MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 3, pp. 262-267, 1991

7,4'-Dihydroxyflavanone Is the Major *Azorhizobium nod* Gene-Inducing Factor Present in *Sesbania rostrata* Seedling Exudate

Eric Messens, Danny Geelen, Marc Van Montagu, and Marcelle Holsters

Laboratorium voor Genetica, Rijksuniversiteit Gent, B-9000 Gent, Belgium.
Received 12 December 1990. Accepted 25 January 1991.

Exudate from *Sesbania rostrata* seedlings contains signaling compounds that induce the common *nodABC* operon of the bacterial symbiont *Azorhizobium caulinodans* ORS571. An *Azorhizobium* strain harboring a *nodA*::*lacZ* reporter fusion was used to monitor the *nod*-inducing activity of crude exudate fractions that were separated by reversed-phase chromatography. The major inducer was shown, by spectroscopic analysis and by comparison with chemically synthesized compounds, to be 7,4'-dihydroxyflavanone (liquiritigenin). Newly synthesized analogues, 7,3'-dihydroxyflavanone and 7,2'-dihydroxyflavanone, have only poor inducing activity.

Additional keywords: gas chromatography, leguminous plants, mass spectrometry, symbiosis.

Phenolic compounds that are derived from seeds or roots of leguminous plants have been shown to serve as inducers of *nod* gene expression in *Rhizobium* and *Bradyrhizobium* species (reviewed in Peters and Verma 1990). Examples are the flavone luteolin in the alfalfa-*R. meliloti* Dangeard interaction (Peters *et al.* 1986) and the isoflavonoids genistein and daidzein in the soybean-*B. japonicum* (Buchanan) Jordan interaction (Kosslak *et al.* 1987). In each case, induction is mediated by one or more regulatory NodD proteins that bind to a conserved promoter consensus sequence located upstream of inducible *nod* operons (Hong *et al.* 1987; Fisher *et al.* 1988). The specificity of the interaction between a particular NodD protein and its inducer contributes to the determination of host specificity (Horvath *et al.* 1987). Besides inducers of *nod* gene expression, competitive inhibitors of induction may also be exuded by host plants (Firmin *et al.* 1986). It has been suggested that the nature and amount of these compounds differ during development of the plant root and according to the position on the root, such that the differential production of inducers and inhibitors helps the soil bacteria to nodulate particular infection sites or infectible regions (Peters and Long 1988).

In strain ORS571 of *Azorhizobium caulinodans* (Dreyfus *et al.* 1988), a newly described genus of soil bacteria nodulating roots and stems of the tropical leguminous plant *Sesbania rostrata* Brem (Dreyfus and Dommergues 1981), we have shown the presence of essential nodulation genes, which are related in sequence and organization to the common *nodABC* genes of (brady)rhizobia (Goethals *et al.* 1989). By means of *lacZ* fusions, *nodABC* gene expression was shown to be induced in the presence of host plant exudates or by the flavanone naringenin, and this activation was dependent upon a single *Azorhizobium nodD* gene (Goethals *et al.* 1990). In this study, we describe the purification and identification of the major *nod*-inducing compound present in *S. rostrata* seedling exudates.

Address correspondence to E. Messens: Laboratorium voor Genetica, Rijksuniversiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium.

MATERIALS AND METHODS

Bacterial strains and plasmids. An *A. caulinodans* ORS571 (Dreyfus *et al.* 1988) derivative harboring a *nodA*::*lacZ* reporter plasmid pRG290-12::T20 (Goethals *et al.* 1989) was used throughout this study.

Preparation and purification of exudate. *S. rostrata* seeds were surface-sterilized (Vincent 1970) and germinated on YEB (Vervliet *et al.* 1975) agar plates for 2 days in the dark at 33° C. Seedlings were incubated (one seedling per 2 ml) in distilled water for 24 hr at 33° C. Three hundred milliliters of exudate was dried by rotary evaporation and resuspended in 1:20 volume of 0.1% trifluoroacetic acid (F_3CCOOH). As a first purification, this solution (15 ml) was loaded on prepacked C18 cartridges (Sep-Pak, Waters Associates, Milford, MA; preactivation of the cartridge was done by consecutive washings with 20 ml of acetonitrile, water, and aqueous 0.1% F_3CCOOH). Biological activity eluted between 20 to 40% acetonitrile in aqueous 0.1% F_3CCOOH. The active fractions were pooled, dried, and redissolved in 5 ml of 8% acetonitrile in 0.1% F_3CCOOH.

The enriched batch was loaded on a Pep-RPC (RPC, reversed-phase chromatography) prepacked column (5 × 50 mm; HR 5/5 [Pharmacia-LKB, Uppsala, Sweden], preequilibrated with aqueous 0.1% F_3CCOOH) and eluted at 1 ml/min using a Pharmacia-LKB fast-protein liquid chromatography (FPLC) system equipped with the LCC 500 chromatographic programmer. A single-path UV monitor (214 nm) was used. The eluant contained a 30-min gradient of 10 to 25% acetonitrile in aqueous 0.1% F_3CCOOH, and 1-min fractions were collected. Minor

biological activity (less than 10%) was eluted at 11, 17.5, and 20% acetonitrile. Major biological activity eluted at about 15% acetonitrile.

The material in the major activity peak was dried and purified to homogeneity with a gradient over 30 min, 1 ml/min, 12 to 18% acetonitrile, on the HR 5/5 column. UV and biological activity monitoring coincided at 15.2% acetonitrile by two additional separations. The dried material was suspended in methanol to about 50 μM. Shift reagents and acid hydrolysis were as described previously (Markham and Mabry 1975; Markham 1982). Gas chromatography/mass spectrometry (GC/MS) was done with a Finnegan 4000 instrument interfaced with an Incos data system. Samples were derivatized by the treatment of dried material (1–20 μg) with 0.1 ml of *N,O*-bis(trimethylsilyl)trifluoroacetamide (TMS) (Alltech Associates, Deerfield, IL) in 0.1 ml of acetonitrile. For GC, 0.1 to 1.0 μl of freshly derivatized samples (TMS-der.) were injected directly onto a 0.25 mm × 20 m OV-1 column ("on column") and eluted over a temperature gradient of 5° C per minute from 70–250° C at 3.5 ml of helium per minute.

Flavonoids. Naringenin and naringin (naringenin-7-glucoside) were purchased from Roth Inc., Karlsruhe, Federal Republic of Germany.

The synthesis of 7,4′-dihydroxyflavanone or liquiritigenin was done as described previously (Van Hulle *et al.* 1971; Wagner and Farkas 1975). The intermediate 4,2′,4′-trihydroxychalcone or isoliquiritigenin yields 22% bright yellow crystals upon recrystallization from toluene and has an mp of 218–220° C. RPC-FPLC elution was at 21.1% acetonitrile. In accordance with Ingham (1979), UV absorption with shift reagents is as follows: λ_{max}^{MeOH} 370 nm (log ϵ = 4.20); λ_{max}^{NaOMe} 430 nm; λ_{max}^{NaOAc} 414 nm; $\lambda_{max}^{NaOAc/H3BO3}$ 380 nm; λ_{max}^{AlCl3} 423 nm; and $\lambda_{max}^{AlCl3/HCl}$ 416 nm. MS, mass-to-charge ratio (m/z) from $(TMS)_3$-der., was (relative intensity) M^+ 474 (33), 309 (15), 267 (28), 222 (20), 192 (52), 179 (100), 177 (38), 147 (22), 133 (26), and 73 (66). GC retention time of $(TMS)_3$-der. was 17.9 min. Batches of about 5 mg of final crude reaction mixture containing both 7,4′-dihydroxyflavanone and 4,2′,4′-trihydroxychalcone in equilibrium were separated by RPC-FPLC with a gradient 10 to 25% proportions of acetonitrile in aqueous 0.1% F_3CCOOH. White crystalline liquiritigenin with an mp of 208–210° C (cf. 208–210° C; Jurd and Wong 1984) formed in the peak center fraction eluted at 15.0% proportion of acetonitrile. All peak fractions were dried. Overall yield was about 58%. In accordance with Ingham (1979), UV absorption with shift reagents is as follows: λ_{max}^{MeOH} 274.8 nm (log ϵ = 4.14) and 313 nm (log ϵ = 3.84); λ_{max}^{NaOMe} 335.5 nm; λ_{max}^{NaOAc} 333.8 nm; $\lambda_{max}^{NaOAc/H3BO3}$ 277.0 and 315 nm; λ_{max}^{AlCl3} 274.8 nm; and $\lambda_{max}^{AlCl3/HCl}$ 274.2 nm. MS, m/z from $(TMS)_2$-der., was (relative intensity) M^+ 400 (58), 385 (15), 235 (22), 209 (10), 193 (26), 192 (86), 185 (25), 179 (100), 177 (52), and 73 (70). GC retention time of $(TMS)_2$-der. was 16.6 min.

Synthesis of 7,3′-dihydroxyflavanone was as follows: 2,4-dihydroxyacetophenone (3 g) and 3-hydroxybenzaldehyde (2.4 g) were dissolved in methanol (5 ml). After cooling to 0° C, a solution (42 ml) of 60% potassium hydroxide in water was added. The mixture was kept at room temperature for 24 hr before pouring into cold diluted hydrochloric acid. The pale yellow precipitate 3,2′,4′-trihydroxychalcone was washed with water, dried at 100° C, and recrystallized bright yellow from toluene. The yield was 17%, the mp was 238–240° C, and RPC-FPLC elution was at 23.6% proportion of acetonitrile. UV absorption with shift reagents is as follows: λ_{max}^{MeOH} 356 nm (log ϵ = 4.18); λ_{max}^{NaOMe} 395 nm; λ_{max}^{NaOAc} 391 nm; $\lambda_{max}^{NaOAc/H3BO3}$ 361 nm; λ_{max}^{AlCl3} 362 nm; and $\lambda_{max}^{AlCl3/HCl}$ 360 nm. GC/MS was not determined. The chalcone (0.5 g) was dissolved in methanol (25 ml) containing 5% concentrated hydrochloric acid and heated for 24 hr under reflux. After evaporation of the methanol, the water layer was extracted with diethylether, the ether was evaporated, and the residue was redissolved in 8% acetonitrile in aqueous 0.1% F_3CCOOH. Batches of about 5 mg of crude material were purified by RPC-FPLC gradient (10–25%) as described above. 7,3′-Dihydroxyflavanone eluted at 15.6% acetonitrile. The yield was 66%. UV absorption with shift reagents is as follows: λ_{max}^{MeOH} 275.6 nm (log ϵ = 4.14) and 313 nm (log ϵ = 3.84); λ_{max}^{NaOMe} 333.4 nm; λ_{max}^{NaOAc} 333.4 nm; $\lambda_{max}^{NaOAc/H3BO3}$ 278 and 315 nm; λ_{max}^{AlCl3} 375.6 nm; and $\lambda_{max}^{AlCl3/HCl}$ 275.4 nm. MS, m/z from $(TMS)_2$-der., was (relative intensity) M^+ 400 (100), 235 (99), 209 (22), 192 (50), 177 (56), and 73 (70). GC retention time of $(TMS)_2$-der. was 15.7 min.

Synthesis of 7,2′-dihydroxyflavanone was done as above starting from 2-hydroxybenzaldehyde (2.4 g). Condensation for 16 hr at 4° C yielded a deep yellow 2,2′,4′-trihydroxychalcone intermediate (21%) upon crystallization from toluene. The mp was 199–200° C, and RPC-FPLC elution was at 24.5% acetonitrile. UV absorption with shift reagents is as follows: λ_{max}^{MeOH} 369 nm (log ϵ = 4.20); λ_{max}^{NaOMe} 433 nm; λ_{max}^{NaOAc} 394 nm; $\lambda_{max}^{NaOAc/H3BO3}$ 377 nm; λ_{max}^{AlCl3} 416 nm; and $\lambda_{max}^{AlCl3/HCl}$ 370 nm. GC/MS was not determined. 7,2′-Dihydroxyflavanone and 2,2′,4′-trihydroxychalcone in the reaction mixture were purified, and the 7,2′-dihydroxyflavanone was eluted at 17.0% acetonitrile. Peak fractions were dried. The yield was 21.5%. UV absorption with shift reagents is as follows: λ_{max}^{MeOH} 275.4 nm (log ϵ = 4.14) and 313 nm (log ϵ = 3.84); λ_{max}^{NaOMe} 333.8 nm; λ_{max}^{NaOAc} 334.0 nm; $\lambda_{max}^{NaOAc/H3BO3}$ 276 and 313 nm; λ_{max}^{AlCl3} 276.2 nm; and $\lambda_{max}^{AlCl3/HCl}$ 275.2 nm. MS, m/z from $(TMS)_2$-der., was (relative intensity) M^+ 400 (85), 385 (15), 343 (20), 311 (15), 281 (12), 235 (42), 209 (35), 193 (18), 192 (40), 179 (30), 177 (56), 166 (26), 161 (24), 151 (35), and 73 (100). GC retention time of $(TMS)_2$-der. was 14.9 min.

Synthesis of 7-hydroxy-4′-methoxyflavanone was done as above starting from 4-anisaldehyde (2.7 g). Condensation for 48 hr at room temperature yielded the intermediate 2′,4′-dihydroxy-4-methoxychalcone (4′-*O*-methylisoliquiritigenin) (23%) as large orange crystals upon recrystallization from toluene. The mp was 186–187° C, and RPC-FPLC elution was at 25.0% acetonitrile. UV absorption with shift reagents is as follows: λ_{max}^{MeOH} 365 nm (log ϵ = 4.18); λ_{max}^{NaOMe} 396 nm; λ_{max}^{NaOAc} 392.5 nm; $\lambda_{max}^{NaOAc/H3BO3}$ 372.5 nm; λ_{max}^{AlCl3} 415.5 nm; and $\lambda_{max}^{AlCl3/HCl}$ 369 and 411 nm. The 7-hydroxy-4′-methoxyflavanone (4′-*O*-methylliquiritigenin) was isolated by elution from RPC-FPLC at 20.1% acetonitrile. UV absorption with shift reagents is as follows: λ_{max}^{MeOH} 274 nm (log ϵ = 4.16) and 313 nm (log ϵ = 3.86); λ_{max}^{NaOMe} 333.4 nm; λ_{max}^{NaOAc} 332.8 nm;

$\lambda_{max}^{NaOAc/H3BO3}$ 277 and 315 nm; λ_{max}^{AlCl3} 275.0 nm; and $\lambda_{max}^{AlCl3/HCl}$ 274.0 nm. MS, *m/z* from the TMS-der., was (relative intensity) M^+ 342 (47), 235 (15), 209 (9), 193 (12), 134 (100), 122 (49), 119 (18), 91 (16), and 73 (20). The *m/z* = 134 (100) is $H_2C{=}CH{\cdot}C_6H_4{\cdot}OCH_3$. GC retention time of the TMS-der. was 15.5 min. These data are in accordance with those of the natural isolates (Achenbach *et al.* 1988).

Bioassays. Samples from RPC-FPLC fractionation were dried *in vacuo* and resuspended in water. Flavonoid concentrations of standards and isolates were determined according to their respective molar extinction coefficients at λ_{max}. Strain ORS571 (pRG290-12::T20) was grown to log phase in YEB medium (Vervliet *et al.* 1975), diluted 10-fold in fresh medium, and incubated for 14 hr in the presence of dilutions of samples to be tested or of a known flavonoid concentration. β-Galactosidase activity was quantified using *o*-nitrophenyl-β-D-galactoside as substrate according to Miller (1972).

RESULTS

Identification of a natural inducer from seedling exudate. Previously we have shown by an *in situ* plate assay (Goethals *et al.* 1989) that seedlings of *S. rostrata*, as well as dormant stem-located root primordia, produce signals which diffuse in the surrounding medium and induce the expression of *Azorhizobium nod* genes. To further characterize these *nod*-inducing compounds, exudate from 2-day-old *S. rostrata* seedlings was fractionated by RPC. The *nod*-inducing activity was monitored by measuring the induction levels of a *nodA::lacZ* fusion in strain ORS571 (pRG290-12::T20).

RPC fractionation showed a major peak of biological activity eluting at about 15% acetonitrile. Three other fractions, corresponding to elution at about 11, 17.5, and 21% acetonitrile, respectively, contained only very weak activity and were not analyzed further. The material in the most active peak was pooled and purified to homogeneity. The structure 7,4′-dihydroxyflavanone (liquiritigenin) was assigned to the purified compound on the basis of the following data.

The stability and increase in intensity of the bathochromic shift in the UV spectrum under basic conditions suggest a flavanone or dihydroflavonol (Markham and Mabry 1975; Markham 1982) (Fig. 1A). The NaOMe-induced spectrum is identical to the NaOAc-induced one and is indicative of the absence of vicinal hydroxyl groups and the presence of a free 7-hydroxyl group. The unchanged methanol spectrum after the addition of aluminum chloride indicates no or a substituted 5-hydroxyl group (data not shown).

After treatment of the purified compound with 1 M trifluoroacetic acid for 30 min at 100° C or with 2 M HCl in aqueous 50% methanol for 1 hr at 100° C, the UV spectrum, chromatographic behavior, and biological activity remained identical. Thus, the inducer cannot be an *O*-glycosylated flavonoid (data not shown).

The GC/MS spectrum of the purified compound was obtained using a trimethylsilyl-derivatized sample. Panels B and C of Figure 1 show the mass spectra and the gas chromatograms, respectively. The molecular ion occurs at *m/z* 400, consistent with a diderivatized (2 × 73) dihydroxyflavanone (256) with a molecular formula of $C_{15}H_{12}O_4$. The most abundant ion, at *m/z* 179, is $^+CH_2{\cdot}C_6H_4{\cdot}OSi(CH_3)_3$. The ion at *m/z* 192 corresponds to $^+CH_2{=}CH{\cdot}C_6H_4{\cdot}OSi(CH_3)_3$, and in addition, the ion at *m/z* 235 is in agreement with a flavanone with one hydroxyl group on the A-ring.

From these data only the position of the hydroxyl group on the 7-place of the A-ring could be assigned. For the assignment of the position of the hydroxyl group on the B-ring, a comparison with analogues was required (Fig. 1). Because they were not commercially available, analogues were synthesized by condensation of 2,4-dihydroxyacetophenone with the appropriate aromatic aldehydes (para, meta, or ortho hydroxy and para methoxy). First the intermediate chalcones were prepared by aldol condensation, and subsequently the flavanones were obtained from their corresponding chalcones by acid-catalyzed ring closure (Wagner and Farkas 1975). The pure compounds were isolated by RPC-FPLC. The UV spectra, mass spectra, and gas chromatogram of the purified inducer were identical to synthetic or naturally occurring 7,4′-dihydroxyflavanone (Van Hulle *et al.* 1971; Achenbach *et al.* 1988). Moreover, the natural inducer and 7,4′-dihydroxyflavanone coeluted under conditions that allowed a clear separation of all our synthetic isomers from an RPC column (7,4′-dihydroxyflavanone at 15.0% acetonitrile, 7,3′-dihydroxyflavanone at 15.6% acetonitrile, 7,2′-dihydroxyflavanone at 17.0% acetonitrile, and 7-hydroxy-4′-methoxyflavanone at 20.1% acetonitrile). Approximately 5 μg of purified natural inducer was obtained from 300 ml of crude exudate.

In Figure 2 the biological activities of the synthesized analogues, the intermediate 4,2′,4′-trihydroxychalcone or isoliquiritigenin, and commercially purchased naringenin (5,7,4′-trihydroxyflavanone) are compared. Naringenin is the flavanone that was previously shown (by screening of a series of phenolic compounds) to be a good *nod* inducer for *Azorhizobium* (Goethals *et al.* 1989). Induction levels with liquiritigenin and naringenin are almost identical. Efficient induction by isoliquiritigenin was dependent on higher concentrations. The other synthetic compounds tested, although structurally very related, were very poor inducers. The activity of the purified inducer from *S. rostrata* exudate was measured at two concentrations and found to be *nod*-inducing at the range of naringenin and synthetic liquiritigenin.

During screening for antagonists of induction, only 7,2′-dihydroxyflavanone, by itself devoid of any significant inducing activity, was found to have a notable inhibitory effect: in the presence of 10 μM 7,2′-dihydroxyflavanone, the induction of ORS571 (*nodA::lacZ*) by 1 μM liquiritigenin was reduced threefold.

DISCUSSION

The data presented here show that the major molecule which is responsible for the azorhizobial *nod* gene-inducing activity of *S. rostrata* seedling exudate is 7,4′-dihydroxyflavanone, better known as liquiritigenin. The

trivial name originated from the first isolation of the compound from "liquorice" roots (Shinoda and Ueeda 1934). Liquiritigenin was also shown to be present in alfalfa root exudate (where it was found to be only a poor inducer in the specific *R. meliloti*-alfalfa interaction; Maxwell *et al.* 1989), and accordingly, it has been observed that the

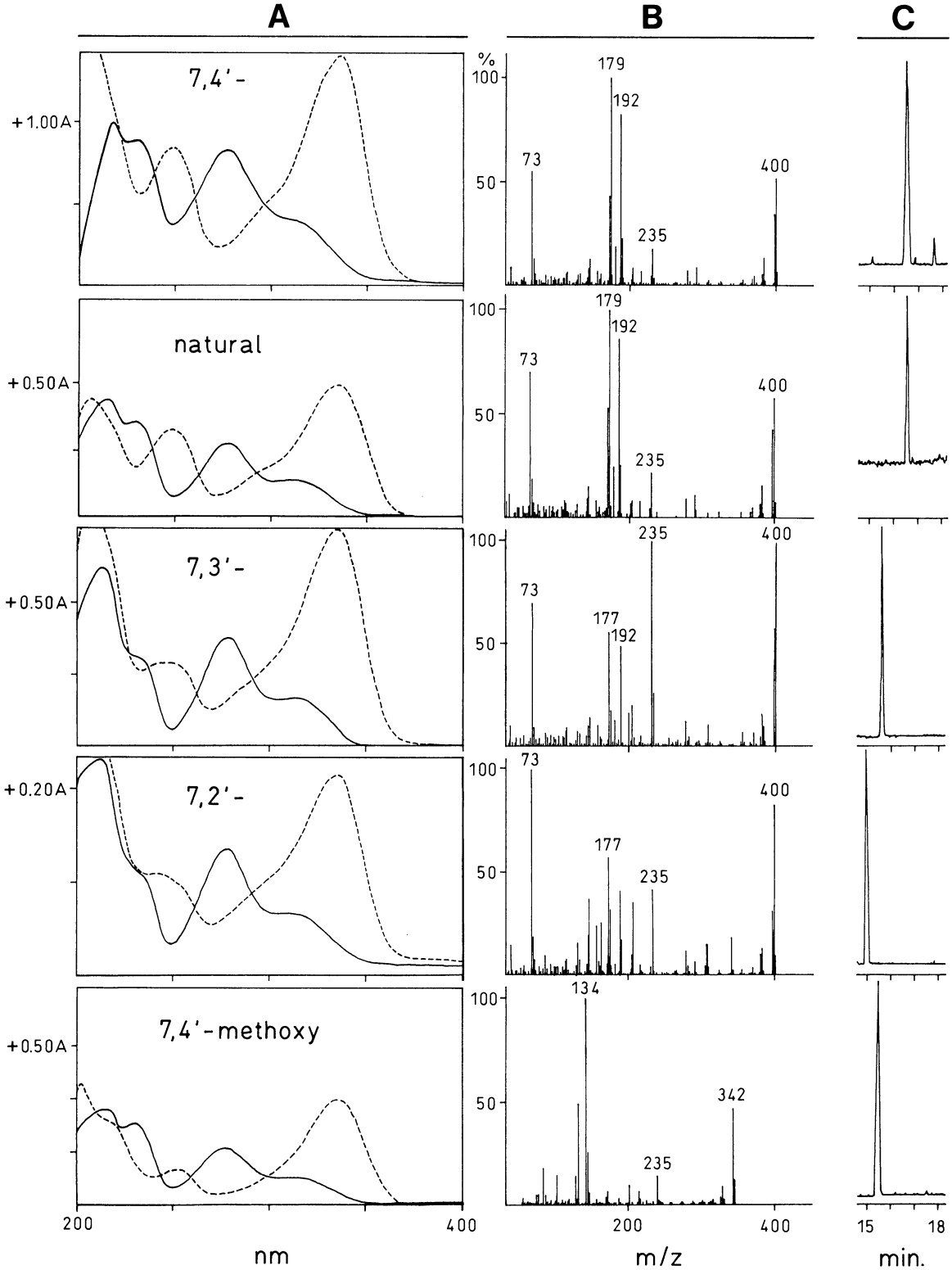

Fig. 1. Identification of *Sesbania rostrata nod* gene-inducing factor as 7,4'-dihydroxyflavanone by gas chromatography/mass spectrometry and UV spectral analyses. **A**, Scanning UV spectra, determined in methanol (—) and methanol/sodium methoxide (- - - -) for natural compound and synthetic dihydroxyflavanone analogues. **B**, Mass spectra of trimethylsilyl-derivatized natural compound and synthetic analogues. **C**, Respective gas chromatograms of 1–100 ng of freshly derivatized material directly injected onto a 0.25 mm × 20 m OV-1 column and eluted over a temperature gradient of 5° C per minute from 70–250° C at 3.5 ml of helium per minute.

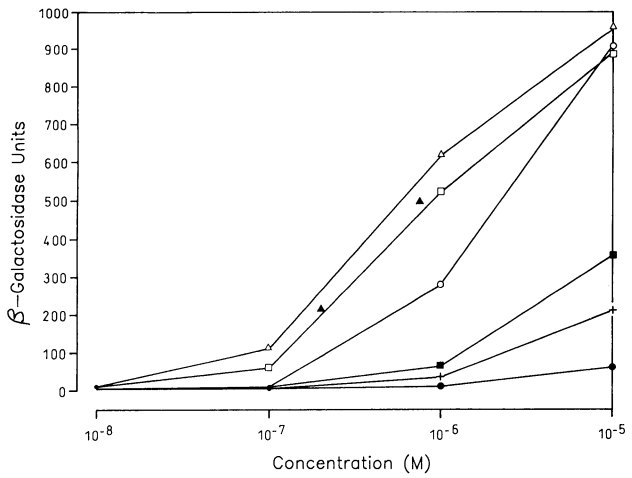

Fig. 2. Comparison of *nod* gene-inducing activity of the natural compound, naringenin, liquiritigenin, and synthetic analogues. Units of β-galactosidase activity induced in the *nodA::lacZ* tester strain ORS571 (pRG290-12::T20) are plotted against the concentration of inducer, which was determined from absorbance measured at its respective λ_{max}. Symbols indicate the following: □, naringenin; △, liquiritigenin; ○, isoliquiritigenin; ▲, liquiritigenin purified from *Sesbania rostrata*; ■, 7,3′-dihydroxyflavanone; ●, 7,2′-dihydroxyflavanone; and +, 7-hydroxy-4′-methoxyflavanone.

expression of a *nodA::lacZ* fusion in ORS571 was very efficiently induced by alfalfa seedling exudate. The same results were obtained with mung bean (M. Holsters, unpublished data). Neither mung bean nor alfalfa, however, is a natural host for nodulation by *A. caulinodans*. Factors other than the lack of efficient *nod* gene induction must therefore play a role in limiting the host range of *Azorhizobium* to a few *Sesbania* species.

Preliminary indications suggested the inducer to be a triglycosylated apigenin. This apigenin triglycoside has structure assignment 7-*O*-α-L-rhamnopyranosyl-4′-*O*-rutinosyl-apigenin and is present in high amounts in exudates (estimated 1 μg per 24 hr per seed; Messens *et al.* 1989). Under certain conditions it elutes from RPC at positions close to 7,4′-dihydroxyflavanone. However, better adjustment of the experimental conditions allows the two to separate well. It is clearly the 7,4′-dihydroxyflavanone, much less abundant in crude exudates and estimated 40 ng per 24 hr per seed (25-fold less abundant than the apigenin triglycoside), that is responsible for *nod*-inducing activity.

Apart from liquiritigenin, naringenin (Goethals *et al.* 1989) and the 4,2′,4′-trihydroxychalcone or isoliquiritigenin were also found to be comparably good inducers of ORS571 *nod* gene expression. A previous screening of phenolic compounds (Goethals *et al.* 1989) together with the present study of analogues suggest that the spectrum of molecules which is able to induce efficiently the ORS571 *nod* genes is rather limited.

As has been described in many rhizobial *nodD*-flavonoid interactions, a 7-OH group is also essential for biological activity in *A. caulinodans*. Neither naringin, which carries an *O*-glycosylation at the 7-OH position, nor 4′-OH flavanone, which lacks this 7-OH group, could mediate induction (Goethals *et al.* 1989; D. Geelen, unpublished data).

Azorhizobium has been shown to harbor a single functional *nodD* gene that is responsible for the plant-induced early gene expression (Goethals *et al.* 1990), and the encoded NodD protein appears to be fairly selective in its interaction with flavonoids. This should be kept in mind when speculating on the nature of the inducing compound(s) exuded by stem-located root primordia that are the infection sites for aerial nodule development. We have indeed shown with an *in situ* plate assay that induction of bacterial gene expression occurs in a semicircular area around these root primordia with the same homogeneous intensity of induced β-galactosidase activity as seen around the young *S. rostrata* seedlings. The adult root system produces far less inducing activity (M. Holsters, unpublished data). These observations, together with the fact that there is only one ORS571 *nodD* gene essential for root as well as "stem" nodulation and featuring a selectivity for only a few flavonoids, suggest that the same inducer will be produced by both seedlings and stem-located root primordia. However, this point remains to be proven. To do so, exudate prepared from *S. rostrata* stems should be analyzed. A suitable control for such a preparation may be provided by the stems of an *S. rostrata* mutant (B. Dreyfus, personal communication) that lacks the stem-located root primordia.

ACKNOWLEDGMENTS

We thank Ir. M. Schelfaut (Rijksuniversiteit Gent) for GC/MS spectra, K. Tomekpe and Guy Van den Eede for help with bioassays of exudates, Chris Bowler for critical reading of the manuscript, Martine De Cock for typing it, and Vera Vermaercke and Stefaan Van Gijsegem for the artwork. This work was supported by grants from the Services of the Prime Minister, Brussels (UIAP 12OC0187), the Commission of the European Communities, Brussesls (TS2-0135-B), the "Vlaams Ontwikkelingsfonds," Brussels, and the "Ministerie voor Vlaamse Gemeenschap," Brussels, and by a collaborative research grant from the North Atlantic Treaty Organization. M. Holsters is a research associate of the National Fund for Scientific Research (Brussels), and D. Geelen is indebted to the Instituut ter aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw (Brussels) for a predoctoral fellowship.

LITERATURE CITED

Achenbach, H., Stöcker, M., and Constenla, M. A. 1988. Flavonoid [illegible] other constituents of *Bauhinia manca*. Phytochemistry 27:1835-18[illegible]

Dreyfus, B., and Dommergues, Y. R. 1981. Nitrogen-fixing nodules induced by *Rhizobium* on the stem of the tropical legume *Sesbania rostrata*. FEMS Microbiol. Lett. 10:313-317.

Dreyfus, B., Garcia, J. L., and Gillis, M. 1988. Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. Int. J. Syst. Bacteriol. 38:89-98.

Firmin, J. L., Wilson, K. E., Rossen, L., and Johnston, A. W. B. 1986. Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. Nature (London) 324:90-92.

Fisher, R. F., Egelhoff, T. T., Mulligan, J. T., and Long, S. R. 1988. Specific binding of proteins from *Rhizobium meliloti* cell-free extracts containing NodD to DNA sequences upstream of inducible nodulation genes. Genes Dev. 2:282-293.

Goethals, K., Gao, M., Tomekpe, K., Van Montagu, M., and Holsters, M. 1989. Common *nodABC* genes in Nod locus *1* of *Azorhizobium caulinodans*: Nucleotide sequence and plant-inducible expression. Mol. Gen. Genet. 219:289-298.

Goethals, K., Van den Eede, G., Van Montagu, M., and Holsters, M. 1990. Identification and characterization of a functional *nodD* gene in *Azorhizobium caulinodans* strain ORS571. J. Bacteriol. 172:2658-2666.

Hong, G.-F., Burn, J. E., and Johnston, A. W. B. 1987. Evidence that DNA involved in the expression of nodulation (*nod*) genes in *Rhizobium* binds to the product of the regulatory gene *nodD*. Nucleic Acids Res. 15:9677-9690.

Horvath, B., Bachem, C. W. B., Schell, J., and Kondorosi, A. 1987. Host-specific regulation of nodulation genes in *Rhizobium* is mediated by a plant-signal, interacting with the *nodD* gene product. EMBO J. 6:841-848.

Ingham, J. L. 1979. Isoflavonoid phytoalexins of the genus *Medicago*. Biochem. Syst. Ecol. 7:29-34.

Jurd, L., and Wong, R. Y. 1984. Diarylheptanoid and other phenolic constituents of *Centrolobium* species. Aust. J. Chem. 37:1127-1133.

Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H. E., and Appelbaum, E. R. 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. Proc. Natl. Acad. Sci. USA 84:7428-7432.

Markham, K. R. 1982. Ultraviolet-visible absorption spectroscopy. Pages 36-51 in: Techniques of Flavonoid Identification. K. R. Markham, ed. Academic Press, London.

Markham, K. R., and Mabry, T. J. 1975. Ultraviolet-visible and proton magnetic resonance spectroscopy of flavonoids. Pages 45-77 in: The Flavonoids. J. B. Harborne, T. J. Mabry, and H. Mabry, eds. Chapman and Hall, London.

Maxwell, C. A., Hartwig, U. A., Joseph, C. M., and Phillips, D. A. 1989. A chalcone and two related flavonoids released from alfalfa roots induce *nod* genes of *Rhizobium meliloti*. Plant Physiol. 91:842-847.

Messens, E., Van Montagu, M., De Bruyn, A., and Jans, A. W. H. 1989. A new glycosylated flavonoid, 7-*O*-α-L-rhamnopyranosyl-4′-*O*-rutinosylapigenin, in the exudate from germinating seeds of *Sesbania rostrata*. Carbohydr. Res. 186:241-253.

Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 466 pp.

Peters, N. K., and Long, S. R. 1988. Alfalfa root exudates and compounds which promote or inhibit induction of *Rhizobium meliloti* nodulation genes. Plant Physiol. 88:396-400.

Peters, N. K., and Verma, D. P. S. 1990. Phenolic compounds as regulators of gene expression in plant-microbe interactions. Mol. Plant-Microbe Interact. 3:4-8.

Peters, N. K., Frost, J. W., and Long, S. R. 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science 233:977-980.

Shinoda, J., and Ueeda, S. 1934. Über das flavanon-glucosid in *Glycyrrhiza glabra* L. var. *glandulifera*, Regel et Herder. Chem. Ber. 67:434-440.

Van Hulle, C., Braeckman, P., and Vandewalle, M. 1971. Isolation of two new flavonoids from the root of *Glycyrrhiza glabra* var. *typica*. Planta Med. 20:52-60.

Vervliet, G., Holsters, M., Teuchy, H., Van Montagu, M., and Schell, J. 1975. Characterization of different plaque-forming and defective temperate phages in *Agrobacterium* strains. J. Gen. Virol. 26:33-48.

Vincent, J. M. 1970. A Manual for the Practical Study of Root-Nodule Bacteria. IBP Handbook 15. Blackwell Scientific Publications, Oxford. 164 pp.

Wagner, H., and Farkas, L. 1975. Synthesis of flavonoids. Pages 127-213 in: The Flavonoids. J. B. Harborne, T. J. Mabry, and H. Mabry, eds. Chapman and Hall, London.