample, dried at 70°C for 48 h before weighing (total nodule dry weight was determined from the known total fresh weight and the measured fresh weight:dry weight ratio of the subsample). These plant fractions were milled, subsamples were weighed, and the total N content was determined (see below).

Measurements of Nodule Gas-Exchange Parameters

ANA and root respiration of intact, undisturbed plants were measured simultaneously and continuously using a flow-through gas system (Minchin et al., 1983) housed in a controlled-environment cabinet (Fisons Ltd., Altringham, UK), providing conditions identical to those described earlier. Nodulated root systems were sealed into the growth pots and allowed to stabilize for 18 to 21 h in a flow of air. At the start of the measurement period H_2 and CO_2 production were determined for nodulated roots in air. After 5 min the root systems were exposed to a gas stream containing 79% (v/v) Ar and 21% (v/v) O_2. H_2 production was measured using an electrochemical H_2 sensor (City Technology Ltd., Portsmouth, UK), as recently described by Witty and Minchin (1998). Respiratory CO_2 production was measured by IR gas analysis. Maximum rates of ANA were determined from the maximum predecline rate of H_2 production (i.e. before any Ar-induced decline; Minchin et al., 1983).

After new steady-state rates were established in the Ar/O_2 gas stream, the O_2 concentration was increased over the range of 30% to 60% (12.27–24.54 mol O_2 m^{-3}) in steps of 10% O_2. This was achieved by supplementing with pure O_2 using mass-flow controllers (ASM, Bilthoven, The Netherlands) and by monitoring O_2 concentration in the gas stream with an O_2 sensor (model KE25, Envin Scientific Products, Gloucester, UK). After each increment in O_2 concentration, there was a 25- to 30-min equilibration period to allow the plant response to stabilize before rates of H_2 evolution were measured again.

Measurement of Nitrogenase Activity in Isolated Bacteroids

Bacteroids were isolated anaerobically from nodules of 4-week-old plants in an anaerobic chamber (Mark 3 system supplied by Don Whitley Scientific Limited, West Yorkshire, UK) and flushed with N using a method modified

from Suganuma et al. (1998). Buffers were saturated with Ar before use. Freshly harvested nodules were placed in 50 mM K-phosphate isolation buffer (pH 7.0) containing 200 mM sorbitol and 10 mM DTT, vacuum evacuated, and saturated with Ar. The nodules were homogenized with a mortar and pestle in the same buffer. The homogenate was filtered through Miracloth prewetted with the isolation buffer. The filtrate was centrifuged at 3000*g* for 5 min, and the pellet was washed once with 50 mM Mops-KOH buffer (pH 7.0) containing 200 mM sorbitol, 4 mM $MgCl_2$, and 10 mM DTT. All operations were carried out at 4°C. The resulting pellet was re-suspended in an assay buffer (see below) and used to determine ARA (see below) and bacteroid soluble protein content (Lowry et al., 1951).

The acetylene-reduction activity of isolated bacteroids was measured in sealed vials in the presence of an assay buffer consisting of 50 mM Mops-KOH (pH 7.0) containing 200 mM sorbitol, 4 mM $MgCl_2$, 5 mM succinate, and 0.2 mM myoglobin. Aliquots of bacteroid suspension (0.2 mL containing approximately 0.5 mg of protein) and 1 mL of the assay buffer were placed into 17-mL vials and sealed with rubber caps under anaerobic conditions. Air was injected to give a final O_2 concentration of 0.2% (v/v) in the gas phase, and the assay was initiated by injecting acetylene to a final concentration of 10% (v/v). The vials were incubated at 20°C for 20 min with shaking, and then gas samples (1 mL) were taken for the assay of ethylene using a gas chromatograph (model 610, Unicam Analytical, Cambridge, UK) fitted with a 1.2-m × 3-mm column (Porapak R, Hewlett-Packard) and a flame-ionization detector.

An optimal O_2 concentration of 0.2% (v/v) was determined by measuring the acetylene reduction activity of WT bacteroids at a range of O_2 concentrations from 0% to 1.3% (v/v). The reaction was linear for at least 30 min. No activity was found when O_2 was not added.

Extraction of Host Plant and Bacteroid Proteins

Nodules were homogenized in a mortar and pestle with 50 mM Mops-KOH (pH 7.0), 4 mM $MgCl_2$, 20 mM KCl, 200 mM sorbitol, and 10 mM DTT at 0°C to 2°C (5 mL/g fresh weight). The homogenate was centrifuged at 20,000*g* for 30 min at 2°C. Samples (50 μL) of the supernatant fraction were retained for immediate PEPC assay, and 1-mL aliquots were desalted by low-speed centrifugation (180*g* for 1 min) through 5-mL columns of Bio-Gel P6DG (Bio-Rad) equilibrated with 50 mM Mops-KOH (pH 7) and 4 mM $MgCl_2$. The desalted extract was used to assay for soluble protein (Lowry et al., 1951), Lb (Appleby and Bergersen, 1980), and several enzymes. PEPC, SS, AI, GS (EC 6.3.1.2), and AAT (EC 2.6.1.1) assays were as described in Gonzalez et al. (1995). MDH, PFK (EC 2.7.1.11), and PPi-dependent, Fru-6-P phosphotransferase (EC 2.7.1.90) were assayed as described in Gordon (1991). NADH-GOGAT (EC 1.4.1.13) was assayed using the method of Groat and Vance (1981). PDC (EC 4.1.1.1) and ADH (EC 1.1.1.1) were assayed according to the method of John and Greenway (1976).

To measure SS activity in the Suc synthesis direction, the production of UDP was coupled to the oxidation of NADH in the presence of pyruvate kinase and lactate dehydrogenase. The decrease in A_{340} was measured spectrophotometrically. Reaction mixtures in a final volume of 1 mL of 50 mM imidazole buffer (pH 8.5) contained 2 units of pyruvate kinase, 10 units of lactate dehydrogenase, an aliquot of the desalted enzyme extract, 5 mM $MgCl_2$, 1 mM PEP, 0.2 mM NADH, 15 mM Fru, and 2 mM UDP-Glc. All assays were performed at 30°C.

Extraction of Bacteroid Proteins

Nodules were extracted and the homogenate centrifuged as described above. The 20,000*g* pellet containing intact bacteroids was washed three times by resuspension in extraction buffer and recentrifugation at 20,000*g* for 30 min at 2°C. The washed pellet was re-suspended a fourth time in the same buffer lacking sorbitol and the bacteroids were broken by sonication (two 30-s pulses at 0°C). The supernatant obtained after a further centrifugation step (as before) was retained for soluble protein determination (see above). SDS-PAGE and immunoblotting of nitrogenase proteins are described below.

SDS-PAGE and Immunoblotting

Soluble host plant protein and bacteroid protein extracts were denatured and prepared for electrophoresis and blotting as described in Gordon and Kessler (1990) and Cresswell et al. (1992). Nitrocellulose membranes onto which host plant proteins were blotted were probed with antibodies specific for SS (Gordon et al., 1992), Lb (Gordon and Kessler, 1990) and GS (provided by J.V. Cullimore, Warwick University, UK). Bacteroid proteins were detected using antibodies specific for nitrogenase components 1 and 2 (provided by P.W. Ludden, University of Wisconsin, Madison). Color development using a secondary antibody conjugated to horseradish peroxidase was as described in Gordon and Kessler (1990).

Nodule Extraction for Carbohydrates and Total Amino Acids and Amides

Nodules (approximately 0.2 g fresh weight) were extracted four times in a total of approximately 50 mL of boiling 80% (v/v) ethanol. The ethanol-soluble extracts were dried under vacuum, and the soluble compounds were redissolved in 4 mL of distilled water and centrifuged at 20,000*g* for 10 min. The supernatant fluid was frozen in liquid N and stored at −80°C for later analysis. The ethanol-insoluble residue was extracted for starch as in MacRae (1971), and Glc was analyzed as described below.

Soluble Carbohydrate Analysis

Glc, Fru, and Suc were determined using a plate reader (model EL340, Bio-Tek Instruments, Winooski, VT) at 340 nm in enzymic reactions coupled to the production of NADH. Samples (up to 50 μL) in the wells of a 96-well

plate were assayed for Glc after incubation with 200 μL of buffer (50 mM imidazole, pH 7.0, 1 mM $MgCl_2$, 0.75 mM NAD, and 0.85 mM ATP) containing 0.04 unit of Glc-6-P dehydrogenase from *Leuconostoc mesenteroides* and 0.1 unit of hexokinase. Fru and Suc were estimated in the same way after further additions of phospho-Glc isomerase (0.4 unit/well) and acid invertase (20 units/well), respectively.

Nitrogenous Compounds

Total free amino acids were assayed according to the method of Vogels and Van der Drift (1970). The total N content of dried, milled plant tissue samples was determined using a mass spectrometer (model 20/20, Europa Scientific, Cheshire, UK) coupled to a solid/liquid preparation module.

RESULTS

Plant Growth

Plants of the *rug4* mutant of pea, grown with an adequate supply of nitrate, were visually indistinguishable from WT plants, apart from the wrinkled appearance of the seed, which was the means by which they were originally selected.

When seeds were inoculated with an effective *R. leguminosarum* culture and supplied with a nutrient solution lacking N, the growth of mutant plants was severely impaired after 3 to 4 weeks (Fig. 1). The large seeds of pea provided sufficient N for the plants to develop healthy leaves during the early stages of growth, and 2 weeks after planting both mutant and WT plants were similar in appearance. Thereafter, however, mutant plants were chlorotic and total dry weight remained constant over the

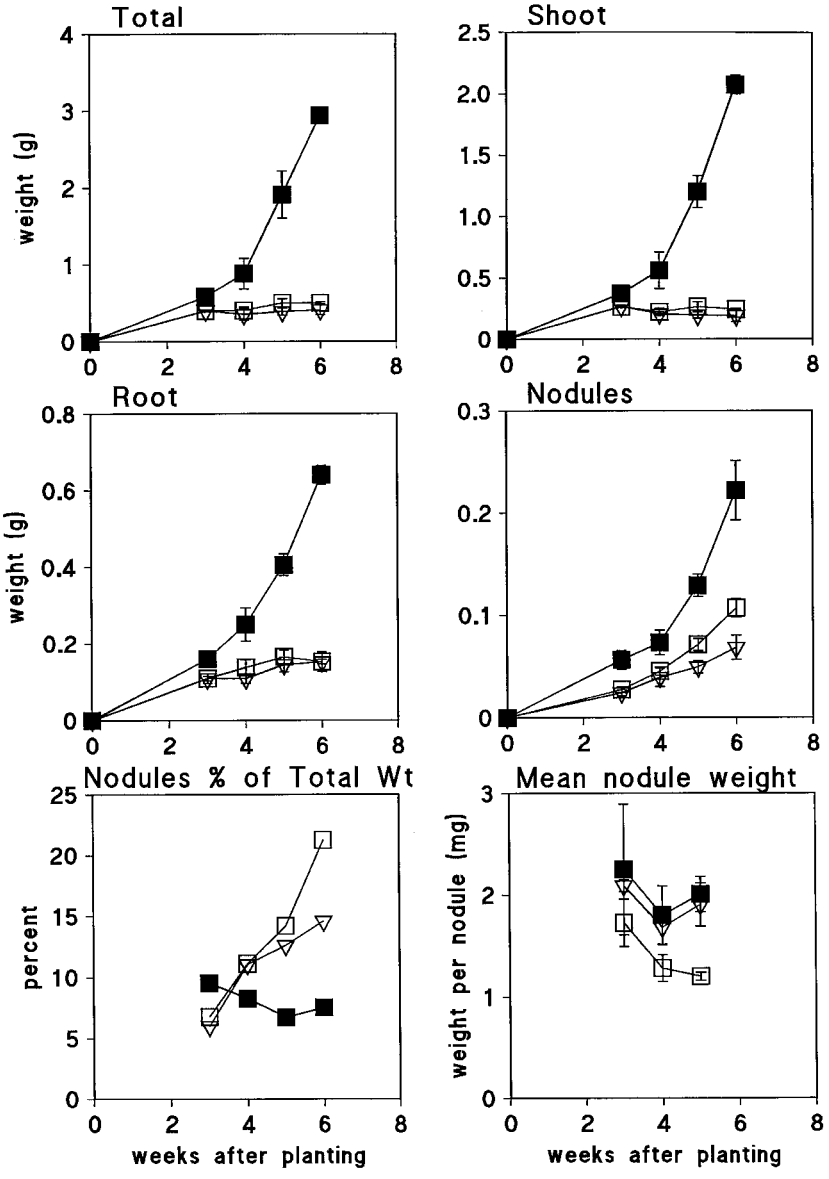

Figure 1. Growth parameters of WT and *rug4* mutants during 6 weeks of growth from seed (0 time) in a controlled-environment cabinet. Growth was dependent on nodule N fixation. ■, WT; ▽, *rug4-a*; □, *rug4-b*. Values are means ± SE (n = 3).

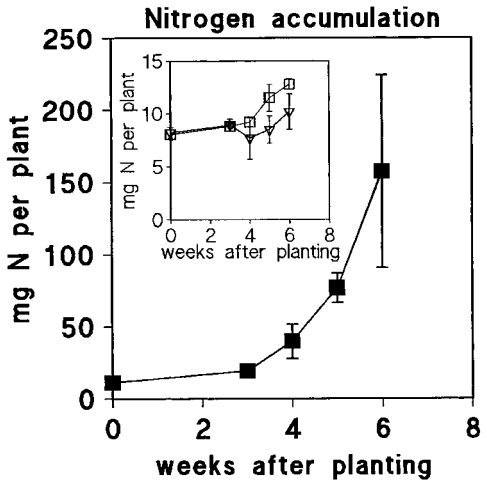

Figure 2 Accumulation of total N in WT and *rug4* mutant plants during 6 weeks of growth from seed (0 time) in a controlled environment cabinet. Growth was dependent on nodule N fixation. Initial seed N contents were: WT, 11.2 ± 0.6 mg seed^{-1}; *rug4-a*, 8.2 ± 0.5 mg seed^{-1}; and *rug4-b*, 8.0 ± 0.5 mg seed^{-1}, (n = 15–27). ■, WT; ▽, *rug4-a*; □, *rug4-b*. Values are means ± SE (n = 3).

period 3 to 6 weeks after sowing (Fig. 1). In contrast, the weight of WT plants increased exponentially with time and no chlorosis was observed. There was one major difference in the partitioning of resources. Total nodule dry weight in mutant plants continued to increase throughout the growth period, in contrast to shoot and root dry weights which remained essentially constant after 3 weeks. In the mutant plants, nodule weight as a proportion of total plant weight increased from 5% to 15% to 20% (over a period of 3–6 weeks) compared with a gradual decline in the nodule component in WT plants (Fig. 1). The average dry weight per nodule was similar in WT and *rug4-b* plants, although weight per nodule in *rug4-a* plants was lower (Fig. 1).

N Accumulation

Despite the fact that mutant plants produced healthy-looking nodules, the implication from the growth data was that they were unable to fix sufficient N to sustain normal plant growth. This was confirmed by measuring total N content per plant over the growth period and also by assessing the ANA of whole plants and of isolated bacteroids.

N Content

N accumulated in WT plants from an initial value of 11 mg per seed to approximately 160 mg per plant at 6 weeks (Fig. 2). In contrast, mutant seeds contained somewhat lower amounts of N (8 mg per seed) and accumulation during growth was severely impaired. In *rug4-a*, in particular, the N content of the whole plant at 6 weeks appeared to be no higher than that present in the original seed.

Nodule ANA

ANA of mutant plants was barely detectable and was only about 3% of WT rates at 5 weeks (Table I). The effect of raising external O_2 concentration was to increase ANA somewhat in the mutant plants. However, the fact that ANA was still extremely low compared with WT, even under elevated O_2, indicates that mutant nodules lacked sufficient metabolic capacity to function at higher rates. Exposing nodulated roots of intact WT plants to elevated O_2 levels tended to decrease ANA.

Nitrogenase Activity of Isolated Bacteroids

Although little ANA was detected in the mutants with whole plant assays, nitrogenase proteins were present in the bacteroids (see below), and it remained a possibility

Table I. *Parameters of nodule nitrogenase activity in intact plants*

Values are means of measurements of three plants ± SE.

Age and Plant Type	Nitrogenase Activity	Maximum Nitrogenase Activity under Elevated O_2	Increase in Activity by Increasing O_2
	$\mu mol\ H_2\ min^{-1}\ plant^{-1}$		%
3 weeks			
rug4-a	0.011 ± 0.001	0.016 ± 0.003	44.9 ± 12.5
rug4-b	0.018 ± 0.001	0.020 ± 0.001	14.9 ± 7.5
WT	0.322 ± 0.051	0.218 ± 0.034	−32.3 ± 1.2
4 weeks			
rug4-a	0.018 ± 0.005	0.026 ± 0.001	46.7 ± 12.5
rug4-b	0.021 ± 0.003	0.038 ± 0.006	79.9 ± 23.3
WT	0.251 ± 0.055	0.205 ± 0.048	−17.8 ± 4.9
5 weeks			
rug4-a	0.018 ± 0.005	0.029 ± 0.007	60.5 ± 8.9
rug4-b	0.017 ± 0.005	0.024 ± 0.006	46.9 ± 6.9
WT	0.737 ± 0.058	0.540 ± 0.026	−26.4 ± 2.3

that nitrogenase was active, but was limited by C substrates and/or O_2. However, anaerobically isolated bacteroids of mutant nodules from 23-d-old plants displayed no measurable nitrogenase activity when assayed in the presence of optimal O_2 concentrations (determined with bacteroids isolated from WT nodules) and with succinate as the C substrate. In contrast, nitrogenase activity of isolated bacteroids from control WT plants of the same age was substantial (253 ± 13 nmol acetylene h^{-1} mg^{-1} bacteroid protein; $n = 19$).

Biochemistry

The precise effect of the mutation was assessed by analyzing protein, Lb, and SS levels (Fig. 3), maximum catalytic activities of a selection of nodule enzymes (Fig. 4), and by immunoblotting using specific antibodies (Fig. 5).

Nodule Enzymes

In assays of host plant enzymes we confirmed that the nodules of mutant plants contained much reduced activities of SS (in the Suc cleavage and Suc synthesis directions) compared with activities in WT nodules (Figs. 3 and 4). The 90% reduction in activity (expressed g^{-1} fresh weight) measured here compares with about 95% reduction in activity when these plants were grown in less favorable conditions (Craig et al., 1999). Associated with this reduced SS activity, total host plant soluble protein content of nodules declined from approximately 70% of WT levels at 3 weeks to approximately 40% of WT levels at 6 weeks (Fig. 3). Lb was present at less than 20% of WT levels and, in addition to reduced SS activity, may have contributed to the inability of mutant nodules to fix N_2.

The data in Figure 4 (expressed per milligram of protein) indicate that despite the diminished activity of SS in the mutant nodules, there was no indication that AI activity was increased to compensate for reduced SS activity. In fact, AI was also lower in mutant nodules compared with the WT (particularly when expressed in the more physiologically meaningful units per gram fresh weight).

Mutant nodules also displayed a number of other pleiotropic effects. There were reduced activities of other nodule enzymes involved with carbohydrate, carboxylic acid, and amino acid metabolism. Most notable were PFK, PEPC, GS, GOGAT, and AAT. In contrast to these low enzyme activities, PPi-dependent, Fru-6-P phosphotransferase, PDC, and ADH activities were generally higher in mutant nodules than in WT nodules. The high activity of ADH in particular suggests that the internal environment in mutant nodules was relatively anaerobic compared with WT nodules.

Bacteroid extracts were prepared from nodules of 4-week-old plants. Bacteroid protein contents of these extracts were 11.25 ± 0.37 (WT), 10.41 ± 0.27 (*rug4-b*), and 9.66 ± 0.68 (*rug4-a*) mg g^{-1} nodule fresh weight.

Western Blotting

Immunoblotting provided a more direct measure of the amounts of specific polypeptides associated with Lb and some enzymes (Fig. 5). The low GS and Lb levels indicated by direct assay were reflected in the lower amounts of protein recognized by specific antibodies. However, the SS polypeptide content of the two mutants was not consistently related to measured enzyme activity. The amount of SS subunit in *rug4-b* nodules appeared to be about the same as that in WT nodules, although activity was much lower (Figs. 4 and 5). In *rug4-a* nodules, in contrast, the lower SS polypeptide content indicated by immunoblotting more closely matched the low enzyme activity. Therefore, the reason for the low SS activity in vitro does not appear to be the same for these two mutants.

We also used antibodies to the two nitrogenase components to indicate whether the low ANA values of the mutants were due to interference with bacteroid development. The presence of similar levels of nitrogenase

Figure 3. Nodule total host soluble protein (mg g^{-1} fresh weight), SS activity in the Suc cleavage direction (μmol Suc metabolized min^{-1} g^{-1} fresh weight), and amount of Lb (mg g^{-1} fresh weight) during growth of WT (■), *rug4-a* (▽), and *rug4-b* (□) pea plants in a controlled-environment cabinet for 6 weeks.

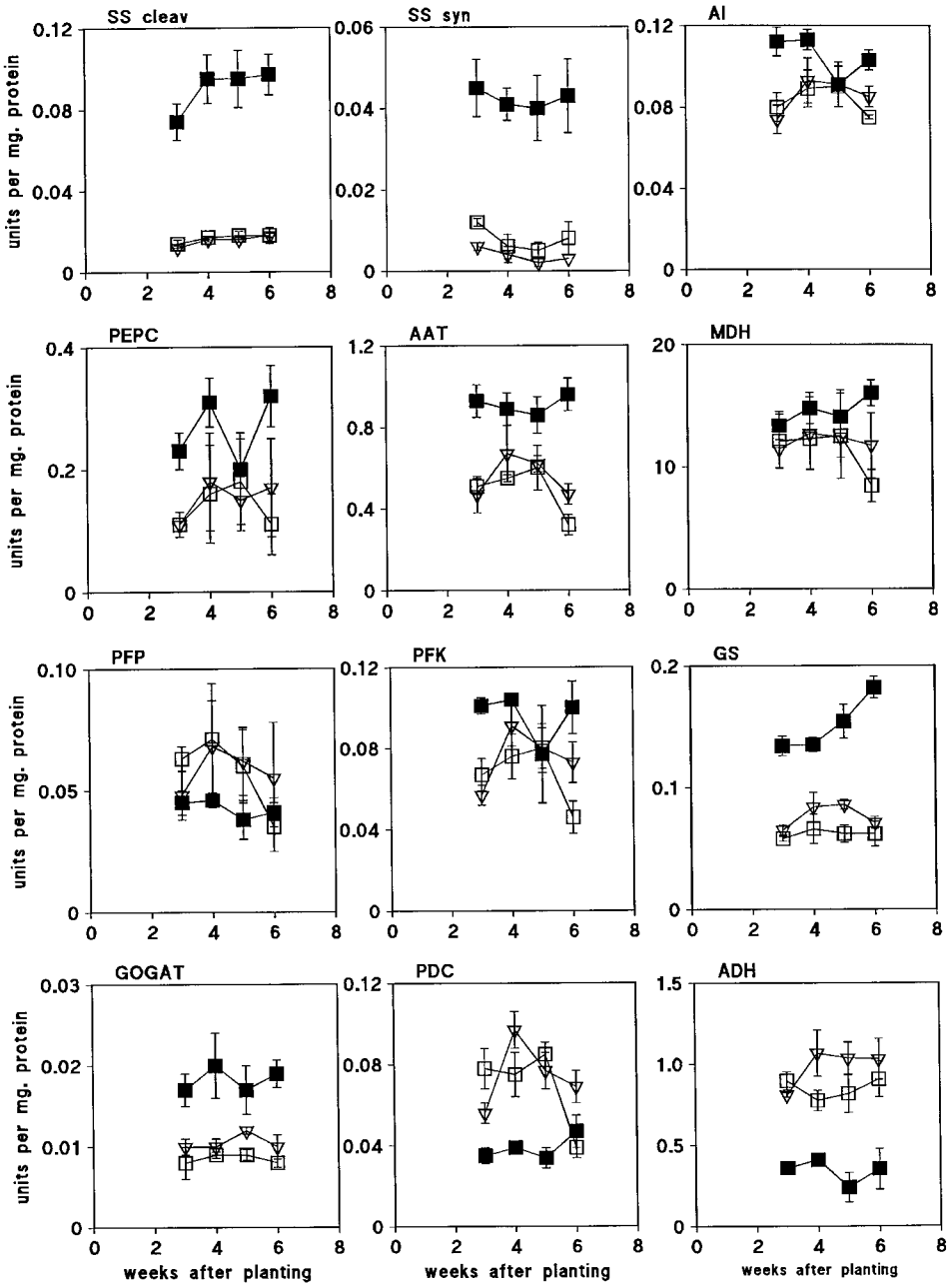

Figure 4. Nodule enzyme activities during growth of WT (■), *rug4-a* (▽), and *rug4-b* (□) pea plants in a controlled-environment cabinet for 6 weeks. Values are means ± SE (n = 3). SS syn, Activity of SS in the Suc-synthesis direction; SS cleav, activity of SS in the Suc cleavage direction.

protein in mutant and WT nodules suggests that bacteroids from mutant nodules had the normal complement of nitrogenase.

Metabolite Levels

The data in Figure 6 are expressed on a starch-free dry-weight basis to eliminate the large but variable contribution that starch made to nodule dry weight. Starch-free dry weight corresponds to nodule structural dry weight. During growth between 3 and 6 weeks, there were no significant differences in the Suc content of WT and mutant nodules, which declined from about 150 to 50 mg g^{-1} nodule dry weight (Fig. 6). Glc levels were consistently higher in the mutant nodules, whereas Fru, present at about 10% of Glc levels, showed no trends. Starch levels were very high in WT nodules, whereas the amount in mutant nodules, although substantial, was always lower. Starch levels declined in both mutant and WT nodules over the time course. Total amino acids levels in nodules also showed a declining trend, but WT levels were always higher than in mutant nodules.

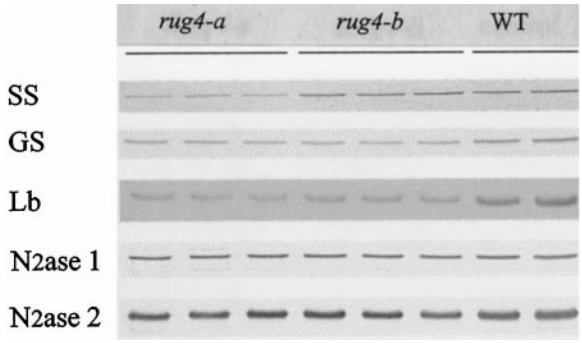

Figure 5. Immunoblots of key proteins in nodule extracts prepared 4 weeks after sowing seeds. Three replicate samples from different plants are shown for *rug4-a* and *rug4-b*, and duplicates are shown for WT. N2ase 1 and 2, Nitrogenase components 1 and 2. Equal amounts of protein were loaded in each lane.

DISCUSSION

The two allelic *rug4* mutants used in this study were selected and genetically characterized on the basis of their wrinkled seed phenotype (Wang and Hedley, 1993). The mutant plants have been shown to have reduced SS activity in embryos and nodules compared with WT plants (Craig et al., 1999; data in this paper). The SS gene cosegregates with the *rug4* locus, and in one of the mutant SS gene sequences so far examined (*rug4-b*), the base at position 1757 was different from the WT sequence and resulted in a change in amino acid from Arg to Lys (Craig et al., 1999). The SS gene in *rug4-a* has now also been sequenced and involves a different single amino acid change than that in *rug4-b* (P. Barratt and A. Smith, personal communication). The seeds used in this work were from the backcross-6 F_2 generation, hence the description "near-isogenic." It is extremely unlikely that identical secondary mutations, cosegregating with SS through six backcrosses, were present in the two allelic mutants studied here. It is most likely that the mutants differ from the WT only with respect to the change induced in the SS gene and the resulting dysfunctional SS polypeptide.

From the work described here, both mutants had low SS activity in both the Suc cleavage and the Suc synthesis directions. However, western blotting indicated that the amounts of the SS subunit were different in the two mutants; *rug4-b* nodules had almost WT levels, whereas *rug4-a* had much reduced levels of SS subunit. At present it is not known how the changes in amino acids in *rug4-a* and *rug4-b* SS result in reduced activity and differences in the amount of polypeptide.

The results reported here indicate that normal nodule development and function depends on the expression of sufficient active SS. AI, although present in both mutant and WT nodules, did not compensate for the reduced activity of SS. In WT nodules, maximum catalytic activity of AI was equal to or greater than that of SS, but was actually reduced in mutant nodules. If this enzyme were to compensate for the lower metabolic activity caused by the reduced activity of SS, then activity of AI should be increased (compare with Zrenner et al., 1995). The decline in AI in the mutant nodules, therefore, implies that AI does not contribute significantly to the provision of substrates for bacteroid metabolism. The SS reaction may be favored over the AI route because the products of the former reaction are less dependent on ATP for further metabolism (Gordon, 1992), and in the O_2-limited environment of the nodule host cell cytosol, ATP is likely to be in short supply (de Lima et al., 1994; Kuzma et al., 1995, 1999).

Many other pleiotropic effects were noted in mutant nodules, which suggests that an active SS is required for normal development of the nodule. Total host plant soluble protein levels were much lower in mutant nodules than in those from WT plants. In terms of specific proteins, one of the most important effects was the much reduced expression of Lb (less than 20% of WT levels). This could limit the flux of O_2 to the bacteroids in the center of infected cells and could also permit the buildup of inhibitory concentrations of O_2 at the periphery of the central infected zone of nodules.

Other enzymes involved in supplying C substrates to bacteroids (e.g. PFK, MDH, and PEPC) and processing ammonia exported from bacteroids (e.g. GS, GOGAT, and AAT) were also present in lower amounts in mutant compared with WT nodules. PDC and ADH activities were generally higher in mutant than in WT nodules, suggesting that anaerobic metabolism may be induced in circumstances of inactive nitrogenase and low Lb levels (compare with Suganuma and LaRue, 1993; Romanov et al., 1995, 1998; Gordon and James, 1997). The connection between SS activity and the expression of normal levels of Lb and these general "housekeeping" enzymes is not known at present, but does imply that the expression of an active SS is a necessary precondition for normal nodule development.

In other studies where SS expression and activity have been altered either through mutation (Singletary et al., 1997) or antisense techniques (Zrenner et al., 1995), significant changes in the activities of other enzymes were also noted. However, additional doses of the *sh1* allele in maize, which caused up to a 92% reduction in SS activity, resulted in increases in activities of a number of enzymes of carbohydrate metabolism (Singletary et al., 1997). In transgenic potato tubers, acid and neutral invertase activities were increased substantially in those tubers showing a 90% reduction in SS activity (Zrenner et al., 1995). Our results contrast with these findings.

The effect of low SS activity in *rug4* nodules on other enzymes also contrasts with the data for enzymes in the embryos of this mutant, where no pleiotropic effects were noted (Craig et al., 1999). However, for nodules, the data presented here are consistent with our findings for other mutants in which the lesions have not been defined (Romanov et al., 1995, 1998), and set a clear precedent in demonstrating that a single mutation in one enzyme can have far-reaching pleiotropic effects on the development of functional nodules.

One surprising finding was that nitrogenase proteins were present in normal amounts, but were essentially inactive, as judged by assays of intact plant root systems and anaerobically isolated bacteroids, and the fact that little or no N accumulated in mutant plants over the 6-week

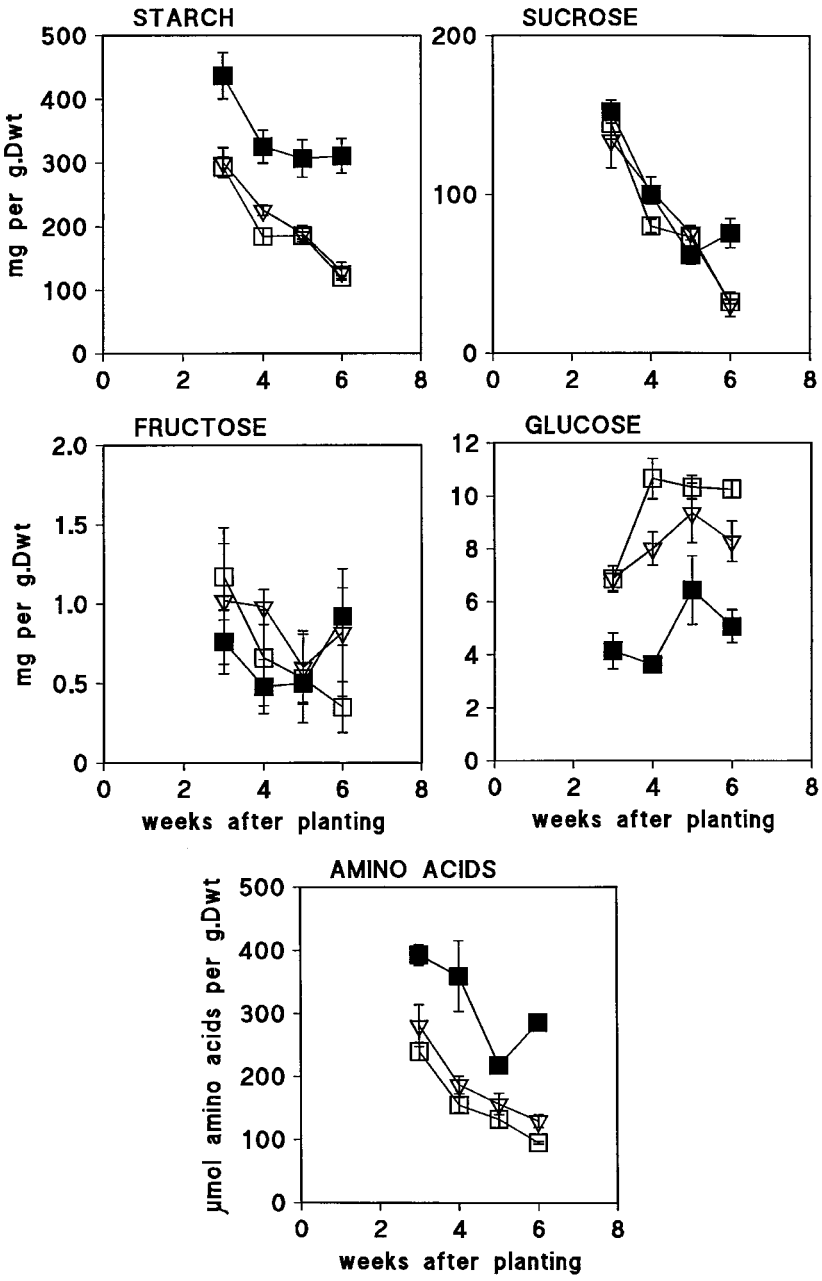

Figure 6. Levels of starch, Suc, Fru, Glc, and total amino acids in nodules of WT (■), *rug4-a* (▽), and *rug4-b* (□) pea plants during growth in a controlled-environment cabinet for 6 weeks. Values are means ± SE ($n = 3$). Dwt, Dry weight.

growth period. In other nonfixing pea mutants studied, nitrogenase proteins were also inactive but were generally present in very small amounts (Romanov et al., 1995; Suganuma et al., 1998). The implication from this study is that SS is not required for the expression of nitrogenase genes but is essential for the maintenance of nitrogenase activity. However, whether this is due to a direct effect of reduced C flux to bacteroids or to the pleiotropic effects caused by low SS activity is unclear at this stage. The substantially lower amount of Lb in *rug4* mutant nodules and the effect this may have on O_2 concentrations in the central region of the nodule could be the reason for inactive nitrogenase (compare with Soupene et al., 1995). In another pea mutant (FN1; Romanov et al., 1998), Lb was present at approximately 40% of WT levels, but nitrogenase activity was detectable, despite the fact that one of the nitrogenase components was present in much lower amounts.

We have previously hypothesized that the control of N fixation may be partially mediated by control of the activity and expression of SS (Gonzalez et al., 1995; Gordon et al., 1997). The results presented here provide additional support for this hypothesis. Since it is demonstrated that nodule function is impaired by low SS activity, it follows that if SS is down-regulated because of stress-mediated signaling, then the resulting decline of SS activity could render nodules less capable of N fixation. Further loss of SS activity would ultimately cause the complete cessation of N fixation.

We conclude that SS is essential for normal nodule development and function. It is apparent that reduced SS activity is not compensated for by increased AI activity, and indeed that normal levels of this enzyme and a number of other essential proteins (key enzymes and Lb) are also dependent on SS activity. It appears, however, that although nitrogenase is expressed normally, active SS may be required for the maintenance of nitrogenase activity.

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