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Short Communication

Carbohydrate Pools in Nodules of «Nonnodulating» and «Supernodulating» Soybean (*Glycine max* L. Merr. cv. Bragg) Mutants

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Summary

Nodulation mutants of soybean (*G. max* L. Merr.) forming less (nod 49, nod 139) or more (nts 382, nts 1116) nodules than the corresponding wildtype (cv. Bragg) were infected with *Bradyrhizobium japonicum* 61-A-101 and grown in sterilized Leonard jars under optimal phytotron conditions. After 1 month, the nodules were harvested and their carbohydrate pools were analyzed. The pools of pinitol, sucrose and starch were greatly increased in the nodules of the weakly nodulating mutants nod 49 and nod 139 as compared with the wildtype. The amount of the disaccharid trehalose, produced by the microsymbiont, was more than 50% lower in nodules from both weakly nodulating and supernodulating plants in comparison to the wildtype. The activity of trehalase, an enzyme stimulated in nodules, was about 60% lower in nodules of the supernodulating mutant nts 382 than in nodules of the isogenic wildtype.

Key words: Autoregulation, carbohydrate metabolism, nodulation, nodules, soybean, symbiosis, trehalase.

Abbreviations: ARA = acetylene reduction activity (assay for nitrogenase); dw = dry weight; fw = fresh weight; MES = 2-(N-morpholino) ethane-sulfonic acid; SE = standard error.

Introduction

In soybean and other legumes, mutants are known with altered developmental patterns of nodulation (Gresshoff and Caetano-Anollès, 1992). In soybean, «nonnodulating» and «supernodulating» plants are known for nearly a decade. The «nonnodulating» nod mutants of the soybean cultivar Bragg form only a very small number of nodules per plant even under growth conditions favouring nodulation (Carrol et al., 1986). The nitrate tolerant or supernodulating mutants (nts), in contrast, form nodules in much higher numbers than wildtype plants, and they do so even under conditions where nodulation is repressed in wildtype plants, e.g. if nitrate is present in high amounts in the culture medium (Carrol et al., 1985). These mutants appear to be defective in genes controlling the feed-back inhibition of nodule initiation both by the presence of nitrate in the soil and by the activity of already established nodules. Therefore, they are referred to as «autoregulation mutants» (Carrol and Mathews, 1989). Grafting experiments have shown that this inhibition seems to require a factor synthesized or modified by the shoot (Gresshoff and Caetano-Anollés, 1992).

It is interesting to investigate whether aspects of metabolism of mature nodules are also affected by these autoregulation mutations. Yet, only little is known about the physiology of the nodules of these mutants (Hansen et al., 1992). Here, we present data about the carbohydrate pools in nodules of «nonnodulating» and «supernodulating» soybean mutants (*G. max* L. Merr. cv. Bragg).

Material and Methods

Plant material

Seeds of soybean (*G. max* L. Merr. cv. Bragg) wildtype, «nonnodulating» (nod 49, nod 139), and «supernodulating» (nts 382, nts 1116) mutants were kindly provided by P. Gresshoff (Knoxville, TN). The seeds were surface-sterilized by immersion in 30% H_2O_2

for 20 min followed by washing with sterile tap-water. They were grown for 3 days at 27 °C in the dark on plates containing 1 % agar in half-concentrated nutrient solution (Werner et al., 1975). Then, the seedlings were grown in sterilized Leonard jars filled with perlite and nutrient solution (Staehelin et al., 1992) amended with 1 mM KNO_3 in a phytotron (14 h day at 300 $\mu E\, m^{-2} s^{-1}$ and 26 °C, 10 h night at 20 °C, 60 % rH). About 1 week after planting, the plantlets were infected with *Bradyrhizobium japonicum* 61-A-101, a strain yielding effective, nitrogen-fixing nodules (Stripf and Werner, 1978). The bacteria had been grown to stationary phase in 20E-medium (Werner et al., 1975). Nodules were harvested one month after infection, used fresh or immediately frozen and lyophilized or stored at −20 °C.

Carbohydrate analysis

Carbohydrates were analyzed as described previously (Müller et al., 1994). Briefly, nodules (about 10 mg dw) were chilled and lyophilized immediately after harvesting. The soluble carbohydrates were extracted by grinding the nodules in methanol (80 % [v/v]; 50 mL g^{-1} dw) containing 1 % insoluble polyvinylpyrrolidone and mannoheptulose (50 g/sample) as internal standard. The homogenized samples were incubated at 60 °C during 10 min followed by centrifugation (13,000 × g, 10 min). The extraction was repeated three times, and the supernatants were collected and vacuum-dried. After resuspending the pellets in 0.6 mL distilled water, charged compounds were removed using a mixed-bed ion exchanger (Serdolit micro blue and red 2 : 1 (v/v), SERVA, Heidelberg, Germany). 50 µL of the wet mixture were added to the samples. After vortexing and centrifugation of the samples, the supernatants were lyophilized. After redissolving and transferring the pellets to gas chromatography vials, silylation and gas chromatography of the silylized carbohydrates was performed as described (Müller et al., 1994 a).

After removing the soluble carbohydrates, starch in the lyophilized nodules was solubilized with dimethylsulfoxide and quantified after digestion with glucoamylase (Boehringer, Mannheim, Germany) by measuring the glucose released with the glucose-peroxidase method using a test kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions (Müller et al., 1994 b).

Enzyme assays and protein determination

Crude enzyme extracts were obtained by grinding frozen soybean nodules or uninfected roots in ice-cold 0.1 M MES (K^+), pH 6.3 (2 mL per g fw nodules or 0.5 mL per g fw of roots) containing 1 mM phenylmethyl-sulfonylfluoride, 2 mM ethylenediaminetetraacetic acid and insoluble polyvinylpyrrolidone (10 mg per g fw). The homogenate was centrifuged (13,000 g, 10 min). The supernatant was used for the enzyme activity assays.

Trehalase activity was measured by estimating the glucose produced by hydrolysis of trehalose (Müller et al., 1992). The reaction mixture contained 10 mM trehalose and 50 mM MES (K^+), pH 6.3, and was incubated at 37 °C for 30 min. The reaction was stopped by boiling for 2 min and the glucose released was measured with the glucose oxidase-peroxidase-test kit (Boehringer, Mannheim, Germany).

Nitrogenase activity was quantified as acetylene reduction activity as described earlier (Turner and Gibson, 1980). Nodulated roots were put into a flask of known volume (600 mL) containing an atmosphere of 10 % v/v acetylene. Gas samples (1 mL) were taken at different time points (up to 30 min) and their ethylene content was assayed by gas chromatography. The ARA was expressed in terms of nodule fresh weight, determined by harvesting and weighing all nodules at the end of the experiment.

Results and Discussion

Under the favourable growth conditions used in the present experiments, the nodule biomass on wildtype plants was nearly as high as on supernodulating mutants. The mutants described as «nonnodulating» under less optimal conditions also produced a small amount of nodules (Table 1). The specific nitrogenase activity in nodules of the autoregulation mutants nod 49, nod 139 and nts 382 was lower than in wildtype nodules (Table 1). The nodules of nts 1116, a supernodulating mutant with an intermediate phenotype (Carrol and Mathews, 1989) had a wildtype-like nitrogenase activity.

Apparently, none of the mutant nodules were subjected to a lower carbohydrate supply. The sucrose pool was even higher in the two «nonnodulating» mutants and in nts 382 than in the wildtype nodules (Fig. 1 A). The amount of starch was more than three times higher in the nodules of «nonnodulating» mutants than in the wildtype and supernodulating plants (Fig. 1 B). This indicates that the suppression of nodulation in the nod mutants is not due to an altered assimilate allocation to the nodules. In the nodules of nts 382 and nts 1116, the amount of starch was slightly lower than in the wildtype nodules. This may be due to a higher respiration activity observed in nodules of this kind (Hansen et al., 1992). Whereas the inositol content was lowered in nodules of all mutants, the nodules of the weakly nodulating mutants nod 49 and nod 139 contained about 5 times more pinitol than wildtype nodules (Fig. 1 C). The role of pinitol in nodules is unknown, but recent data about pinitol in leaves of some legumes suggest that it may be involved in osmoprotection (Keller et al., 1993). Interestingly, we found that in nodules colonized by some ineffective strains (Müller et al., 1994 a) and in nodules grown in the presence of inhibitory amounts of nitrate (Müller et al., 1994 b) both pinitol and sucrose were increased.

In nodules of all autoregulation mutants, the amount of the bacterial carbohydrate trehalose was reduced to 50 % or less of the wildtype level (Fig. 1 D). These data suggest that the assimilate allocation between the plant and the bacteroids may be altered inside the nodule. This is similar to our observations with nodules exposed to high amounts of nitrate (Müller et al., 1994 b).

Table 1: Nodulation parameters of soybean (*G. max* L. Merr.) «nonnodulating» (nod 49, nod 139) and «supernodulating» (nts 382, nts 1116) mutants and their corresponding wildtype (cv. Bragg). Nodules were harvested 1 month after infecting the plants with *B. japonicum* 61-A-101. Each Leonard jar contained three plants. Mean values and SE are given for three independent replicates.

Soybean line	Nodule number per jar	Nodule biomass per jar (mg fw)	ARA (nkat [g fw]$^{-1}$)
Bragg wt	185±37	2443±275	0.86±0.09
nod 49	15± 4	226± 82	0.48±0.05
nod 139	12± 3	112± 17	0.60±0.07
nts 382	>1000e	2701±368	0.48±0.04
nts 1116	>>200e	2732±276	0.82±0.10

e, estimated.

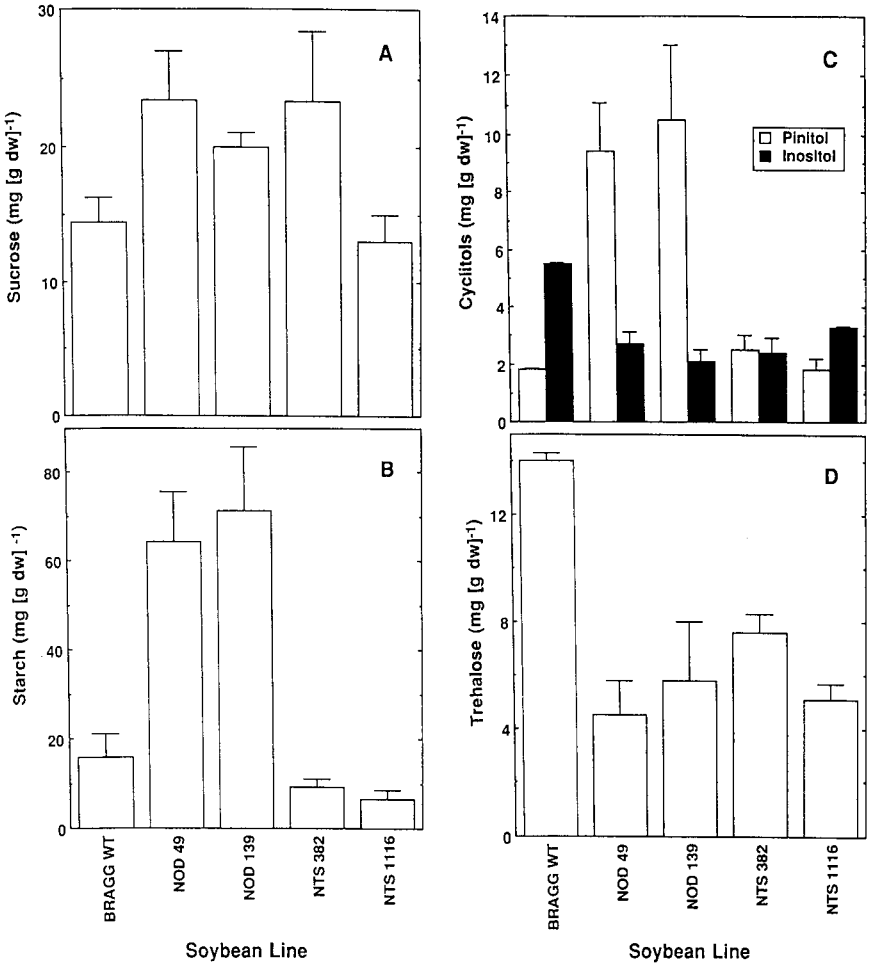

Fig. 1: Main carbohydrate pools in nodules from wildtype soybean (*G. max* L. Merr. cv. Bragg) and in the autoregulation mutants (nod 49, nod 139, nts 382, nts 1116. Nodules were harvested 1 month after infecting the plants with *B. japonicum* 61-A-101. Mean values and SE are given for three independent replicates. Note the different scales of abscisses. A, sucrose; B, starch; C, cyclitols; D, trehalose.

Table 2: Trehalase activity (pH 6.3, 37 °C) in nodules and uninfected, 4 days old roots of wildtype soybean (*G. max* L. Merr. cv. Bragg) and in the «nonnodulating» (nod 49, nod 139) and «supernodulating» (nts 382, nts 1116) mutants. Nodules were harvested 1 month after infecting the plants with *B. japonicum* 61-A-101. Mean values and SE are given for three independent replicates.

Soybean line	Trehalase activity (nkat [g fw]$^{-1}$)	
	Nodules	Sterile roots
Bragg wt	12.6±0.5	0.18±0.04
nod 49	11.4±0.3	0.12±0.01
nod 139	11.3±0.4	0.23±0.03
nts 382	5.0±0.8	0.18±0.03
nts 1116	11.3±0.9	0.21±0.03

Interestingly, the activity of the nodule stimulated enzyme trehalase was altered only in the nodules of nts 382 (Table 2). All other nodules had activity levels of about 10–12 nkat (g fw)$^{-1}$, values which were also found in other soybean nodules (Müller et al., 1994 a).

In conclusion, our data show that the mutations of autoregulation do not only affect the initiation of nodules in soybean but also have some consequences on metabolism in established nodules. They lead to a change in carbohydrate transfer from the plant to the microsymbiont, although the assimilate supply from the shoot to the nodules does not appear to be reduced.

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