Bradyrhizobium japonicum nodD1 Can Be Specifically Induced by Soybean Flavonoids That Do Not Induce the nodYABCSUIJ Operon*

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Besides genistein and daidzein, which are active inducers of the nodYABCSUIJ operon in Bradyrhizobium japonicum, soybean seeds also excrete compounds that are not inducers of the nodYABCSUIJ genes but enhance induction of this operon in the presence of a suboptimal genistein concentration. This synergism was studied in detail, and specific compounds were identified in seed exudate which specifically induce the nodD1 gene but not the nodYABCSUIJ operon. Therefore, our current hypothesis is that the observed synergism is caused by a specific induction of nodD1. The specific nodD1 inducers from soybean seed extract have been purified and characterized chemically. They appear to be derivatives of genistein, glycitein, and daidzein with glucose, malonyl, and acetyl groups attached. Both root and seed exudate appear to contain these compounds, with the seed being the major source. No hydrolysis of these compounds to their aglycones was detected in the presence of B. japonicum. A model for nod gene induction in B. japonicum is discussed.

Bradyrhizobium and Rhizobium species infect leguminous plants in a process leading to nitrogen-fixing nodules on the roots. Rhizobial species differ in their host spectrum; e.g., Rhizobium leguminosarum biovar viciae nodulates Vicia and Pisum, R. leguminosarum bv. trifolii nodulates Trifolium species, and Rhizobium meliloti nodulates species of Medicago and Melilotus. Bradyrhizobium japonicum is the symbiont of Glycine species. Research into the establishment of these plant-bacterium interactions has shown the importance of intercellular communication between the microbe and the host plant (reviewed in Halverson and Stacey, 1986; and Long, 1989). Several bacterial nodulation genes are involved in the early stages of nodule formation, of which the nodABC genes in Trifolium pratense, R. leguminosarum, Pisum, R. leguminosarum biovar viciae, and Medicago spp. are the most intensively studied. Recently, LeRouge et al. (1990) presented evidence that the nodABC genes in conjunction with the nodH and Q genes in R. meliloti are responsible for the synthesis of a substituted tetrasaccharide which acts as a signal to the plant. A compound similar but not identical to this has been identified in R. leguminosarum (Spanik et al., 1990). The nod genes are activated by the NodD protein(s) (Mulligan and Long, 1985; Rossen et al., 1985), which binds to the nod-box promoter sequence preceding these inducible genes (Hong et al., 1987; Fisher et al., 1988). Successful activation of these inducible operons depends on the presence of the right inducer molecules, which were identified as specific flavonoids (Peters et al., 1986; Kosslak et al., 1987; Zaat et al., 1987a; Hartwig et al., 1990; Banflavi et al., 1990).

Despite numerous genetic and biochemical data, not much is known about how the plant inducer and the NodD protein interact. Since flavonoids were found to accumulate in the inner membrane in which NodD was also localized, it has been proposed that they may interact directly at that site (Schlaman et al., 1989; Recourt et al., 1989). Moreover, the identification of certain domains in the primary structure of NodD responsible for flavonoid activation and recognition points to a direct interaction between the two (Spanik et al., 1989).

Some rhizobia contain more than a single nodD gene (Mulligan et al., 1986; Johnston et al., 1989). For example, B. japonicum has two nodD genes (Gottfert et al., 1989), of which the nodD1 gene is the most intensively studied and was found to be essential for flavonoid induction of the nod genes (Banflavi et al., 1988). The main inducers of the nodABC genes were isolated from soybean, the common host plant of this bacterium, and identified as the isoflavones genistein and daidzein (Banflavi et al., 1988; Gottfert et al., 1988; Kosslak et al., 1987). In contrast to most other rhizobia, the nodD1 gene in B. japonicum is also inducible, and genistein and daidzein appeared to be good inducers (Banflavi et al., 1988). In this study we report the presence of a number of other compounds excreted by soybean seeds and roots which are able to induce specifically the nodD1 gene but not the nodYABCSUIJ genes. These inducers were purified from soybean seeds and characterized chemically. Our current model for nod gene induction in B. japonicum is discussed.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions—** B. japonicum USDA135, obtained from H. Keyser (USDA, Beltsville, MD) was used as wild-type strain. B. japonicum USDA135 harboring a nodDl-lacZ fusion (plasmid pZB22) and USDA135 harboring a plasmid with a lacZ fusion in the nodY gene, which precedes the nodABC genes in B. japonicum (plasmid pZB32) (Banflavi et al., 1986; Nieuwkoop et al., 1987), were used to analyze inducing compounds from soybean Glycine max cv. Essex. The bradyrhizobia were maintained on RGY (Rhizobium defined yeast medium)-agar plates (Bishop et al., 1976) and cultured in liquid-RGY. Strains harboring plasmids were grown on...
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Biological Activities—The nod-inducing activities of seed extract and exudate, root exudate, and other compounds was assayed as β-galactosidase activity using the strains described above. β-Galactosidase assays were performed as described previously (Banfalvi et al., 1988; Miller 1972), using chlorophenol red-β-galactopyranoside (Boehringer Mannheim) as substrate in place of 2-nitrophenyl-β-D-galactopyranoside. Daidzin (Carl Roth KG, Karlsruhe, Federal Republic of Germany) and genistein (ICN Biomedicals, Cleveland, OH) were used as controls. Since inducibility of the bradyrhizobia was found to be optimal at low cell densities, the bacterial cultures were grown in RY to an A600 of 0.5-1.0, and prior to induction diluted in RY without tetracycline to an A600 of 0.1. Tetracycline was not added since β-galactosidase levels were higher and more consistent in the absence of the antibiotic during induction. The original optical density before dilution did not significantly affect maximum induction values as long as the cultures were diluted to 0.1. Time of induction was chosen between 8 and 20 h, since β-galactosidase levels reached a maximal level after 6-8 h and then remained constant (data not shown).

Preparation of Exudates and Extracts—Soybean seeds were surface sterilized by treatment with 1% sodium hypochlorite solution, commercial grade, for 10 min following five successive washings with sterile deionized water. Subsequently they were incubated for 10 min in 0.01 N HCl for 10 min and washed 10 times with sterile deionized water. Seed extract and seed exudate were obtained from seeds that were not surface sterilized prior to incubation in water. In these cases, exudates were obtained after 4 and 18 h of incubation in sterile deionized water. In other cases exudate was obtained from cotyledons and seed coat only (again, one cotyledon or seed coat/ml) to determine which of these was the major source of inducers. Root exudate was obtained by excising axenically grown seedlings. Seeds were first surface sterilized and germinated in the dark for 2 days on wet Whatman type 1 filter paper. Subsequently, 100 germinated seeds were transferred to a support of steel wire netting located at 5 mm above 400 ml of deionized water in a 2-liter beaker and covered with aluminum foil with the roots in the dark. After 4-7 days the seedlings were removed, and the root exudate was collected and filtered through a 0.45-μm membrane filter (Millipore Corp., Milford, MA).

Purification of Inducers—Soybean seed extract samples were thawed and passed through a 0.45-μm membrane filter (Millipore Corp.). The sample from 1,000 seeds was loaded in several subsamples of Sep-Pak C18 cartridges (Waters, Milford, MA). Subsequently, the cartridges were washed with 3 ml of 10% ethanol and eluted with 3 ml of 50% ethanol. The eluent of each separation was dried in a vacuum centrifuge (Savant Speed-Vac). The dried SE fraction was resuspended in 100 μl of 50% ethanol, representing an approximately 200-fold concentration in comparison with the starting material.

Aliquots of 50 μl were loaded onto a Waters HPLC® system (Millipore Corp.) fitted with a reverse phase μ-Bondapak C18 column (Waters, Millipore Corp.; dimensions 300 × 3.9 mm) and eluted at 1.0 ml/min from 0 to 5 min with an isocratic solvent system of 25% methanol (Milli-Q water, Millipore Corp.) and from 5 to 25 min with a linear gradient to 55% methanol followed by a linear gradient to 75% in 5 min. Subsequently, the column was washed with 100% methanol and reequilibrated with 25% methanol. Eluting compounds were monitored with a Waters model 484 absorbance detector at 280 nm.

Fractions were collected on a time basis, dried by vacuum evaporation, redissolved in 50% ethanol, and assayed for nod gene inducing ability using the nodD1-lacZ and nodY-lacZ fusion strains. Purity of the compounds was assayed by running the samples under identical conditions, as well as running under isocratic conditions with 25% acetonitrile (Mallinkrodt, HPLC grade) as eluent. Retention times for daidzin, daidzein (ICN Biomedicals), genistein, genistin (Spectrum Chemical Manufacturing Corp., Gardenia, CA), and coumestrin (Sigma) were used as reference. Purified compounds were stored at −20 °C.

Seed and root exudates were filtered and partly purified using Sep-Pak C18 cartridges essentially as described above and analyzed by HPLC.

β-Glucosidase and Acid Hydrolysis Treatments of Compounds—β-Glucosidase (Sigma) treatments of samples were performed in the presence of 100 μg/ml enzyme in 10 mM phosphate buffer, pH 5.5, for 3-18 h. After treatment, the samples were centrifuged for 5 min in an Eppendorf centrifuge at maximum speed and analyzed by HPLC under the same conditions as described above for purification of inducers. Blotting patterns were compared with known standards and untreated fractions.

Seed extract and purified compounds were subjected to acid hydrolysis for 1 h at 100 °C in 2 N HCl and analyzed by HPLC. Daidzin and genistin were used as controls for both β-glucosidase treatment and acid hydrolysis. A number of treated samples were also tested for induction of the nodY-lacZ fusion.

β-Glucosidase Activity of B. japonicum—β-Glucosidase activity of USDA135 was tested by incubation of genistin, daidzin, seed extract, and purified compounds with 1, 5, and 50 μg/ml enzyme in 10 mM phosphate buffer, pH 5.5, for 6-8 h. After incubation, the cultures were harvested and extracted with 50% ethanol. Cell debris was removed by centrifugation in an Eppendorf centrifuge for 10 min at 15,000 g. The supernatant was subsequently dried in a vacuum centrifuge and resuspended in 50 μl of 50% ethanol. These solutions were clarified by centrifugation for 10 min before 10-μl aliquots of each were analyzed by HPLC.

UV Spectrophotometry—UV spectra were analyzed using a Shimadzu UV-160 dual beam spectrophotometer. Authentic standards (genistin, daidzin, genistein, and daidzein) were compared with the peaks of interest. All spectra were taken in 50% ethanol.

Proton Magnetic Resonance Spectroscopy (NMR) Samples were dissolved or suspended in D2O and lyophilized. This procedure was repeated twice before the samples were dissolved in D2O or in deuterium oxide for NMR analysis. Spectra were taken at 300 K on either a Bruker AM250 or AM500 instrument.

Mass Spectrometry—Fast atom bombardment mass spectroscopy (FAB-MS) was performed on a VG ZAB-SE instrument. The spectra were taken in the positive mode with an accelerating voltage of 70 eV. Thioglycerol was used as the matrix. Gas liquid chromatography-mass spectroscopy (GC-MS) was performed on a Hewlett-Packard 5890/5970 GC/MSD system using a 30-m DB-1 column from J&W Scientific.

Chemical Analysis—Determination of glycosyl components was made by methanolysis with 1 M HCl in methanol at 100 °C for 18 h, followed by conversion of trimethylsilyl derivatives to Tri-Sil (Fisher Chemical Co.), and GC analysis using a 30-m DB-1. Details of trimethylsilyl methyl glycoside preparation were as described previously (York et al., 1985). Malonate was determined by methanolysis with 1 M HCl for 1 h at 80 °C followed by GC-MS analysis using a DB-1 column. The temperature program was from 50 to 200 °C at 4 °C/min. Under these conditions the retention time of the resulting dimethyl malonate was about 10 min. Authentic dimethyl malonate was used as a standard.

RESULTS

In contrast to Rhizobium spp., the nodD1 gene in B. japonicum is inducible. Fig. 1 shows activation of both the nodY-lacZ as well as the nodD1-lacZ fusion by addition of genistein. Approximately 0.5–1.0 μg/mg genistein is required for optimal induction of either fusion, and maximum induction for both fusions was found to be approximately 40-fold above background although the actual levels were quite different (see Banfalvi et al., 1988). When HPLC fractions from soybean seed extract were tested for induction ability on the nodY-lacZ fusion, induction was only found in fractions migrating with the known inducers genistein and daidzein. However, when the same fractions were tested on the nodD1-lacZ fusion, it appeared that several fractions were able to induce this fusion (see below). This result pointed to the presence of compounds other than genistein and daidzein. Since retention

1 The abbreviations used are: HPLC, high pressure liquid chromatography; FAB, fast atom bombardment; MS, mass spectroscopy; GC, gas chromatography.
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Fig. 1. Induction of nodD₁ and nodY by genistein. Induction of nod genes was measured as β-galactosidase activity from a nodD₁-lacZ (left axis, ○) and a nodY-lacZ (right axis, ●) fusion, respectively, in B. japonicum USDA135.

Fig. 2. Enhancement of nodY-lacZ induction by fractions from soybean seed extract separated by reverse phase chromatography (C₃₈) in the presence of suboptimal genistein (0.1 μM) concentrations. The induction activity of 0.1 μM genistein is shown as control.

Fig. 3. Panel A, separation of isoflavones from soybean seed extract by reverse phase chromatography (HPLC, C₃₈). Compounds 1–8 were collected for testing of induction ability and chemical analysis. Panel B, inducing activity of compounds 1–8 with the nodY-lacZ fusion. Panel C, Inducing activity of the compounds with the nodD₁-lacZ fusion. Controls are background β-galactosidase activity when no inducer was added and the activity after induction with 2 μM genistein. Compounds were added in concentrations of approximately 10 μg/ml. See “Results” for details on solubility.

with the nodD₁-lacZ fusion in contrast to the nodY-lacZ observation that several fractions showed inducing activity which might result in a higher induction of the nodD₁ fusion, led us to the hypothesis that there are inducers present in soybean seed extract which specifically induce the nodD₁ gene but not the nodYABCSUIJ operon. nodD₁ induction would likely lead to an enhanced level of NodD₁ protein, which might result in a higher induction of the nod·YABCSUIJ genes in the presence of suboptimal genistein concentrations (Fig. 2). These specific nodD₁ inducers were subsequently purified and chemically characterized.

Purification of the nodD₁-specific Inducers—To purify the nodD₁-specific inducers, the conditions for separation were optimized and adapted for larger quantities (Figs. 2 and 3). The soybean extract was purified and concentrated, prior to separation by HPLC reverse phase chromatography, by loading of the sample on Sep-Pak C₃₈ cartridges. The cartridges were washed and eluted with 50% ethanol as described under “Experimental Procedures.” Subsequently the sample was loaded on a reverse phase C₃₈ HPLC column, and peak fractions were collected. This was repeated several times to obtain enough material for chemical analysis.

The improved separation conditions resulted in the purification of eight major compounds according to absorbance at 262 nm (Fig. 3A). Samples of these peak fractions (especially peak 4), when tested for enhancement of nodY-lacZ expression in the presence of suboptimal levels of genistein, resulted in increased β-galactosidase activity (data not shown). These eight compounds were collected and tested for inducing ability on the nodY- or nodD₁-lacZ fusions. Fig. 3B shows the inducing activity of the compounds with the nodY-lacZ fusion, and Fig. 3C shows the activity with the nodD₁-lacZ fusion. In all cases comparable amounts were added (in the range of 1–10 μg/ml) in the assays; however, since the compounds 1–6 were not completely soluble in 50% ethanol, we could not establish the exact activity per μM. Therefore, these data should be viewed more qualitatively than quantitatively. For commercially available genistin and daidzin it was found that the sensitivity of both fusions was approximately 5 × lower when compared with genistein and daidzein, respectively, as inducer. Compounds 7 and 8 had retention times identical to daidzein and genistein, respectively, which was also confirmed by the addition of genistein and daidzein to the seed extract samples as internal standards. Both were active inducers on both the nodY-lacZ as well as the nodD₁-lacZ fusion. Peak 6 was found also to be active on both fusions although induction of the nodY-lacZ fusion was lower than found for daidzein and genistein. Compounds 2 and 5 showed only a very weak induction of nodD₁-lacZ and had no activity on nodY-lacZ.
Compounds 1, 3, and 4 showed background activity with the nodY-lacZ fusion; however, they were found to be active inducers of the nodD1 gene. Remarkably, addition of the void volume fraction resulted in a decrease of induction, which might point to the presence of induction-inhibiting compound(s) in this fraction.

Chemical Characterization of Purified Inducers—The HPLC-purified compounds were chemically analyzed to reveal their structures. UV absorption spectra of purified compounds revealed that the spectra of compounds 1, 4, and 7 show strong similarities to spectra taken from the reference compounds daidzein and daidzin. Compounds 3, 6, and 8 are almost identical to the spectra of genistein and genistin. The spectra of compounds 2 and 5 differ from the other spectra but are almost identical to each other (Fig. 4).

Acid hydrolysis as well as β-glucosidase treatment of soybean seed extract and purified compounds 1–6 resulted in an increase in induction activity when tested with the nodY-lacZ fusion. Moreover, HPLC analysis of these treated samples showed a shift in retention times to those corresponding to daidzein and genistein (Fig. 5). A small peak with a retention time between the retention times of daidzein and genistein was increased also when compounds 2 and 5 and total seed extract were treated. Interestingly, compounds 1–5 appeared to be more resistant to β-glucosidase treatment than compound 6, since they required substantially longer incubation times for successful hydrolysis. These results corroborate results obtained by UV spectroscopy in that they point to compounds 1, 3, 4, and 6 as being derivatives of genistin and daidzein. Compounds 2 and 5 might degrade to a compound with a retention time between that of daidzein and genistein. Indeed, this might be the retention time for glycitein (see below), but that could not be confirmed because of the lack of pure glycitein.

Comparison of NMR spectra of the eight compounds from soybean seeds with the spectra for daidzein (4',7-dihydroxyisoflavone), genistein (4',5,7-trihydroxyisoflavone) and the published spectral data for glycitein (4',7-dihydroxy-6-methoxyisoflavone) (Markham and Mabry, 1975) revealed that all eight compounds are derivatives of these three isoflavones (Figs. 6 and 7). In fact the NMR spectra for compounds 7 and 8 are identical to those for daidzein and genistein, respectively, thereby identifying these two compounds (data not shown). A review of these isoflavones as well as many other flavonoid compounds has been published (Harborne, 1988).

The NMR spectra of all eight compounds show that each compound has a singlet between 6.80 and 8.5, which is characteristic for the C-2 proton of isoflavones (Markham and Mabry, 1975). The C3 proton of flavones resonates between 6.60 and 6.5 (Markham and Mabry, 1975).

Compounds 1–6 are all glycosylated. Preparation and analysis of trimethylsilyl methyl glycosides revealed that the only glycosyl component was glucose. Integration of the proton NMR spectra for these compounds showed that the daidzein derivatives, compounds 1 and 4, have an isoflavone/glucosyl anomeric proton ratio of 8:1 whereas the genistein and glycine-tein derivatives, compounds 2, 3, 5, and 6, have a ratio of 7:1. Thus all the glucosylated derivatives have 1 glucosyl residue/isoflavone. In each case the coupling constant of the anemic proton is between 7.0 and 7.5 Hz, which shows that the glucosyl residue in each isoflavone is β-linked.

The Daidzein Derivatives—The NMR spectra for standard daidzin (7-glucosyl/daidzin) and compound 1 are shown in Fig. 7, A and B, respectively. The assignments for the aglycone protons (between 6.5 and 8.5) of each molecule are based on the literature values for daidzein (Markham and Mabry, 1975). Slight differences in the chemical shifts of the aglycone protons between daidzin and compound 1 are because the solvent for daidzin was CD3OD and for compound 1, D2O. As with daidzin, the H6 and H8 protons of compound 1 are shifted downfield by 0.4 ppm when compared with daidzin, H6 = 6.96 and H8 = 8.65 (spectrum not shown). This downfield shift is characteristic for isoflavones which are

![Fig. 4. UV absorption spectra of compounds 1–8, with genistein, genistin, daidzein, and daidzin as controls.](image-url)
The compounds were identified as daidzin (Fig. 7A) except for the chemical shifts and characteristic coupling constants, 63.95 and 4.35. This downfield shift is characteristic of glycosyl malonyl derivatives of flavonoid glycosides (Beck and Knox, 1990).

The NMR spectrum of genistin and compound 3 are shown in Fig. 7, C and D. The proton assignments for genistein are based on the literature (Markham and Mabry, 1975). The resonances at 6.35 and 6.50 for compound 3, and 6.55 and 6.70 for genistin, are doublets with coupling constants of about 2.5 Hz, characteristic of the meta coupling between H6 and H8. The 0.2- and 0.3-ppm downfield shift of H6 and H8 for both molecules when compared with those protons from the glycone genistein, H6 = 6.15 and H8 = 6.35 (spectrum not shown), shows that the glycosyl residue is at O7 for both isoflavonoids. As with the malonylated daidzin molecule discussed above, the 0.5-ppm downfield shift of the glycosyl H6' protons indicates that compound 3 contains an acyl group at C6 of the glycosyl residue. This was confirmed by GC-MS identification of dimethylmalonate released by mild methanolation. FAB-MS analysis (Fig. 8B) shows that compound 3 has ions of m/z = 519 (M+H+) and 537 (M+K+). The 519 ion is 86 mass units greater than the respective ion for genistin, M+H' = 433 (spectrum not shown) and is consistent with a malonyl substituent. Thus compound 3 is 6''-O-malonylgenistin. This compound, as with 6''-O-malonyldaidzin, was recently reported to be present in soybean tissue by Graham and co-workers (Graham et al., 1990).

The Glycitein Derivatives—The NMR spectra of compounds 2 and 5 are shown in Fig. 7, E and F, respectively. In both cases the "A-ring" protons, the singlets at about 7.4 and 7.7, are shifted 1.0 ppm downfield compared with the A-ring protons (H6 and H8) of genistin. The fact that these resonances do not show doublet characteristics of the H6 and H8 meta coupling observed for genistein and its derivatives shows that the A-ring protons for compounds 2 and 5 are located para to one another. Thus compounds 2 and 5 contain substituents at C6 and C7 and protons at C5 and C8. For both compounds, the proximity of H5 to the C4 carbonyl function would cause it to resonate downfield relative to H8. Thus the singlets at about 7.75 for compounds 2 and 5 can be assigned to the H5 protons for these molecules. This assignment is also consistent with published spectral data for other 6, 7, 4'-trihydroxyisoflavones (Markham and Mabry, 1975). Additionally, both compounds 2 and 5 have a sharp singlet at 63.95 which can be assigned to an aromatic methoxyl group. The chemical shifts of the "B-ring" protons are consistent with isoflavones that do not contain substituent groups at the C4' hydroxyl group (Markham and Mabry, 1975). Thus the glycosyl and methoxyl substituents are located on the A-ring of compounds 2 and 5. The exact position of the methoxyl and glycosyl substituents (i.e. at C6 and C7, respectively) was elucidated by NMR spectroscopy in which the nuclear Overhauser effect between H6 and H5 and the methoxyl protons was determined. Irradiation at 87.75, assigned to H5, results in a nuclear Overhauser effect at 83.95, and irradiation at 83.95 causes a nuclear Overhauser effect at 87.75. A nuclear Overhauser effect was not observed between H6 and the methoxyl protons. This result shows that the methoxyl group is in close proximity to H5, not H6, and therefore must be located at C6, not C7.

The spectrum of compound 2 is different from that of glycosylated at O7 (Markham and Mabry, 1975). The H6" protons of the daidzin glycosyl residue are apparent from their chemical shifts and characteristic coupling constants, 63.95 (J = 12, 2.5 Hz) and 83.73 (J = 12, 5.7 Hz). In the case of compound 1, these resonances are shifted downfield to 64.55 and 4.35. This downfield shift is characteristic of glycosyl residues which are acylated at C6 and has been reported for malonyl derivatives of flavonoid glycosides (Beck and Knox, 1971). The presence of malonate was confirmed by GC-MS analysis of dimethylmalonate released from compound 1 after mild methanolation. FAB-MS analysis of compound 1 (Fig. 8A) gives ions at m/z = 503 (M+H+), 525 (M+Na+) and 541 (M+K+). The 503 ion is 86 mass units greater than the respective ion for genistin, M+H' = 433 (spectrum not shown) and is consistent with a malonyl substituent. Thus compound 3 is 6''-O-malonylgenistin. This compound, as with 6''-O-malonyldaidzin, was recently reported to be present in soybean tissue by Graham and co-workers (Graham et al., 1990).
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FIG. 7. NMR spectra of the isoflavone nod inducers isolated from soybean. A, standard daidzin. B, 6'-O-malonyldaidzin (peak 1). C, genistin (peak 6). D, 6'-O-malonylgenistin (peak 3). E, glycitin (peak 5). F, 6'-O-malonylglycitin (peak 2).

compound 5 in that the glucosyl H6" protons are shifted downfield by 0.8 ppm compared with those protons from compound 5. As with compounds 1 and 3 (see above results), this indicates that compound 2 is an acylated derivative of compound 5. FAB-MS of compound 5 shows an m/z of 447 (M+H+) (spectrum not shown). FAB-MS of compound 2 (Fig. 8C), shows ions at m/z = 533 (M+H+), 555 (M+Na+) and 571 (M+K+). The increase in molecular weight of 86 for compound 2 compared with compound 5 is consistent with the addition of a malonyl group. Thus the data indicate that compound 5 is glycitin (7-O-glucosylglycitein) and compound 2 is 6'-O-malonylglycitin. Glycitin has been reported in soybean tissues (Eldridge, 1982); however, to our knowledge, this is the first report of its malonyl derivative.

Are the nodD1 Inducers Also Present in Root and Seed Exudate?—Since an ethanol extract of the seeds might be different from seed exudate and since it has been reported that seed exudates and root exudate from certain plants differ significantly (Hartwig et al., 1989; Maxwell et al., 1989), we tested both seed and root exudate for the presence of the above described compounds. Soybean seed exudates, obtained after 4-h as well as after 18-h imbibition of the seeds in sterile water, were analyzed by HPLC and compared with seed extract. As shown in Fig. 9, the chromatograms were very similar, which makes it very likely that compounds 1-8 are also present in seed exudate. Similarly, when root exudate was analyzed by HPLC for the presence of these compounds, it was again found that the patterns were similar to seed extract (Fig. 9C). Moreover, when root and seed exudate were treated with β-glucosidase and subsequently analyzed by HPLC, it appeared that the presumptive glycosylated compounds were indeed hydrolyzed, since they were strongly
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FIG. 8. FAB-MS spectra of the malonyl isoflavone derivatives. A, 6"-O-malonyldaidzin (peak 1). B, 6"-O-malonylgenistin (peak 3). C, 6"-O-malonylglycitin.

decreased and the genistein and daidzein peaks were increased after treatment (data not shown). Exudate collected from the seed coat and cotyledons separately, and analyzed by HPLC, revealed that cotyledons were the major source of inducers, based on peak height/mg of material. The results indicate that compounds similar to compounds 1-8 are present in both seed and root exudate of soybeans. The actual amount of inducer present in root and seed exudate differed significantly based on HPLC elution data as well as induction data, indicating that the seed most likely is a major source of inducers during germination of the soybean seedling.

A comparison between root exudate obtained from axenically grown soybean seedlings and root exudate from seedlings inoculated with B. japonicum USDA135 showed high similarity based on HPLC analysis, indicating that the root exudate composition appears not to be significantly affected by the presence of B. japonicum bacteria (data not shown).

Does B. japonicum Hydrolyze the Specific nodD₁ Inducers into Their Aglycone Form?—B. japonicum USDA135 was tested for its possible ability to produce β-glucosidases, which could hydrolyze the specific nodD₁ inducers to their aglycone forms. Purified compounds and commercially available daidzin and genistin, as controls, were incubated with B. japonicum. After 18 h of incubation, ethanol was added to the culture to a final concentration of 50%, the suspension was clarified by centrifugation, and the extract was analyzed by HPLC. It appeared that neither of the nodD₁ inducers nor any of the control compounds was affected in their retention time, and no peaks were observed with retention times of the aglycones as a result of incubation with B. japonicum (data not shown). These results corroborate the induction data (Fig. 3) since, if significant hydrolysis occurred in the presence of B. japonicum, one would expect the induction of nodY-lacZ by the aglycone inducers. Therefore, the results indicate that B. japonicum apparently does not possess significant β-glucosidase activity under these conditions.

DISCUSSION

Results from this study show clearly that soybean seed extract contains derivatives of the known inducers genistein
and daidzein, which are specific inducers of the nodD1 gene but do not induce the nodYABCSUIJ operon. These specific inducers have been purified and characterized as glucosides of genistin, daidzein and glycitein (Fig. 6). Interestingly, genistin (compound 6), the 7-O-glucoside of genistin, could also act as an inducer of the nodYABCSUIJ operon whereas 6-malonyl-genistin is not active. Apparently, the size of the side chain or the chemical characteristics of the malonyl group itself prevent this compound from being an inducer. The same was found for daidzin, which is also active on the nodY-lacZ fusion, and its derivatives compounds 1 and 4. These compounds were found only to be active inducers on the nodD1-lacZ fusion.

Although amounts of inducer used in the assays seem to be rather high, the specific induction of the nodD1-lacZ fusion does not result from impurities or chemical degradation of the glycosylated compounds into their aglycone form by B. japonicum, since if that would have occurred, the nodY-lacZ fusion would also be induced. β-Glucosidase activity of bacteria was found to play an important role in virulence of the closely related species Agrobacterium tumefaciens although not all strains tested showed this activity (Morris and Morris, 1990). However, it was found that B. japonicum USDA135 does not possess β-glucosidase activity under the conditions used since it is not able to hydrolyze the glycosylated soybean seed extract compounds nor the commercially available daidzin and genistin. In addition, the nodD-specific inducers also appeared to be less sensitive to β-glucosidase treatment.

The differential induction of the nodD1 and the nodYABCSUIJ genes points to functional differences between the nod boxes of these genes. The nod box preceding the nodD gene was found to be a divergent but functional one (Nieuwkoop et al., 1987; Wang and Stacey, 1991) when compared with the consensus nod box (Spanik et al., 1987). Recently, Wang and Stacey (1991) proposed a new consensus for the nod box as a tetramer of a 9-base pair sequence, arranged in two doublets. When compared with this new consensus sequence, the nodD1 nod box has only two of these repeats, whereas the nodYABCSUIJ nod box is highly homologous to the complete 9-base pair tetramer nod box. These differences in nod box structure might explain differences in activation by inducers. A model could be proposed in which NodD binds to the different nod box sequences preferentially depending on which inducer is present. Functional exchange of nod boxes or mutations in the nod box preceding the nodYABCSUIJ operon should clarify whether this difference explains the activation by the glycosylated compounds found in soybean extracts/exudates.

HPLC analysis of soybean seed and root exudates revealed that the glycosylated nodD1 inducers are not only present in seed extract but also in seed and root exudate. No strong differences in composition between root and seed exudate were observed as described for alfalfa (Hartwig et al., 1989). A comparison between the amount of inducer found in seed and root exudate showed that the seed is most likely the major source of inducer during germination of the soybean seedling. Interestingly, it appeared that the soybean cotyledons contained a relatively larger amount of inducers compared with the seed coat whereas in the case of peanut seeds an opposite result was found (Smit et al., 1990).

No significant differences were found in the composition of soybean root exudate in the presence or absence of B. japonicum. This result corroborates data from Mathews et al. (1989), who did not find differences in the level of induction. In the case of Vicia, it appeared that the levels as well as the composition of the root exudate were affected in the presence of its symbiont R. leguminosarum (Van Brussel et al., 1990; Recourt et al., 1990). It remains to be determined whether such differences are plant specific or dependent on test conditions.

A model consistent with our current knowledge of nod gene induction in B. japonicum is shown in Fig. 10. nodD1 is expressed at a low level in the absence of inducers. Its expression can be strongly increased in the presence of flavonoid inducers. These inducers can be the same ones that are required for activation of the nodYABCSUIJ operon, daidzein and genistin, or derivatives of these, as characterized above. The higher level of nodD1 expression would likely lead to higher levels of NodD1 protein in the cell, which could enhance the induction of the other nodD1-dependent genes in the bacterium. Glycosylated isoflavones were found to be able to induce nodD1 specifically, presumably leading to an increased level of NodD1 protein in the cell. Since the actual amount of genistin needed for optimal induction of the nodYABCSUIJ operon is high in comparison with other rhizobias (e.g. Zaat et al., 1987a, 1987b; Hartwig et al., 1990) as well as in comparison with other Bradyrhizobias (Smit et al., 1990), the function of this elevated level of NodD1 protein might be to enhance the expression of the nodYABCSUIJ operon under suboptimal genistin concentration. However, nod gene regulation in B. japonicum is complex, and the model shown in Fig. 10 is certainly incomplete but still useful as a guide for further experiments. For example, other functions might be linked to specific nodD1 induction, e.g. chemotaxis as found in R. meliloti (Caetano-Annoles et al., 1988). Initial characterization of the nodD1-specific inducers revealed that they do not show an increased chemotaxis when compared with genistin in a standard assay. However, the nodD1-specific inducers are more hydrophilic, and therefore it can be argued that they are able to diffuse more easily under natural (soil) conditions.

It should be noted that the two promoter regions of the nodD1 gene and the nodYABCSUIJ operon show an overlap. Whether this overlap plays a role in the specific expression of these genes is not known, but it is potentially important for an understanding of nod gene regulation. Both fusions used in this study contain the whole region between the nodD1 and the nodY genes, indicating that any possible interference between the two promoters would also occur in the test strain. Future research should focus on the role of specific nodD1 regulation in the nodulation process and the functional requirements of the nod box for the response to nodD1-specific inducers.

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