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Diverse Flavonoids Stimulate NodD1 Binding to *nod* Gene Promoters in *Sinorhizobium meliloti*

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NodD1 is a member of the NodD family of LysR-type transcriptional regulators that mediates the expression of nodulation (*nod*) genes in the soil bacterium *Sinorhizobium meliloti*. Each species of rhizobia establishes a symbiosis with a limited set of leguminous plants. This host specificity results in part from a NodD-dependent upregulation of *nod* genes in response to a cocktail of flavonoids in the host plant's root exudates. To demonstrate that NodD is a key determinant of host specificity, we expressed *nodD* genes from different species of rhizobia in a strain of *S. meliloti* lacking endogenous NodD activity. We observed that *nod* gene expression was initiated in response to distinct sets of flavonoid inducers depending on the source of NodD. To better understand the effects of flavonoids on NodD, we assayed the DNA binding activity of *S. meliloti* NodD1 treated with the flavonoid inducer luteolin. In the presence of luteolin, NodD1 exhibited increased binding to *nod* gene promoters compared to binding in the absence of luteolin. Surprisingly, although they do not stimulate *nod* gene expression in *S. meliloti*, the flavonoids naringenin, eriodictyol, and daidzein also stimulated an increase in the DNA binding affinity of NodD1 to *nod* gene promoters. In vivo competition assays demonstrate that noninducing flavonoids act as competitive inhibitors of luteolin, suggesting that both inducing and noninducing flavonoids are able to directly bind to NodD1 and mediate conformational changes at *nod* gene promoters but that only luteolin is capable of promoting the downstream changes necessary for *nod* gene induction.

The soil bacterium *Sinorhizobium meliloti* and the leguminous plant alfalfa form a symbiotic relationship that results in the differentiation of *S. meliloti* into nitrogen-fixing bacteroids that reside in nodules formed on alfalfa roots. To initiate the symbiosis, alfalfa roots and seeds excrete a cocktail of nodulation-inducing molecules composed predominantly of flavonoids (15, 34, 60). In response, NodD proteins in *S. meliloti* activate transcription of *nod* genes, which encode the enzymes responsible for the synthesis of Nod factor, a lipochito-oligosaccharide signal required for symbiotic development in alfalfa (17). The genome of *S. meliloti* encodes three NodD polypeptides, NodD1, NodD2, and NodD3, that share greater than 77% amino acid identity (29, 32, 48, 67). NodD1 and NodD2 require plant-derived inducers for activity (49), while NodD3, when overexpressed, is active in the absence of flavonoids (33, 48).

Each species of rhizobia establishes a symbiosis with a limited set of host plants depending in part on the cocktail of flavonoids in the plant exudate. For example, *S. meliloti* nodulates alfalfa, while *Rhizobium leguminosarum* bv. trifolii nodulates clover (64). High-performance liquid chromatography (HPLC) analysis demonstrates that most legume seed and root exudates contain approximately 10 different flavonoid compounds (59, 81). Flavonoids that do not stimulate *nod* gene induction can act as inhibitors that are capable of antagonizing *nod* gene expression (13, 84). In the soil, rhizobia are exposed to a variety of flavonoids, and so *nod* gene expression is likely affected by a combination of stimulatory and inhibitory interactions (12, 13, 15, 30).

No direct biochemical evidence has been reported for flavonoid binding to NodD, but several lines of genetic evidence indicate that NodD is directly involved in flavonoid perception. First, a functional *nodD* gene is both necessary and sufficient to mediate *nod* gene induction in the presence of flavonoids (49, 83). Second, point mutations in NodD from *R. leguminosarum* bv. trifolii and *Rhizobium leguminosarum* bv. viciae result in *nod* gene transcription in response to inducers which are normally inactive (7, 45). Furthermore, data from several studies suggest that NodD controls the response of rhizobia to flavonoids in a species-specific manner (4, 34). Spaink and colleagues directly tested this hypothesis by introducing plasmids carrying *S. meliloti nodD1*, *R. leguminosarum* bv. viciae *nodD*, or *R. leguminosarum* bv. trifolii *nodD* into a strain of *R. leguminosarum* bv. trifolii and assaying for *nod* gene induction in response to a spectrum of purified flavonoids (73, 81). By changing the source of *nodD*, *nod* gene transcription was initiated in response to distinct sets of flavonoid inducers. For example, when *S. meliloti nodD1* was expressed, *nod* gene transcription was initiated only in response to the alfalfa-derived inducer luteolin. The interpretation of these results is complicated by the fact that the *nodD* genes were expressed under their native promoters, carried on plasmids containing multiple *nod* genes, and assayed in a Sym$^-$ strain of *R. leguminosarum* bv. trifolii that potentially lacks genes involved in flavonoid perception. For example, both *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii NodD proteins have been shown to autoregulate their expression (45, 65), while *S. meliloti* NodD1 does not (49). In addition, *S. meliloti* NodD1 showed only 25% of the levels of *nod* gene expression mediated by NodDs from other species of rhizobia (73). Thus,

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Rm 1021	*S. meliloti* SU47 str-21 Sm^r	46
A2105	Rm 1021 *nodD1*::Tn*5(9B7) nodD2*::*tm nodD3*::*sp/g-1 nodC'-'lacZ*	33
ANU843	*R. leguminosarum* bv. trifolii wild type	63
Rlv 300	*R. leguminosarum* bv. viciae wild type	37
Plasmids		
pBS SK	Cloning vector, Ap^r	Stratagene
pCRII	Cloning vector, Ap^r	Invitrogen
pRF771	pTE3 with modified polylinker	79
pTE3	IncP, Tc^r, *Salmonella enterica* serovar Typhimurium *trp* promoter	14
pNodD1-ST	In-frame fusion of *nodD1* to the strep-tag	80
pRmE43	*S. meliloti nodD1* in pTE3	14
pGroESL	*E. coli groESL* operon in pTG10	50
pRF796	*nodF nod* box in pCRII	This study
pMP50	*S. meliloti nodD1* in pCRII	This study
pMP51	*R. leguminosarum* bv. trifolii *nodD* in pCRII	This study
pMP52	*R. leguminosarum* bv. viciae *nodD* in pCRII	This study
pMP52a	*R. leguminosarum* bv. viciae *nodD* in pBS SK^-	This study
pMP53	*S. meliloti nodD1* in pRF771	This study
pMP54	*R. leguminosarum* bv. trifolii *nodD* in pRF771	This study
pMP55	*R. leguminosarum* bv. viciae *nodD* in pRF771	This study

unequal levels of NodD could explain the differential levels of *nod* gene induction in response to purified flavonoids.

NodD is a member of the LysR family of transcriptional activators. Members of the LysR family are ~35 kDa in size, have an N-terminal helix-turn-helix DNA binding domain, frequently show autorepression, and usually require inducers for activity (68). Inducers have been found to influence the function of LysR-type activators at several distinct steps during transcription initiation, thus directly translating extracellular signals into gene transcription (68). Specifically, inducers can alter the interaction with DNA by stimulating a change in DNA binding affinity (11, 77) or in promoter architecture (36, 53, 61, 74). Inducers also increase the degree of multimerization, which can extend the region of promoter DNA that the activator binds (10, 23, 54). Finally, inducers can change the conformation of activators to expose domains that interact with RNA polymerase (RNAP), facilitating formation of the closed transcription complex (1, 40, 78). Two LysR family members, CysB and DntR, have been crystallized as dimers, with the two monomers enclosing a cavity postulated to bind ligands (71, 75). Supporting this hypothesis, both proteins cocrystallize with small molecules that mimic anti-inducers in the putative ligand binding cavity.

During transcriptional activation, NodD binds to a 55-bp highly conserved sequence in *nod* gene promoters termed the *nod* box (18, 66), which can be divided into two half-sites that lie on the same face of the DNA helix (19). In cross-linking and bacterial two-hybrid studies, NodD1 shows dimerization (R. F. Fisher and S. R. Long, unpublished data), although it is unclear if it acts as a higher-order oligomer in vivo. Circular permutation analysis has demonstrated that the binding of NodD3 to *nod* gene promoters induces a bend of between 52° and 68° at the center of the *nod* box (19). Genetic studies revealed that the chaperonin GroEL is required for *S. meliloti* NodD activity in vivo, and the DNA binding activity of NodD is decreased in cell extracts from a *groESL* mutant (50).

The molecular mechanism by which flavonoids stimulate NodD1 to activate transcription remains unclear. However, recent studies have provided the first in vitro evidence of an effect of the flavonoid luteolin on NodD1 activity (80). NodD1 isolated from *S. meliloti* or from *Escherichia coli* copurifies with the chaperonin GroEL (80). In the presence of GroES and ATP, which are required for GroEL to stimulate protein folding, luteolin stimulated the binding of purified NodD1 to the *nod* box (28, 80). Furthermore, a size exclusion chromatography (SEC)-HPLC fraction of purified NodD1 containing GroEL bound to *nod* gene promoters only in the presence of GroES, ATP, and luteolin (80). The authors hypothesized that the addition of GroES and ATP to the NodD1-GroEL complex facilitated correct folding of NodD1 with luteolin, resulting in an increase in DNA binding activity (80).

In this study, we present genetic and biochemical data in support of the hypothesis that luteolin directly interacts with Nod1 to promote *nod* gene transcription. We expressed orthologous NodD family members from several rhizobial species in an *S. meliloti* strain where all endogenous NodD family members have been genetically disabled. Induction of *nod* gene transcription was then assayed using several different flavonoids to determine if the profile of flavonoids capable of mediating *nod* gene induction is dependent on the source of NodD. To understand the biochemical effects of the luteolin-NodD1 interaction, we assayed the DNA binding activity of purified NodD1 treated with luteolin either in vivo or in vitro. We also examined whether the DNA binding activity changed in the presence of flavonoids other than luteolin. Based on these results and on data from in vivo competition assays between inducing and noninducing flavonoids, we suggest a model for luteolin stimulation of NodD1 activity in *S. meliloti*.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. Bacterial cultures were grown in LB or TY

(tryptone yeast extract) (5) medium supplemented with the appropriate antibiotics (ampicillin, 50 to 100 μg/ml; spectinomycin, 50 μg/ml; tetracycline, 10 μg/ml; streptomycin, 500 μg/ml; neomycin, 50 μg/ml). *E. coli* was grown at 37°C and *S. meliloti* at 30°C. Plasmids were introduced into *S. meliloti* by triparental mating using the helper plasmid pRK2013 (26).

Chemicals. Luteolin and eriodictyol were obtained from Atomergic Chemicals (Farmingdale, NY). Daidzein, 7-hydroxyflavone, and naringenin were obtained from Sigma Chemical Co. (St. Louis, MO). Flavonoids were dissolved in *N*,*N*-dimethylformamide (DMF) to make a concentrated stock solution and diluted in TED_{100} to make a working solution as previously described (80).

DNA manipulation and sequencing. Rhizobial genomic DNA was isolated from Rm1021, ANU843, and Rlv300 using the Puregene kit (Gentra Systems, Inc., Minneapolis, MN). Plasmid DNA was isolated using the QIAGEN Mini-Prep kit (QIAGEN, Inc., Valencia, CA) or Wizard DNA purification system (Promega, Madison, WI). DNA was purified from agarose gel slices using the Geneclean kit (Bio 101, Irvine, CA). Fluorescent DNA sequencing was carried out on an Applied Biosystems Prism 310 machine (Perkin-Elmer, Inc., Wellesley, MA).

Plasmid construction. A 1.1-kb fragment amplified by PCR from *S. meliloti* genomic DNA using an N-terminal primer (NodD N′, ATTGATTGTTTGGAT) and C-terminal primer (Rm-NodD1-C′, GAAAAGCGAGGAGT) to *nodD1* was cloned into pCRII to create pMP50. Similarly, a 1.2-kb fragment amplified by PCR from *R. leguminosarum* bv. trifolii genomic DNA using NodD N′ and Rt NodD-C′ (CCTCATCAAAACTAA) was cloned into pCRII to create pMP51. *R. leguminosarum* bv. viciae genomic DNA was used as a template with Rlv NodD N′3 (GAAAGATTGGTAAAATTG) and Rlv NodD-C′ (GTAATACCT CCTTT) to amplify by PCR a 1.1-kb fragment that was cloned into pCRII to create pMP52. Each N-terminal primer is based on DNA sequence 20 to 40 bp upstream of the *nodD* translation start site, and each C-terminal primer is based on DNA sequence 35 bp downstream of the translation stop site. Insert orientation was determined by DNA sequencing.

We reversed the orientation of *R. leguminosarum* bv. trifolii *nodD* with respect to the polylinker of pMP52 by cloning an EcoRI fragment from pMP52 into EcoRI-digested pBS SK^-, creating pMP52a. SpeI/EcoRV-digested inserts from pMP50, -51, and -52a were cloned into XbaI/ScaI-digested pRF771 to yield pMP53, -54, and -55, respectively.

Purification of NodD1. Streptavidin-tagged NodD1, referred to as affinity-purified NodD1 in the text, was isolated from cultures grown in the presence of DMF or 3 μM flavonoid on a streptavidin-agarose affinity column as described previously (80). SEC-HPLC-purified NodD1 was isolated from affinity-purified NodD1 separated on a Zorbax Bio series GF-250 column using a Dionex DX500 system as previously described (80). Strep-tagged NodD1-activated *nod* gene expression was similar to activation by wild-type NodD1 in a strain of *S. meliloti* carrying insertional mutations in *nodD1*, *nodD2*, and *nodD3* (data not shown), demonstrating that the Strep tag does not interfere with NodD1 function.

Total protein concentration of the purified NodD1 containing GroEL was determined using a modified Bradford assay (Bio-Rad, Hercules, CA). To determine the individual concentrations of NodD1 and GroEL in each protein sample, Coomassie blue R250 (Sigma-Aldrich, St. Louis, MO)-stained sodium dodecyl sulfate-polyacrylamide (12.5%) gels were scanned on an AlphaImager 2000 (Alpha Innotech, San Leandro, CA), and the amount of protein in each band was quantified in relationship to the total amount of protein in the sample. The only two protein species visible on a Coomassie-stained gel were NodD1 and GroEL. Both affinity-purified and SEC-HPLC-purified protein samples contained 4.5 NodD1 monomers per molecule (14-mer) of GroEL.

In vitro GroESL incubation and inducer treatment. SEC-HPLC-purified NodD1 was preincubated for 10 min with the GroESL cycling system prior to a 5-min incubation with 10 μM of the indicated inducer, as previously described (80).

Electrophoretic mobility shift assay (EMSA). An end-labeled *nodF nod* box DNA fragment was prepared by digesting pRF796 with EcoRI, filling in the ends of the digestion products with [α-^{32}P]dATP and dTTP, and isolating the insert DNA containing the 250-bp *nodF nod* box on a polyacrylamide gel. DNA was extracted from the gel slice with the Qiaex II kit (QIAGEN, Inc., Valencia, CA). The recovery of labeled *nodF nod* box fragment was estimated at 70%.

NodD1 (0 to 7.3 μM) was incubated with end-labeled *nodF nod* box DNA (6 fmol, unless otherwise indicated) as previously described (18). NodD1-DNA complexes and free DNA were separated on 5% Tris-borate-EDTA–polyacrylamide gels. Bands representing unbound DNA and the NodD1-DNA complex were quantified on a phosphorimager (GS-363; Bio-Rad, Hercules, CA). The fraction bound represents the fraction of NodD1-DNA complex per total input DNA.

Data analysis. Because individual protein preparations varied in total activity, we used NodD1 protein samples that showed consistent DNA binding activity in the absence versus presence of luteolin to compare the effects of noninducing flavonoids. We identified and discarded outliers using Dixon's or Grubb's test ($P < 0.05$) (62, 72). We analyzed DNA binding by NodD1 that was isolated from cells grown in the presence or absence of flavonoids at each protein concentration by the Wilcoxon two-sample test. We determined significance by calculating a two-tailed t_s for tied variates (62, 72). In the box plots, the boxes are divided at the median and the top and bottom are drawn at the upper and lower quartiles (25). Top and bottom whiskers represent 1.5 interquartile ranges of the top and bottom, respectively. Observations beyond these limits are plotted as individual points.

DNase I footprinting. NodD1 purified from cells grown in the absence or presence of 3 μM luteolin was subjected to EMSA. Both unbound DNA and the NodD1-DNA complex were subjected to DNase I cleavage in the gel slice, as previously described (16).

β-Galactosidase assays. *S. meliloti* A2105 cultures expressing the indicated plasmid were grown for 3 to 20 h with the specified compounds. No difference was seen in *nod* gene induction levels from bacteria grown for 3 h versus 20 h (data not shown). β-Galactosidase activity of 300 to 750 μl of each culture was measured as described elsewhere (47).

RESULTS

Genetic evidence that NodD is the flavonoid sensor. To determine whether changing the source of *nodD* in *S. meliloti* alters the sensitivity of the bacterium to the spectrum of flavonoids capable of inducing *nod* gene expression, we expressed the open reading frames (ORFs) encoding *S. meliloti nodD1*, *R. leguminosarum* bv. viciae *nodD*, and *R. leguminosarum* bv. trifolii *nodD* from a constitutive promoter in an *S. meliloti* background carrying insertional mutations in *nodD1*, *nodD2*, and *nodD3* (14, 33, 69, 70). Consequently, the only source of NodD in these experiments is the cloned ORF that is being expressed. Since the three ORFs are all in the same expression context, we assume that they are all being expressed at indistinguishable levels. For each experiment, *nod* gene induction was assayed by measuring β-galactosidase levels from an *S. meliloti nodC′-′lacZ* reporter. Because *nod* boxes from different rhizobia show a high degree of homology, NodDs from *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii are predicted to bind to the *S. meliloti nodABC* promoter (16). Flavonoids were selected based on their reported presence in compatible host plant exudates and on previous work demonstrating their ability to stimulate NodD activity (15, 56, 60). To reflect the estimated concentration in the rhizosphere, *nod* gene induction was assayed at a 3 μM flavonoid concentration (30, 57).

S. meliloti expressing its native copy of *nodD1* induced *nod* gene expression 15-fold in the presence of luteolin but, as described previously (56, 73, 81), NodD1 failed to respond to the other inducers (Fig. 1B). *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii NodDs also mediated *nod* gene transcription in response to luteolin but, unlike *S. meliloti* NodD1, they were also activated by naringenin (Fig. 1C and D). The two strains were distinguished by their responses to eriodictyol and 7-hydroxyflavone, respectively. Eriodictyol was the most potent stimulator of *R. leguminosarum* bv. viciae NodD but failed to stimulate *R. leguminosarum* bv. trifolii NodD. In contrast, 7-hydroxyflavone was the most potent stimulator of *R. leguminosarum* bv. trifolii NodD but only slightly stimulated *R. leguminosarum* bv. viciae NodD. Daidzein, previously shown to be unable to activate *nod* gene transcription in the three species tested (81), was also inactive in our assay (Fig. 1). It is clear that NodD is responsible for activation of the *nodC′-′lacZ* fusion, because in the absence of NodD, we observed no *nod* gene induction in the presence of any of the

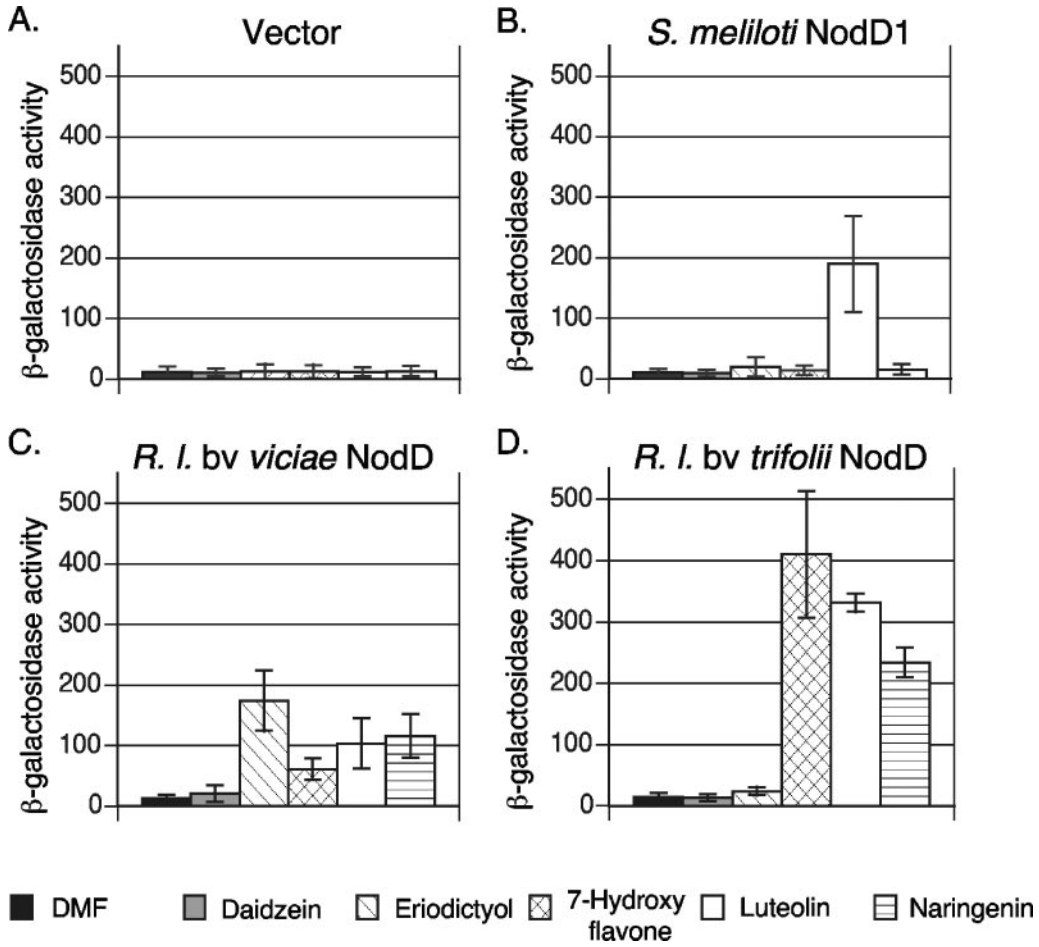

FIG. 1. Genetic evidence that NodD is the flavonoid sensor. *nodC'-'lacZ* reporter activity was assayed in *S. meliloti* A2105 expressing the indicated *nodD*. Cultures were grown in the absence or presence of 3 μM flavonoid in DMF. (A) Vector control (pRF771). (B) *S. meliloti* NodD1. (C) *R. leguminosarum* bv. viciae NodD. (D) *R. leguminosarum* bv. trifolii NodD. Data are an average of at least eight independent assays and are plotted as means ± standard deviations.

flavonoids tested (Fig. 1A). Furthermore, increasing the concentration of flavonoid did not result in increased levels of *nod* gene induction for any of the NodDs tested (data not shown). Thus, when we expressed either *R. leguminosarum* bv. viciae or *R. leguminosarum* bv. trifolii *nodD* in *S. meliloti*, *nod* gene expression was induced in response to a wider structural range of flavonoids than observed in the same strain expressing *S. meliloti nodD1*.

Luteolin stimulates the DNA binding activity of affinity-purified NodD1. Because our genetic studies suggested that NodD1 is the flavonoid sensor, we wanted to study the molecular mechanism by which luteolin stimulates NodD1 to activate *nod* gene transcription. A previous study showed that incubation of luteolin with affinity-purified NodD1 in the presence of a GroESL cycling system stimulated NodD1 binding to the *nod* box in vitro (80). Thus, we wanted to determine if luteolin would also stimulate NodD1 binding to the *nod* box in vivo. Because attempts to assay NodD1 binding to the *nod* box in vivo were unsuccessful (data not shown), we affinity purified NodD1 from cells that were grown in the absence or presence of 3 μM luteolin to use in our analysis. As previously reported (80), NodD1 copurifies with the chaperonin GroEL; therefore, all affinity-purified NodD1 used in these assays also contains GroEL. Although we have not demonstrated biochemically that NodD1 isolated from cells grown with luteolin still contains bound luteolin, for purposes of clarity we will refer to this fraction as NodD1 plus luteolin.

We incubated increasing amounts of affinity-purified NodD1 or NodD1 plus luteolin with *nodF nod* box DNA and analyzed binding to the *nodF nod* box by EMSA. NodD1 isolated from cells grown with luteolin reproducibly showed a greater binding affinity for the *nodF nod* box than did NodD1 isolated from cells grown without luteolin (Fig. 2). We used the amount of protein required to bind 50% of the input DNA as a measure of DNA binding affinity. NodD1 and NodD1 plus luteolin required approximately 2.9 μM and 1.5 μM protein, respectively, to bind 50% of the *nodF nod* box DNA in the reaction.

Luteolin stimulates the DNA binding activity of SEC-HPLC-purified NodD1. Our previous study demonstrated that an SEC-HPLC-purified fraction of NodD1 (0.5 μM) containing GroEL binds to the *nodF nod* box only when preincubated with luteolin, GroES, and ATP (80). To determine whether SEC-HPLC-purified NodD1 can bind to the *nodF nod* box in the absence of luteolin, as observed with affinity-purified

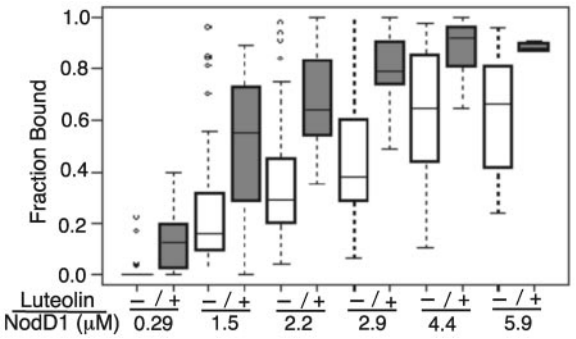

FIG. 2. Luteolin stimulates the DNA binding activity of NodD1. Increasing amounts of affinity-purified NodD1 containing GroEL isolated from cells grown in the absence (open boxes) or presence (shaded boxes) of 3 μM luteolin were incubated with labeled *nodF nod* box DNA as described in Materials and Methods. Data are presented as a box plot (see Materials and Methods). Data represent ≥10 independent protein purifications and ≥14 independent DNA binding reactions for all samples except 5.9 μM NodD1+L ($n = 5$ independent protein purifications and 5 independent DNA binding reactions). A P value of <0.02 was found at each protein concentration tested.

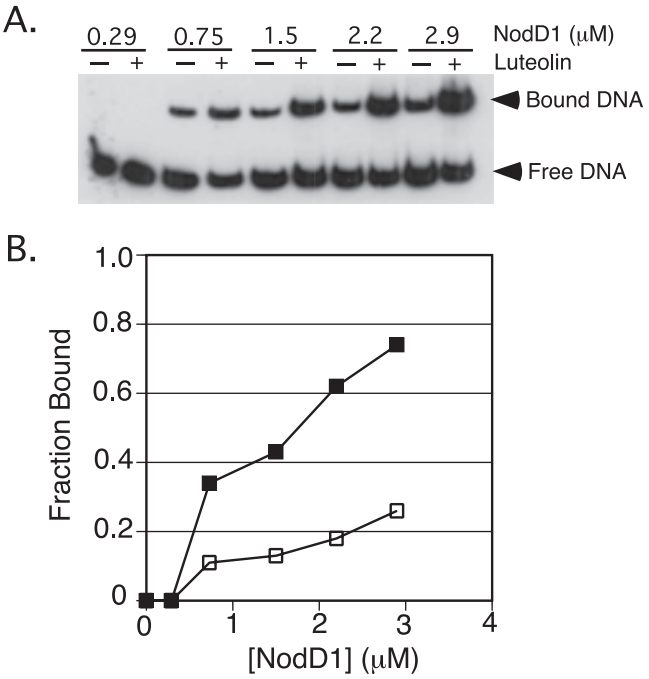

FIG. 3. Luteolin stimulates the DNA binding activity of SEC-HPLC-purified NodD1. Increasing amounts of SEC-HPLC-purified NodD1 containing GroEL were incubated with GroES and ATP for 10 min prior to the addition of DMF (−, □) or 10 μM luteolin (+, ■). The samples were then incubated with labeled *nodF nod* box DNA and analyzed as described in Materials and Methods. $n \geq 8$ for each concentration tested. (A) Representative experiment of NodD1 with or without luteolin binding to the *nodF nod* box. Arrows indicate the NodD1-DNA complex (bound DNA) and free DNA. (B) Quantitation of NodD1 binding to the *nodF nod* box from the experiment shown in panel A.

NodD1, we incubated increasing concentrations of SEC-HPLC-purified NodD1 containing GroEL with GroES and ATP with or without 10 μM luteolin and assayed levels of *nodF nod* box DNA binding (Fig. 3). Clearly, at concentrations above 0.75 μM, NodD1 preincubated with only GroES and ATP bound to the *nodF nod* box. Moreover, when the preincubation also included luteolin, binding to the *nod* box increased three- to fourfold (Fig. 3B). Thus, while elevated levels of NodD1 alone resulted in the formation of a NodD-*nod* box complex after preincubation with GroES and ATP, addition of luteolin during the preincubation dramatically stimulated NodD1-*nod* box complex formation. Similar trends were observed in all experiments using SEC-HPLC-purified fractions of NodD1 with or without luteolin, although there was a substantial variation in the absolute levels of NodD1-*nodF nod* box DNA binding between assays (data not shown). This variation is due to differences in the amount of active NodD1 present between SEC-HPLC-purified fractions and the limitation that each fraction only provides enough protein to be used for a single titration experiment (data not shown).

Luteolin does not significantly alter the footprint of affinity-purified NodD1 at a *nod* box. Having established that the DNA binding behaviors of affinity-purified NodD1 isolated from cells that were grown in the absence and presence of luteolin are biochemically distinct, we tested whether luteolin alters the footprint pattern of NodD1 on *nod* box DNA. We detected only minor changes between the DNase I footprints of NodD1 that had been isolated from cells grown with or without luteolin (Fig. 4A). Specifically, in the presence of luteolin, NodD1 displayed a slightly shorter footprint on the top strand (Fig. 4B) of the transcription start site distal half of the *nod* box, and there was a new hypersensitive cleavage site on the same strand between the two halves of the *nod* box. These two differences are consistent with an alteration in the bending angle between the two halves of the *nod* box in the presence of NodD1 plus luteolin. Overall, the data agree with previously published DNase I footprints using immunoaffinity-purified NodD1 (16).

Noninducing flavonoids stimulate the DNA binding activity of NodD1. Because luteolin may alter NodD1 activity at multiple steps during *nod* gene induction, such as affecting NodD1-DNA and NodD1-RNAP interactions, it is possible that only one of these steps is regulated by luteolin in a species-specific manner. In other words, multiple flavonoids may bind to NodD1 at *nod* gene promoters, but only luteolin is capable of initiating the later steps required for transcription. Therefore, we asked whether NodD1 isolated from cells that had been grown with noninducing flavonoids would show an altered binding affinity to the *nodF nod* box. Although these compounds do not promote NodD1-mediated *nod* gene induction, they are structurally similar to luteolin (Fig. 5A) and, in other species, mediate *nod* gene transcription by binding to NodDs that share greater than 84% similarity to NodD1 (3, 4, 15, 83).

We isolated affinity-purified NodD1 from *E. coli* that had been grown in the presence of 3 μM daidzein, eriodictyol, or naringenin and assayed for *nodF nod* box binding activity. As shown in Fig. 5B and C, daidzein and eriodictyol stimulated an increase in the DNA binding affinity of NodD1 to the *nodF nod* box. We observed a similar trend with naringenin-induced NodD1 DNA binding to the *nodF nod* box, although the differences were not as large (data not shown). Comparing Fig. 2

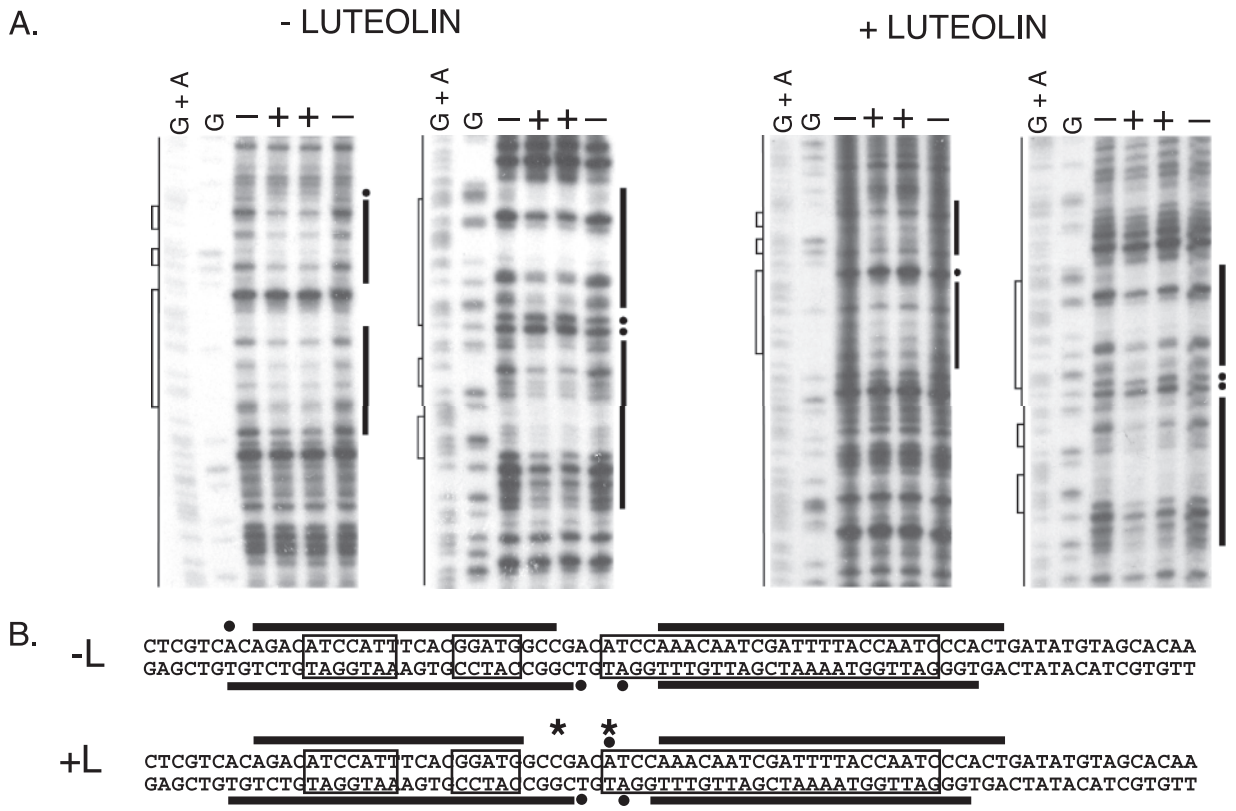

FIG. 4. Luteolin does not significantly alter the footprint of affinity-purified NodD1 at the *nodF nod* box. (A) Free DNA fragments and NodD1-*nod* box complexes were subjected to DNase I footprinting within the polyacrylamide gel slice as previously described (16). G+A and G chemical sequencing reactions are labeled. The position of the *nod* box is indicated schematically to the left of each panel. The lines to the right of each panel indicate segments protected from cleavage; a ● indicates hypersensitive cleavage sites. The left panel of each pair shows the results when the top strand shown in panel B was labeled, and the right panel of each pair shows the same when the bottom strand was labeled. − is the product of DNase I cleavage of the free DNA fragment, and + is the product of cleavage of the NodD1-*nod* box shifted complex. (B) Summary of footprinting reactions shown in panel A in the absence (-L) and presence (+L) of luteolin. The consensus *nod* box sequence is boxed. The thick lines above and below the sequence indicate segments protected from cleavage, and a ● indicates hypersensitive cleavage sites. An asterisk indicates changes in the footprint in the presence versus absence of luteolin.

and 5, it is clear that the NodD1 DNA binding affinities at *nod* gene promoters are similar for NodD1 isolated from cells grown in the presence of daidzein, eriodictyol, or luteolin.

To determine if noninducing flavonoids added to NodD1 in vitro can also stimulate binding to the *nodF nod* box, we incubated an SEC-HPLC-purified fraction of NodD1 containing GroEL with GroES, ATP, and 10 μM daidzein, eriodictyol, or naringenin. As shown in Fig. 6, each flavonoid stimulated NodD1 binding to the *nodF nod* box to a level that was indistinguishable from that observed with luteolin. From these findings we conclude that the increase in DNA binding affinity of NodD1 at *nod* gene promoters is not luteolin specific.

Noninducing flavonoids compete with luteolin for binding to NodD1. Several studies support the hypothesis that inducing flavonoids mediate the conversion of NodD to a positive regulatory form, while noninducing compounds (termed anti-inducers) compete for and occupy the same inducer binding site, resulting in poor or no activation of NodD (12, 57, 84). Genetic data imply that this inhibition takes place at the level of active versus inactive NodD binding to the same *nod* box DNA binding site (82). Consistent with this hypothesis, mutations in *nodD* that led to *nod* gene induction in response to anti-inducers have been isolated from both *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii (7, 45). Further support for the role of anti-inducers comes from the demonstration that noninducing flavonoids are capable of decreasing luteolin-mediated, NodD1-dependent *nod* gene induction in *S. meliloti* (57). Finally, the same flavonoid can act as an inducer or anti-inducer, depending on the source of NodD (84).

To determine whether daidzein, eriodictyol, and naringenin directly antagonize luteolin-mediated NodD1-dependent *nod* gene induction, we measured *nodC'-'lacZ* expression in *S. meliloti* overexpressing *nodD1* under conditions of increasing amounts of noninducing compounds in the presence or absence of luteolin. In agreement with previous studies (57), each noninducing compound inhibited NodD1-mediated *nod* gene induction (Table 2). For example, in the presence of naringenin, the most effective antagonist, luteolin-induced *nod* gene induction was reduced by 88% (Table 2). When naringenin was chosen as a representative of these noninducing flavonoids and examined in greater detail, we found that increasing the concentration of luteolin could overcome the inhibitory effects of naringenin on *nod* gene

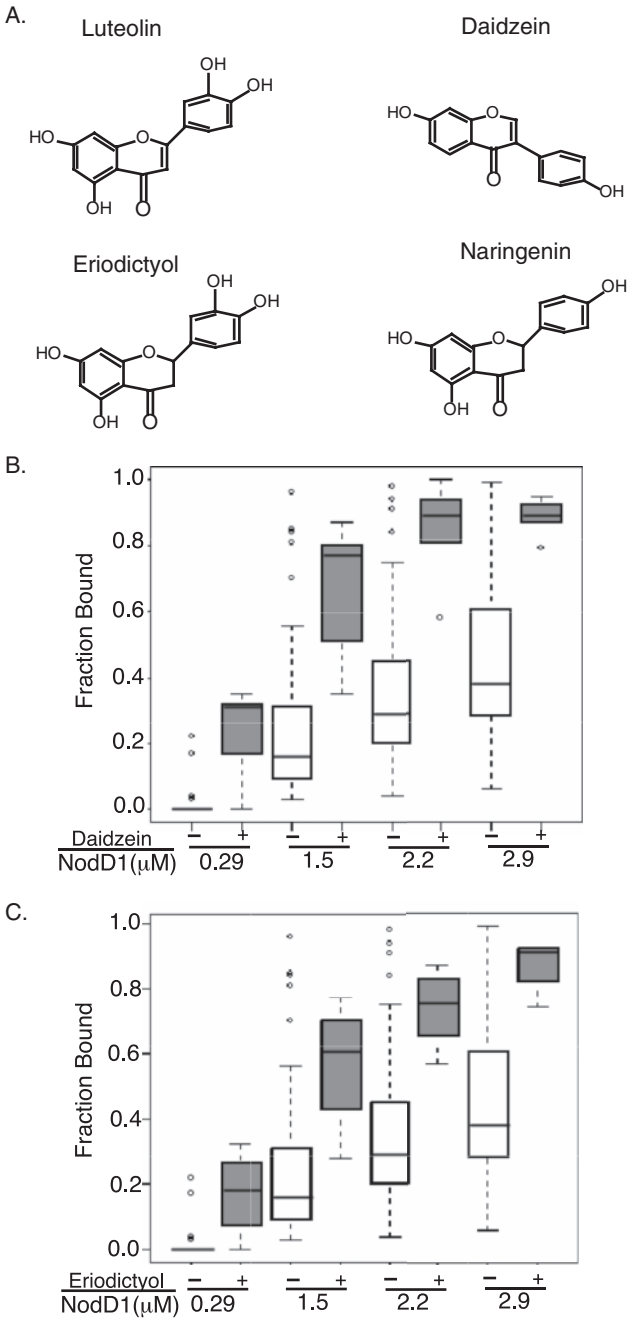

FIG. 5. Noninducing flavonoids stimulate the DNA binding activity of NodD1. (A) Structures of flavonoids used in these assays. (B and C) Increasing concentrations of affinity-purified NodD1 isolated from cells that had been grown in the absence (open boxes) or presence (shaded boxes) of 3 μM daidzein (B) or eriodictyol (C) were incubated with labeled *nodF nod* box and analyzed as described in Materials and Methods. Data are presented as a box plot (see Materials and Methods). Each point represents at least four independent experiments. A P value of <0.05 was found at each protein concentration except at 1.5 and 2.2 μM eriodictyol, where P was <0.10.

expression (data not shown). This is consistent with the hypothesis that noninducing flavonoids are acting as competitive inhibitors of inducing flavonoids, preventing NodD1 from activating *nod* gene transcription.

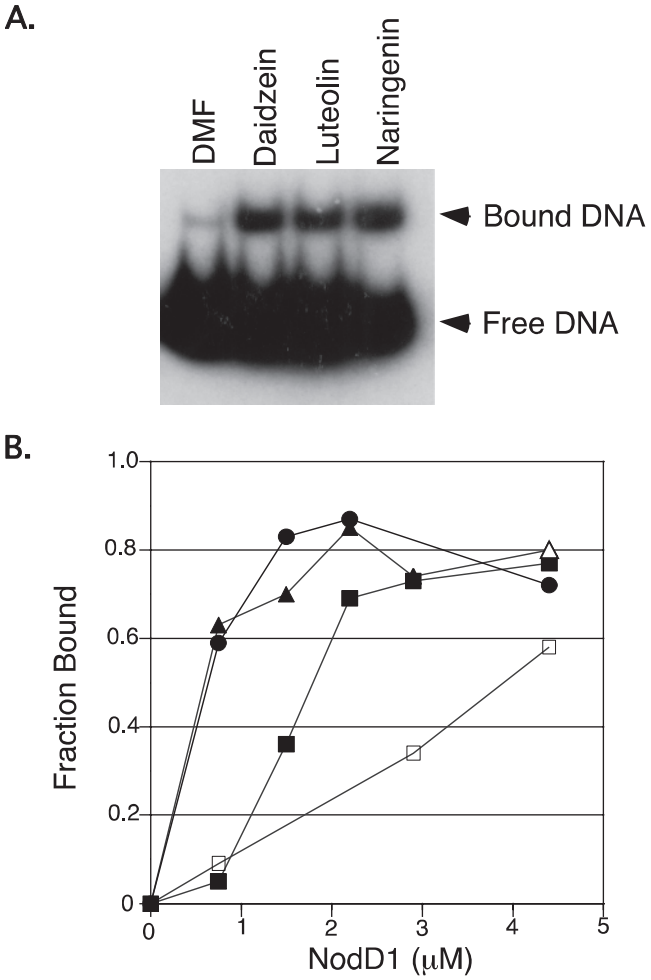

FIG. 6. Noninducing flavonoids stimulate the DNA binding activity of SEC-HPLC-purified NodD1. SEC-HPLC-purified NodD1 containing GroEL was preincubated with GroES and ATP for 10 min prior to the addition of DMF or 10 μM flavonoid. The sample was then mixed with labeled *nodF nod box* DNA (1.3 fmol) as described in Materials and Methods. (A) Representative gel shift analysis of 0.44 μM SEC-HPLC-purified NodD1 incubated with DMF, daidzein, luteolin, or naringenin. Arrows indicate the NodD1-DNA complex (bound DNA) and free DNA. (B) Increasing amounts of SEC-HPLC-purified NodD1 were incubated with DMF (□), luteolin (■), eriodictyol (▲), or naringenin (●) and assayed for *nodF nod* box binding. A representative assay is shown.

DISCUSSION

The genetic studies presented in this work strongly imply that NodD directly interacts with flavonoids to activate *nod* gene transcription. We showed, in experiments targeting a single ORF, that changing the source of NodD from *S. meliloti* NodD1 to *R. leguminosarum* bv. viciae or *R. leguminosarum* bv. trifolii NodD alters the response of the host cell to a set of flavonoid inducers. By using a constitutive promoter in a strain of *S. meliloti* with insertional mutations in *nodD1*, *nodD2*, and *nodD3*, we were able to compare the behaviors of equivalent levels of NodD protein in an otherwise wild-type background. *S. meliloti* NodD1 shows the most restrictive flavonoid recognition pattern, activating *nod* gene transcription only in the presence of luteolin, while *R. leguminosarum* bv. viciae and *R.*

TABLE 2. Noninducing flavonoids act as competitive inhibitors of *nod* gene induction in *S. meliloti*

Competitor (concn [μM])	Activity (Miller units)[a]	
	Without luteolin	With luteolin[b]
None	6.8 ± 3.6	163.2 ± 45.3
Daidzein (3)	7.5 ± 3.1	140.9 ± 39.2
Daidzein (6)	6.9 ± 2.1	106.8 ± 36.8
Daidzein (9)	7.5 ± 2.0	89.5 ± 32.0
Eriodictyol (3)	24.8 ± 7.4	116.8 ± 13.3
Eriodictyol (6)	24.7 ± 8.4	81.5 ± 16.6
Eriodictyol (9)	22.5 ± 7.8	70.7 ± 9.5
Naringenin (3)	14.5 ± 2.9	25.6 ± 4.3
Naringenin (6)	13.0 ± 5.2	23.5 ± 3.5
Naringenin (9)	12.5 ± 5.0	20.6 ± 5.8

[a] *nodC′-′lacZ* activity in A2105 plus pRmE43 (wild-type NodD1). Data are expressed as means ± standard deviations and represent at least four independent assays. Vector alone showed <16 units of β-galactosidase activity under all tested conditions.
[b] Luteolin concentration of 3 μM.

leguminosarum bv. trifolii NodDs induce *nod* gene transcription in response to a broader range of inducers. These data are consistent with previous studies in which *nodD* genes were expressed under their native promoters in a Sym$^-$ strain of *Rhizobium* sp. (15, 60, 73). Our finding that normally nonactive flavonoids stimulate *nod* gene transcription in *S. meliloti* in the presence of heterologous NodDs does not support the hypothesis that differential and structurally specific uptake of flavonoids is a regulatory mechanism for flavonoid recognition (82). In other words, our data argue that there is not a dedicated transporter specific to each flavonoid.

How might flavonoids stimulate NodD-mediated activation of *nod* gene transcription? Studies of other protein regulators show that small molecule inducers can affect binding of the activator to DNA and/or the conformation of the bound protein, thus influencing DNA architecture or the interaction with RNAP (1). Recent protein folding theories suggest, contrary to the induced fit model (39), that proteins constantly convert between inactive and active conformations: ligands serve to shift the population towards the active state by selectively stabilizing the active conformer, not by inducing a conformational change in the protein (41, 76). Based on studies with the transcription factor NtrC from *E. coli*, Buck and Rosen hypothesize that the stimulation of multiple regulatory steps by inducers, such as increasing DNA binding affinities and promoting interactions with RNAP, would be an advantage to proteins that are "off" in their inactive states but respond to ligands with rapid activation (6). By requiring multiple regulatory steps that each modulate the conformational distribution, the equilibrium would rarely shift enough to drive a protein through the multiple conformations required for activation, except in the presence of inducers (42).

Our biochemical studies demonstrate quantitatively that luteolin stimulates the DNA binding affinity of purified NodD1 to *nod* gene promoters. Specifically, NodD1 containing GroEL purified from cells grown with luteolin demonstrates increased affinity for the *nodF nod* box compared to the same protein purified from cells grown without luteolin. We hypothesize that NodD1 actively folds around luteolin in vivo, because the NodD1 protein that we isolated from cells exposed to luteolin immediately prior to cell lysis failed to show increased binding to the *nod* gene promoter (data not shown). In addition, SEC-HPLC-purified NodD1 containing GroEL demonstrated increased affinity for the *nodF nod* box in the presence of luteolin, GroES, and ATP compared to incubation with GroES and ATP alone. The affinity-purified NodD1 from cells grown in the presence of luteolin showed almost indistinguishable DNA binding properties from SEC-HPLC-purified NodD1 incubated with luteolin (Fig. 2 and 3). Thus, luteolin, whether added to growing cells or directly to purified protein, stimulates NodD1 binding to *nod* gene promoters. Although we observed only a three- to fourfold increase in the affinity of NodD1 for the *nod* box, this effect could be significant in vivo given that wild-type *S. meliloti* only shows a two- to threefold induction of *nod* genes in the presence of luteolin (29, 48).

Supporting these results, studies from other species of rhizobia demonstrate that flavonoids influence DNA binding during *nod* gene activation. In *Sinorhizobium fredii*, crude extracts isolated from cells grown in the presence of genistein demonstrated *nod* gene binding activity, while extracts isolated from cells grown in the absence of inducer failed to bind to *nod* gene promoter DNA (43). Studies in *R. leguminosarum* bv. phaseoli and *Azorhizobium caulinodans* showed that in vitro addition of naringenin to crude extracts was required for or significantly stimulated NodD binding to *nod* gene promoters (27, 31).

In our current model of NodD1-mediated transcription, luteolin binds to NodD1 and increases each dimer's affinity for the *nod* box but does not significantly alter the region of bound DNA. Our work with both affinity-purified NodD1 and the SEC-HPLC-purified fraction of NodD1 containing GroEL argues that the interaction between NodD1 and luteolin is direct. In other words, our work does not support the hypothesis that intermediary proteins are involved in the flavonoid-NodD1 interaction. Although it is still a formal possibility that luteolin acts on NodD1 indirectly through the chaperonins GroES and/or GroEL, this possibility is inconsistent with the observed species specificity of flavonoid action, given that a nonhomologous GroEL preparation (from *E. coli*) was used in our in vitro experiments. Luteolin may be able to interact with NodD1 that is already bound to *nod* gene promoters to stabilize the DNA-bound conformation of the protein. Alternatively, in combination with the chaperonin GroEL, luteolin may stabilize the folding of unbound NodD1, thus resulting in more "active" available protein. Support for this hypothesis comes from studies of the transcription factor LuxR, which requires the presence of autoinducer and GroEL to fold into an active conformation (22). Luteolin may also alter the NodD1-induced DNA bend in the *nod* box. Supporting this hypothesis, NodD1 plus luteolin induces the appearance of an additional hypersensitive cleavage site between the two halves of the *nod* box compared to the footprint of NodD1 alone.

In the case of NodD1, luteolin likely acts both to increase the affinity of NodD1 for *nod* gene promoters and at additional downstream steps to confer specificity to *nod* gene activation. Specifically, of the inducers we tested, only luteolin is capable of activating in vivo *nod* gene transcription in *S. meliloti*. However, the noninducing flavonoids daidzein, naringenin, and eriodictyol all stimulated NodD1 binding to the *nodF nod* box at levels greater than or equal to the levels of binding stimulated by luteolin. Competition experiments in which daidzein, erio-

dictyol, and naringenin each decreased the ability of NodD1 to upregulate *nod* gene transcription in the presence of luteolin suggest that these noninducing flavonoids compete with luteolin for direct binding to NodD1. In future experiments, it will be interesting to assay the effects of both luteolin and noninducing flavonoids on promoter bending, RNA polymerase loading, and promoter clearance.

Based on the studies presented in this paper, we propose a model in which both inducing and noninducing flavonoids stabilize conformers of NodD1 bound to *nod* gene promoters but that only luteolin-bound NodD1 is capable of undergoing the conformational changes required to initiate *nod* gene transcription. Evidence from several LysR systems supports this model, demonstrating that inducers must do more than simply stimulate DNA binding of the activator protein to target promoters. For example, mutants in the OccR DNA binding site that lock the protein into an "activated" footprint do not cause constitutive activity (2). Similarly, full occupancy of either the CysB or TrpI binding sites is not sufficient for transcription to occur (23, 24, 36). *R. leguminosarum* bv. viciae mutants in the *nodFEL* promoter were isolated that allowed *nod* box binding but not transcription activation (51). Our studies provide the first in vitro evidence addressing the mechanism by which noninducing flavonoids may antagonize *nod* gene induction. We suggest that nonactivating flavonoids function as competitive inhibitors of nodulation not only by preventing luteolin from interacting with NodD1 but also by directly stimulating unproductive NodD1-promoter interactions, that is, promoter binding without transcription initiation. To account for the ability of NodD1 to bind to multiple ligands, Ma and colleagues argue that proteins exist in a distribution of conformational isomers around the native state, presenting to incoming ligands a range of binding site shapes (42). Binding to the incoming ligand will then shift the equilibrium in favor of that conformer. Other LysR proteins, such as NahR, CysB, and TrpI, also bind to structural analogs of their inducers, thus reflecting the promiscuous nature of the protein domain that interacts with these small molecules (8, 9, 35, 52).

Plants may have evolved to regulate the host-symbiont recognition process by controlling the spectrum of flavonoids that they release (44, 58). Specifically, production of nonactivating flavonoids may be a mechanism by which legumes prevent overnodulation (38, 64). It will be interesting to test whether our results can be extended to explain a recent report that chemicals that disrupt estrogen signaling in mammalian cells, such as organochlorine pesticides, can inhibit *nod* gene transcription in *S. meliloti* and nodule formation in alfalfa (20, 21, 55). In other words, do these chemicals also increase the DNA binding affinity of NodD to *nod* gene promoters to inhibit *nod* gene transcription? Understanding the detailed mechanism of flavonoid interactions with members of the NodD family thus may provide further insight into evolution and diversity in the *Rhizobium*-legume symbiosis.

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