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Communication

Regulation of the Soybean-*Rhizobium* Nodule Symbiosis by Shoot and Root Factors[1]

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ABSTRACT

The availability of soybean mutants with altered symbiotic properties allowed an investigation of the shoot or root control of the relevant phenotype. By means of grafts between these mutants and wild-type plants (cultivar Bragg and Williams), we demonstrated that supernodulation as well as hypernodulation (nitrate tolerance in nodulation and lack of autoregulation) is shoot controlled in two mutants (nts382 and nts1116) belonging most likely to two separate complementation groups. The supernodulation phenotype was expressed on roots of the parent cultivar Bragg as well as the roots of cultivar Williams. Likewise it was shown that non-nodulation (resistance to *Bradyrhizobium*) is root controlled in mutant nod49. The shoot control of nodule initiation is epistatically suppressed by the non-nodulation, root-expressed mutation. These findings suggest that different plant organs can influence the expression of the nodulation phenotype.

The development of N-fixing nodules on legume roots upon invasion of *Rhizobium* (or *Bradyrhizobium*) bacteria is subject to regulation by factors both external and internal to the plant host. In particular, the extent of nodulation is restricted by a process termed autoregulation, in which the formation of nodules on one part of the root systemically inhibits subsequent nodule formation in other root regions (3, 15). Nodulation is also severely restricted by the presence of nitrate in the soil (7). Our laboratory has recently isolated several soybean mutants with altered symbiotic features, including some tolerant to nitrate (nts[3]) which also supernodulate (3, 4), and others which do not form any nodules (nod$^-$) (1, 8).

Clearly nodulation is subject to control by plant factors; the sites of this control are unknown, although some experimentation has implicated shoot-root interactions (11–13). By means of grafts between these mutant and wild-type plants, we show here that supernodulation in soybean is controlled in two separate mutants by the shoot and that the genotype of the root controlled the non-nodulation phenotype of the plant. These findings will facilitate further biochemical and molecular analysis of these mutants at a tissue-specific level. Crosses made between these mutants and the wild-type parental cultivar Bragg indicated that the altered genes in the nts mutants were different between the two nts mutants used and that one, from nts1116, is at least a partial dominant, whereas the other, from nts382, is a recessive (8).

MATERIALS AND METHODS

Plant Material. The isolation and preliminary characterization of the mutants was described by Carroll *et al.* (3, 4). This study used *Glycine max* cultivar Bragg (wild-type, nod$^+$, fix$^+$), and the derived mutants nts382 (nitrate tolerant, supernodulating, nod^{+++}, fix$^+$), nts1116 (nitrate tolerant, but only hypernodulating, nod^{++}, fix$^+$) and nod49 (non-nodulating with normal inoculant doses (approximately 10^7 bacterial per plant) of *Bradyrhizobium japonicum* strain USDA110 or CB1809, allelic to rj_1, Table II). Genetic analysis of all three mutants has shown that the lines have bred true for a minimum of 5 generations. Preliminary analysis suggests that nts382 and nts1116 are in separate complementation groups. Mutant lines were selected from M2 families derived from ethyl methanesulfonate-treated M1 seeds.

Growth Conditions and Nodulation Tests. Plants used for Table I were inoculated with *B. japonicum* strain USDA110 (approximately 10^7 bacteria per plant) and were cultured in 25 cm pots filled with vermiculite:sand mixture (1:2 ratio). Glasshouse temperatures were held between 14 and 30°C and incandescent 100 W bulbs extended the photoperiod to 16 h near summer conditions). The pots received 1.2 L of nutrient solution as described by Herridge (10) three times a week (Table I). The nutrient solution was either N-free (nitrate absent) or was supplemented with 5 mM KNO_3 (nitrate present). Plants were harvested 45 d after planting and nitrogenase activity was determined as described by Carroll *et al.* (3).

Grafted plants used for Tables III and IV were also grown under glasshouse conditions of 2 per pot in a 3:1 mixture of sterilized sand and vermiculite. Data are thus compatible with those of Table II. Grafting occurred 10 d after sowing using a wedge-shaped graft with the cotyledons left on the scion. Grafts were held in place by a polythene sleeve covering the whole graft union and plants were placed immediately after grafting under an intermittent, automatic misting system for 10 d to prevent desiccation before the grafts had functionally rejoined. Plants were then transferred to a glasshouse, inoculated with *B. japonicum* strain USDA110 as a slurry of bacteria, peat and water (at approximately 10^7–10^8 bacteria per plant). Watering was daily with a complete nutrient solution containing 7.5 mM KNO_3 to run off. Plants were harvested 9 weeks after sowing, nodules were picked and counted from each plant and dry weights obtained after material was oven dried. Plants for Table V were grown as

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[3] Abbreviation: nts, nitrate tolerant symbiosis.

Table I. *Herridge's Nutrient Solution (Full-Strength)*

Composition of Herridge's nutrient medium, as developed in Ref. 15 and referred to in Ref. 1. Formulation is included here for improved accessibility.

Chemical[a]	Final Concentration
	mg L^{-1}
KH_2PO_4[b]	17.0
K_2HPO_4	21.8
KCl[b]	18.7
$MgSO_4 \cdot 7H_2O$[b]	123.3
$CaCl_2$	27.7
Ferric monosodium salt of EDTA[c]	8.7
H_3BO_3[d]	71.5×10^{-2}
$MnCl_2 \cdot 4H_2O$[d]	45.3×10^{-2}
$ZnCl_2$[d]	2.8×10^{-2}
$CuCl_2 \cdot 2H_2O$[d]	1.3×10^{-2}
$NaMoO_4 \cdot 2H_2O$[d]	0.6×10^{-2}

[a] Chemicals were prepared as stock solutions and diluted in tap water. [b] Administered from 1 M stock solutions. [c] Administered from a 4000 times stock solution. [d] These chemicals were collectively prepared in a 4000 times stock solution.

those for Tables III and IV, except that 3 to 5 mM nitrate was added, autumn/winter growth conditions were present and harvesting was at 6 weeks after sowing. Individual experimental sets as grouped by the tables are thus internally controlled, but may vary between sets due to different harvesting times, nitrate supplementation concentration and general growth season.

RESULTS AND DISCUSSION

The symbiotic features of the three soybean mutants isolated from cultivar Bragg are summarized in Table I. Of the two nts mutants, isolate nts382 is a supernodulator with many times more nodules than the wild type and with more nodules when grown in the presence than in the absence of nitrate. In contrast mutant nts1116 is only hypernodulated and remains somewhat sensitive to nitrate, although less so than Bragg wild type. Both mutants have higher nitrogen fixation capacities as judged by acetylene reduction activity (Table II) and higher organic nitrogen content (5) than Bragg. The increased nodulation in the absence of nitrate suggests a mutational alteration of the autoregulation system and indicates that nitrate sensitivity and autoregulation of nodule development involve closely related processes.

Mutant nod49 is a symbiotically defective mutant which does not form nodules in soil with normal inoculant doses (up to 10^9 bacteria per plant) of any *Rhizobium* or *Bradyrhizobium* strain tested so far. Neither mutant nod49 nor the nts mutants are defective in uptake or utilization of nitrate (1, 3).

Experiments in which shoots of one soybean line were grafted onto root stocks of another were carried out to determine whether control of the nts or nod$^-$ phenotypes resided in the shoot or the root. In the experiment summarized in Tables III and IV, mutants nts382 and nts1116 were grafted with two commercially available wild-type cultivars, Bragg and Williams. In every case, the wild-type shoots gave 'normal' (wild-type) nodulation patterns on roots, with nodule number per plant ranging from 12 to 19 (note that these nodule numbers and corresponding masses were low due to the application of high nitrate levels). Shoots from mutant nts382 induced supernodulation on all root stocks including on roots of nts1116. Shoots from mutant nts1116 (a hypernodulator) resulted in intermediate numbers of nodules on all root stocks including those from the supernodulator nts382. Separate measurements confirmed that the nodules on grafted root stocks were active in N fixation (*cf.* Ref. 8).

These results clearly demonstrated that the extent of nodulation on the roots was strictly controlled by the shoots. The reciprocal effects of mutants nts1116 and nts382 was further supported by the conclusion reached from genetic studies that two complementation groups (possibly genes) at least are involved in the regulation of nodule development in soybean (8).

We have available an additional 10 nts mutants derived by similar chemical mutagenesis and genetic and developmental studies may indicate further complexity. The ability of shoots from nts mutants derived from cultivar Bragg to induce supernodulation or hypernodulation appropriately on another cultivar (Williams) suggests that a common shoot factor is involved in nodulation control of all soybean cultivars. Cultivar Bragg and Williams basically differ in their maturity group ranking and the fact that cultivar Bragg is a determinate variety (vegetative tip growth ceases upon flowering), whereas Williams is indeterminate (no cessation of growth) may indicate that they represent two major classes of commerically used soybeans.

Split root experiments by Kosslak and Bohlool (11) have shown that the autoregulation phenomenon involves a root response which triggers a transmitted signal which results in an inhibition of nodulation (but not infection) on one side of the root system by developing (but not yet N fixing) nodules on the other side. Since the nts mutants discussed here display a normal autoregulatory response when grafted to wild-type shoots (Tables III and IV), the root factors involved in such a response must be unaltered. Rather it seems that the shoots of mutants nts382 and nts1116 respond differently to root signals generated after bacterial infection and early nodule development. Whether the mutant shoot response involves failure to translocate an inhibitory signal or the elicitation of a positively acting factor cannot yet be judged. Nor have we, as yet, ascertained the source of the shoot response (although cotyledon removal did not prevent it). The suggested interaction between shoot and root factors implicates a role for translocatable growth substances (8). In this context it is worth noting that initial lateral root formation and shoot to root ratios are different in mutant nts382 relative to

Table II. *Symbiotic Characteristics of Supernodulation, Hypernodulation, and Non-Nodulation Mutants and Cultivar Bragg*

Data are expressed per plant and each entry is the mean ± SD of 3 to 6 plants. Plants were raised as described in "Materials and Methods."

Soybean Genotype	Nodule No.		Nodule Dry Weight		Nitrogenase Activity	
	Nitrate absent	Nitrate present	Nitrate absent	Nitrate present	Nitrate absent	Nitrate present
			mg		*nmol* $C_2H_4 \cdot min^{-1}$	
Bragg (parent cultivar)	26 ± 6	19 ± 7	31 ± 10	5 ± 3	71 ± 13	1 ± 1
*nts*382 (nitrate tolerant, supernodulating)	576 ± 77	1007 ± 154	166 ± 9	193 ± 35	119 ± 35	69 ± 11
*nts*1116 (nitrate tolerant, hypernodulating)	101 ± 26	74 ± 45	66 ± 12	30 ± 12	85 ± 17	23 ± 10
*nod*49 (non-nodulating)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table III. *Control of Supernodulation by the Shoot of Cultivar Bragg*

Plants were raised and harvested as described in "Materials and Methods." Each entry is the mean ± SD of 4 plants.

Graft (Shoot/Root)	Nodule No. per Plant	Nodule Mass
		mg dry wt nodule g^{-1} dry wt plant
nts382/nts382	249 ± 90	139 ± 101
nts1116/nts382	71 ± 18	110 ± 5
Bragg/nts382	11 ± 5	2 ± 1
nts382/nts1116	251 ± 46	182 ± 16
nts1116/nts1116	64 ± 6	14 ± 5
Bragg/nts1116	8 ± 3	3 ± 1
nts382/Bragg	182 ± 35	56 ± 28
nts1116/Bragg	48 ± 4	9 ± 2
Bragg/Bragg	8 ± 1	2 ± 1

Table IV. *Control of Supernodulation by the Shoot of Cultivar Williams*

Plants were raised and harvested as described in "Materials and Methods." Each entry is the mean ± SD of 4 plants.

Graft (Shoot/Root)	Nodule No. per Plant	Nodule Mass
		mg dry wt nodule g^{-1} dry wt·plant
Williams/Williams	19 ± 4	2 ± 1
Williams/nts382	14 ± 4	2 ± 2
nts382/Williams	409 ± 76	125 ± 52
Williams/nts1116	12 ± 2	0.1 ± 0.1
nts1116/Williams	100 ± 11	27 ± 5
Bragg/Williams	16 ± 3	2 ± 1
Williams/Bragg	14 ± 5	2 ± 1

Table V. *Control of Non-Nodulation by the Root*

Plants were raised and harvested as described in "Materials and Methods." Each data point is the mean ± SD of 8 plants.

Graft (Shoot/Root)	Nodule No. per Plant	Nodule Mass
		mg dry wt nodule g^{-1} dry wt·plant
Bragg/Bragg	26 ± 8	49 ± 9
Bragg/nod49	0	0
Bragg/nts382	87 ± 15	53 ± 17
nod49/nod49	0	0
nod49/Bragg	23 ± 7	41 ± 9
nod49/nts382	69 ± 17	53 ± 9
nts382/nts382	284 ± 96	88 ± 25
nts382/nod49	0	0
nts382/Bragg	177 ± 40	127 ± 23

Bragg, even in the absence of *Bradyrhizobium* (5).

Nitrate sensitivity of nodule formation is a complex, yet unexplained phenomenon which involves both localized root effects and possible transmitted effects (2). Since the grafting experiments of Tables II and IV were carried out in the presence of high concentrations of nitrate, it appears that the shoot also plays a role in the sensitivity of the symbiosis to nitrate. To date we have isolated 12 supernodulating soybean mutants, each from a separate mutagenic event, and all display some tolerance to nitrate. Taken together, the results show that nitrate inhibition and autoregulation of nodulation are functionally if not generally linked. Mediation of the nitrate response may involve the autoregulatory shoot signal implicated by the data of Tables II and IV.

In contrast to the nts mutants, the inability of our non-nodulation mutant to nodulate is strictly determined by the root (Table V). Regardless of whether the grafted shoot was that of Bragg or nts382, mutant nod49 root stocks failed to develop nodules. Mutant nod49 itself seems to be blocked at the root hair curling stage (hac^-) and no infection events are normally seen (14). Grafting mutant nod49 shoots onto Bragg roots did not alter nodulation significantly compared to Bragg controls (23 *versus* 26 nodules per plant) and mutant nod49 shoots inhibited nodulation on mutant nts382 roots to a similar extent as did Bragg shoots (69 *versus* 87 nodules per plant). Thus the ability of mutant nod49 shoots to autoregulate nodulation is unaltered. Instead, the root itself expresses the mutation which prevents bacterial invasion. Similar root control of non-nodulation was previously reported in a naturally occurring soybean line rj_1 (6).

The results from these simple grafting and hybridizing experiments demonstrate that the development of the soybean-*Bradyrhizobium* symbiosis is under the control of both shoot and root factors and that these factors interact. They furthermore indicate that the molecular analysis of the symbiosis should not only target onto the affected organ, but that other plant parts and perhaps commonly used developmental pattern and signals do play an important role in the regulation of the symbiosis (also see Ref. 8.).

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