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SPATIAL EXPRESSION OF SUCROSE SYNTHASE GENE IN *LOTUS JAPONICUS* ROOT NODULE DEVELOPMENT

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Key words: *Lotus japonicus*, in situ hybridization, sucrose synthase

Abstract: In situ hybridization was performed in order to assess the location of sucrose synthase transcripts in developing Lotus japonicus nodules. Sucrose synthase transcripts were present at high levels in all cell types of young nodules, whereas in mature nodules were detected in vascular bundles and inner cortex

INTRODUCTION

Lotus japonicus is a temperate legume that has been proposed as a model for the study of nitrogen metabolism in this group of plants due to its biological characteristics, among them its small genome size (400 Mb) and its amenability for Agrobacterium transformation (Handberg and Stougaard, 1992). The cloning of genes involved in nitrogen assimilation is the first step in the understanding and manipulation of this process.

Sucrose plays a central role in plant growth and development. It is a major end product of photosynthesis and function as a primary transport sugar and in some cases as a direct or indirect regulator of gene expression (Farrar et al., 2000; Winter and Huber, 2000).

Legume nodules are primarily dependent on the import and metabolism of sucrose to provide the energy and carbon skeletons for biological nitrogen fixation, the assimilation of ammonia and the export of nitrogenous fixation products. Sucrose is synthesized in the leaves and exported in the phloem to sinks such as the nodules. Once unloaded in the nodule cortex, sucrose must diffuse into the infected region of the nodule to be metabolized. The products of sucrose catabolism (usually malic acid) (Udvardi and Day, 1997) are then used by bacteroids to fuel the fixation of nitrogen (Vance and Heichel, 1991; Gordon, 1995). Ammonia is exported from 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.

Sucrose arrives at the developing nodule through the nodular vascular system and is translocated apoplastically and/ or symplastically into the cells, where it is hydrolyzed predominantly by sucrose synthase (Day , DA and Copeland, L., 1991)

THE INVESTIGATIONS AIM

We have examined the spatial accumulation of gene transcript coding for the enzyme involved in sucrose breakdown (sucrose synthase). The data obtained suggest that the sites of sucrose breakdown may vary according to the stages of nodule development.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Lotus japonicus (Cultivar Gifu B-129) seeds were kindly provided by Dr. Jens Stougaard (University of Aarhus, Denmark). The plants were grown in a controlled environment with a 18-h-day / 6h-night cycle, a 22□C day / 18□C night regime and 70% humidity (Handberg and Stougaard, 1992). Prior to germination, seeds were soaked for 5 min with H_2SO_4 and then sterilized for 20 min in a solution containing 2% NaOCl- 0.02% Tween 20. Seeds were pregerminated at 18□C in the dark for 72 h and the small plants were grown in Holland nutrient solution. For the inoculation with rhizobia, 72h seedlings were inoculated with a 0.1 OD_{600} suspension culture of Mesorhizobium loti (strain E1R.pMP2112) and the plants were grown in nitrogen-free BXD nutrient solution (Broughton and Dilworth, 1971). The day of inoculation was considered day 0.

IN SITU HYBRIDIZATION

Lotus japonicus nodules harvested at 15, 21 and 28 d.p.i. (days post inoculation) with Mesorhizobium loti (strain E1R.pMP2112) were fixed in 4% paraformaldehyde supplemented with 0,25% glutaraldehyde in 10mM phosphate buffer (pH = 7) for 4 h in a vacuum aspirator (Flemetakis et al., 2002). Fixed tissues were dehydrated through ethanol series , embedded in paraffin and 7-10 □m sections were cut (van de Wiel et al., 1990). Nodules were block-stained with 0,5% safranin.

Antisense and sense RNA probes were obtained from pBlueScript SK+ plasmid vector, by in vitro transcription with T3 and T7 RNA polymerase (Promega). The RNA was labeled by non-radioactive method, using DIG- 11-rUTP (Boehringer Mannheim, Germany). The probes were partially degraded to an average length of 150 nucleotides. Sections (7 to 10 □m) were prepared for hybridization according to Scheres et al (1988) and were put to hybridize overnight at 42□C in 50% formamide, 300mM NaCl, 10 mM Tris –HCl, 1 mM EDTA, 0.02% Ficoll, 0.02% polivinylpyrrolidone, 0.025% bovine serum albumine (BSA), 10% dextran sulfate, 60 mM dithiothreitol (DTT), 500 □g of poly (A) RNA per ml and 150 □g of yeast tRNA per ml. The label was detected using anti-digoxigenin antibodies. To visualize probe hybridization, the antibodies were conjugated with alkaline phosphatase. The location of alkaline phosphatase- conjugated antibody was visualized by incubation with BCIP NBT (5-bromo-4chloro- 3-inolylphosphate / nitroblue tetrazolium) substrate, generating a purple-blue precipitate. The signal detection was performed as described by Papadopoulou et al. 1996.

RESULTS AND DISCUSSIONS

Since sucrose is the main carbon source for nodule maintenance and function, the nodular sites of sucrose synthase gene expression are expected to provide information on the distribution of activities of the sucrose synthase in developing nodules.

In young nodules (15 d.p.i.), high levels of sucrose synthase transcripts were found in the vascular bundle as well as in the part of the vascular bundle connecting the nodule lobe to the root. High levels of expression were also observed in parenchimateous cells, particularly in those around the central tissue and the vascular bundles. No signal was observed in the cells of the outer cortex (Figure 1).

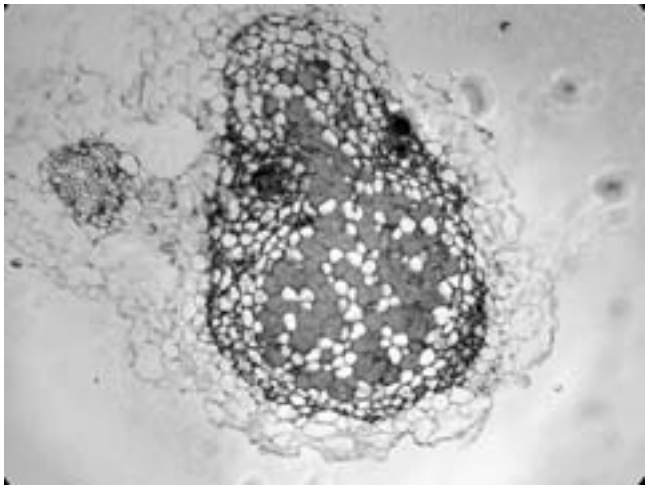

Figure 1. In situ localization Lj sucrose synthase gene transcript in *Lotus japonicus* root nodules. Transverse 7 µm thin sections of nodules at 14 days post-infection with *Mesorhizobium loti*

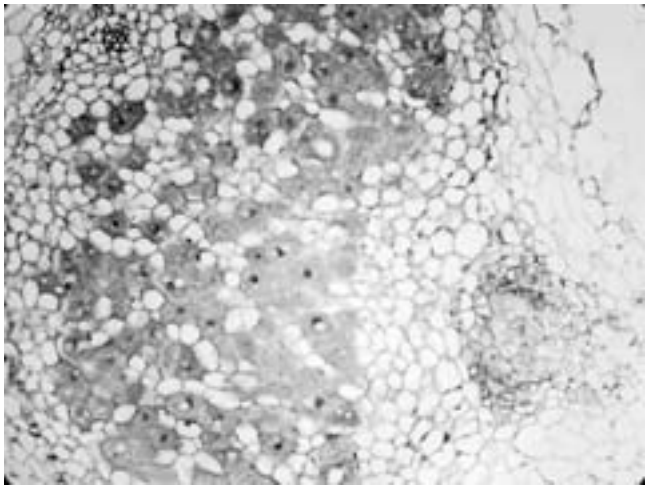

Figure 2. In situ localization Lj sucrose synthase gene transcript in *Lotus japonicus* root nodules. Transverse 7 µm thin sections of nodules at 21 days post-infection with *Mesorhizobium loti*

At nodules of 21 d.p.i., the hybridization signal was detected in vascular bundles and also, in all the cells of the central tissue (Figure 2).

In mature nodules (28 d.p.i.) was registered a strong hybridization signal in the cells of parenchyma around central tissue and also in vascular bundles. It is not clear whether the signal can be seen in uninfected cells. The level of sucrose synthase transcript was low comparing with that in young nodules and lower in the central tissue than in parenchyma (Figure3).

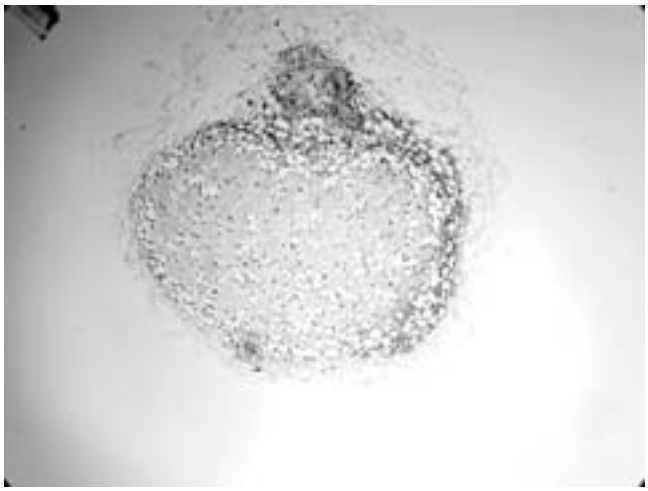

Figure 3. In situ localization Lj sucrose synthase gene transcript in *Lotus japonicus* root nodules. Transverse 7 μm thin sections of nodules at 28 days post-infection with *Mesorhizobium loti*

In legume nodules, carbon skeletons derived from the breakdown of sucrose are used in numerous metabolic processes including amino acid production, plant and bacterial respiration and the biosynthesis of starch and cellulose. Thus, the sites of sucrose breakdown and the fate of the breakdown products are expected to be dictated, in each developmental stage, by the level of various enzymes and this is expected to be a reflection of the corresponding mRNA levels, including sucrose synthase mRNAs.

The data showed that the level of sucrose synthase transcripts was high in young nodules while it is reduced in mature nodules, with the decline being more visible in the infected cells of the central tissue. These data corroborated earlier work that demonstrated that sucrose synthase activity increased in developing nodules and then decreased during the course of nodule maturation (Anthon and Emmerich, 1990; Thummler and Verma, 1987). However, in situ hybridization studies on the localization of sucrose synthase transcripts in Phaseolus vulgaris nodules have shown that the level of transcripts is much higher in mature than in young nodules (van Ghelue et al., 1996),

suggesting that sucrose metabolism in soybean and common bean might differ in the patterns of sucrose synthase expression.

The data demonstrate that in Lotus japonicus nodules, the distribution and concentration of the sucrose synthase transcripts in various cell types and at various developmental stages differ.

These indications are further support for the idea that, in mature nodules, sucrose breakdown is taking place mainly in cells other than those located in the central tissue and that carbon compounds needed for malate production are mobilized symplastically from other nodular cell types than the infected cells.

The nodule vascular system and in particular the pericycle cells appear to be the site of high expression of genes coding for enzymes involved in carbon and nitrogen metabolism (Charrier et al., 1998).

Imunological and RNA blot analyses of several legumes indicated that sucrose synthase gene expression is enhanced during effective nodule development while it remains low in ineffective nodule development (Anthon, GE and Emmerich, DW, 1990; Vance et al., 1997).

Accumulation of sucrose synthase gene transcripts was detected in infected and uninfected cells of the symbiotic zone in indeterminate nodules (de la Pena et al., 1997) and the central tissues as well as the vascular system of determinate nodules (van Ghelue et al., 1996). An exception are the older nodules, where expression of the sucrose synthase gene appears to be restricted to the infected cells and to the pericycle of the vascular bundles (van Ghelue et al., 1996).

CONCLUSIONS

By using in situ hybridization technique, we showed that irrespective of the developmental stage, the level of sucrose synthase mRNA transcripts in the vascular bundle of developing Lotus japonicus root nodules is high.

The strong expression of sucrose synthase gene in the vascular system of mature nodules indicates that in nitrogen-fixing nodules the pericycle cells of the nodular vascular system are likely to be very active in metabolizing sucrose to oxaloacetate and thus providing substrates for energy production. This energy demand is expected to be higher in the vascular bundles of mature nodules since they have to simultaneously import sucrose and export ureides and/ or amides.

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