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Induction of the *nodA* Promoter of *Rhizobium leguminosarum* Sym Plasmid pRL1JI by Plant Flavanones and Flavones

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An expression vector containing the *Rhizobium leguminosarum nodA* promoter cloned in front of the *Escherichia coli lacZ* gene was used to characterize the properties of the *R. leguminosarum nodA* gene-inducing compound(s) present in sterile root exudate of the host plant *Vicia sativa* L. subsp. *nigra* (L.). The major inducing compound was flavonoid in nature, most likely a flavanone. The commercially available flavonoids naringenin (5,7,4′-trihydroxyflavanone), eriodictyol (5,7,3′4′-tetrahydroxyflavanone), apigenin (5,7,4′-trihydroxyflavone), and luteolin (5,7,3′,4′-tetrahydroxyflavone) induced the *nodA* promoter to the same level as the root exudate. On the basis of chromatographic properties, it was concluded that none of these compounds is identical to the inducer that is present in root exudate. The induction of the *nodA* promoter by root exudate and by the most effective inducer naringenin was very similar, as judged from the genetic requirements and the kinetics of induction.

Bacteria of the genus *Rhizobium* form nitrogen-fixing root nodules on leguminous plants. The nodulation process is a host-specific interaction in that each species of *Rhizobium* nodulates only one or a limited number of host plants. Many bacterial nodulation genes reside on so-called Sym (biosis) plasmids (2, 3, 6, 9). Based on complementation analysis of transposon insertion or deletion mutants and cloned fragments of the *nod* gene region, it was concluded that the genes *nodA*, *nodB*, *nodC*, and *nodD* are common, i.e., are functionally interchangeable between different species of *Rhizobium*, whereas other genes code for host-specific nodulation functions (7, 8, 17, 22).

The symbiosis between *Rhizobium* sp. and its host plant is established in a sequence of events of which bacterial adhesion to plant root hairs, and the subsequent curling of these root hairs, followed by the development of an infection thread are the first to be observed microscopically (22). Recently, it was shown that the common *nodA*, *nodB*, and *nodC* genes, which are required for root hair curling, as well as several host-specific *nod* genes, require a plant product for induction. The regulation of *nod* genes was studied after fragments of the *nod* region were cloned in front of the *Escherichia coli lacZ* structural gene. It appeared that *nodD* is expressed constitutively (8, 14) and is subject to autoregulation (16). None of the other *nod* promoters studied was expressed in batch culture. In the presence of plant root exudates or seed exudates, however, promoter activity of common *nod* genes was observed in *R. meliloti* (14) and *R. leguminosarum* (16), and promoter activity of common as well as host-specific *nod* genes was observed in *R. trifolii* (8). In all cases tested, the presence of a functional *nodD* gene was a prerequisite for induction (14, 16).

In this study we started with the preliminary characterization of some properties of the inducer of the *R. leguminosarum nodA* promoter that is present in *Vicia sativa* root exudate. Because these results suggested that the inducer was flavonoid in nature, a large number of commercially available putative inducers were screened for activity with a *nodA-lacZ* expression vector. The results showed that the flavanones eriodictyol and naringenin and the flavones apigenin and luteolin are active in nanomolar concentrations. Although these plant products appeared to be different from the natural inducer, our findings indicate that the expression of *nod* genes can be studied in the absence of root exudate, which is an easily contaminated and chemically very complex mixture of compounds.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this study are listed in Table 1. The transcriptional fusion vector pMP190 is a 15-kilobase derivative of the broad-host-range, mobilizable plasmid pKT214 of the IncQ incompatibility group (1). The vector contains a multiple cloning sequence that is derived from pIC20H (12), the Shine-Dalgarno sequence from the *E. coli* chloramphenicol acetyltransferase gene, and the structural gene *lacZ* of *E. coli* β-galactosidase without its promoter (10). Details on the construction of this plasmid will be published elsewhere. Plasmids pMP154 and pMP158 are derived from this expression vector. In pMP154 and pMP158 a 114-base-pair *BglII-BclI* fragment of the *nod* region of pRL1JI with the promoter of *nodA*, and a 2-kilobase *BclI* fragment with the entire *nodD* gene as well as the promoter of *nodA*, respectively, were inserted into the *BglII* site of the multiple cloning sequence preceding the *lacZ* gene of pMP190. (For the exact localization of the mentioned inserted fragments within pRL1JI, see reference 17.)

Cells to be used for induction experiments were pregrown at 28°C on solid YMB medium containing yeast extract and mannitol (5). For stable maintenance of the recombinant plasmids, the medium was supplemented with streptomycin (1 mg/ml) and chloramphenicol (5 μg/ml). After growth for 4 days the plates were stored for a maximum period of 5 days at 4°C. Prior to the induction assay cells were suspended from the plate in induction medium to an A_{660} of 0.15. Induction medium consisted of deposit-free Jensen medium, which is a mineral medium without fixed nitrogen (21), supplemented with 20% thiamine-free medium (mannitol-nitrate) (18) and streptomycin (1 mg/ml) and buffered to a final pH of 6.0 as described previously (19). The suspension

TABLE 1. Strains and plasmids used in this study

Strain and plasmid	Relevant characteristics[a]	Reference or source
Strain		
LPR5045	*R. trifolii* RCR5 *rif* cured of Sym plasmid pRtr5a	P. J. J. Hooykaas
RBL5560	LPR5045(pJB5JI)	This study
RBL5561	LPR5045(pRL602)	This study
Plasmid		
pJB5JI	*R. leguminosarum* Sym plasmid pRL1JI, *cin*::Tn*5*	9
pRL602	pRL1 *nodD2*::Tn*5*	23
pKT214	IncQ, Mob$^+$ Cm^r Sm^r	1
pMP190	15-kilobase derivative of pKT214 *lacZ* (promoterless) Mob$^+$ Cm^r Sm^r	This study
pMP154	Promoter *nodA-lacZ* fusion in pMP190, Cm^r Sm^r	This study
pMP158	*nodD*, promoter *nodA-lacZ* fusion in pMP190, Cm^r Sm^r	This study

[a] Abbreviations: *cin*, bacteriocin production; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Mob, mobilization; *nod*, nodulation; Sm^r, streptomycin resistance.

was incubated under vigorous aeration at 28°C for 18 h, after which an A_{660} of approximately 0.25 was reached.

Plant cultivation and preparation of sterile root exudates. Surface sterilization and germination of *V. sativa* L. subsp. *nigra* (L.) seeds (20) and subsequent cultivation of the plants (19) were carried out as described previously. Root exudates were prepared from 150 3-day-old *V. sativa* plants grown in 750 ml of Jensen medium and sterilized as described previously (20). Only sterile root exudates were used in additional tests.

Ultrafiltration of root exudate. *V. sativa* root exudate was concentrated fivefold by vacuum evaporation at 45°C. Five milliliters of this concentrate was passed through ultrafilters with decreasing pore diameter at 35 lb/in^2 by using an ultrafiltration cell (type 8010; Amicon Corp., Danvers, Mass.) with Ym10, YM5, and YM2 filters. These filters allowed permeation of molecules with apparent molecular weights below 10,000, 5,000, and 1,000, respectively. After each filtration step, 0.4 ml of the filtrate was tested for *nodA* promoter-inducing ability as described below.

Solvent partitioning of the promoter *nodA*-inducing activity present in exudate. One liter of *V. sativa* exudate was concentrated approximately 100-fold by vacuum evaporation at 45°C and freeze-dried. The lyophilisate was extracted three times with 10 ml of 70% ethanol for 2 h at room temperature. The ethanol fractions were pooled, passed through a glass fiber filter (GF/A; Whatman, Maidstone, United Kingdom), and mixed with 30 ml of petroleum ether. After 18 h at 4°C both the aqueous and the organic phases were evaporated to dryness and solubilized in 4 ml of ethanol. A total of 8 μl of each fraction was tested for *nodA* promoter-inducing ability as described below.

Thin-layer chromatography of ethanolic root exudate extract. The lyophilisate of 1 liter of *V. sativa* exudate was extracted three times with 10 ml of 96% ethanol for 2 h at room temperature. The extract was concentrated to 1 ml (by evaporation at 45°C) and stored at 4°C. By using this procedure, the inducer of the *nodA* promoter was extracted quantitatively, as judged from the results of the induction assay.

For thin-layer chromatography on cellulose 5552 or 5574 (Merck, Darmstadt, Federal Republic of Germany), 2 μl of extract was applied to the plate if *nodA* promoter-inducing ability was to be determined (see below), whereas 50 μl was used if plates were to be inspected under UV light (wavelength, 366 nm). The fluorescence indicator present in thin-layer chromatographic plates (no. 5574) did not influence the induction of the *nodA* promoter. For chromatography of commercially available inducers, 1 μg was applied in ethanolic solution and detected under UV light at a wavelength of 366 nm. Two-dimensional chromatography was performed with solvent 1 (*t*-butanol–acetic acid–water [3:1:1; vol/vol/vol]) and solvent 2 (15% acetic acid in water) in the first and second dimensions, respectively. This system is commonly applied for the analysis of flavonoid patterns (11). Solvent 3 (chloroform-acetic acid-water [10:9:1; vol/vol/vol]) as well as solvents 1 and 2 were used for one-dimensional thin-layer chromatography.

Assay of *nodA* promoter-inducing activity. Inducer consisted of sterile exudate, an ethanolic extract of the exudate, exudate fractions after various treatments, or a solution of one of the commercially available putative inducers in 70% ethanol. Prior to their use in induction experiments, ethanolic solutions were always prediluted in induction medium such that the final ethanol concentration during the assay never exceeded 0.1% (final concentrations of 0.5% or higher progressively inhibited the induction of β-galactosidase production).

Fresh cells grown in induction medium to an A_{660} of 0.25 that were pregrown on solid medium as described above were used for the induction assay. Induction was started by adding 1 ml of this suspension to 3 ml of induction medium containing the appropriate inducer. Unless otherwise indicated the suspensions were incubated for 18 h at 28°C on a

TABLE 2. Genetic requirements for induction of the *nodA* promoter of *R. leguminosarum*[a]

Strain	*nod* genotype for:		β-Galactosidase activity (U) after induction by:			
	Sym plasmid	Clone fragment[b]	Exudate[c]	Control[d]	Eriodictyol[e]	Naringenin[e]
RBL5560(pMP154)	pJB5JI	Promoter *nodA*	20,200	300	18,500	19,200
LPR5045(pMP154)	Absent	Promoter *nodA*	300	300	300	200
RBL5561(pMP154)	pJB5JI *nodD*::Tn*5*	Promoter *nodA*	300	200	300	300
LPR5045(pMP158)	Absent	*nodD*, promoter *nodA*	19,100	300	18,100	19,100
RBL5560(pMP190)	pJB5JI	None	100	100	100	100

[a] Experiments were carried out as described in the text, with an induction time of 18 h.
[b] Inserted into pMP190 in front of the structural part of *lacZ*.
[c] Sterile *V. sativa* root exudate was prepared as described in the text. A twofold diluted preparation was used.
[d] Control was induction medium without added inducers.
[e] Added at 400 nM.

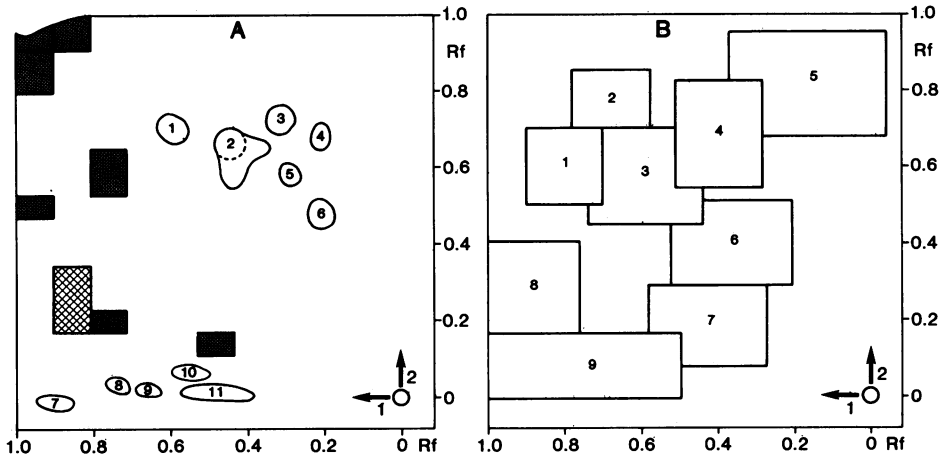

FIG. 1. (A) Two-dimensional chromatographic analysis of *V. sativa* ethanolic root exudate extract on cellulose. Inspection under UV light revealed the following colors. Spots, 1, 5, 9, and 11, blue; spot 2, fluorescent light blue; spots 3, 4, and 10, dull brown; spot 6, pale green; spot 7, pale yellow; spot 8, yellowish green. Fragments of the chromatogram with moderate (300 to 900 U) and high (≥3,500 U) β-galactosidase-inducing activity are presented as dotted and dashed areas, respectively. All other fragments induced less than 300 U of β-galactosidase activity. (B) General distribution of various classes of flavonoids in the same two-dimensional system shown in panel A (11): 1, dihydroflavonol aglycones; 2, dihydroxyflavonol 3-*O*-monoglycosides; 3, flavonol 3-*O*-di- and monoglycosides; 4, isoflavone 7-*O*-mono- and diglycosides; 5, flavonol 3-*O*-mono- and diglycosides, 7-*O*-diglycosides, and 3, 7-*O*-diglycosides; 6, flavone and flavonol 7-*O*-diglycosides; 7, flavone and flavonol 7-*O*-monoglycosides; 8, isoflavone and flavanone algycones; 9, flavone, flavonol, biflavonyl, chalcone, and aurone aglycones.

rotary shaker at 180 rpm. Units of β-galactosidase were determined and expressed as described by Miller (13). For exudate, apigenin, eriodictyol, luteolin, and naringenin, the level of induction obtained under these conditions was proportional to the inducer concentration up to 14,000 U.

When the activity from cellulose chromatograms was to be assayed, the chromatogram was made free of solvent under a stream of warm air for 3 h and subsequently cut into fragments 1 by 1.5 cm or 2 by 1.5 cm, depending on the expected activity. Individual fragments were incubated with 2 ml of freshly grown cells diluted fourfold in induction medium (to an A_{660} of 0.06) as described above. The inducing activity was quantitatively recovered from the chromatogram.

Chemicals. The origin of the chemicals that were tested for their *nodA* gene-inducing ability was as follows. 4-Chromanol, 4-chromanone, fisetin, rutin, and thiochroman-4-ol were obtained from Aldrich Chemie SA, Brussels, Belgium; chromone, flavanone, flavone, and thiochroman-4-on were obtained from Ega, Steinheim, Federal Republic of

TABLE 3. Induction of the *nodA* promoter by flavonoids[a]

Putative inducer[b]	Hydroxylation pattern at the following carbons:					Maximal response (U of β-galactosidase activity)	Inducer (nM) required for:	
	3	5	7	3′	4′		Maximal induction	Half-maximal induction
Flavanones								
Naringenin		OH	OH		OH	19,500	100	15
Eriodictyol		OH	OH	OH	OH	18,900	200	60
Flavones								
Apigenin		OH	OH		OH	19,200	100	16
Luteolin		OH	OH	OH	OH	18,800	150	40
Chrysin		OH	OH			2,000	500	100
7-Hydroxyflavone			OH			10,100	150	35
5-Hydroxyflavone		OH				300		ND[c]
Flavonols								
Kaempferol	OH	OH	OH		OH	600	240	ND
Quercetin	OH	OH	OH	OH	OH	200		ND
Isoflavones								
Genistein		OH	OH		OH	200		ND
Control[d]						200		

[a] Experiments were carried out as described in the text, with an induction time of 18 h.
[b] The following compounds were inactive at up to 5 μM: flavanone; flavone; the flavonols fisetin, kaempherid, morin, myricetin, and rutin; the isoflavones daidzein, genistin, prunetin, and 6,7,4′-thrihydroxy isoflavone; and the miscellaneous phenolic compounds catechin, 4-chromanol, 4-chromanone, chromone, epicatechin, thiochroman-4-ol, and thiochroman-4-on.
[c] ND, Not determined.
[d] Control was induction medium without added inducers.

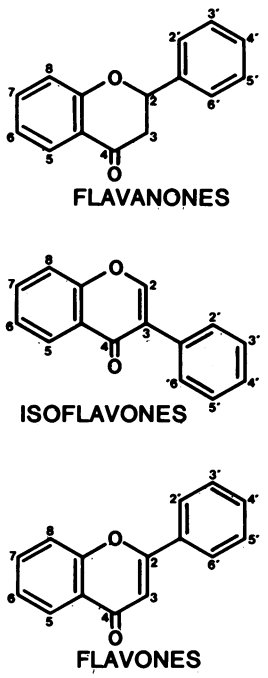

FIG. 2. Flavonoid structures. In flavanones and flavones the B ring is attached to C-2, and in isoflavones it is attached to C-3.

Germany; kaempherid was obtained from Fluka AG, Buchs, Switzerland. Apigenin, eriodictyol, and luteolin were purchased from Carl Roth GmbH & Co., Karlsruhe, Federal Republic of Germany; daidzein, genistein, genistin, prunetin, and 6,7,4'-trihydroxy-isoflavone were purchased from Sarsyntex, Merignac, France; catechin, chrysin, epicatechin, kaempferol, morin, myricetin, naringenin, and quercetin were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Preliminary characterization of *nodA* promoter-inducing factor(s) present in *V. sativa* root exudate. By using strain RBL5560(pMP154), which contains the *R. leguminosarum* Sym plasmid pJB5JI and the promoter of *nodA* cloned in front of the structural *lacZ* gene, it was shown that *V. sativa* root exudate contains promoter *nodA*-inducing activity (Table 2). The results with strain LPR5045(pMP154) show that the presence of the Sym plasmid is required for induction. Induction was abolished when the *nodD* gene of the Sym plasmid was inactivated [strain RBL5561(pMP154) in Table 2]. The results with strain LPR5045(pMP158), in which the Sym plasmid was replaced by a cloned *nodD* gene, show that the requirement of the Sym plasmid for induction can be completely fulfilled by the *nodD* gene.

The approximate molecular weight of the *nodA* promoter-inducing factor(s) that was present in exudate was estimated by ultrafiltration. The activity passed through all of the filters that were used, indicating a molecular weight of less than 1,000. The inducing activity of exudate was not influenced by heating for 20 min at 100°C. The activity was recovered from the aqueous phase of a biphasic water-ethanol-petroleum ether (3:7:10; vol/vol/vol) mixture.

Based on these properties of the inducer and on the notion that the chemotaxonomy of members of the family *Leguminosae* is partly derived from their flavonoid content (4), we decided to investigate the possibility that the inducer was a flavonoid. Two-dimensional thin-layer chromatography on cellulose and subsequent testing of the chromatogram fragments for *nodA* promoter-inducing activity revealed that over 95% of the activity was present in one spot (Fig. 1). Comparison of its chromatographic mobility with the distribution of various classes of flavonoids in this test system (11) indicated the possibility that the inducer was a flavanone or isoflavone (Fig. 1).

Naringenin, eriodictyol, apigenin, and luteolin induce the *nodA* promoter. A number of commercially available flavanones, isoflavones, and other related flavonoids or phenolic compounds were tested for their ability to induce the *nodA* promoter (Table 3). Relevant structures are shown in Fig. 2. Maximal induction of the promoter by *V. sativa* root exudate corresponded to 19,000 ± 1,500 U of β-galactosidase activity and was obtained with a fivefold-diluted preparation. The flavanones naringenin and eriodictyol and the flavones apigenin and luteolin appeared to be the most active inducers among the commercially available compounds that were tested (Table 3). They induced the *nodA* promoter to the same level as the exudate, and half-maximal and maximal induction as observed at low concentrations (Table 3). 7-Hydroxyflavone also gave half-maximal induction at a low concentration, but it induced a lower maximal response than exudate. The flavone chrysin is a poor inducer at up to 2 μM. The flavonol kaempferol induced a response that was twice the background activity at concentrations of 240 nM to 5 μM. All other compounds tested in this study were inactive (Table 3).

Since naringenin was the most active inducer, this flavanone was used to study induction in more detail. The response of the *nodA* promoter to increasing concentrations of naringenin was linear from 4 to 25 nM. Half-maximal and maximal induction required 15 and 100 nM, respectively (Fig. 3). A significant increase of β-galactosidase activity was observed at concentrations as low as 2.5 nM (Fig. 3, insert).

Comparison of induction of the *nodA* promoter by naringenin and by *V. sativa* root exudate. The time course of

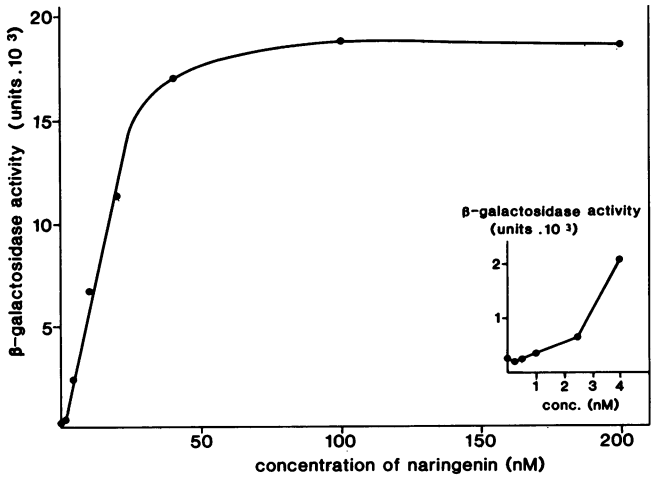

FIG. 3. Induction of promoter *nodA-lacZ* as a function of the naringenin concentration. Values represent averages of three measurements. The insert shows the values measured at low inducer concentrations. Incubation was for 18 h.

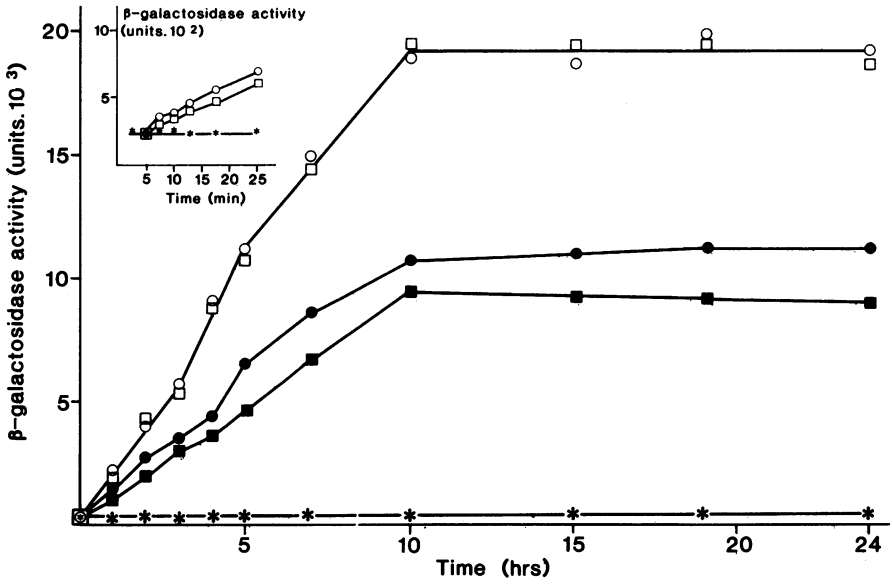

FIG. 4. Time course of the induction of the *nodA* promoter by naringenin and *V. sativa* root exudate. Values represent averages of three measurements. The insert shows the first 25 min of induction. Symbols: ●, 20 nM naringenin; ○, 100 nM naringenin; ■, exudate diluted 10-fold in induction medium; □, exudate diluted twofold in induction medium; *, induction medium.

induction of the *nodA* promoter by naringenin and *V. sativa* exudate was monitored for 24 h. The promoter appeared to be induced after only 5 min (Fig. 4, insert). In each case β-galactosidase levels increased linearly in time, reaching a plateau after approximately 10 h of incubation (Fig. 4). The induction characteristics of 2-fold-diluted exudate and 100 nM naringenin were almost identical, whereas 10-fold-diluted exudate was slightly less active than 20 nM naringenin, indicating that the concentration of inducer present in exudate is equivalent to 160 to 180 nM naringenin.

The genetic requirements for *nodA* promoter induction by naringenin and eriodictyol were the same as for induction by root exudate (Table 2). In addition to the cloned *nodA* promoter, the *nodD* gene of Sym plasmid pJB5JI was required and was sufficient, as was concluded from the results with strains RBL5561(pMP154) and RBL5560 (pMP158), respectively (Table 2).

Comparison of the chromatographic behavior of the major *nodA* promoter-inducing compound in exudate and the most active flavonoids. The chromatographic behavior of naringenin, eriodictyol, apigenin, and luteolin was compared with that of an ethanolic exudate extract on cellulose thin-layer chromatograms (Table 4). In solvent 2, the R_f value of exudate inducer differed significantly from that of luteolin and apigenin and only slightly from that of naringenin. In solvent 3, however, the R_f values of all four commercially available inducers were significantly lower than that of the natural inducer from *V. sativa* root exudate, indicating that the natural inducer is distinct from, but probably closely related to, the tested commercial preparations.

DISCUSSION

Induction of the *R. leguminosarum nodA* promoter by plant flavonoids. Nodulation genes of *Rhizobium* spp. can be induced by products of their respective host plants (8, 14, 16). Using the *nodA* promoter of *R. leguminosarum* in an expression vector, we monitored the behavior of the inducing substance(s) present in *V. sativa* root exudate. The physical properties of the major inducing compound and its chromatographic behavior suggested that is flavonoid in nature, probably a flavanone or isoflavone (Fig. 1). Of the large number of commercially available flavanones, isoflavones, and other related compounds that were tested, naringenin and apigenin, and to a less extent eriodictyol and luteolin, were found to be very powerful inducers of the *nodA* promoter (Table 3).

Structural requirements for inducers of the *nodA* promoter. The data obtained on the biological activity of naringenin, apigenin, eriodictyol, and luteolin (Table 3) indicate that the C-2–C-3 double bond of the flavones, which is absent in the flavanones (Fig. 2), is not important for *nod* gene induction. On the other hand hydroxylation of C-3 decreased the inducing activity substantially (compare the flavanones naringenin and eriodictyol and the flavones apigenin and

TABLE 4. R_f values of commercially available *nodA* promoter inducers and of the major inducing activity present in *V. sativa* root exudate extract[a] on cellulose thin-layer chromatograms

Inducer	R_f value of[b]:		
	Solvent 1	Solvent 2	Solvent 3
Naringenin	0.86	0.35	0.78
Eriodictyol	0.79	0.28	0.54
Apigenin	0.81	0.06	0.69
Luteolin	0.68	0.04	0.39
Exudate	0.86	0.27	0.87

[a] Thin-layer chromatography and preparation of ethanolic exudate extract were carried out as described in the text.

[b] R_f values for naringenin, eriodictyol, apigenin, and luteolin were calculated from the centers of spots visible under UV light. The localization of the inducer from exudate was determined by using the promoter induction assay described in the text.

luteolin in Table 3). The isoflavone genistein only differs from the flavone apigenin in that the B ring is attached to C-3 instead of C-2 (Fig. 2). As genistein is not able to provoke a significant promoter response (Table 3), which is in contrast to apigenin, we conclude that the attachment of the B ring to C-2, as is found in flavones and flavanones, is of crucial importance for induction.

The loss of the C-4′ hydroxyl group results in a dramatic decrease in the *nodA* promoter-inducing ability (compare apigenin and chrysin in Table 3). Although 7-hydroxyflavone also lacks the C_4' hydroxyl group, it is active. It differs from the inactive chrysin in that it lacks the C-5 hydroxyl group. 5-Hydroxyflavone is also inactive. Thus, hydroxylation of C-7 is essential, whereas an additional hydroxyl group at C-5 reduces biological activity considerably, unless C-4′ is also hydroxylated. Hydroxylation of C-5, C-7, and C-4′ (see apigenin in Table 3) in fact is more favorable than hydroxylation at C-7 only (see 7-hydroxyflavone in Table 3).

The inducing substances directly or indirectly activate the *nodA* gene in a *nodD*-mediated process. In discussing structural requirements of the inducing substance, it should be indicated that we do not know whether these structural requirements are necessary for uptake, possible alteration of the external inducing substance to a product that is the intracellular inducer, or interaction with the *nodD* gene product.

Naringenin can replace root exudate as a *nodA* gene inducer. The inducer present in exudate has physical and chromatographic properties of flavanone or isoflavone aglycones (Fig. 1). As isoflavones are apparently not able to induce the *nodA* promoter (Table 3), the exudate compound is presumably of a flavanone nature. The most likely candidates, the active flavanones naringenin and eriodictyol, however, differ from the exudate inducer, as judged from their chromatographic behavior (see solvent 3 in Table 4).

A characteristic of the induction of the *nodA* promoter by exudate is that the presence of a functional *nodD* gene is both required and sufficient (Table 2); these are observations that are consistent with similar investigations on the induction of *R. leguminosarum nodA*, *nodB*, and *nodC* (16) and of *R. meliloti nodC* (14). The genetic requirements for promoter induction by naringenin are exactly the same as those for exudate (Table 2). Thus, although naringenin and the exudate inducer are not identical, the kinetics of induction (Fig. 4) and the genetic requirements are similar. These findings, together with the physical and chromatographic characteristics of the inducer in exudate, strongly suggest that the latter substance is of a flavanone nature, probably closely resembling naringenin (and eriodictyol). Consistent with this notion are recent results on the nature of the natural inducers of inducible *nod* genes of *R. meliloti*, which has been identified as luteolin (15), and *R. trifolii*, which is also of a flavone nature (J. Redmond and B. Rolfe, personal communication).

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ADDENDUM IN PROOF

Recently, the *R. trifolii nod* gene-inducing compounds from *Trifolium repens* seedlings were isolated. They were identified as 7,4′-dihydroxyflavone, 7,4′-dihydroxy-3′-methoxyflavone (geraldone), and 4′-hydroxy-7-methoxyflavone (in order of decreasing inducing ability) (J. W. Redmond, M. Batley, M. A. Djordjevic. R. W. Innes, P. L. Kuempel, and B. G. Rolfe, Nature **323**:632–635, 1986).

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