MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 4, pp. 356-364, 1991

Diverse Signal Sensitivity of NodD Protein Homologs from Narrow and Broad Host Range Rhizobia

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Received 12 December 1990. Accepted 19 March 1991.

The narrow host range plant endosymbiont *Rhizobium meliloti* exhibited diverse symbiotic properties (nodulation, nitrogen fixation) in concert with 10 host plant species from the genera *Medicago*, *Melilotus*, and *Trigonella*. These plant species released divergent but overlapping sets of compounds from their seeds. Several constituents of the plant exudates exerted inductive or inhibitory effects on nodulation gene expression in *R. meliloti*, depending on the plasmid-borne NodD protein homolog (NodD1, NodD2, and NodD3 of *R. meliloti*) introduced into the test cell. These three types of NodD proteins, as well as NodD of the broad host range strain *Rhizobium* sp. MPIK3030 diverged in terms of specific recognition of flavonoid standards. The $NodD_{MPIK3030}$ protein required only the hydroxylation of the C7 atom on the flavonoid ring for a substantial nodulation gene induction ability. Consequently, the $NodD_{MPIK3030}$ protein was sensitive to a far broader range of flavonoids than either of three NodD homologs of *R. meliloti* which, in turn, required the appropriate constellation of additional substitutions on the flavonoid skeleton for effectivity. The aurone sulphuretin was an extremely potent inducer of nodulation genes even without plasmid-borne NodD in *R. meliloti*. Examination of the effects of individual substitutions and different ring structures suggests that 7,3′,4′-hydroxyflavone can be an ideal inducer of nodulation genes in *R. meliloti*. We conclude that the diverse signal sensitivity of NodD proteins correlates with the narrow or broad host range properties of the rhizobial strains.

Additional keyword: flavonoid specificity.

Rhizobia are characterized by their ability to establish nitrogen-fixing endosymbiosis with a limited number of dicotyledonous plants, mainly legumes. The host range of rhizobia is confined to one or a few plant genera (e.g., in the case of *Rhizobium leguminosarum* and *R. meliloti*) or extends to several plant taxons (e.g., for *R. fredii*, *Rhizobium* spp. NGR234 and MPIK3030). The host range is primarily determined at early stages of the bacterium-plant interaction, which is governed by the rhizobial *nod*(ulation) genes. Some of these genes affect host specificity (*hsn* genes), whereas others (common *nod* genes) perform general functions necessary for nodulation of any hosts (reviewed by Kondorosi and Kondorosi 1986).

The induction of *nod* genes requires the regulatory gene *nodD* in conjunction with host plant-derived signal molecules (flavonoids; reviewed by Long 1989). The *nodD* gene product (NodD protein) binds to the inducible *nod* promoters (Kondorosi *et al.* 1989). Genetic evidence suggests that a direct interaction with plant signals converts NodD into its positive transcriptional regulator form by a proposed conformational shift of the protein, referred to as "activation" (Burn *et al.* 1987, Horvath *et al.* 1987, Djordjevic *et al.* 1987, McIver *et al.* 1989, Kondorosi *et al.* 1989). NodD exhibits sequence similarity to bacterial and vertebrate regulatory proteins with analogous functions (Henikoff *et al.* 1988, Györgypal and Kondorosi, in press).

The NodD proteins have a potential role in determining host specificity, because they are divergent with respect to their ability to recognize specific signals (Horvath *et al.* 1987, Spaink *et al.* 1987). Flavonoid activators of NodD proteins have been characterized for *Bradyrhizobium japonicum*, *Rhizobium* sp. NGR234, *R. fredii*, *R. meliloti*, *R. leguminosarum* bvs. *viciae*, *trifolii*, and *phaseoli* (LeStrange *et al.* 1990, Zaat *et al.* 1989, Djordjevic *et al.* 1987, Redmond *et al.* 1986, Firmin *et al.* 1986, Sadowsky *et al.* 1988, Peters and Long 1988, Kosslak *et al.* 1987, Davis and Johnston 1990). Our knowledge is, however, rather limited with regard to the specific inhibition of *nod* gene expression by certain flavonoids (Firmin *et al.* 1986, Djordjevic *et al.* 1987, Peters and Long 1988).

The majority of rhizobia contain more than one allele of the *nodD* gene (Rodriguez-Quinones *et al.* 1987). *R. meliloti*, which infects *Medicago*, *Melilotus*, and *Trigonella* plants, has three NodD homologs (Göttfert *et al.* 1986, Honma and Ausubel 1987). NodD1, NodD2, and NodD3 interact differently with plant exudates (Györgypal *et al.* 1988, Mulligan and Long 1989, Honma *et al.* 1990, Maillet *et al.*, in press). The flavones luteolin and chrysoeriol, isolated from seeds, were found to be the major *nod* gene inducers interacting with NodD1 (Peters *et al.* 1986, Hartwig *et al.* 1990), whereas 7,4′-hydroxyflavone, 7,4′-hydroxyflavanone, and 4,4′-hydroxy-2′-methoxychalcone constituted the major inducers in roots of the host plant alfalfa (*Medicago sativa* L.) (Maxwell *et al.* 1989). Limited data are available on the signal specificity of the *R. meliloti* NodD2 and NodD3 proteins, except that the flavonoids luteolin and 4,4′-hydroxy-2′-methoxychalcone have been shown to have inducing activity in concert with NodD3 and NodD2, respectively (Dusha *et al.* 1989, Hartwig *et al.* 1990).

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Here, we report the isolation of various *nod* gene inducers and inhibitors from 10 host plants of the narrow host range symbiont *R. meliloti*, which interact diversely with the three NodD homologs of the bacterium. With authentic flavonoids, a systematic study was performed to compare the signal specificities of the three *R. meliloti* NodD homologs with that of the single functional NodD protein of the broad host range strain *Rhizobium* sp. MPIK3030 (Horvath *et al.* 1987). Structure-activity relationships for both inducing and inhibiting activities were established and the *nod* gene regulatory ability of aurone-type flavonoids was ascertained as well. Our results show that the NodD protein of strain MPIK3030 is sensitive to a broader spectrum of activating signals than the three *R. meliloti* NodD proteins. This property is correlated with the fairly high number of plant species nodulated by MPIK3030.

MATERIALS AND METHODS

Bacteria, plasmids and microbiological techniques. JM57 is a derivative of *R. meliloti* 1021 carrying a *nodC::lacZ* fusion (Mulligan and Long 1985). pKSK5 (Kondorosi *et al.* 1984) and pMG108 (Göttfert *et al.* 1986) are derivatives of plasmid pRK290 (Ditta *et al.* 1980), carrying the *nodD1* and *nodD2* loci of *R. meliloti* 41. A derivative of plasmid pNID6 (Györgypal *et al.* 1988) was used as the source of *nodD3*. This plasmid also carried Tn*5* insertion in the *syrM* locus. The plasmids were introduced into JM57 by triparental matings (Ditta *et al.* 1980).

Symbiotic tests. The nodulation experiments, each containing 25 parallel tubes, were performed in triplicate as described (Kondorosi *et al.* 1977). The plants were examined for nodules every day during the first 2 wk after inoculation. After this period, nodules were scored in 5-day intervals. The plant species used were: *Medicago sativa* L. 'Nagyszenasi,' *M. coerulea* Less., *M. media* Pers., *M. quasifalcata* R. et F., *M. truncatula* Gaertner 'Jemalong,' *M. varia* Martyn 'Kisvardai,' *Melilotus alba* Medik. 'Kecskemeti,' *M. officinalis* (L.) Lam. 'Norgold,' *Trigonella coerulea* (L.) Ser., *T. foenum-graecum* L.

Plant factor preparation. One gram of plant seeds, surface-sterilized with ethanol and 0.1% $HgCl_2$, was soaked overnight in 5 ml of sterile water. The seed washes were extracted twice with 1.5 ml of *n*-butanol. The butanol phases were dried, redissolved in 0.2 ml of ethanol, and chromatographed on silica gel 60 F_{254} TLC plates (Merck & Co., Inc., Rahway, NJ), with chloroform/acetic acid/water (10:9:1, v/v) as the solvent. Discrete bands were isolated from the layer, redissolved in 0.2 ml of ethanol, and the purification procedure was repeated. By using this method, 81 ± 9% (mean ± standard error) of the biological activity in the original seed washes could be recovered.

Nodulation gene induction assay. Derivatives of *R. meliloti* JM57 (Mulligan and Long 1985), which harbored one of the three *nodD* genes of strain Rm41 on a recombinant plasmid were examined. Bacteria were diluted at an OD_{600} of 0.02 in 1 ml of YTB medium (Orosz *et al.* 1973), containing the test substances at the concentrations described in the text. After incubation for 16 hr at 30° C, the cultures were monitored for β-galactosidase activity of the *nodC::lacZ* fusion as described (Miller 1972). All assays were repeated at least three times, giving a variation of 23%. The sources of flavonoid standards were: Aldrich, Steinheim, Germany (biochanin A); Roth, Karlsruhe, Germany (7-hydroxyflavone, luteolin, fisetin, morin, naringenin, naringin, hesperetin, daidzein, formononetin, sulphuretin, maritimein); G. Hrazdina, Cornell University, Ithaca, NY (techtochrysin, 7,4′-hydroxyflavone, chrysin, acacetin, apigenin, myricetin, aromadendrin, fustin, 4′-hydroxy-5,7-methoxyflavanone, hesperidin, 5,7-hydroxy-8,4′-methoxy-isoflavone, 5,7-hydroxy-8-methoxy-isoflavone, 7,4′-hydroxy-5,8-methoxy-isoflavone, 7-hydroxy-5,8-methoxy-isoflavone, 4,6,4′-hydroxyaurone, aureusidin); G. Litkey, Kossuth University, Debrecen, Hungary (apigenin-7-glycoside, rhamnetin, kaempferid, kaempferol, quercetin, 7-methoxyflavanone, 7-glycosyloxy-flavanone, taxifolin, 7-methoxy-isoflavone, 7-hydroxy-isoflavone, genistein, sakuranetin, flavonol, 4′-hydroxy-isoflavone, isoflavone, 4′-hydroxy-7-methoxy-isoflavone, 7,4′-methoxy-isoflavone).

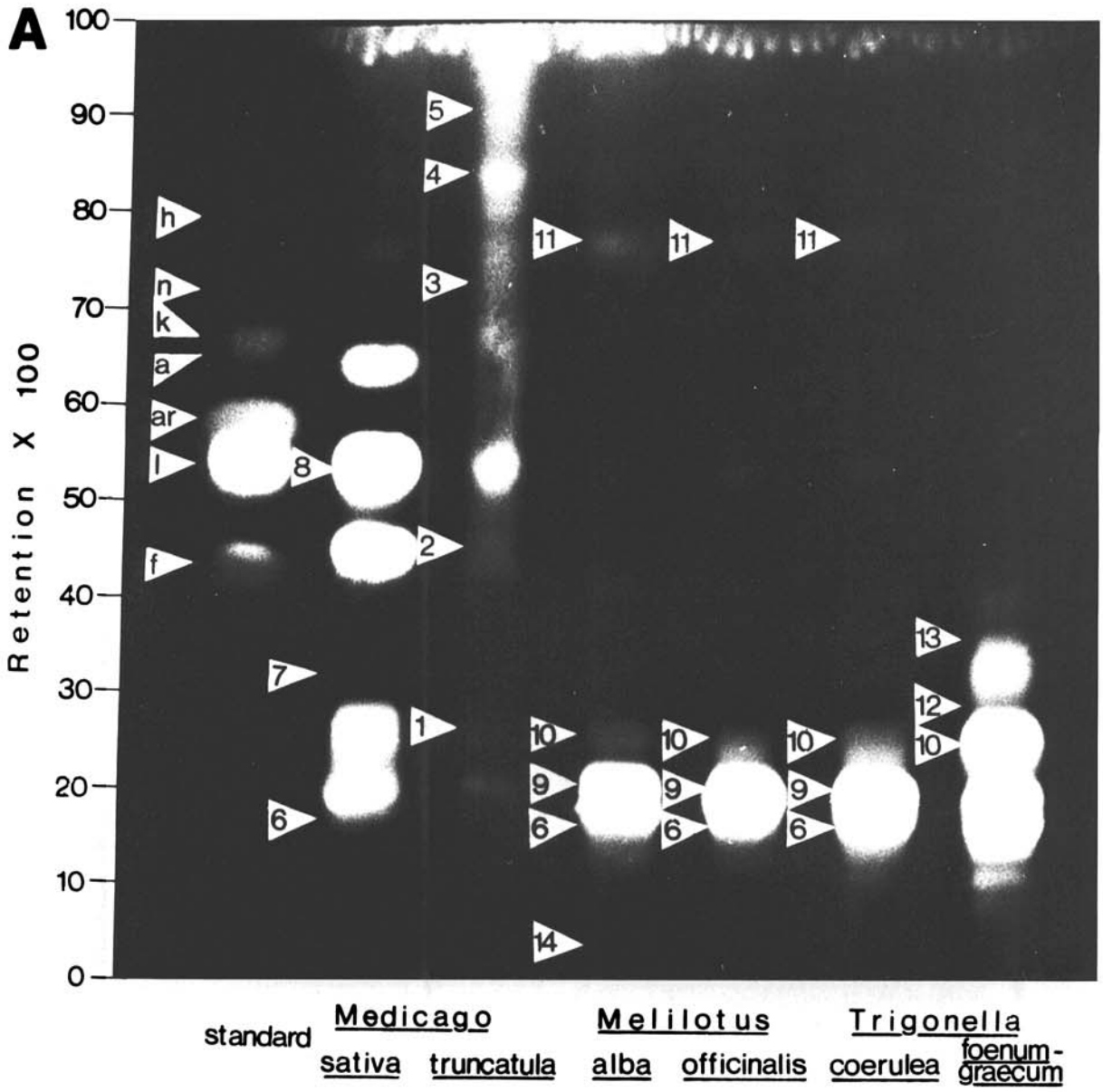
RESULTS

Diverse symbiotic properties of *R. meliloti* on host plants. Ten host plant species from all three genera infected by *R. meliloti* 1021 were tested for nodulation ability, nitrogen fixation efficiency, and *nod* gene-inducing capacity (Table 1). Five closely related *Medicago* spp. established a rapidly developing, effective symbiosis and exhibited relatively high levels of *nod* gene-inducing activity. The more distantly related legume *M. truncatula* was much less efficient in terms of the three phenotypes studied. The nodulation and nitrogen fixation on two *Melilotus* spp. was observed at nearly the same rate as on the five *Medicago* spp., whereas their *nod* gene-inducing abilities were lower. The two

Table 1. Symbiotic phenotypes of *Rhizobium meliloti* on host plants used in this study

Host plant	Nodulation[a]	Nitrogen fixation[b]	Fold induction of *nodC::lacZ* by seed exudate[c]
Medicago spp.			
M. coerulea	36	356	4.0[d]
M. media	61	370	2.9[d]
M. quasifalcata	25	381	2.2[d]
M. sativa	50	324	3.5[d]
M. varia	67	268	2.6[d]
M. truncatula	5	23	1.2
Melilotus spp.			
M. alba	26	267	1.5
M. officinalis	62	170	1.4
Trigonella spp.			
T. coerulea	5	30	1.3
T. foenum-graecum	0[e]	38	1.1

[a] Data are the percentages of nodulated plants on the eighth day after inoculation.
[b] Data are the dry weight increases of inoculated plants on the 55th day after inoculation, expressed as percentages of the weight of the uninoculated control.
[c] Measured in strain JM57 after cultivation with whole seed exudates derived from 50 mg of seeds. Data are fold inductions over the background *nodC::lacZ* activity of 7.3 units in strain JM57 grown without exudate.
[d] Significant induction of *nodC::lacZ* ($P < 0.01$).
[e] Nodulation occurred after the eighth day.



B

Plasmid-borne NodD in JM57	Regulatory effects on nodC-lacZ activity of the given plant exudate components													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
NodD1	−	−	−	+	ns	+	+	+	ns	ns	+	+	+	ns
NodD2	ns	ns	ns	+	+	ns	ns	ns	+	ns	+	+	ns	−
NodD3	ns	ns	+	−	+	+	ns	+	ns	+	+	+	ns	ns

Fig. 1. Signal compounds from host plants of *Rhizobium meliloti*. **A,** Seed exudates separated by thin-layer chromatography. The chromatogram was prepared as described in text, sprayed with Natural Product Reagent A (Roth, Karlsruhe, Germany), and photographed under $UV_{366\,nm}$ illumination. Compounds influencing *nod* gene expression are numbered (see panel **B**); other substances appearing on the chromatogram were inactive. Abbreviations for the components of the standard mixture are: a, apigenin; ar, aromadendrin; f, fustin; h, hesperetin; k, kaempferol; l, luteolin; n, naringenin. **B,** Effects on nodulation gene expression. The plant exudate components are identified by the numbers shown in panel A. Derivatives of the strain JM57 (containing a *nodC::lacZ* fusion) were grown in the presence of plant compounds derived from 12.5 mg of seeds. +, Significant (2.2- to 25.8-fold; $P < 0.01$) induction of *nodC::lacZ*; −, significant (49–89%; $P < 0.01$) inhibition of the *nodC::lacZ* induction achieved by 3.3 μM of fisetin; ns, no significant effect. Control activities are given in Table 2.

Trigonella spp. examined exhibited very low levels of *nod* gene induction and nodulation, and the nitrogen fixation capacity was not significant (Table 1).

Differential interactions of natural signal compounds with the NodD proteins of *R. meliloti*. The different host plants of *R. meliloti* were examined for their seed exudate

Table 2. Effects of flavonoids on *nod* gene expression

Flavonoid (trivial names)	Substitution pattern on ring positions[a]								Effect on *nodC*::*lacZ* activity[b] in conjunction with NodD			
	3	5	7	8	2′	3′	4′	5′	1-Rm	2-Rm	3-Rm	MPIK3030
Flavones												
Techtochrysin		O	M						ns	ns	ns	ns
—[c]			O						0.05	3.3	0.32	58.3
DHF			O				O		6.5	5.3	3.9	62.6
Chrysin		O	O						0.00	0.05	ns	60.9
Acacetin		O	O				M		0.00	0.05	0.08	54.1
Apigenin		O	O				O		22.0	3.1	10.6	61.1
—		O	G				O		12.5	3.5	2.6	62.5
Luteolin		O	O			O	O		88.8	ns	10.7	53.7
Flavonols												
Flavonol	O								ns	ns	ns	ns
Rhamnetin	O	O	M			O	O		ns	ns	ns	ns
Kaempferid	O	O	O				M		0.36	0.52	ns	39.2
Kaempferol	O	O	O				O		0.44	0.68	ns	60.2
Quercetin	O	O	O			O	O		ns	ns	ns	20.5
Myricetin	O	O	O			O	O	O	ns	ns	ns	7.6
Morin	O	O	O		O		O		5.7	ns	2.5	43.3
Fisetin	O		O			O	O		105.6	12.5	18.1	49.9
Flavanones												
—			M						ns	ns	ns	ns
—			G						0.60	ns	ns	11.9
—		M	M				O		ns	ns	ns	ns
Sakuranetin		O	M				O		ns	ns	ns	ns
Naringenin		O	O				O		0.03	0.10	2.9	50.8
Naringin		O	R				O		ns	ns	0.55	ns
Hesperetin		O	O			O	M		0.09	0.16	4.0	58.9
Hesperidin		O	U			O	M		ns	ns	ns	ns
Flavanonols												
Aromadendrin	O	O	O				O		3.6	0.08	0.41	48.1
Taxifolin	O	O	O			O	O		2.9	2.6	ns	9.5
Fustin	O		O			O	O		4.0	2.7	ns	11.9
Isoflavones												
Isoflavone				(no substitution)					ns	ns	ns	ns
—			M						ns	ns	ns	ns
—			M				M		ns	ns	ns	ns
—			M				O		ns	ns	ns	ns
—							O		ns	ns	ns	ns
—			O						ns	0.65	2.3	50.0
Daidzein			O				O		ns	0.83	0.42	51.5
Formononetin			O				M		ns	0.77	1.7	40.6
Genistein		O	O				O		0.57	0.02	0.74	57.1
Biochanin A		O	O				M		0.71	0.40	3.2	43.4
—		O	O	M			M		ns	ns	0.65	11.4
—		O	O	M					ns	ns	3.0	10.8
—		M	O	M			O		ns	ns	0.43	ns
—		M	O	M					ns	ns	0.71	ns
Aurones												
—		O	O				O		25.8	ns	9.0	11.0
Aureusidin		O	O			O	O		ns	ns	ns	10.8
Sulphuretin			O			O	O		215.1	ns	41.0	58.2
Maritimein			G	O		O	O		ns	ns	2.5	ns
Controls (*nodC*::*lacZ* activity [units])												
No inducer (for induction tests)									1.3	8.9	7.0	11.5
3.3 μM of fisetin (for inhibition tests)									68.2	24.6	21.1	417.0

[a] For comparability, the flavone-type numbering is applied in the table for aurones as well, although the numbering of the carbon atoms in aurones is different from that in other flavonoids (e.g., C6 in aurones corresponds to C7 in others; see Fig. 2). Abbreviations for substitutions are: O, hydroxyl; M, methoxyl; G, glycosyl; R, rhamnoglycosyl; U, rutinosyl.

[b] Derivatives of the strain JM57 (containing a *nodC*::*lacZ* fusion) were cultured in the presence of the listed substances at a concentration of 10 μM. In inhibition tests, 3.3 μM of fisetin was applied as the competitive inducer. Values higher and lower than 1.0 represent fold inductions and fold inhibitions (residual activities), respectively, of the control activities (see the bottom of the table). Only significant effects are shown ($P < 0.05$; others are marked by ns, not significant). Rm, *Rhizobium meliloti*.

[c] —, No trivial name.

composition by thin-layer chromatography. A representative chromatogram is shown in Figure 1A. Note that only a part of the substances detected and studied is visible on Figure 1A, and compounds with similar retentions are not necessarily identical. The exudate composition of *M. truncatula* was distinct from those of the other five *Medicago* spp. (Fig. 1A), which released nearly uniform sets of compounds (data not shown). *Melilotus* and *Trigonella* spp. excreted a third type of exudate that overlapped with the pattern of the five *Medicago* spp. (see compound no. 6 in Fig. 1; other substances common for *Medicago* and *Melilotus* are invisible in Fig. 1A).

The purified seed extract components were tested individually for their ability to influence *nod* gene expression in conjunction with the three *R. meliloti* NodD homologs. The derivatives of the *nodC::lacZ* fusion strain JM57 that harbored extra *nodD* copies on recombinant plasmids were used. Facilitated by the lack of the *nod*-repressor (Kondorosi *et al.* 1989), the induction levels of *nodC* in such strains increase significantly when the plasmid-borne extra NodD interacts with the added plant factors (Mulligan and Long 1985).

In addition to a number of ineffective compounds, 28 different substances were found, which either stimulated or repressed the *nod* gene expression in conjunction with at least one of the three NodD types. All these compounds were identified as putative flavonoids according to their purification profile, chromatographic behavior, and color reactions (data not shown). The presence of the known *nod* gene-inducing flavone, luteolin (Peters *et al.* 1986; compound no. 8 in Fig. 1), in all *Medicago* spp. studied (excluding *M. truncatula*) was verified by multiple cochromatography with a standard luteolin sample by using various solvent systems (data not shown).

Fourteen of the active compounds (appearing in Fig. 1A) are listed in Figure 1B, along with their effect on *nodC-lacZ* induction in the presence of different NodD proteins. The distribution of the inducers and inhibitors varied in the exudates of different plants. Twelve out of fourteen compounds exerted different induction/inhibition activities depending on the NodD present (Fig. 1B). These results suggest that the *nod* gene expression level in *R. meliloti* is influenced by a complex interaction between the different NodD copies and the host-dependent set of inducers and inhibitors.

Divergent flavonoid specificity of NodD proteins from narrow and broad host range rhizobia. The interactions of NodD proteins with signal compounds were examined with authentic flavonoid standards. In these studies, we compared the three NodD homologs of the narrow host range bacterium *R. meliloti* with the single functional NodD protein of the broad host range strain *Rhizobium* sp. MPIK3030 (Horvath *et al.* 1987). Forty-five representative flavonoids were tested for their capacity to either induce the *nodC-lacZ* expression in JM57 or antagonize the effect of the inducer, fisetin (Table 2). The compounds classified as inducers stimulated *nod* gene expression when applied alone. Inhibitors competitively decreased the inducing effect of fisetin but did not exhibit an inducing ability by themselves. Weak inducers were also able to competitively reduce the effect of fisetin (data not shown).

All effective compounds contained a hydroxyl moiety at least at the C7 position of the flavonoid ring (Fig. 2; Table 2). In contrast, substances containing a methoxyl group or containing no substitution at the C7 atom were found to be ineffective. Also, the substitution of the C7 position with sugar moieties was generally deleterious for the activity of the molecule (Table 2). Roughly half of the authentic flavonoids tested exhibited inducing or inhibitory activity with each of the NodD forms studied here (Table 2). However, the distribution of the inducers, inhibitors, and ineffective compounds was distinct depending on the actual NodD type. As an example, 7-hydroxy-isoflavone was an ineffective compound in the presence of NodD1, whereas it was an inhibitor with NodD2, a weak inducer with NodD3, and a strong inducer with $NodD_{MPIK3030}$. The ratio between the number of inducers and inhibitors was 0.6, 1.1, and 1.7 for NodD2, NodD1, and NodD3, respectively, whereas all effective compounds were inducers for $NodD_{MPIK3030}$. Regarding the latter type of NodD, hydroxylation of the C7 position was sufficient for induction to substantial levels, irrespective of the ring skeleton structure (Table 2). Further substitutions at other positions (except for at position C4′) were found to be unfavorable.

The effects of flavonoids in conjunction with the NodD homologs of *R. meliloti* (Table 2) were influenced by the substitution pattern of the ring skeleton in a more complex manner than in the case of $NodD_{MPIK3030}$. 7-Hydroxyflavone was an inhibitor with NodD1 and NodD3, and an additional substitution at C4′ rendered the molecule to be an inducer. Further hydroxyl groups at C5 and C3′ were generally favorable for interacting with NodD1 but often unfavorable for NodD3. 7-Hydroxyflavone was found to be an inducer with NodD2, and any change of

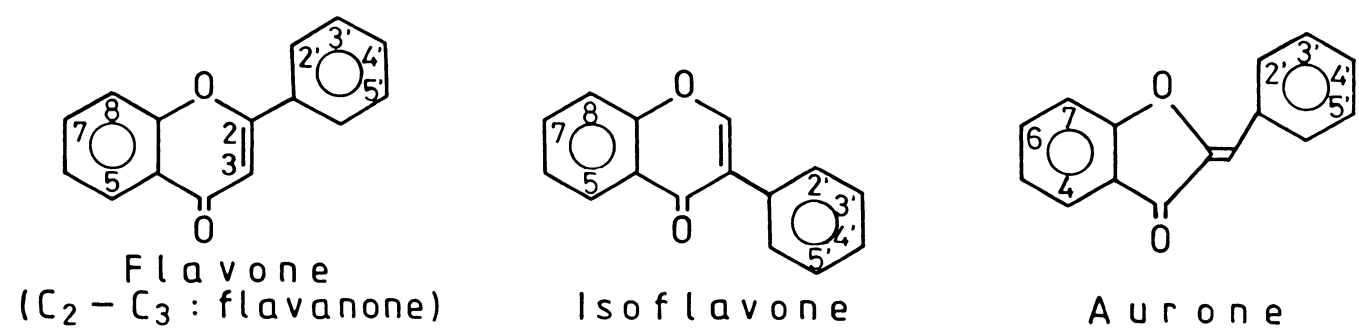


Fig. 2. Ring skeletons of the flavonoid subclasses.

this structure (except hydroxylation at C4') was deleterious for the interaction. The isoflavone structure was beneficial for NodD3, whereas it was detrimental for the activity with NodD1 and NodD2 (Table 2). The effects of alterations in the ring skeleton type, however, also depended on the actual substitution pattern of the molecule.

Aurones as a novel class of *nod* gene inducers. Four representatives of the minor flavonoid subclass, aurones (Fig. 2), were included in our studies. Sulphuretin and 4,6,4'-trihydroxyaurone were found to be inducers in conjunction with NodD1, NodD3, and $NodD_{MPIK3030}$. Aureusidin interacted exclusively with $NodD_{MPIK3030}$ and maritimein with NodD3 (Table 2). Sulphuretin was the most potent inducer among those interacting with the NodD1 and NodD3 proteins. This feature explains the unique ability of sulphuretin to attain high levels of *nod* gene activity in *R. meliloti*, in the absence of plasmid-encoded extra *nodD* copies (Fig. 3).

DISCUSSION

Involvement of the *R. meliloti* NodD homologs in signal recognition. The presence of three diverged NodD homologs in *R. meliloti* may offer an advantage for the bacterium, when receiving signal molecules from a potential host plant (Honma and Ausubel 1987, Györgypal *et al.* 1988). We observed that *Medicago*, *Melilotus*, and *Trigonella* plants exhibit diverse symbiotic abilities and release (partly) divergent sets of signal compounds. Our results suggest a dependence of the *R. meliloti* nodulation efficiency on the *nod* gene-inducing activity of the respective plant species' seed exudate. This possibility is supported by the observation that nodulation of a specific alfalfa line was limited by its low luteolin content (Kapulnik *et al.* 1987). In addition to seed flavonoids, which are both chemoattractans and *nod* gene inducers (Caetano-Annolles *et al.* 1988, Peters *et al.* 1986, Hartwig *et al.* 1990), a recent study proves a contribution of a distinct set of root-derived signal molecules to *nod* gene regulation (Maxwell *et al.* 1989). We propose that the overall effect of a plant exudate on *nod* gene induction depends on its ability to activate the heterogenous NodD pool in *R. meliloti*, which is determined by the inherent inducer/inhibitor ratio for each indigenous NodD homolog.

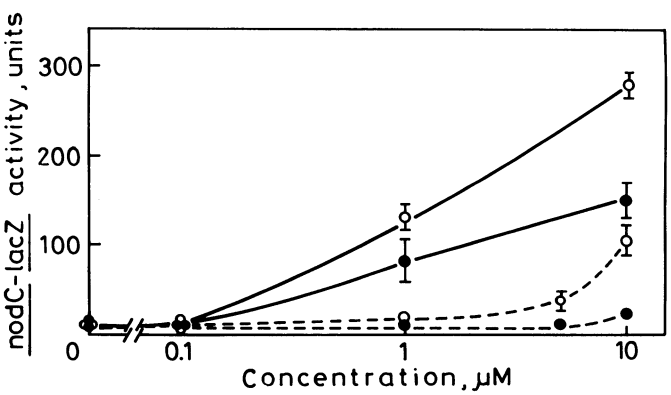


Fig. 3. Induction of *nodC-lacZ* by sulphuretin (○) and luteolin (●) in the presence (——) and absence (- - - -) of plasmid-borne copies of the *nodD1* gene in strain JM57. The means ± standard errors derived from three replica experiments are shown.

It has been established that all three NodD types of *R. meliloti* are functional in nodulation (Honma and Ausubel 1987, Györgypal *et al.* 1988, Mulligan and Long 1989). We found that, similar to NodD1 (Mulligan and Long 1985, Peters *et al.* 1986, Zaat *et al.* 1989), both NodD2 and NodD3 regulate *nod* genes in conjunction with natural plant signal compounds (Fig. 1) as well as distinct flavonoids (Table 2). The three NodD homologs of *R. meliloti* show functional divergence in a fashion similar to the differences found between the single NodD proteins of other rhizobia (LeStrange *et al.* 1990, Zaat *et al.* 1989, Djordjevic *et al.* 1987, Redmond *et al.* 1986, Firmin *et al.* 1986, Sadowsky *et al.* 1988, Peters and Long 1988, Kosslak *et al.* 1987). A divergent range of compounds stimulate or repress the common ability of the NodD homologs to activate *nod* gene expression.

The regulation of *nodD3* is distinct from other *nodD* genes because its expression is controlled by the product of the adjacent *syrM* gene (Barnett and Long 1990). The flavonoid-independent expression of *nodC*, caused by plasmid-borne *syrM* copies (Mulligan and Long 1989), was avoided in our studies by inactivating *syrM* on the plasmid harboring *nodD3*. The NodD3 protein of Rm41 responded to a relatively wide spectrum of signal compounds (Table 2), contrary to NodD3 of Rm1021, which is not activated by flavonoids (Mulligan and Long 1989, Honma *et al.* 1990, Maillet *et al.*, 1990). It will be interesting to compare the sequences of NodD3 from the two strains because the expected differences between them may point to residues important in the interaction with signal molecules.

Structure-activity relationships of the flavonoid-NodD interaction. Specificity of the aromatic signal molecules with NodD proteins is due to their ring structure and substitution pattern. The inferences of several individual structural characteristics of the flavonoids on their biological activity are summarized in Figure 4. The structural elements found to be beneficial for the activity were the hydroxylation of the C7 and C4' atoms and the flavone-type ring skeleton. In contrast, hydroxylation of the C3' position, methoxylation of the C8 atom, and ring skeletons other than the flavone structure were generally unfavorable for the activity. As an exception, isoflavones were potent inducers with $NodD_{MPIK3030}$, and many of them were also able to interact with NodD3. The effects of hydroxylations at the C5, C2', and C3' positions and the effects of alterations of the ring structure depended on the NodD protein present (Fig. 4). Hence, the structural elements mentioned can provide the molecule with specific characteristics in the interaction with NodD.

The effects of hydroxyl substitutions at the C5, C3' and C4' positions showed a strong dependence on substitutions at other positions and/or the actual ring structure (Fig. 4). Despite the observation that sulphuretin was the most potent inducer for NodD1 and NodD3 (Table 2; Fig. 3), the aurone-type ring skeleton does not seem to be an ideal structure for the interaction with NodD proteins. Our results predict that 7,3',4'-hydroxyflavone could be an extremely strong inducer of *nod* genes, as it has an ideal structure with respect to both ring skeleton type and sub-

stitution pattern. A recent study by Maillet *et al.* (1990) verified this deduction because 7,3′,4′-hydroxyflavone induced the *R. meliloti nod* genes even more than luteolin when interacting with NodD1.

We observed that the lack of a hydroxyl group at certain positions (e.g., at the C4′ atom in 7-hydroxyflavones when NodD1 or NodD3 is present) converted inducers to inhibitors (Table 2). In contrast, the lack of hydroxylation at the C7 atom rendered the molecules ineffective even in inhibition assays. A possible explanation of the phenomenon is that the C7-hydroxyl moiety is involved in the hypothetical binding of a signal molecule to NodD

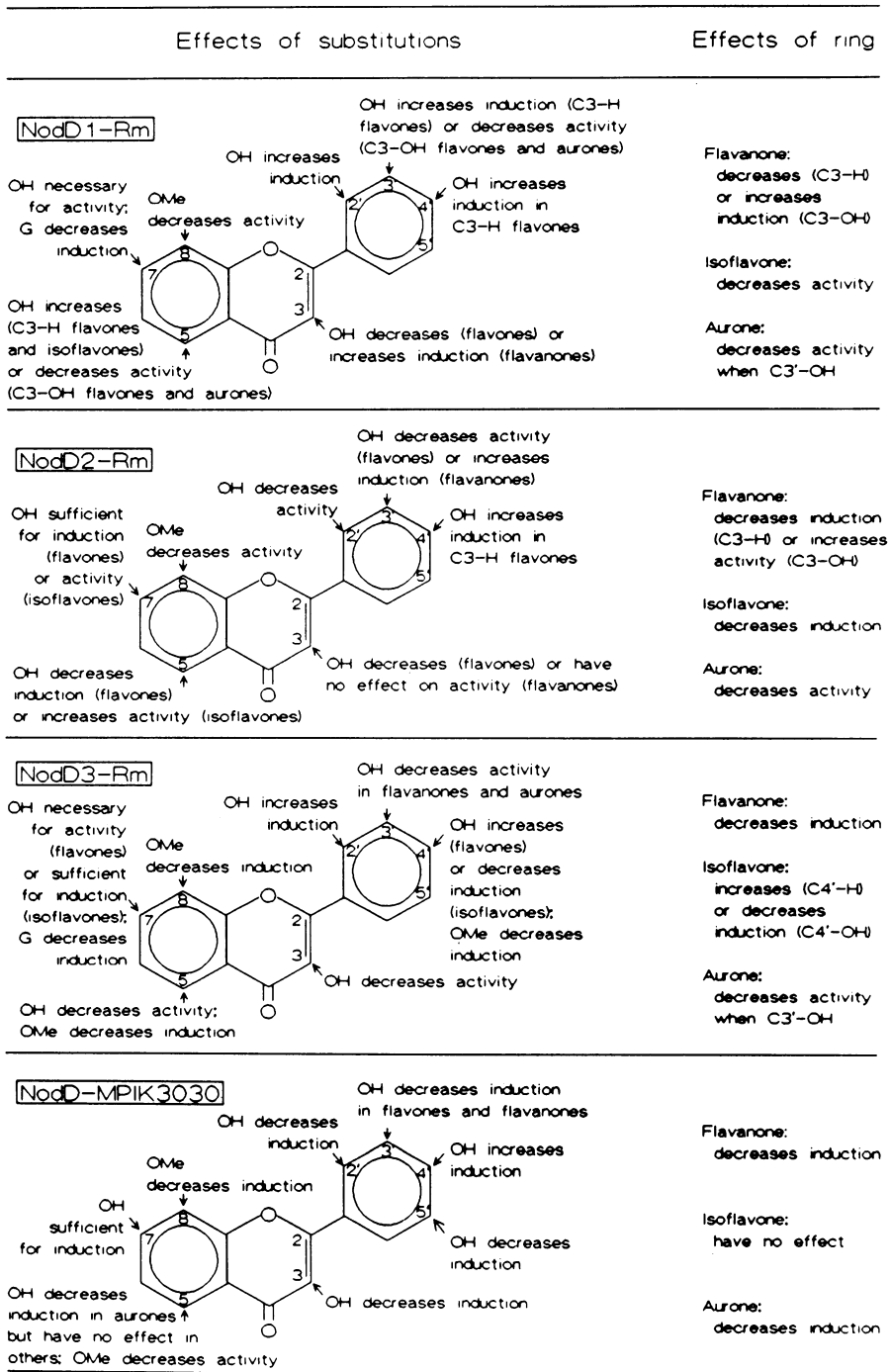


Fig. 4. Structure-activity relationships of the flavonoid-NodD interaction. The figure shows the effects of various flavonoid structural elements on the interactions of the molecule with four NodD homologs as inferred from the data presented in Table 2. On the left, flavone rings are displayed showing the effects of substitutions at specified carbon atoms. Conclusions were deduced from comparisons of the effects of those flavonoids that differ only by the presence or absence of the substitution in question. On the right, the influences of the ring skeleton types are presented. Conclusions were deduced from comparisons of the activity of molecules having different ring systems but the same substitution patterns. Statements on "activity" mean that not only induction but even inhibition ability of a molecule are affected. Abbreviations for substitutions are: OH, hydroxyl; OMe, methoxyl; G, glycosyl. Rm, *Rhizobium meliloti.*

(Djordjevic *et al.* 1987, Kondorosi *et al.* 1989, Zaat *et al.* 1989, Long 1989, Györgypal and Kondorosi, in press). Binding of a flavonoid might be necessary but not sufficient for the putative protein activation event (possibly involving the C4′-hydroxyl group), which subsequently results in the induction of *nod* genes.

Signal sensitivity of NodD as an adaptation to host plants. The relative accessibility of the NodD homologs for their interaction with signal molecules was found to be distinct (Table 2). Hydroxylation at the C7 atom of flavonoids alone conferred the ability to activate $NodD_{MPIK3030}$, and no inhibitors for that NodD homolog were found. It seems that activation of $NodD_{MPIK3030}$ takes place simply on interaction with a 7-hydroxyflavonoid. In contrast, the putative binding and activation events seem to be separate in the cases of the NodD homologs of *R. meliloti*. The NodD protein of *Rhizobium* sp. NGR234, which is closely related to strain MPIK3030, is also capable of responding to a large number of signal compounds including even monocyclic molecules (LeStrange *et al.* 1990).

It is apparent that the breadth of flavonoid specificity of the endogenous NodD proteins correlates with the host range when comparing strain MPIK3030 and *R. meliloti*. We speculate that the enhanced signal sensitivity of the $NodD_{MPIK3030}$ protein is an adaptation to the fairly large number of signal molecules exuded by a broad range of its host plants. This type of NodD can be considered as a primitive one whose only function is to switch on *nod* genes when a 7-hydroxyflavonoid is present. As the other extremity, the more refined inhibition-sensitive triple NodD system of *R. meliloti* is adapted to a strictly limited number of plant hosts; its NodD proteins may provide *R. meliloti* with the ability to select between plants by stimulating *nod* gene expression only when the signals characteristic for natural host plants are recognized.

ACKNOWLEDGMENTS

We are indebted to S. R. Long for providing strain JM57 and to G. Hrazdina and G. Litkey for supplying flavonoid standards. We thank T. Timar for his contribution to the plant factor analysis; B. Polyak for his help in TLC techniques; Z. Liptay for skilled assistance; A. Borka, B. Dusha, and G. Nöllenburg for preparing the illustrations; and L. Bögre, I. Dusha, M. Nöllenburg, and P. Putnoky for critical review of the manuscript.

This work was supported by grants IM 19650/1988, OMFB, OKKFT(Tt)/1986 and OTKA553.

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