*Symbiosis*, 22 (1997) 241–254
Balaban, Philadelphia/Rehovot



# Down-Regulation of Sucrose Synthase Expression and Activity in Transgenic Hairy Roots of *Lotus Japonicus*

LEIF SKØT*, ANTHONY J. GORDON, EMMA TIMMS, CARON L. JAMES, K. JUDITH WEBB, and SUE MIZEN
*Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Ceredigion, SY23 3EB, Wales, UK.*
*Tel. +44-1970-828255, Fax. +44-1970-828357, E-mail. leif.skot@bbsrc.ac.uk*

Received September 26, 1996; Accepted December 21, 1996

**Abstract**

Sucrose synthase is central to carbon metabolism in roots and nodules of legumes. In order to analyze its role we have used antisense RNA strategies to down-regulate its expression in the model legume *Lotus japonicus*. In contrast to other legumes the sucrose synthase gene from *Lotus* was expressed highly not only in nodules, but also in roots. This enabled us to use transgenic hairy roots to evaluate the potential of the antisense RNA strategy. The sucrose synthase sense message was drastically reduced in 4 out of 5 antisense sucrose synthase hairy root lines. Sucrose synthase protein levels were significantly reduced, and sucrose synthase enzyme activities declined to between 20% and 25% of the controls. By contrast, activities of UDPGlucose pyrophosphorylase, fructokinase, glucokinase and acid invertase remained unchanged, while unexpectedly, alkaline invertase activity declined in the antisense sucrose synthase lines. The significantly higher fresh weight of the control cultures compared to the antisense hairy roots observed after 14 days of growth, suggest that the changes in enzyme activities affected the growth rate. Overall, this work demonstrates that the antisense RNA strategy can be used successfully in *L. japonicus* to alter gene expression.

Keywords: Antisense RNA, hairy roots, *Lotus japonicus*, nitrogen fixation, sucrose

Presented at the 8th International Symposium on Molecular Plant-Microbe Interactions, July 14–19, 1996, Knoxville, TN.

*The author to whom correspondence should be sent.

**Abbreviations**

kb: kilobase; MES: (2-[Morpholino] ethanesulfonic acid); MOPS: 3-(N-Morpholino)-propanesulfonic acid; NAD: Nicotineamide adenine dinucleotide; PCR: Polymerase chain reaction; PMSF: Phenyl-methyl-sulphonyl-fluoride; SDS: Sodium dodesyl sulphate; Tris: (Tris[hydroxymethyl]aminomethane.

## 1. Introduction

In most plants sucrose is the major carbohydrate translocated in the phloem from photosynthesizing leaves to sink tissues. One of the factors contributing to the sink strength of a given plant tissue is believed to be the activity of the sucrose synthase (SS) enzyme (UDP-Glucose: D-fructose-2-glycosyl transferase, EC 2.4.1.13) (Zrenner et al., 1995). SS mobilises sucrose by hydrolysing it to UDP-Glucose and fructose. The products can then be metabolised further via the glycolytic pathway or used as precursors in the biosynthesis of starch and cell wall polymers. In nitrogen fixing legumes the root nodules are powerful sinks. This is due, partly to the large amounts of ATP and reductants required to drive the reduction of $N_2$ to $NH_4^+$, and partly to carbon skeletons, in the form of dicarboxylic acids required to assimilate $NH_4^+$ (Witty et al., 1986; Skøt et al., 1986). A close correlation was found between SS and nitrogenase activity in soybean nodules (Gordon et al., 1997).

The suggested importance of SS in root nodules is further emphasized by the finding of Thummler and Verma (1987) that the soybean nodulin NGm-100 was identical to the subunit of the homo-tetrameric SS enzyme, and 20 times more abundant in nodules than in roots. Küster et al. (1993) isolated the first full length cDNA encoding a nodule enhanced SS from *Vicia faba*, and this was also expressed 10–20 fold more strongly in nodules than in roots.

Sucrose may however, be hydrolysed by either SS or Alkaline Invertase (ALK INV) (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) (Stitt and Sonnewald, 1995). It is perhaps significant that, in contrast to ALK INV, sucrose hydrolysis catalysed by SS has no net requirement for ATP to produce phosphorylated monosaccharide products. This could be advantageous in an oxygen limited environment such as root nodules or flooded roots (Gordon, 1992; Plaxton, 1996). Expression of SS was induced or enhanced in response to low $O_2$ in sugar beet, rice, potato and maize (Hesse and Willmitzer, 1996; Ricard et al., 1991; Salanoubart and Belliard, 1989; Springer et al., 1986).

A partial clone encoding an SS gene was isolated from a cDNA library of *Lotus japonicus* root nodules (Skøt et al., 1996). Although expression was nodule enhanced, our preliminary results suggested that this gene was also expressed highly in roots of *L. japonicus*. The clone was isolated with a view to using it in

antisense strategies to analyze the effect of reduced SS expression on root nodule carbon metabolism and nitrogen fixation, and establish the relative importance of SS and ALK INV. Expression of the SS gene in roots opens up possibilities of studying the effects of down-regulating SS expression in transgenic hairy roots of *Lotus*. Here we present results of the first successful use of antisense RNA technology in the model legume *L. japonicus* expressing the antisense SS gene in hairy root cultures.

## 2. Materials and Methods

*Transformation construct*

A partial cDNA clone of 1 kb encoding the 3' end of the sucrose synthase was isolated as described, from a nodule library of *Lotus japonicus* (Skøt et al., 1996). It was then cloned as a 0.8 kb fragment in the antisense orientation in the binary plant transformation vector pROK2, which is a derivative of pBIN19 (Bevan 1984) containing the constitutive CaMV 35S promoter and the NOS polyadenylylation sequence. The resulting plasmid was used to transform the *Escherichia coli* host strain DH5α. From there the plasmid was transferred to the *Agrobacterium rhizogenes* strain LBA 9402 by triparental mating with the helper plasmid pRK2013. The wild type *A. rhizogenes* strain 8196 (Dessaux et al., 1986) was used as the control in transformations.

*Production of transformed root lines*

Seeds of *Lotus japonicus* cv. Gifu (S9) were originally provided by J. Stougaard, University of Aarhus, Denmark. Seeds were scarified, then surface-sterilised for 30 minutes in sodium hypochlorite (6% v/v available chlorine) with 0.1% Tween80. Seeds were washed 6 times in sterile tap water, imbibed for 18 hours and washed again before sowing on a stack of 10 sterile filter papers soaked in sterile tap water in 90 mm Petri dishes sealed with Nescofilm.

After 7 days germination under a light intensity of 25 μmol $m^{-1}$ $sec^{-1}$ at 25°C, hypocotyls were excised, cut longitudinally and co-cultivated with *Agrobacterium rhizogenes*. Bacteria from 4 day old cultures grown on semi-solid Yeast Mannitol medium (YM, Vincent, 1970) were suspended in liquid YM at $10^8$–$10^9$ bacteria $ml^{-1}$. Hypocotyls plus bacteria were transferred to sterile filter papers soaked in one tenth strength B5 salts (Imperial Laboratories; Gamborg et al, 1968) with vitamins, 5 mM MES and 100 μM acetosyringone and no added sucrose (Handberg and Stougaard, 1992) and co-cultivated in the dark at 20°C for 7 days.

Hypocotyls were transferred to semi-solid half-strength B5 salts with 1.0% agar, 3% (w/v) sucrose, vitamins and 300 mg $l^{-1}$ cefotaxime and to fresh medium every 7 days. After 11 weeks, 2 cm roots were removed from 30 independent lines from each transformation and transferred to separate compartments in repli dishes. After 17 weeks growth, single 5 cm long roots with laterals were transferred to 10 ml half strength B5 liquid medium with 3% (w/v) sucrose and added vitamins in 50 ml flasks sealed with aluminium foil. The cultures were shaken at 120 rpm, at 18°C in the dark. The root cultures were transferred to fresh liquid medium at intervals of 2 weeks.

Replicates were established in 50 ml liquid medium in 250 ml conical flasks sealed with aluminium foil. Tissue for molecular and biochemical analyses was harvested and frozen in liquid nitrogen after 9 days growth.

*Plant growth*

Seeds of *Lotus japonicus* were scarified and surface sterilised as described above. They were then planted in 90 mm diameter pots in vermiculite, inoculated with *Rhizobium loti* NZP 2238 and grown in a growth room with a 16/8 hour 20°C/20°C day/night regime. The plants were watered with a half strength nitrogen free nutrient solution (Ryle et al., 1978).

*DNA manipulations*

Extraction of DNA from *L. japonicus* was performed as described by Handberg and Stougaard (1992). Verification of the presence of the transgene in the hairy root material was obtained by PCR using a primer pair with sequences from the 3' end of the CaMV 35S promoter (5'-CTGATATCTCCACTGAC-3') and from the complementary strand of the 5' end of the NOS terminator (5'-ATTTCTCAACTTGTTTACGT-3'). The PCR reaction was carried out in a 30 µl volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each of all four dNTPs, 0.2 µM of each primer, 0.15 Units of Taq polymerase (Amplitaq, Perkin Elmer Corporation, Norwalk, USA) and 3 µg $ml^{-1}$ of target DNA. The amplification was carried out using a Thermal Cycler (Perkin Elmer 480) with the following program: 95°C for 1 min (hotstart), 94°C 1 min; 55°C 30 sec; 72°C 1 min with 5 sec autoextension for 39 cycles. Aliquots of the PCR reaction were transferred to Nylon membranes (Amersham Life Sciences, UK) after separation by Agarose gel electrophoresis and hybridised at 65°C to a [$^{32}$P]dCTP labelled sucrose synthase cDNA probe. The membranes were washed twice for 20 min with 2xSSC, 0.1% (w/v) SDS, and once with 0.1xSSC, 0.1% SDS at 65°C (1xSSC is 0.15 M NaCl, 0.015 M $Na_3$Citrate, pH 7.5).

*RNA extractions and analysis*

RNA was extracted from plant material using a hot phenol method as described (Skøt et al., 1996), and separated by Agarose/Formaldehyde gel electrophoresis (Sambrook et al., 1989). Northern blot hybridisation was carried out at 42°C after capillary transfer of RNA to Nylon membrane. cDNA probes were labelled with [$^{32}$P]dCTP using the "Prime it" random primer labelling kit (Stratagene, La Jolla, CA, USA). Filters were washed twice for 20 min with 2xSSC, 0.1% (w/v) SDS at 42°C, and once for 20 min with 0.2xSSC, 0.1% SDS at 42°C.

*Extraction and assay of enzymes*

Root samples were homogenised in a mortar and pestle with 50 mM MOPS, pH 7.2, 20 mM KCl, 5 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, at 0–2°C (3 ml $g^{-1}$ fresh weight). The homogenate was centrifuged for 5 min at 20 000 x g, 2°C. Samples (50 µl) of the supernatant were immediately assayed for AGPase activity and 1 ml aliquots were desalted by low speed centrifugation (180 x g, 1 min) through 5 ml columns of Bio Gel P6DG (BioRad) equilibrated with extraction buffer lacking EDTA. The desalted extract was used to assay for the activities of a number of enzymes. Aliquots were precipitated with trichloroacetic acid for later protein determination (Lowry et al., 1951). Sucrose synthase (SS), alkaline invertase (ALK INV) (Gonzalez et al., 1995) and UDPG pyrophosphorylase (UGPase) (Gordon and Kessler, 1990) were assayed as described. The following enzymes were assayed spectrophotometrically in a final volume of 1 ml at 340 nm and 30°C.

AGPase: 50 mM Bicine, pH 8.0, 5 mM $MgCl_2$, 1 mM NAD, 1 mM PPi, 0.01 mM Glu 1,6 bis P and 1 mM ADPGlucose. The coupling enzymes were phosphoglucomutase (PGM) 2 U $ml^{-1}$ and glucose 6-phoshate dehydrogenase (G6PDH) 2 U $ml^{-1}$.

Glucose kinase (GK) and Fructokinase (FK): 50 mM Bicine, pH 8.0, 5 mM $MgCl_2$, 1 mM NAD, 1 mM ATP and 2 mM of either glucose or fructose. The coupling enzymes were phosphoglucose isomerase (PGI) 2 U $ml^{-1}$ and G6PDH 2U $ml^{-1}$.

Acid Invertase (ACID INV) was assayed in a two stage reaction. In step 1, enzyme sample (50 µl) was incubated with 0.25 ml of a reaction mixture composed of 50 mM MES, pH 5.0, 5 mM $MgCl_2$ and 100 mM sucrose at 30°C for 40 min before being stopped by the addition of 1ml 0.1 M imidazole, 5 mM $MgCl_2$ buffer, pH 7.5, and boiling for 2 min. In the second stage, glucose and fructose produced in the first step was quantified in enzyme coupled reactions to produce 6-phosphogluconate and NADH, which was measured by the change in $A_{340}$

after incubation with 0.7 ml 50 mM imidazole, 5 mM $MgCl_2$, pH 7.5, containing 1 mM NAD, 1 mM ATP, 1 U HK, 0.25U PGI and 0.1U G6PDH for 60 min at 30°C.

Acid FK was measured in a two stage reaction. In step 1, enzyme sample (50 µl) was incubated with 0.25 ml of a reaction mixture composed of 50 mM MES, pH 5.0, 5 mM $MgCl_2$, 1 mM ATP and 2 mM fructose at 30°C for 40 min before being stopped by the addition of 1 ml 0.1 M imidazole, 5 mM $MgCl_2$ buffer, pH 7.5, and boiling for 2 min. In the second stage fructose 6-phosphate produced in the first step was quantified in enzyme coupled reactions to produce 6-phosphogluconate and NADH, which was measured by the change in A340 after incubation with 0.7 ml 50 mM imidazole , 5 mM $MgCl_2$, pH 7.5, containing 1 mM NAD, 0.01 mM Glu 1,6 bis P, 0.25 U PGI, 0.25U PGM and 0.1U G6PDH for 60 min at 30°C.

*Western immunoblotting*

Samples of root protein extracts were prepared for SDS-polyacrylamide gel electrophoresis, blotting on to nitrocellulose membranes and treatment with antibodies raised to soybean nodule SS as described in Cresswell et al. (1992) and Gordon et al. (1993). Equal amounts of protein (8 µg) were applied to each track.

## 3. Results

Fig. 1 shows the results of Northern blot hybridisation analyses of RNA from 5 week old untransformed *L. japonicus* plants. No SS message was detected in the leaves. The steady state level of SS mRNA was highest in the nodules, but significant levels of expression were also found in the roots (approximately 30–40% of nodules).

Initially, 30 lines transformed with the chimaeric antisense SS gene, were screened by Northern blot hybridisation analysis for expression of the antisense gene and down-regulation of the indigenous gene. All antisense lines expressed a 0.9 kb message, which is consistent with the size of the antisense SS gene, and expression of the sense SS gene was reduced to varying degrees (data not shown). Five lines with the most intense antisense SS RNA signal, were chosen for further analysis, together with 2 control lines transformed with the wild type *A. rhizogenes* strain.

To further confirm the presence of the transgene, DNA from the selected lines were analyzed by PCR. The primer-pair described in Materials and Methods amplifies any DNA sequence present between the CaMV 35S promoter and the NOS polyA sequence. The size of the PCR product from the antisense SS hairy

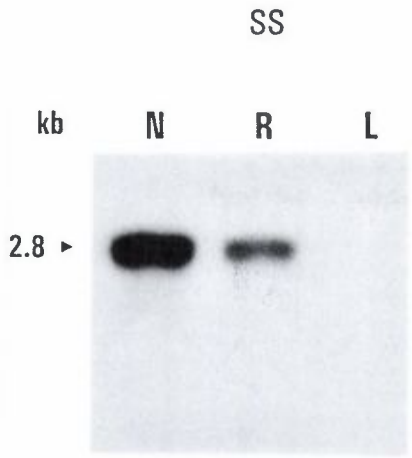


Fig. 1

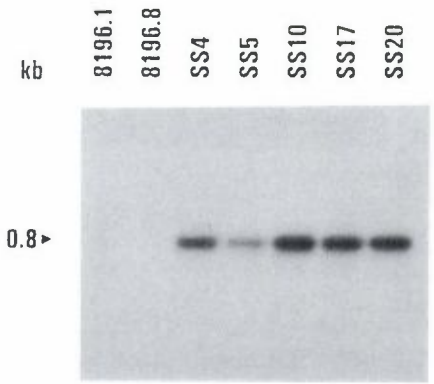


Fig. 2

Figure 1. Northern blot analysis of RNA isolated from nodules (N), roots (R) and leaves (L) of 5 week old *Lotus japonicus* plants, and probed with a sucrose synthase (SS) probe. Approximately 10 μg of RNA was applied to each lane.

Figure 2. Southern blot hybridisation of PCR products resulting from the transgene specific amplification of *Lotus japonicus* DNA. extracted from hairy root cultures. A sucrose synthase cDNA clone was used as probe.

roots was 0.8 kb, which is consistent with the predicted length. The 2 control lines did not contain any CaMV 35S promoter or NOS polyA sequences. Consequently, no PCR product was detected from these lines. Southern analysis clearly showed that the PCR products from the antisense SS lines hybridised with the SS cDNA probe, confirming the presence of this transgene (Fig. 2). It might be argued that the amplified product could have arisen from contaminating *Agrobacterium* in the root culture. If this had been the case, the culture medium would have shown visible signs of bacterial growth after 9 days, so this possibility can be ruled out.

The result of a Northern blot hybridisation analysis of RNA from the 5 selected hairy root lines, using the SS cDNA as a probe, is shown in Fig. 3. It demonstrates that the sense message in the 2 control lines was of the same order of magnitude as in untransformed roots (Figs. 1 and 3). In 4 of the antisense lines the sense SS signal was reduced drastically, and was barely detectable (Fig. 3). In SS20 a visible sense SS signal was present. As the antisense SS gene was smaller than the sense gene, using the SS cDNA as a probe allowed the detection of both the sense and antisense transcripts. A strong message of 0.9 kb

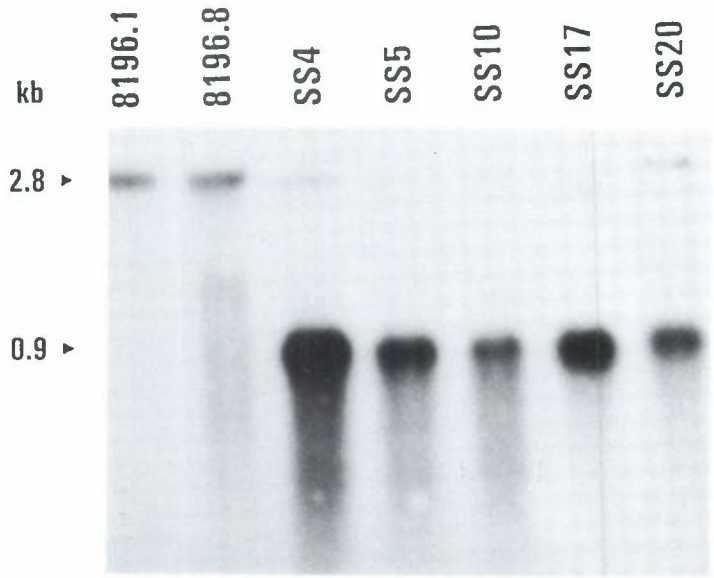


Figure 3. Northern blot hybridisation analysis of RNA from *Lotus japonicus* hairy root cultures using a sucrose synthase cDNA probe. Approximately 10 μg of RNA was applied to each lane. The 2.8 kb fragment represents the SS sense transcript, and the 0.9 kb fragment represents the antisense transcript.


Figure 4. Immunoblot analysis of protein extracts from *Lotus japonicus* hairy root cultures using a sucrose synthase antiserum. Approximately 8 μg of protein was applied to each lane.

was seen in all 5 antisense lines. This is consistent with the predicted size of the antisense transcript. The hairy roots were 9 days old when harvested and thus in the exponential growth phase. Steady state SS message levels were lower in older cultures that had reached the stationary phase after 14 days of growth (data not shown).

Western blot analysis of protein extracts from the hairy root cultures confirm the results of the Northern analysis. Fig. 4 shows that SS protein levels were significantly lower in 4 of the antisense SS cultures compared to the controls, while SS20 had near normal levels. This correlated with the SS sense signal from this line, which was also stronger than in the other antisense lines (Fig. 3).

Activities of enzymes involved in the metabolism of sucrose and its hydrolysis products were measured. The results are summarised in Table 1. In 4 of the antisense lines SS activity was reduced to approximately 20–25% of the controls, while in SS20 it was 50%. UGPase, which catalyses the conversion of UDPGlucose to Glucose-1-P, was not affected by the antisense gene expression. The same was true of glucose kinase, alkaline and acid fructokinase as well as acid invertase (data not shown). In controls and antisense hairy root lines the activity of AGPase was low. There was a suggestion of reduced activity in the antisense lines, but the differences were not significant. Activity of ALK INV in 4 antisense SS lines was reduced to approximately 40–50% of the control lines. ALK INV in SS20 was similar to control levels. For comparison, enzyme activity data for roots of 5 week old untransformed *L. japonicus* plants were included in Table 1. Although enzyme activities in roots probably vary depending on plant age and other factors, for most of the enzymes they were in the same range as the hairy root control lines. The exception was UGPase, the activity of which was about twice that in the hairy root samples.

Table 1. Activity of enzymes in control and antisense SS hairy root lines of *L. japonicus*.

| | SS | AGPase | UGPase | ALK. INV |
|---|---|---|---|---|
| 8196.1 | 63.5 ± 4.6 | 11.0 ± 2.4 | 407.4 ± 30.6 | 35.7 ± 5.6 |
| 8196.8 | 74.0 ± 11.3 | 10.5 ± 3.8 | 399.0 ± 35.0 | 34.3 ± 2.5 |
| SS4 | 14.3 ± 2.2 | 7.8 ± 2.8 | 389.2 ± 35.2 | 11.3 ± 2.0 |
| SS5 | 14.8 ± 3.1 | 5.0 ± 0.1 | 472.4 ± 44.3 | 13.3 ± 3.1 |
| SS10 | 19.0 ± 1.1 | 7.5 ± 0.7 | 428.5 ± 60.4 | 18.1 ± 2.4 |
| SS17 | 17.6 ± 2.4 | 8.3 ± 1.2 | 419.0 ± 76.5 | 12.1 ± 2.3 |
| SS20 | 31.8 ± 0.9 | 5.2 ± 1.2 | 383.0 ± 32.8 | 30.4 ± 4.1 |
| ROOTS | 91.8 ± 7.8 | 8.1 ± 1.4 | 1,060 ± 146 | 47.1 ± 3.5 |

Values are nmoles $min^{-1}$ mg $protein^{-1}$, and represent the mean and standard error of 3 replicate cultures. ROOTS represent the mean and standard error of two replicate root samples (2x10 plants) from 5 week old untransformed plants.

The fresh weights of the hairy roots were also recorded. In the 2 controls they varied between 2.1 and 2.3 g flask$^{-1}$, and in the 5 antisense SS lines values ranged between 0.56 and 0.96 g flask$^{-1}$. Although this suggests an effect of the antisense treatments, caution should be exercised in the interpretation of these data. The *Agrobacterium* strain used as a control is not identical to the one used for the antisense transformations. Secondly, the growth rate of hairy root cultures is dependent on the number of living root tips at the time of subculturing. Although care was taken to minimise this potential source of variation, it can not be ruled out completely.

## 4. Discussion

The high levels of expression of the nodule-enhanced sucrose synthase in the roots of untransformed plants of *L. japonicus* as well as in hairy roots transformed with wild type *Agrobacterium*, distinguishes it from nodule-enhanced SS genes of soybean (Thummler and Verma, 1987) and *Vicia faba* (Küster et al., 1993). It seems reasonable to assume that roots of *L. japonicus* grown in well aerated soil or, in this case, vermiculite are aerobic (John Witty, personal communication). If so, it demonstrates that low concentration of $O_2$ is not required to induce expression of SS in *Lotus japonicus* roots. In contrast Xue et al. (1991) found that SS expression was induced in soybean callus cultures after reducing the $O_2$ concentration to 4% in the environment. It has also been shown that expression of SS genes in other plant species is induced or increased under anoxic conditions (Hesse and Willmitzer, 1996; Ricard et al., 1991; Salanoubat and Belliard, 1989; Springer et al., 1986). Carbohydrate levels can also modulate the expression of SS in potato (Salanoubat and Belliard, 1989) and maize (Koch et al., 1992). Whether SS expression increases in *L. japonicus* roots under anaerobic conditions remains to be seen. Alternatively, it is possible that the root and nodule SS in *L. japonicus* are distinct, but very closely related isoforms, and that the nodule SS is induced by low $O_2$, whereas the root isoform is regulated differently. In other plant species, including maize (Chen and Choury, 1989), rice (Chan et al., 1990) and potato (Salanoubat and Belliard, 1989), a number of different classes of SS genes are present, while in *Vicia faba* only one isoform of SS has been identified (Ross and Davies, 1992). In *L. japonicus* there is evidence from Southern hybridisations to suggest the presence of a small gene family (Skøt et al., 1996). However, the fact that the homology dependent antisense RNA strategy was effective in the hairy roots suggests that the root and nodule SS are identical.

The Northern analysis shows that the antisense SS transgene, consisting of approximately 25% of the full length SS transcript from the 3' end is sufficient

to inhibit sense gene transcription in the hairy roots almost completely. It is also evident that in the antisense SS20 line the reduction in SS message, protein concentration and enzyme activity was less marked compared to the other 4 antisense lines (Figs. 3 and 4; Table 1), but the reason for this is not clear. There was no obvious correlation between the intensity of the antisense RNA signal and the extent of down-regulation of the sense transcript (Fig. 3), so it seems likely that the silencing of the indigenous SS gene is dependent on factors other than copynumber (i.e. dosage) of the transgene.

However, even the high level of inhibition of SS mRNA was not sufficient to eliminate SS protein and enzyme activity (Fig. 4, Table 1). This demonstrates that a very strong inhibition of the sense SS transcript is necessary to eliminate SS enzyme activity completely. A similar result was obtained by Zrenner et al. (1995) with transgenic potato transformed with an antisense SS gene construct. Neither sense nor antisense SS gene transcripts were detectable in tubers from the transgenic plants, but some residual enzyme activity was still present, although it had decreased to between 4 and 44% of wild type levels.

The decrease in SS activity of the antisense SS lines may be expected to have an effect on "downstream" enzymes involved in carbon metabolism. However, neither UGPase nor fructokinase activity were different from the controls. In antisense SS potato tubers Zrenner et al. (1995) also found no change in UGPase protein levels and enzyme activity, although steady state mRNA levels were much lower than in control tubers. The UDPGlucose formed by the SS-catalysed hydrolysis of sucrose can also be used as a precursor in cell wall biosynthesis or, via AGPase, be converted to ADPGlucose which is a precursor in starch biosynthesis. Although the average AGPase activities in the antisense SS lines were lower than in the controls, the differences were not significant. In antisense SS potato tubers a dramatic decrease in AGPase mRNA levels was observed, but no change in enzyme activity was seen (Zrenner et al., 1995).

Since sucrose provides the only significant carbon source for the hairy root cultures, a five fold decrease in SS activity, which was observed in some of the antisense cultures, might be expected to lead to a compensatory increase in other enzymes capable of hydrolysing sucrose. While ACID INV activity did not change significantly from that of the controls, in four of the five antisense SS lines ALK INV had decreased by approximately 50%. This surprising result is difficult to explain. Perhaps the change in SS enzyme activity led to changes in the cytosolic environment which were unfavourable to ALK INV activity. It may even be possible that the ALK INV gene has sufficient homology to the SS gene to be partially "antisensed". At present it is impossible to say whether the inhibition in ALK INV was due to a decrease in transcriptional activity or to post transcriptional regulation. The reduction in SS activity may not have presented the roots with a sufficiently severe limitation in availability of

substrates for glycolysis. Existing levels of invertase activity together with remaining SS activity may have been more than adequate to provide these substrates. The decrease in fresh weight of the antisense hairy root cultures compared to the controls does however, suggest that the inhibition of SS and/or ALK INV activity did limit growth. The ACID INV activity measurements described here do not allow us to establish whether this activity is apoplastic. If so, it implies the presence of hexose uptake systems in the root cells. However, hairy root cultures of *L. corniculatus* do not grow in media where sucrose is replaced by glucose or fructose (Phillip Morris, personal communication). This would suggest a cytoplasmic or vacuolar location of ACID INV.

In conclusion, the work presented here demonstrates that the nodule enhanced sucrose synthase gene in *L. japonicus* differs from related sucrose synthases isolated from other legumes by having substantial levels of expression in the roots as well as in hairy root cultures. Secondly, antisense RNA strategies can be used successfully in *L. japonicus* to bring about significant levels of inhibition of a target gene. How down-regulation of the SS gene affects carbon and nitrogen metabolism in nodules is currently being investigated using transgenic *Lotus* plants containing the antisense SS gene.

**Acknowledgements**

The Institute of Grassland and Environmental Research is grant aided by the Biotechnology and Biological Sciences Research Council. The authors wish to thank Dr. Jens Stougaard, University of Aarhus, Denmark for the *Lotus japonicus* nodule cDNA library and Phillip Morris (IGER) for useful discussions.

REFERENCES

Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research* **12**: 8711–8721.

Chan, H.-Y., Ling, T.-Y., Juang, R.-H., Ting, I.-N., Sung, H.-Y. and Su, J.-C. 1990. Sucrose synthase in rice plants. *Plant Physiology* **94**: 1456–1461.

Chen, Y.C. and Choury, P.S. 1989. Spatial and temporal expression of the two sucrose synthase genes in maize: immunohistological evidence. *Theoretical and Applied Genetics* **78**: 553–559.

Cresswell, A., Gordon, A.J., and Mytton, L.R. 1992. The physiology and biochemistry of cultivar-strain interactions in the white clover-*Rhizobium* symbiosis. *Plant and Soil* **39**: 47–57.

Dessaux, Y., Guyon, P., Farrand, S.K., Petit, A., and Tempé, J. 1986. *Agrobacterium* Ti and Ri plasmids specify enzymic lactonization of mannopine to agropine. *Journal of General Microbiology* **132**: 2549–2559.

Gamborg, O.L., Miller, R.A., and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50**:151-158.

Gonzales, E.M., Gordon, A.J., James, C.L., and Arrese-Igor, C. 1995. The role of sucrose synthase in the response of soybean nodules to drought. *Journal of Experimental Botany* **46**: 1515–1523.

Gordon, A.J. 1992. Carbon metabolism in the legume nodule. In: *Carbon Partitioning*. C.J. Pollock, J.F. Farrar, and A.J. Gordon, eds. BIOS Scientific Publishers, Oxford, pp. 133–162.

Gordon, A.J. and Kessler, W. 1990. Defoliation-induced stress in nodules of white clover. Immunological and enzymic measurements of key proteins. *Journal of Experimental Botany* **41**: 1255–1262.

Gordon, A.J., Minchin, F.R., Skøt, L., and James, C. 1997. Stress-induced declines in soybean $N_2$ fixation are related to sucrose synthase activity. *Plant Physiology*, in press.

Gordon, A.J., Ougham, H.J., and James, C.L. 1993. Changes in levels of gene transcripts and their corresponding proteins in nodules of soybean plants subjected to dark-induced stress. *Journal of Experimental Botany* **44**: 1453–1460.

Handberg, K. and Stougaard, J. 1992. *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. *Plant Journal* **2**: 487–496.

Hesse, H. and Willmitzer, L. 1996. Expression analysis of a sucrose synthase gene from sugar beet (*Beta vulgaris* L.). *Plant Molecular Biology* **30**: 863–872.

Koch, K.E., Nolte, K.D., Duke, E.R., McCarty, D.R., and Avigne, W.T. 1992. Sugar levels modulate differential expression of maize sucrose synthase genes. *Plant Cell* **4**: 59–69.

Küster, H., Frühling, M., Perlick, A., and Pühler, A. 1993. The sucrose synthase gene is predominantly expressed in the root nodule tissue of *Vicia faba*. *Molecular Plant-Microbe Interactions* **6**: 507–514.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**: 265–275.

Plaxton, W.C. 1996. The organization and regulation of plant glycolysis. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**: 185–214.

Ricard, B., Rivoal, J., Spiteri, A., and Pradet, A. 1991. Anaerobic stress induced the transcription and translation of sucrose synthase in rice. *Plant Physiology* **95**: 669–674.

Ross, H.A. and Davies, H.V. 1992. Purification and characterization of sucrose synthase from the cotyledons of *Vicia faba* L. *Plant Physiology* **100**: 1008–1013.

Ryle, G.J.A., Powell, C.E., and Gordon, A.J. 1978. Effect of source of nitrogen on the growth of Fiskeby soybean: the carbon economy of the whole plant. *Annals of Botany* **42**: 637–648.

Salanoubat, M. and Belliard, G. 1989. The steady-state level of potato sucrose synthase mRNA is dependent on wounding, anaerobiosis and sucrose concentration. *Gene* **84** : 181–185.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, New York.

Skøt, L., Hirsch, P.R., and Witty, J.F. 1986. Genetic factors in *Rhizobium* affecting the symbiotic carbon costs of $N_2$ fixation and host plant biomass production. *Journal of Applied Bacteriology* **61**: 239–246.

Skøt, L., Minchin, F.R., Timms, E., Fortune, M.T., Webb, K.J., and Gordon, A.J. 1996. Analysis of the two nodulins, sucrose synthase and ENOD2, in transgenic *Lotus* plants. *Plant and Soil* **186**: 99–106.

Springer, B., Werr, W., Starlinger, P., Bennett, D.C., Zokolica, M., and Freeling, M. 1986. The shrunken gene on chromosome 9 of *Zea mays* L. is expressed in various plant tissues and encodes an anaerobic protein. *Molecular and General Genetics* **205**: 461–468.

Stitt, M. and Sonnewald, U. 1995. Regulation of metabolism in transgenic plants. *Annual Review of Plant Physiology Plant Molecular Biology* **46**: 341–368.

Thummler, F. and Verma, D.P.S. 1987. Nodulin-100 of soybean is the subunit of sucrose synthase regulated by the availability of free heme in nodules. *Journal of Biological Chemistry* **262**: 14730–14736.

Vincent, J.M. 1970. *A Manual for the Practical Study of Root-Nodule Bacteria*. Blackwell Scientific Publications, Oxford.

Witty, J.F., Minchin, F.R., Skøt, L., and Sheehy, J.E. 1986. Nitrogen fixation and oxygen in legume root nodules. *Oxford Surveys of Plant Molecular and Cell Biology* **3**: 275–314.

Xue, Z.-t., Larsen, K., and Jochimsen, B.U. 1991. Oxygen regulation of uricase and sucrose synthase in soybean callus tissue is exerted at the mRNA level. *Plant Molecular Biology* **16**: 899–906.

Zrenner, R., Salanoubat, M., Willmitzer, L., and Sonnewald, U. 1995. Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). *Plant Journal* **7**: 97–107.