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Early Nodulation Signals of the Wild Type and Symbiotic Mutants of Soybean (*Glycine max*)

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Factors affecting *Bradyrhizobium nod* gene expression derived from extracts and root exudates of soybean cultivar Bragg were compared using a bioassay that monitors expression of the plant-inducible *nodYABC* operon from *Bradyrhizobium japonicum* strain USDA110. Wild-type soybean plants (inoculated and uninoculated with *B. japonicum*) were compared with an autoregulatory ("supernodulating") and two nonnodulating mutants. Analyses of extracts from seeds, cotyledons, roots, stems, and leaves showed levels of *nod* gene induction characteristic for each tissue type; however, no significant variation was observed between each plant genotype tested. Extracts from root tips of inoculated and uninoculated plants showed similar abilities to induce the *nodYABC* operon in a time course experiment. Root exudates from wild-type and mutant plants also had similar *nod* gene inducing ability. The validity of the *nod* gene induction bioassay was confirmed using HPLC analysis, which showed that the peak size of the inducer daidzein in fractionated root extracts correlated with *nod* gene inducibility. The results indicate that the autoregulatory and nonnodulating plant mutants do not have significantly different levels of bacterial *nod* gene signaling molecules compared to wild-type plants. It appears unlikely that alterations in levels of plant signals are responsible for the mutant symbiotic plant phenotypes examined.

Additional keywords: symbiosis, isoflavone.

Nodule formation on the roots of soybean plants following invasion by the nitrogen-fixing symbiont *Bradyrhizobium japonicum* (Buchanan) Jordan can be strictly controlled by both the plant and the environment. The plant can restrict nodule number via a process termed "autoregulation" or feedback regulation in which the formation of nodules on one part of the root systemically inhibits subsequent nodule formation in other root regions (Bhuvaneswari *et al.* 1980; Pierce and Bauer 1983; Kosslak and Bohlool 1984; Olsson *et al.* 1989). This host-mediated regulatory response serves to curb excessive nodulation. Many environmental factors also lower nodulation by reducing the overall fitness of the plant (Dart 1974; Lie 1969); exogenous nitrate, however, severely restricts nodule formation without being detrimental to soybean growth (for review, see Carroll and Mathews 1990).

Soybean autoregulatory mutants have been isolated (Carroll *et al.* 1984) that can nodulate profusely in the presence of exogenous nitrate (Carroll *et al.* 1985a, 1985b). These *nts* (nitrate tolerant symbiosis) mutants have up to 10 times the nodule number of wild-type plants (Gresshoff *et al.* 1985). Grafting experiments between shoots and root stocks of wild type and *nts* mutants have shown that the "supernodulating" character of *nts* mutants is controlled by a translocated shoot factor (Delves *et al.* 1986). Developing nodules are thought to release a signal that "activates" the autoregulatory inhibitor in the shoot which, in turn, is translocated back to the roots to arrest infections (Gresshoff and Delves 1986).

In addition to the supernodulating *nts* soybean mutants, two classes of nonnodulating mutants have also been isolated (Carroll *et al.* 1986). Based on physiologic observations and complementation tests, these mutants fall into two phenotypic and genotypic groups (Mathews *et al.* 1987; Mathews *et al.* 1989a, 1989b, 1989c). The first group, exemplified by the nod49 mutant, forms only subepidermal cell divisions after inoculation with normal inoculum doses (approximately 10^7 bacteria per plant). These divisions are not associated with infection threads (Mathews *et al.* 1989a) and are classified as pseudoinfections (Calvert *et al.* 1984). At high inoculum doses, however, more cell divisions are seen and "occasional" nodules can occur, suggesting that these mutants require a higher concentration of a signal or bacterial by-product to nodulate (Mathews *et al.* 1987). The second group, characterized by the nod139 mutant, is affected even earlier in the nodulation process. This mutant initiates no cell divisions in the outer cortex of the root in response to inoculation (Mathews *et al.* 1989a). Neither group shows root hair curling, which normally characterizes early nodulation events (Mathews *et al.* 1987). Grafting experiments have indicated that the nonnodulating phenotype is root-controlled in both groups (Delves *et al.* 1986).

The *Rhizobium*-legume interaction has many features in common with plant parasite infection including binding of microbes, penetration of the host, host response, and redirection of host metabolites (Verma and Nadler 1984). One of the many defense mechanisms plants use against microbial infection is the accumulation of antimicrobial phytoalexins. Phytoalexins are synthesized and accumulated in plant tissue exposed to microorganisms (Darvill

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and Albersheim 1984). In soybean, many phytoalexins are synthesized by the phenylpropanoid pathway, which catalyzes reactions leading to the phytoalexic glyceollins (I, II, and III) and isoflavones (Ribéreau-Gayon 1972). In soybeans, both the isoflavone, daidzein, and the glyceollins are known to accumulate in leaves inoculated with certain phytopathogenic bacteria (Osman and Fett 1983).

The "common" nodulation (*nod*) genes of (*Brady*)-*Rhizobium* (*nodABC*) are essential for nodule initiation (for review, see Appelbaum 1990). Mutations of any of these genes will result in a nonnodulation (Nod^-) phenotype (Djordjevic *et al.* 1985). An additional open-reading frame (*nodY*), of unknown function (Banfalvi *et al.* 1988; Scott 1986), occurs at the start of the *nodABC* operon in some *Bradyrhizobium* species. Expression of the *nod(Y)ABC* operon is repressed in normal culture (Mulligan and Long 1985). Another essential *nod* gene, *nodD*, is constitutively expressed in normal culture (Djordjevic *et al.* 1987b; Bassam *et al.* 1988). The gene product of the *nodD* gene (NodD) interacts with specific plant-derived flavonoids to activate transcription of other *nod* genes (Spaink *et al.* 1987). The isoflavones genistein and daidzein, while acting as inhibitors of *nod* gene induction in other legume-*Rhizobium* symbioses (Firmin *et al.* 1986), are inducers in the soybean-*Bradyrhizobium* symbiosis (Kosslak *et al.* 1987; Banfalvi *et al.* 1988; Bassam *et al.* 1988; Göttfert *et al.* 1988).

In this study, a bioassay that measures the expression of the *nodYABC* operon in *B. japonicum* was first characterized and then used to monitor changes in *nod* gene inducers from different tissues and root exudates of wild-type, autoregulatory, and nonnodulating mutants of soybean cultivar Bragg. This was done to determine if alterations in levels of plant signal compounds contribute to the phenotype of the soybean mutants or whether plant signal levels measurably change in response to inoculation.

MATERIALS AND METHODS

Plant material. Two nonnodulation mutants, nod49 and nod139 (Carroll *et al.* 1986); a supernodulating autoregulatory mutant, nts382 (Carroll *et al.* 1985a, 1985b); and the parent commercial cultivar Bragg were used. Each mutant was derived from ethylmethane sulfonate mutagenesis as described previously (Carroll *et al.* 1985a; Carroll *et al.* 1986).

Measurement of *nod* gene expression. Expression of the *nodYABC* operon was measured using strain ZB977 (Banfalvi *et al.* 1988), a derivative of the soybean inoculum *B. japonicum* strain USDA110. Strain ZB977 has a translational *lacZ* gene fusion to the *nodY* open-reading frame, hence measurement of β-galactosidase activity in this strain reflects expression of the flavonoid-inducible *nod* genes (the effect of native β-galactosidase activity was found to be insignificant, data not shown). Strain ZB977 cells were grown to early logarithmic phase ($OD_{600} \approx 0.1$) in *Rhizobium* fast growth medium (Howitt and Gresshoff 1985) containing 40 μg/ml of tetracycline. When buffered, 5.8 mg/ml of 3-(4-morpholino)-propanesulfonic acid (MOPS; Eastman Kodak Co., Rochester, NY) was added before autoclaving. Cells were induced with plant exudates or extracts, and as standards, the isoflavones genistein and daidzein (7.3 μM) (ICN Biomedicals, Plainview, NY) were used. Incubation was for 8 hr at 30° C. The β-galactosidase assay of Miller (1972) was used to quantify expression of bacterial *nod* genes (measured in "Miller units" of specific β-galactosidase activity). *B. japonicum* strain ZB977 liquid cultures were streaked onto Luria broth plates (Miller 1972) to check for possible contamination.

Plant growth conditions. Seeds of soybean (*Glycine max* (L.) Merr.) cultivar Bragg, its supernodulating mutant nts382, and nonnodulating mutants nod139 and nod49 were surface-sterilized for 2 min in 70% ethanol-30% distilled H_2O (dH_2O), carefully washed in dH_2O for 4 min, imbibed for 2 hr, and then planted in trays of damp vermiculite for 4 or 12 days. The seeds were germinated and grown in a controlled environment growth cabinet with an 18-hr, 26° C day period and a 6-hr, 18° C dark period. Seedlings were watered daily with sterile quarter-strength Herridge's solution (Herridge 1977) and inoculated with 10^7 *B. japonicum* strain USDA110 bacteria per plant at both day 0 and day 1 after planting.

Preparation of plant extracts and exudates. Tissues of 4- or 12-day-old seedlings (approximately 20 plants per flask) were harvested and placed in vacuum flasks with 10 times tissue fresh weight of 80% methanol-dH_2O. Tissue was put under vacuum for 15–20 min, and the pH was adjusted to 5.3 with 0.1 N HCl; it was then shaken at 4° C for 24 hr at 130 rpm. Extracts were filtered through Whatman No. 1 filter paper and rotary evaporated to remove the methanol fraction. The aqueous fraction was freeze-dried, resuspended to equal 10 mg extract dry weight per milliliter of dH_2O, and stored at −20° C until needed.

Seedlings for exudate studies were first grown in sterile sand and watered daily with sterile quarter-strength Herridge's solution (Herridge 1977). Seedlings were inoculated with 10^7 *B. japonicum* strain USDA110 bacteria per plant at day 0 and day 1 after planting. After 12 days, plant roots were washed free of sand and suspended in flasks in sterile dH_2O. The flasks were gently shaken for 24 hr in a laminar flow hood at room temperature. The root liquid was filtered through Whatman No. 1 filter paper and rotary evaporated at 40° C under high vacuum to an equivalent of 1 ml per 500 mg root dry weight. Samples were stored at −20° C.

The effect of inoculation on inducer compound levels in 8- to 11-day-old seedlings was compared to uninoculated controls by extracting the root section from the root tip to the first distinct lateral root (approximately 5-cm sections). Extractions were done upon inoculation with strain USDA110 and at 24-, 48-, and 72-hr intervals.

The levels of inducer compound in different root sections were determined from extracts of different root sections. Seedlings were selected that had uniform 8-cm roots. The apical 2-cm section containing the root tip to the smallest emergent root hair was used as young root. The 2-cm adjacent section was discarded, and the next 2-cm region was used as the more mature root section.

Preparation of extracts for high pressure liquid chromatography (HPLC) analysis. Extracts were phase-

partitioned three times against equal volumes of ethyl acetate, retaining each organic fraction. Dry sodium sulfate was added to absorb the remaining water. The clear ethyl acetate fraction was decanted and rotary evaporated at 30° C until dry and stored at −70° C until required. The sample was redissolved in 1 ml of HPLC grade acetonitrile:chloroform (1:1) and centrifuged for 5 min before use. An LKB 2000 series HPLC system using a linear 0.5% acetic acid-acetonitrile gradient with a ratio of time (min) to acetonitrile (percent) coordinates of 0:20, 6:21, 17:40, 25:70, 28:99, and 38:100 was used with a flow rate of 0.9 ml·min^{-1} to separate compounds within the extract. The 250-nm wavelength was used to detect compounds.

RESULTS

Measurement of *B. japonicum nod* gene induction. Expression of the isoflavone-inducible *nodYABC* operon of *B. japonicum* strain ZB977 was measured by assaying β-galactosidase activity from a *lacZ* translational gene fusion to the *nodY* open-reading frame (Banfalvi *et al.* 1988). Before using the induction of β-galactosidase activity from strain ZB977 as a bioassay for plant signals, parameters affecting the assay were examined in some detail. Induction of β-galactosidase activity in strain ZB977 by the known isoflavone inducers (genistein and daidzein) was tested for a range of concentrations from 0 to 80 μM. Induction of the operon was not linear over this range (Fig. 1) and was maximal at about 10 μM for each isoflavone (giving between 600 and 800 Miller units with genistein and between 250 and 400 Miller units with daidzein).

Daidzein consistently had less than half the inducing ability of genistein at all measured concentrations (Fig. 1). A time course experiment using 7.3 μM genistein to induce expression from the strain ZB977 *nodYABC* operon showed that β-galactosidase activity increased linearly for the first 6 hr, after which it tapered off slowly. Thus, an incubation time of 8 hr was used for measuring induction of the operon (Fig. 1). In addition, it was shown that the yellow color reaction of the β-galactosidase assay could vary from an OD_{420} of 0.1 to 0.6 without affecting calculated specific β-galactosidase activity (data not shown).

Titration curves were made from each different plant extract to ensure that the amount of extract used in comparing samples was in the range most sensitive for the bioassay. At higher extract concentrations, for example, the bioassay can lose accuracy due to saturation of the system with inducer or toxicity of the extract. Cell viability was not affected by the concentrations of extracts used in each assay (that is, less than 70 μg·ml^{-1}, data not shown). This was shown by serial dilution platings of *B. japonicum* cell cultures after a 48-hr incubation period with different concentrations of plant exudates. However, loss of viability was observed when very high exudate levels were used (for example, 700 μg·ml^{-1} caused about 50% cell death).

Factors affecting *nod* gene inducibility. The growth stage of strain ZB977 cells was found to be critical for induction of the *nodYABC* operon. A "window of inducibility" was observed in late lag and early logarithmic phase cells, with inducibility decreasing rapidly as cell growth progressed through logarithmic phase (Fig. 2). Therefore, only cell cultures with an OD_{600} absorption between 0.09 and 0.11 were suitable for use in signal bioassays.

It was found that the pH of the cell culture increased significantly as the inducibility of the cells decreased (Fig. 2). In an effort to extend the window of inducibility of strain ZB977 cells, we buffered the growth medium with MOPS. Cells grown in buffered medium also showed a window of inducibility, but remained inducible throughout the growth cycle of the strain (Fig. 2). In another experiment, strain ZB977 cells grown to early logarithmic phase were incubated at different pH levels during induction with genistein and daidzein. Induction was optimal between pH 5.3 and 6.5, but decreased rapidly outside this range

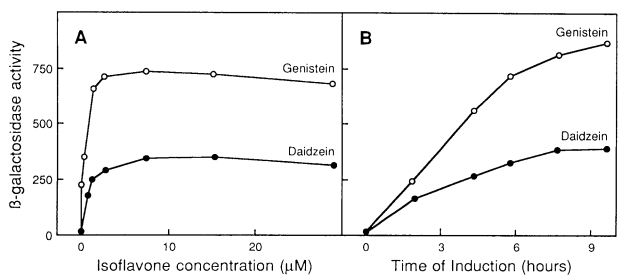

Fig. 1. Induction of the *Bradyrhizobium japonicum nodYABC* operon. Induction of the *B. japonicum nodYABC* operon by different isoflavone concentrations and an 8-hr incubation period (panel **A**) and after different incubation times with 7.3 μM isoflavone (panel **B**). Open circles show the level of induction of the operon with genistein in strain ZB977 cells grown to early logarithmic phase, and closed circles show the level of induction with daidzein. Units of β-galactosidase activity are given in Miller units (Miller 1972). The experiment was repeated three times; the results presented are from the third experiment. Each data point is the average of four replicates. Standard deviation in all cases is less than ±10%.

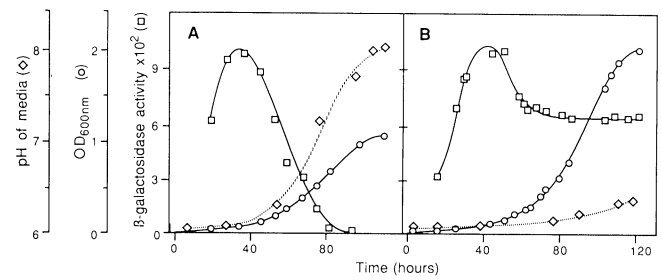

Fig. 2. Growth phase and pH effects on *Bradyrhizobium japonicum nod* gene inducibility. Induction of the *B. japonicum nodYABC* operon by 7.3 μM genistein in strain ZB977 cells is shown at different stages in the growth cycle in both buffered and unbuffered growth media. Open squares show the level of induction of the operon in Miller units of β-galactosidase activity (Miller 1972), and open circles show the OD_{600} of the cell culture at different time points in the growth cycle. Open rhomboids show the pH change in the culture medium. Cells are inducible during early logarithmic/late stationary phase in normal unbuffered liquid fast growth medium (panel **A**). The transient "window" of maximal inducibility is also evident in cells grown in buffered fast growth media (panel **B**), but cells remain inducible into stationary phase. Cells cultured in pH-buffered medium reach a much higher cell density indicating that pH changes in the unbuffered medium quickly limit growth. The curves fitted to the data are approximations only. The experiment was repeated three times; the results presented are from the third experiment. Each data point is the average of four replicates. Standard deviation is less than ±10% (β-galactosidase activity) and less than ±5% (OD_{600}) in all cases.

(Fig. 3). The pH was kept within this range in all plant extract induction assays.

Signal levels from different plant tissue. Extracts were prepared from seeds, cotyledons, roots, stems, and leaves of wild-type Bragg and its autoregulatory and Nod$^-$ mutant derivatives. The relative *nod* gene inducing ability of each plant tissue type varied considerably (from about 25 units in roots to about 300 units in seeds and cotyledons; Table 1). No significant difference in *nod* gene inducing ability was observed between mutant and wild-type soybean plants for each tissue type tested (Table 1). Because the observed level of *nod* gene induction was proportional to the amount of root extract added, the low level of inducing ability from root extracts was not the result of high levels of inhibitors in the extract. Furthermore, no inhibitory effect on *nod* gene induction was observed when different concentrations of root extract added to standard concentrations of genistein and daidzein were used as the inducer (data not shown).

Signal levels from young root tissue. Extracts from the first 2 cm of roots of 8- to 11-day-old cultivar Bragg seedlings were compared at 0, 24, 36, and 72 hr after inoculation with strain USDA110. No change in *nod* gene inducing ability was observed between the young root extracts of these seedlings (Table 2). We found that the condition of the plant material was important to the accuracy of these experiments. Roots suffered extensive browning if they were over-watered and produced extracts with artificially high inducing ability.

In other experiments, we found a twofold increase in *nod* gene inducing ability (from about 60 to 100 units) in younger as compared to older root segments of 4-day-old uninoculated cultivar Bragg seedlings, indicating that younger root tissue had a higher level of *nod* gene inducing signals. A similar result was found with inoculated root extracts.

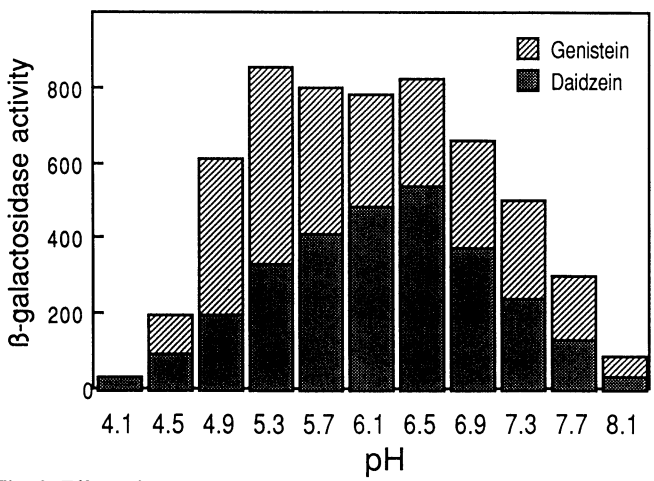

Fig. 3. Effect of pH on *Bradyrhizobium japonicum nod* gene induction. The *nodYABC* operon of *B. japonicum* was induced with 7.3 μM genistein and daidzein at various pH levels. In each assay, strain ZB977 cells were incubated for 8 hr in buffered fast growth medium using 3-(4-morpholino)-propanesulfonic acid to a range of specific pH levels. Units of β-galactosidase activity are given in Miller units (Miller 1972). The experiment was repeated twice; the results presented are from the second experiment. Each data point is the average of four replicates. All bars originate at 0 units. Standard deviation in all cases is less than ±10%.

Signal levels from root exudates. The ability of exudates from wild-type Bragg and Bragg-derived mutants to induce the *Bradyrhizobium* strain ZB977 *nodY::lacZ* gene fusion was compared. The inducing activity was found to be similar in all genotypes tested (Table 3).

HPLC analysis of root extracts. HPLC analysis of further purified root extract fractions from both wild-type cultivar Bragg and the nts382 mutant correlated flavonoid composition with the biological activity measured with the plant signal bioassay (Fig. 4). The proposed daidzein peak eluated at the same time and had the same spectrum as a commercially obtained daidzein standard with absorption maxima at 244 and 296 nm and a trough at 280 nm. β-Galactosidase activity measured with the signal bioassay correlated to the size of the daidzein peaks obtained from root extracts. The response of bacterial *nod* genes to the extracts, after they were further purified for HPLC analysis, showed that the ability to induce early nodulation genes

Table 1. Ability of tissue extracts to induce *Bradyrhizobium japonicum nod* genes[a]

Mutant	Inoculation	Seeds	Cotyledons	Roots	Stems	Leaves
Bragg	−	304 ± 33	225 ± 32	25 ± 3	308 ± 33	343 ± 26
Bragg	+	Not done	232 ± 31	25 ± 4	336 ± 31	368 ± 40
nts382	−	268 ± 18	200 ± 26	24 ± 3	338 ± 31	338 ± 31
nod49	−	321 ± 32	218 ± 29	22 ± 4	316 ± 41	365 ± 26
nod139	−	Not done	202 ± 32	26 ± 3	305 ± 55	328 ± 35

[a]Comparison of the ability of cultivar Bragg wild-type and mutant soybean tissue extracts to induce *B. japonicum nod* genes Inducing ability is expressed as Miller units of β-galactosidase activity per milligram plant tissue dry weight ± SD. The inoculum level was 10^7 *B. japonicum* cells per plant at day 0. Plants were harvested after 12 days. The data presented are mean results of three separate experiments on identical plant material with four repeats per experiment.

Table 2. Effect of inoculation on levels of *nod* gene inducers from young root tissue of soybean[a]

Treatment	Time after inoculation (hr) 0	24	36	72
Uninoculated	31 ± 6	35 ± 6	35 ± 5	34 ± 6
Inoculated	Not done	39 ± 7	33 ± 6	34 ± 5

[a]Induction of *Bradyrhizobium japonicum nod* genes with inoculated vs uninoculated root tip extracts from wild-type cultivar Bragg. Inducing ability is expressed as Miller units of β-galactosidase activity per milligram root dry weight ± SD. The inoculum level was 10^7 *B. japonicum* cells per plant. The data presented are mean results of three separate experiments on identical plant material with four repeats per experiment.

Table 3. Signal levels in root exudates[a]

Plant	Inoculated	Signal level (Miller units)
Bragg	−	1200 ± 182
Bragg	+	1360 ± 260
nod49	−	1264 ± 356
nod139	−	1160 ± 184
nts382	−	988 ± 236

[a]The ability of soybean root exudates from wild-type cultivar Bragg and a supernodulating (nts382) and two nonnodulating (nod49 and nod139) mutant derivatives to induce the *Bradyrhizobium japonicum nodYABC* operon. Plants were prepared as described in the text. Gene expression is measured in Miller units per gram root dry weight ± SD. The data presented are means of four separate experiments on identical plant material with four repeats per experiment.

was retained in the fraction used for HPLC analysis. The chromatograms (250 nm) obtained from root extracts of inoculated and uninoculated wild-type Bragg and the nts382 mutant showed similar composition (Fig. 4). The extract from older root sections showed a reduced level of all compounds present but a similar peak profile compared to younger root tissue in both wild-type and supernodulating soybean nodulation phenotypes.

DISCUSSION

The specific flavonoid molecules that act as early nodulation signals between legumes and their (*Brady*)*Rhizobium* symbiotic partners vary in both kind and relative amount from one type of host plant to another (Kosslak *et al.* 1987; Horvath *et al.* 1987; Djordjevic *et al.* 1987a; Zaat *et al.* 1988). Correspondingly, *nodD*-like genes of different (*Brady*)*Rhizobium* species are activated by flavonoid signals exuded by their preferred plant host (Redmond *et al.* 1986; Peters *et al.* 1986; Firmin *et al.* 1986; Kosslak *et al.* 1987). Recent evidence suggests that flavonoid signal levels can directly affect nodulation quality. Kapulnik *et al.* (1987), for example, reported that the superior nodulation and nitrogen fixation of HP32 alfalfa compared to HP alfalfa were associated with a 77% increase in the amount of plant tissue luteolin (a preferred inducer signal of *R. meliloti nod* genes). Because changes in the amount or type of flavonoid plant signal can have significant effects on the symbiosis, this study aimed to determine the following: 1) whether changes in the level or composition of early plant nodulation signals occurred between (and therefore might be responsible for the phenotypes of) wild-type and symbiotic mutants of soybean; and 2) whether changes in plant signal levels occur in response to inoculation with *Bradyrhizobium*.

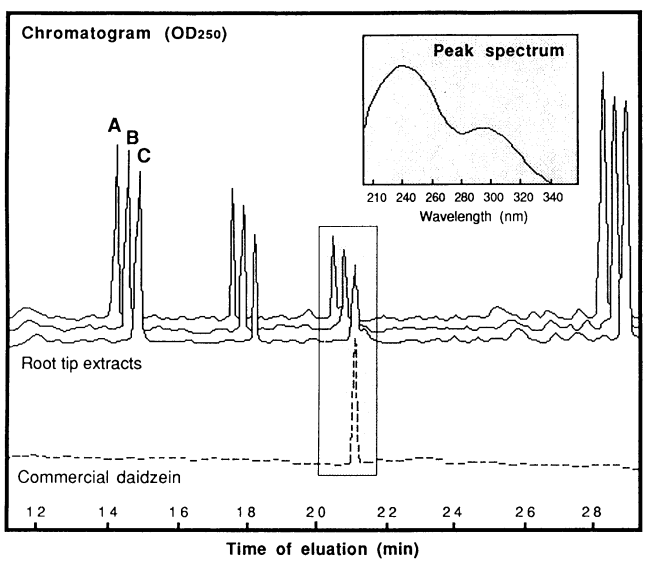

Fig. 4. Comparison of high pressure liquid chromatography (HPLC) chromatograms. Chromatograms (OD_{250}) of root extracts separated by HPLC: **A**, uninoculated wild-type cultivar Bragg; **B**, inoculated wild-type cultivar Bragg; and **C**, supernodulating mutant nts382. The spectra of **A** and **B** have been displaced to the left for clarity. Each spectrum shown was traced from original printouts. The daidzein peak in each spectrum was identified by peak spiking with commercially obtained daidzein (dashed line) and compared with the spectrum of commercial daidzein alone. The identity of the other major peaks shown was not determined. The inset box (upper right) shows a representative expanded spectrum of the daidzein peak obtained from each extract. The highlighted box at around 21 min shows the daidzein peak, as confirmed by *nodY-lacZ* induction of this fraction, the commercial standard and absorption profile (insert, upper right).

In this report, we relied on a bioassay that directly measured plant signal levels as perceived by the normal *Bradyrhizobium* symbiotic partner of soybean. We confirmed the validity of such an assay before relying on any results obtained and found that while the bioassay was sensitive and could provide a high degree of repeatability, only *Bradyrhizobium* cells of a very specific growth phase cultured within a narrow pH range were suitable for use in the bioassay and that great care is needed in selecting plant material. For accurate results, it was also necessary to construct titration curves of inducibility for a particular sample of plant signal in order to avoid saturation of the system with excess signal and to reduce the effect of toxins in the extract.

Using the *nod* gene expression bioassay, we examined the signal levels from the seeds, stems, leaves, cotyledons, young root tissue, older root tissue, and root exudates from wild-type Bragg as well as from each of the soybean mutants. In all of the tissue types tested, we found no significant differences in signal levels between each plant type, suggesting that neither absence nor overproduction of signal is responsible for the mutant phenotypes. These results were confirmed for several of the plant extract signal samples using HPLC analysis.

Significant variation in signal levels was observed between extracts of each tissue type, reflecting variations in the kind as well as the amounts of signal in each. Although the isoflavones genistein and daidzein are recognized as the primary signal molecules between soybean and *Bradyrhizobium nod* genes (Kosslak *et al.* 1987), a variety of other flavonoid compounds can also act as inducers. Both quercetin and kaempferol, which occur in soybean tissue (Buttery and Buzzell 1973), are known inducers of *B. japonicum nod* genes, having 10 and 29%, respectively, of the reported genistein activity (Kosslak *et al.* 1987).

Our results concur with those of Mathews *et al.* (1989b) who compared whole seedling extracts from 3-day-old nonnodulating nod49 and supernodulating nts382 mutants using a similar bioassay of *nod* gene inducibility and found that the extracts had the same ability to induce as did wild-type Bragg extracts. The uniformity of plant signal levels between the soybean nodulation mutants indicates that the level of plant signals within the plant makes little or no contribution to each mutant phenotype. This is, perhaps, not surprising considering that while levels of flavonoid signals vary little within a particular cultivar, as demonstrated here, significant differences in signal level which do not affect total nodule number can be observed between cultivars. Mathews *et al.* (1989b), for example, showed that 3-day-old seedlings of soybean cultivar Williams have a 10 times greater ability to induce *Bradyrhizobium nod* genes than do 3-day-old cultivar

Bragg seedlings, although mature plants have the same nodulation frequency.

As with tissue extracts, we found the level of signal from wild-type and mutant soybean root exudates to be similar. Since it is by means of the root-exuded signals that soil (*Brady*)*Rhizobium* sense their prospective hosts (Caetano-Anollés *et al.* 1988), this result is strong evidence that differences in plant-bacterium signaling are not involved in the nodulation phenotype of the soybean mutants studied here. Mathews *et al.* (1989b) cocultivated the nonnodulating mutants with either wild-type cultivar Bragg or its supernodulating derivative under sterile conditions in which the root systems of both phenotypes were tightly intertwined. They showed no effect on either the nonnodulating or the supernodulating phenotype, although the "cross-feeding" of exudates must clearly have occurred. Peters and Long (1988) have reported that exudates from a nonnodulating alfalfa mutant also had the same ability to induce *R. meliloti nod* genes as did exudates from wild-type plants.

It has been previously shown that phytoalexin accumulation can occur in response to inoculation with incompatible *Rhizobium* strains. Weinman *et al.* (1988) reported that inoculation of white clover with incompatible *Rhizobium* strains led to an accumulation of flavonoid-inducing compounds in seedling extracts, whereas infection with *R. trifolii*, the normal microsymbiont, gave a less pronounced accumulation. The soybean phytoalexins, glyceollin I, II, and III, as well as their precursor, daidzein, have been reported to accumulate in soybean leaves in response to infection with certain pathogenic bacteria (Osman and Fett 1983; Fett and Jones 1984). We found no difference in the levels of flavonoid plant signals in extracts from uninoculated controls and inoculated root tissue up to the maximum tested time of 72 hr post-inoculation. HPLC analysis of fractionated inoculated vs uninoculated root extracts showed similar levels of the potent signal daidzein. The results obtained for both wild-type and mutant soybeans are thus consistent with a compatible host-symbiont interaction in which a general host defense response to invasion does not occur.

Although it seems unlikely that flavonoid signals are involved in the plant autoregulatory response, it is possible that regulation of root flavonoid levels might affect nodulation susceptibility. The zone between the smallest emerging root hairs and the root tip has been found to be the location of infectible cells on the primary root, inferred from the position of subsequent nodule development (Bhuvaneswari *et al.* 1980). Some legumes release *nod* gene stimulatory compounds in high concentrations from the zone of emerging root hairs (Redmond *et al.* 1986). We found that soybean root extracts from the root tip section had a twofold increase in *Bradyrhizobium nod* gene inducing ability compared to more mature root extracts. The lowered levels of substances in the mature root may be due to a greater "extractability" of noninducing compounds or may reflect a lower level of inducing compounds in the tissues. It has also been found that *Trifolium repens* (white clover) secretes compounds from cells located immediately behind the root tip which repress *nod* gene transcription (Djordjevic *et al.* 1987b). Djordjevic *et al.* (1987a) suggest that fluctuations in the concentrations of the inhibitory and stimulatory compounds may affect *nod* gene expression in the infection thread, with high inhibitor levels repressing *nod* gene transcription and arresting further nodule development.

Our results show that although plant signals to the *nod* genes in the soybean-*Bradyrhizobium* symbiosis are involved in the control of early infection events, they have little or no involvement with autoregulation and thus nodulation frequency in cultivar Bragg. Furthermore, changes in *nod* gene signal levels are not responsible for the phenotypes of the nonnodulating and supernodulating mutants used in this study.

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LITERATURE CITED

Appelbaum, E. R. 1990. The *Rhizobium*/*Bradyrhizobium*-legume symbiosis. Pages 131-158 in: The Molecular Biology of Symbiotic Nitrogen Fixation. P. M. Gresshoff, ed. CRC Press, Boca Raton, FL.

Banfalvi, Z., Niewkoop, A., Schell, M., Besl, L., and Stacey, G. 1988. Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. 214:420-424.

Bassam, B. J., Djordjevic, M. A., Redmond, J. W., Batley, M., and Rolfe, B. G. 1988. Identification of a *nodD*-dependent locus in the *Rhizobium* strain NGR234 activated by phenolic factors secreted by soybeans and other legumes. Mol. Plant-Microbe Interact. 1:161-168.

Bhuvaneswari, T. V., Turgeon, B. G., and Bauer, W. D. 1980. Early events in the infection of soybean (*Glycine max* (L.) Merr.) by *Rhizobium japonicum*. I. Location of infectible root cells. Plant Physiol. 66:1027-1031.

Buttery, B. R., and Buzzell, R. I. 1973. Varietal differences in leaf flavonoids of soybeans. Crop Sci. 13:103-106.

Caetano-Anollés, G., Christ-Estes, D. K., and Bauer, W. D. 1988. Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. J. Bacteriol. 170:3164-3169.

Calvert, H. E., Pence, M. K., Pierce, M., Malik, N. S. A., and Bauer, W. D. 1984. Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. Can. J. Bot. 62:2375-2384.

Carroll, B. J., McNeil, D. L., and Gresshoff, P. M. 1984. Breeding soybeans for increased nodulation in the presence of external nitrate. Pages 43-50 in: Symbiotic Nitrogen Fixation, Vol. 1. B. S. Ghai, ed. USG Publishing Co., Ludhiana, India.

Carroll, B. J., McNeil, D. L., and Gresshoff, P. M. 1985a. Isolation and properties of soybean (*Glycine max* (L.) Merr.) mutants that nodulate in the presence of high nitrate concentrations. Proc. Natl. Acad. Sci. USA 82:4162-4166.

Carroll, B. J., McNeil, D. L., and Gresshoff, P. M. 1985b. A supernodulation and nitrate tolerant symbiotic (*nts*) soybean mutant. Plant Physiol. 78:34-40.

Carroll, B. J., McNeil, D. L., and Gresshoff, P. M. 1986. Mutagenesis of soybean (*Glycine max* (L.) Merr.) and the isolation of non-nodulating mutants. Plant Sci. 47:109-119.

Carroll, B. J., and Mathews, A. 1990. Nitrate inhibition of nodulation in legumes. Pages 160-180 in: The Molecular Biology of Symbiotic Nitrogen Fixation. P. M. Gresshoff, ed. CRC Press, Boca Raton, FL.

Dart, P. J. 1974. The infection process. Pages 381-429 in: The Biology of Nitrogen Fixation. A. Quispel, ed. North-Holland Publishing Co., Amsterdam.

Darvill, A. G., and Albersheim, P. 1984. Phytoalexins and their elicitors – A defense against microbial infection in plants. Annu. Rev. Plant Physiol. 35:243-275.

Delves, A. C., Mathews, A., Day, D. A., Carter, A. S., Carroll, B. J., and Gresshoff, P. M. 1986. Regulation of the soybean-*Rhizobium*

symbiosis by shoot and root factors. Plant Physiol. 82:588-590.
Djordjevic, M. A., Schofield, P. R., and Rolfe, B. G. 1985. Tn*5* mutagenesis of *Rhizobium trifolii* host-specific nodulation genes results in mutants with altered host range ability. Mol. Gen. Genet. 200:463-471.
Djordjevic, M. A., Gabriel, D. W., and Rolfe, B. G. 1987a. *Rhizobium* - The refined parasite of legumes. Annu. Rev. Phytopathol. 25:145-168.
Djordjevic, M. A., Redmond, J. W., Batley, M., and Rolfe, B. G. 1987b. Clovers secrete specific phenolic compounds which either stimulate or repress *nod* gene expression in *Rhizobium trifolii*. EMBO J. 6:1173-1179.
Fett, W. F., and Jones, S. B. 1984. Stress metabolite accumulation, bacterial growth and bacterial immobilization during host and nonhost responses of soybean to bacteria. Physiol. Plant Pathol. 25:277-296.
Firmin, J. L., Wilson, K. E., Rossen, L., and Johnston, A. W. B. 1986. Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. Nature 324:90-92.
Göttfert, M., Weber, J., and Hennecke, H. 1988. Induction of a *nodA-lacZ* fusion in *Bradyrhizobium japonicum* by an isoflavone. J. Plant Physiol. 132:394-397.
Gresshoff, P. M., Carroll, B. J., and McNeil, D. L. 1985. Symbiotic mutants of soybean capable of supernodulation in the absence and presence of nitrate. Pages 86-88 in: Advances in the Molecular Genetics of the Bacteria-Plant Interaction. A. A. Szalay and R. P. Legocki, eds. Media Services, Cornell University, Ithaca, NY.
Gresshoff, P. M., and Delves, A. C. 1986. Plant genetic approaches to symbiotic nodulation and nitrogen fixation in legumes. Pages 159-206 in: Plant Gene Research III. A Genetical Approach to Plant Biochemistry. A. D. Blonstein and P. J. King, eds. Springer-Verlag, Vienna.
Herridge, D. F. 1977. Carbon and nitrogen nutrition of two annual legumes. Ph.D. thesis, University of Western Australia, Perth.
Horvath, B., Bachem, C. W. B., Schell, J., and Kondorosi, A. 1987. Host-specific regulation of nodulation genes in *Rhizobium* is mediated by a plant-signal, interacting with the *nodD* gene product. EMBO J. 6:841-848.
Howitt, S. M., and Gresshoff, P. M. 1985. Ammonia regulation of glutamine synthetase in *Rhizobium* species ANU289. J. Gen. Microbiol. 131:1433-1440.
Kapulnik, Y., Joseph, C. M., and Phillips, D. A. 1987. Flavone limitations to root nodulation and symbiotic nitrogen fixation in alfalfa. Plant Physiol. 84:1193-1196.
Kosslak, R. M., and Bohlool, B. B. 1984. Suppression of nodule development of one side of a split root system of soybeans causes by prior inoculation of the other side. Plant Physiol. 75:125-130.
Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H., and Appelbaum, E. 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. Proc. Natl. Acad. Sci. USA 84:7428-7432.
Lie, T. A. 1969. The effect of low pH on different phases of nodule formation in pea plants. Plant Soil 31:391-407.
Mathews, A., Carroll, B. J., and Gresshoff, P. M. 1987. Characterisation of non-nodulation mutants of soybean (*Glycine max* (L.) Merr.): *Bradyrhizobium* effects and absence of root hair curling. J. Plant Physiol. 131:349-361.
Mathews, A., Carroll, B. J., and Gresshoff, P. M. 1989a. Development of *Bradyrhizobium* infections in a supernodulating and non-nodulating mutant of soybean *Glycine max* (L.) Merr. Protoplasma 150:40-47.
Mathews, A., Kosslak, R. M., Sengupta-Gopalan, C., Appelbaum, E. R., Carroll, B. J., and Gresshoff, P. M. 1989b. Biological characterization of root exudates and extracts from nonnodulating and supernodulating soybean mutants. Mol. Plant-Microbe Interact. 2:283-290.
Mathews, A., Carroll, B. J., and Gresshoff, P. M. 1989c. A new non-nodulation gene in soybean. J. Hered. 80:357-360.
Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mulligan, J. T., and Long, S. R. 1985. Induction of *Rhizobium meliloti nodC* expression by plant exudate requires *nodD*. Proc. Natl. Acad. Sci. USA 82:6609-6613.
Olsson, J. E., Nakao, P., Bohlool, B. B., and Gresshoff, P. M. 1989. Lack of systemic suppression of nodulation in split root systems of supernodulating soybean (*Glycine max* (L.) Merr.) mutants. Plant Physiol. 90:1347-1352.
Osman, S. F., and Fett, W. F. 1983. Isoflavone glucoside stress metabolites of soybean leaves. Phytochemistry 22:1921-1923.
Peters, N. K., Frost, J. W., and Long, S. R. 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science 233:977-980.
Peters, N. K., and Long, S. R. 1988. Alfalfa root exudates and compounds which promote or inhibit induction of *Rhizobium meliloti* nodulation genes. Plant Physiol. 88:396-400.
Pierce, M., and Bauer, W. D. 1983. A rapid regulatory response governing nodulation in soybean. Plant Physiol. 73:286-290.
Redmond, J. W., Batley, M., Djordjevic, M. A., Innes, R. W., Kuempel, P. L., and Rolfe, B. G. 1986. Flavones induce expression of nodulation genes in *Rhizobium*. Nature 323:632-635.
Ribéreau-Gayon, P., ed. 1972. Plant Phenolics. Hafner Publishing Co., New York.
Scott, K. F. 1986. Conserved nodulation genes from the non-legume symbiont *Bradyrhizobium* sp. (*Parasponia*). Nucleic Acids Res. 14:2905-2919.
Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E., and Lugtenburg, B. J. J. 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol. Biol. 9:27-39.
Verma, D. P. S., and Nadler, K. 1984. Legume-*Rhizobium* symbiosis: Host's point of view. Pages 57-93 in: Genes Involved in Microbe-Plant Interactions. D. P. S. Verma and T. L. Horn, eds. Springer-Verlag, Vienna.
Weinman, J. J., Djordjevic, M. A., Sargent, C. L., Dazzo, F. B., and Rolfe, B. G. 1988. A molecular analysis of the host range genes of *Rhizobium trifolii*. Pages 33-34 in: Molecular Genetics of Plant-Microbe Interactions. R. Palacios and D. P. S. Verma, eds. American Phytopathological Society, St. Paul, MN.
Zaat, S. A., Wijffelman, C. A., Mulders, I. H. M., Van Brussel, A. A. N., and Lugtenberg, B. J. J. 1988. Root exudates of various host plants of *Rhizobium leguminosarum* contain different sets of inducers of *Rhizobium* nodulation genes. Plant Physiol. 86:1298-1303.