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A Plant Flavone, Luteolin, Induces Expression of *Rhizobium meliloti* Nodulation Genes

N. KENT PETERS, JOHN W. FROST, SHARON R. LONG*

The symbiotic interaction of *Rhizobium meliloti* and alfalfa results in the formation of nitrogen-fixing root nodules. *Rhizobium meliloti nodABC* genes are required for the early host responses of cortical cell divisions and root hair curling. The induction of *nodABC* expression by alfalfa exudates demonstrates host-symbiont signaling at an early stage in nodule development. The inducer molecule for *nodABC* expression was isolated from plant exudate by constructing a *nodABC-lacZ* fusion to monitor the inducing activity. From ultraviolet-visible absorption spectra, proton nuclear magnetic resonance, and mass spectrometry, the inducer was determined to be 3′,4′,5,7-tetrahydroxyflavone (luteolin). Luteolin is a normal secondary plant metabolite found throughout the plant kingdom that may serve to control *nodABC* expression during nodule development. This regulatory role for a flavone contrasts with the function of some flavonoids as defense compounds.

MANY SOIL MICROORGANISMS establish nutritionally beneficial symbiotic or parasitic relationships with plants. Bacteria of the genus *Rhizobium* fix nitrogen in root nodules on host plants of the family Leguminosae. This symbiosis develops through a complex and ordered sequence of events, including cell division and differentiation of both partners, which suggests the need for signaling between host and symbiont. A few bacterial and plant genes specifically required for this symbiosis have been identified, including those coding for bacterial nitrogenase and for plant leghemoglobin (*1*). The functions of other genes remain unknown, but can be assigned to specific stages of nodule development (*2*). For example, the *Rhizobium* common nodulation genes (*nodDABC*) are responsible for stimulating the earliest detectable host responses of cortical cell division (*3*) and root hair curling (*4, 5*). These genes are induced by compounds in exudates of plant hosts (*6–8*).

Another example of a bacterial response to plant compounds is the induction of the virulence (*vir*) genes of the opportunistic pathogen *Agrobacterium tumefaciens*. These genes are induced by acetosyringone (*9*) and other phenolic compounds (*10*) whose presence in plant exudates is increased when the plant has been wounded (*9*). In this report, we demonstrate that *R. meliloti nodABC* genes are induced by luteolin, a flavone that is present in exudates of alfalfa seeds.

Our initial observation that expression of *nodC* in *R. meliloti* can be induced by an exudate from alfalfa roots or seeds (*6*) prompted us to isolate and characterize the molecule or molecules responsible for this activity. The inducer was monitored during purification by assaying induction of a *nodABC-lacZ* translational fusion borne on plasmid pRmM57 (*6*). Preliminary tests of alfalfa seed exudate suggested that the inducer molecule was a small aromatic compound. Activity of the plant exudate was not affected by treatment with heat, protease, or nucleases. However, activity was removed from exudates by treatment with activated charcoal or by dialysis through membranes with a molecular weight exclusion limit of 2000 daltons (*11*). Because exposure of exudates to bacteria caused loss of their activity, it was necessary to keep exudates sterile; seeds were routinely used as a source to facilitate aseptic handling of plant material.

The exudate from alfalfa seeds was fractionated by high-pressure liquid chromatography (HPLC) using a reverse-phase C_{18} column. Fractions were assayed for *nodABC-lacZ* inducing activity (Fig. 1A). Activity eluted as a broad peak between 95 and 100% methanol, indicating that the inducing molecule had significant hydrophobic character. The inducer molecule could be partially purified by extracting the inducing factor into diethyl ether. These ether-extracted compounds were separated by HPLC, and fractions containing the inducing activity again eluted between 95 and 100% methanol, along with several ultraviolet (UV)-absorbing compounds (Fig. 1B). To resolve the inducer molecule from these coeluting compounds, we fractionated the ether-extracted materials on a methanol-water gradient of 50 to 100% (Fig. 1C). Under these conditions, the various components were resolved and the majority of inducing activity correlated with a single absorbance peak eluting at 90 to 95% methanol. Other UV-absorbing compounds with little inducing activity eluted between 70 and 75% methanol. Fractions containing the most activity (90 to 95% methanol) were pooled for structural analysis (Fig. 1C,*).

N. K. Peters and S. R. Long, Department of Biological Sciences, Stanford University, Stanford, CA 94305.
J. W. Frost, Department of Chemistry, Stanford University, Stanford, CA 94305.

*To whom correspondence should be addressed.

We determined the UV and visible absorbance spectra of the yellow colored inducer in methanol and methanol–sodium hydroxide. The band I absorbance of the inducer as measured in methanol displayed a bathochromic shift of 50 nm when strong base was added (Table 1). We speculated that the inducer might be a secondary plant metabolite, so we compared our spectra to various classes of molecules, including flavonoids (*12*). The observed absorption maxima and the magnitudes of the bathochromic shifts in strong base are characteristic of flavonoid molecules, particularly flavones. Further spectral analyses of the unknown compound, both in weak base and when complexed with a metal, are consistent with the reported spectra of 3′,4′,5,7,-tetrahydroxyflavone (luteolin) (Table 1), a compound that had previously been extracted from alfalfa seeds (*13*). We purchased synthetic luteolin, and determined that its spectra are indistinguishable from that of the purified inducer molecule (Fig. 2, A and B).

We analyzed isolated inducer and synthetic luteolin by mass spectrometry (*14*) and nuclear magnetic resonance spectroscopy (NMR). Both synthetic luteolin and isolated inducer share a molecular ion M^+ (*m/e* = 286) and a base peak (*m/e* = 153). The proton (^1H) NMR of isolated inducer (270 MHz, CD_3OD) (Me_4Si), 6.19 (d, J = 2 Hz, 1H), 6.42 (d, J = 2 Hz, 1H), 6.52 (s, 1H), 6.88 (d, J = 9 Hz, 1H), 7.35 (d, J = 2 Hz, 1H), 7.36 (dd, J = 9, 2 Hz, 1H), is identical to synthetic luteolin. Thus, isolated inducer and synthetic luteolin have indistinguishable absorption spectra, mass spectra, and proton NMR.

To test the biological activity of luteolin, we compared its ability to induce *nodABC-lacZ* with that of purified inducer at concentrations ranging over six orders of magnitude. The concentration dependence of the induction by the two compounds is indistinguishable (Fig. 3). Therefore, synthetic luteolin displays the same biological activity as the isolated inducer.

Compounds similar in structure to luteolin were tested for their ability to induce *nodABC-lacZ* (Fig. 4). Of the compounds tested, only apigenin has detectable inducing activity. At 50 μ*M*, which is a hundred times the concentration of luteolin required for full induction, apigenin has only 20% the activity of luteolin (Fig. 3). These results suggest structural features of the B and C rings that are required for inducing activity (Fig. 4). Chrysin's inactivity in the induction assay demonstrates the necessity for hydroxylation at the 3′ or 4′ positions or both of the B ring. Activity of apigenin shows that a hydroxylated 4′ position confers partial function and that 3′ hydroxylation is required for full activity. Failure of

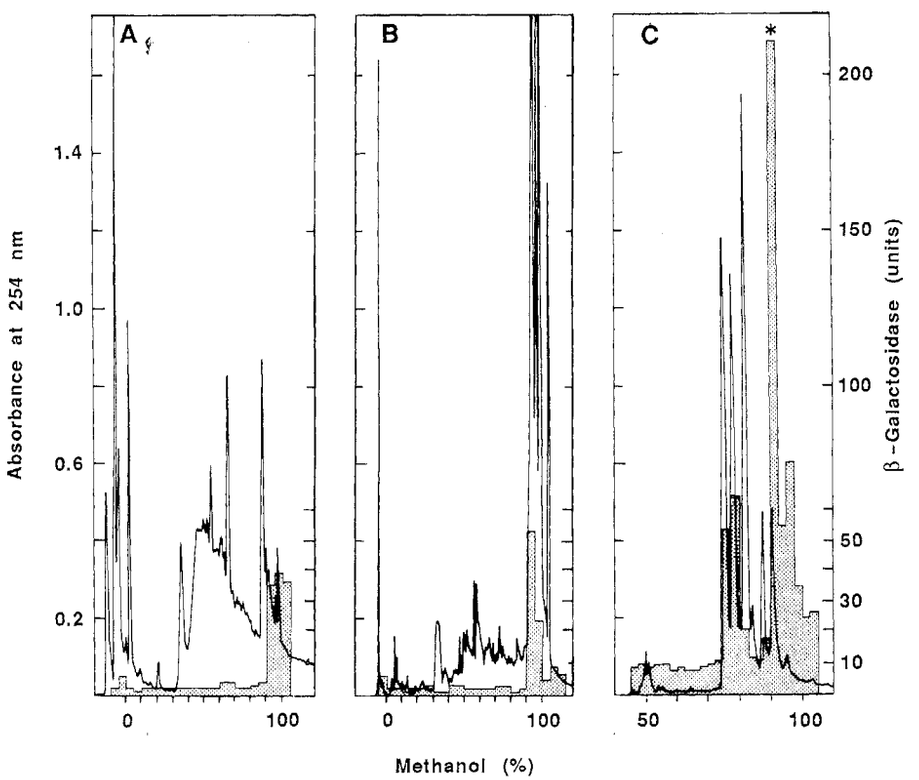


Fig. 1. Fractionation of exudate and ether-extracted compounds by reverse-phase liquid chromatography. The elution profile as monitored by absorption at 254 nm is shown (solid line) for aqueous exudate (A) and ether-extracted compounds (B and C). The β-galactosidase activity assayed in each fraction is shown as a shaded bar graph. Exudates were prepared from a 200-cm^3 packed volume of alfalfa seeds. Seeds were sterilized by treatment in 95% ethanol for 2 hours, and treatment in 4% sodium hypochlorite for 15 minutes. Seeds were rinsed in several volumes of sterile distilled water and soaked overnight in 4 volumes of sterile water. The exudate was decanted after 16 to 24 hours and the seeds were discarded. Greater than 90% of the activity was extracted into ethyl ether after three sequential partitionings with equal volumes of ether, provided the *p*H was less than 10. The pooled ether phases were subsequently dried in a rotary evaporator. The original aqueous exudate (A) and the concentrated ether extract (B) were analyzed with a Waters HPLC fitted with a 3.9 mm × 30 cm Bondapak C_{18} column. The sample was injected onto the column preequilibrated in water. Products were eluted at 1.0 ml/min for 4 minutes before increasing the methanol linearly to 100% over a 20-minute period. Fractions of 1.0 ml were collected, dried, resuspended in 100 μl of distilled H_2O, and assayed for inducing activity. For preparative isolation, seeds were extracted in boiling methanol. The extracted material was dried by rotary evaporation and resuspended in H_2O. The resuspended material was extracted with ether and processed as above. Extraction of the seeds with methanol was more efficient than preparing aqueous exudates and gave similar elution profiles of UV-absorbing compounds upon fractionation by HPLC. Preparative fractionations were as in (B), except a larger (7.8 mm × 30 cm) Bondapak C_{18} column was first equilibrated in water-methanol (50:50) and products eluted at 2.0 ml/min. Induction of *nodABC-lacZ* was assayed with a *nodABC-lacZ* translational fusion, Rm 1021/pRmM57, constructed as described (*6*). Assays were performed as described (*26*) and modified (*6*).

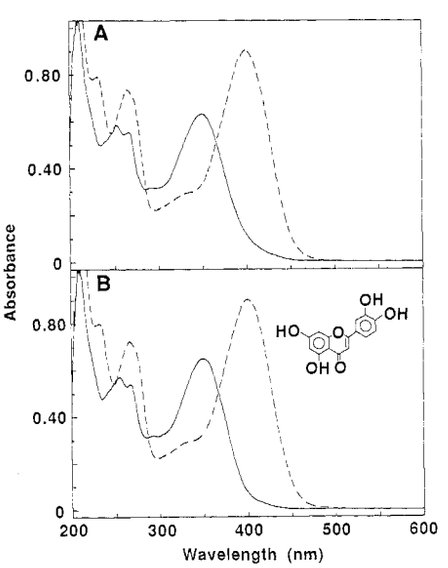


Fig. 2. Ultraviolet and visible absorption spectra of purified inducer and synthetic luteolin. The absorption spectra of purified inducer (A) and synthetic luteolin (B) are depicted as measured in methanol (——) and in methanol plus NaOH (--------). The structure of luteolin is shown in panel (B). The inducer was purified as described in Fig. 1. The purity of synthetic luteolin (ICN K&K Chemicals) was confirmed by UV-visible spectroscopy, and by ^1H-NMR and mass spectroscopic analysis. The absorption spectrum of each was first measured in methanol, then 10 μl of NaOH was added to a 1.0 ml sample and the absorption spectrum was remeasured.

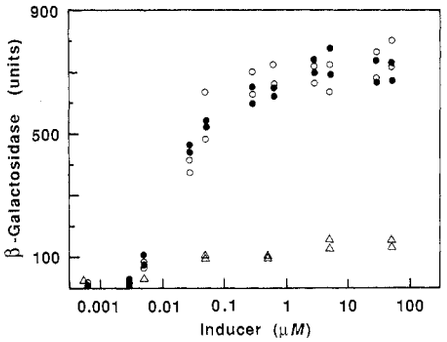


Fig. 3. Comparison of inducing activity of purified inducer and synthetic luteolin. The relative units of β-galactosidase activity are plotted on the vertical axis versus the concentration of purified inducer (●), synthetic luteolin (○), and apigenin (△). The concentrations of inducer, luteolin, and apigenin were determined by absorbance. For luteolin and inducer an extinction coefficient of $10^{4.2}$ at 256 nm was used, and for apigenin an extinction coefficient of $10^{4.3}$ at 269 nm was used. The induction assays were performed as described in Fig. 1. Duplicate points are from a single experiment.

morin and quercetin to induce *nodABC-lacZ* demonstrates that hydroxylation either at the 2′ position or the 3 position renders the molecule ineffective.

The concentration dependence of *nodABC-lacZ* induction by luteolin resembles that observed for other inducible systems such as the lactose operon (*15*) and the arabinose operon (*16*); however, full induction of the *nodABC* operon occurs at inducer concentrations three to five orders of magnitude lower than the inducers of the metabolic lactose and arabinose operons. This greater sensitivity to inducer may reflect the concentrations of luteolin available in the rhizosphere.

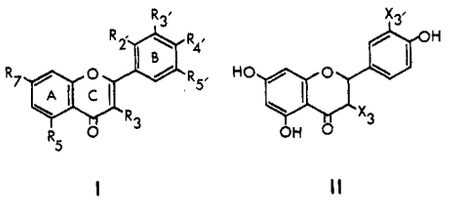
The identification of the inducer molecule reported here will permit more detailed study of the *nodABC* gene activation mechanism. Induction of *nodABC* requires not only the plant inducer, but also the gene product of *nodD* (*6*). Synthesis of radiolabeled inducer will make it possible to study the interaction of luteolin with the *nodD* gene product or other possible receptor molecules, and to follow the fate of luteolin in the bacterium.

Luteolin and apigenin are among hundreds of identified flavonoid compounds found in plants (*17, 18*). Most flavonoids are not well characterized functionally, although many are pigments functioning in reproduction as positive signals to pollinators. Other flavonoids have attracted interest because of their role in plant defense. Some isoflavonoids, for example, function as phytoalexins in legumes (*19*). It has been shown that flavonoid synthetic enzymes, such as phenylalanine ammonia lyase and chalcone synthase, increase in activity in response to infection by pathogens (*20*). It is interesting that normal infection by *Rhizobium* does not elicit a similar response (*21*), although *R. japonicum* mutant 61A24 causes accumulation of the phytoalexin glyceollin I in nonfixing soybean nodules (*22*).

Alfalfa exudates appear to contain a mixture of inducing compounds; of these, luteolin is the most active (Fig. 1C). The less active compounds are structurally related to luteolin, although their identities have not been determined. One active component may be apigenin, which has been found in extracts of alfalfa seeds (*13*).

Rhizobium meliloti successfully forms nodules on a few genera of legumes, including *Medicago* (alfalfa), *Melilotus* (sweet clover), and *Trigonella* (fenugreek) (*2*). Leguminous plants outside the *R. meliloti* cross-inoculation group also yield exudates that activate *R. meliloti nodABC* (*23*), but we have not identified the compound or compounds responsible for the activity. It will be of interest to know whether luteolin or other compounds are responsible for the inducing activity reported in other legume-*Rhizobium* symbiotic pairs (*7, 8*). Since the *nodD* gene product is required for induction and can complement across species, we predict that luteolin or a similar compound will act as an inducer for other symbiotic pairs.

Because the *nodABC* genes are required for a specific *Rhizobium*-legume interaction (*3–5*), we expected the molecule responsible for their induction to be specific to legumes. Luteolin, however, is widespread in the plant kingdom, having been identified in a variety of vascular plants and ferns (*24*).



I II

Fig. 4. The structure of compounds tested for inducing activity. Flavone (I) $R_{2'} = R_{3'} = R_3 = R_{4'} = R_{5'} = R_5 = R_7 = H$; flavanol (I) $R_{2'} = R_{3'} = R_{4'} = R_{5'} = R_5 = R_7 = H$, $R_3 = OH$; chrysin (I) $R_{2'} = R_{3'} = R_3 = R_{4'} = R_{5'} = H$, $R_5 = R_7 = OH$; fisetin (I) $R_{2'} = R_{5'} = R_5 = H$, $R_{3'} = R_3 = R_{4'} = R_7 = OH$; morin (I) $R_{3'} = R_3 = R_{5'} = H$, $R_{2'} = R_{4'} = R_5 = R_7 = OH$; myricetin (I) $R_{2'} = H$, $R_{3'} = R_3 = R_{4'} = R_{5'} = R_5 = R_7 = OH$; quercetin (I) $R_{2'} = R_{5'} = H$, $R_{3'} = R_3 = R_{4'} = R_5 = R_7 = OH$; apigenin (I) $R_{2'} = R_{3'} = R_3 = R_{5'} = H$, $R_{4'} = R_5 = R_7 = OH$; dihydroquercetin (II) $X_3 = X_{3'} = OH$; naringenin (II) $X_3 = X_{3'} = H$.

Thus, the specificity of the *Rhizobium*-legume interaction is not a result of the induction of the *nodABC* genes by luteolin.

It is interesting that the *vir* genes of the plant pathogen *A. tumefaciens* are also induced by a compound found in plant exudates. An inducing compound identified in tobacco, acetosyringone, is more prominent in exudates of wounded plants than of unwounded plants (*9*). This compound seems an appropriate molecule to induce the *vir* genes since a wound is required for infection by *A. tumefaciens*. In contrast, *R. meliloti* invades by root hair penetration and develops a stable coexistence with the plant host (*2*). The *nodABC* genes are required early in infection (*3–5*), and immunochemical data indicate that they are also expressed in developing nodules (*25*). The use of luteolin, a normal plant metabolite, as a signal may reflect the system's requirement for regulation throughout a lengthy, ordered developmental process, and underlines the distinction between the symbiotic and pathological strategies.

Table 1. Absorbance maxima (nanometers). Spectral analyses were performed as described (*12*) and data for luteolin were obtained from (*12*). MeOH, methanol; Ac, acetate.

Solvent	Band I		Band II	
	Luteolin	Inducer	Luteolin	Inducer
MeOH	349	351	253, 267	256, 267
MeOH + NaOH	401	405	266	266
MeOH + $AlCl_3$	328, 426	328, 426	271	274
MeOH + $AlCl_3$ + HCl	355	357	274	275
MeOH + NaAc	384	384	269	269
MeOH + NaAc + H_3BO_3	370	372	259	259

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Immortalization of Human T Lymphocytes After Transfection of Epstein-Barr Virus DNA

MARIO STEVENSON, BARBARA VOLSKY, MONA HEDENSKOG, DAVID J. VOLSKY*

Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, has the ability to transform human B lymphocytes. No other cell type has been experimentally transformed by EBV, either by intact virions or naked viral DNA and subgenomic fragments. Two immortalized human T-lymphoblastoid cell lines have now been established by transfecting cord blood lymphocytes with purified B95-8 viral DNA enclosed in fusogenic Sendai virus envelopes (RSVE) and then exposing the cells to EBV from a P3HR-1 cell subclone. One of these lines, which has been fully characterized, is termed HBD-1. This line is positive for EBV DNA and expresses surface OKT11, OKT4, and Tac receptors, but not M-1, μ immunoglobulin chains, EBV receptors, or B-1 surface markers. The cells contain fully rearranged T-cell receptor genes and germline immunoglobulin genes. The karyotype of the cells is normal, they do not require interleukin-2 for growth, and do not contain human T-lymphotropic virus type I. However, the HBD-1 cells contain incomplete EBV genomes and express several EBV-determined antigens, including the early antigen type D, membrane antigens, but not EBV-determined nuclear antigen (EBNA). This association of the EBV genome with permanently growing hematopoietic cells of non B-cell lineage should prove useful in studies on the mechanism of EBV-mediated cell transformation.

EPSTEIN-BARR VIRUS (EBV) IS A HUman herpesvirus that causes infectious mononucleosis and is believed to be etiologically associated with Burkitt's lymphoma and nasopharyngeal carcinoma (*1*). One of the major distinguishing features of EBV has been its inability to infect any cells other than human B lymphocytes. Only mature B lymphocytes expressing specific viral receptors have been infected by the virus in vitro (*2*). The infection immortalizes the cells and the resulting lymphoblastoid cell lines carry latent viral genomes, but do not support virus replication. The latently infected cells express EBV-determined nuclear antigens (EBNA) that are considered to be the first products of the viral cycle and to be important for cell transformation. However, we have now established two EBV DNA-positive but EBNA-negative human T-lymphoblastoid cell lines. These cell lines were obtained after cord blood lymphocytes (CBL) were transfected with purified B95-8 viral DNA enclosed in fusogenic Sendai virus envelopes and then exposed to EBV from a P3HR-1 cell subclone. The cells were negative for retrovirus, had normal karyotypes, and expressed several antigens associated with the EBV replicative cycle. The lack of EBNA expression suggests that this antigen might not be an essential indicator of EBV infection. The demonstration that EBV genomes can be maintained indefinitely in human T lymphocytes may have important implications with regard to the role of EBV in the pathogenesis of certain human diseases.

The EBV genome-positive T-cell lines were established when we were attempting to map the regions of the EBV genome involved in B lymphocyte immortalization (*3*). While studying the expression of cloned EBV DNA fragments in freshly isolated CBL, we found that the EBV DNA region located within the Bam HI–D1 fragment stimulated cell growth but did not induce EBNA or cell immortalization (*3*). Because this region contains sequences deleted in the nontransforming P3HR-1 (P-EBV) virus DNA (*4*), we investigated whether freshly isolated lymphocytes could be transformed by concomitant exposure to P3HR-1 virus and either purified EBV DNA or the cloned Bam HI–D1 region that is missing from the P-EBV genome. We introduced the EBV DNA into CBL by a recently developed technique in which gene transfer is mediated by reconstituted Sendai virus envelopes (RSVE) (*3, 5, 6*). The DNA was encapsulated in fusogenic Sendai virus envelopes during envelope reconstitution. The DNA-containing RSVE were then fused with target cells and exposed to P3HR-1 virus of the HH514-16 strain (HH-EBV) (*6*). We chose this viral isolate because it was shown to induce little or no EBV-determined early antigen (EA) upon superinfection of Raji cells (*7*) and thus could be expected to be less cytotoxic than the parent P3HR-1 strain. The DNA-transfected, HH-EBV–exposed cells were cultured on an irradiated feeder layer of normal human fibroblasts (from explanted tonsil tissue) at 37°C in 5% CO_2 and tested weekly for EBNA induction and morphologic transformation. No EBNA induction or transformation was observed in cells coinfected with Bam HI–D1 fragment and HH-EBV. In contrast, cells transfected with purified EBV DNA from the transforming B95-8 strain and exposed to HH-EBV rapidly proliferated, then declined to few living cells about 4 weeks after infection, and finally grew out into a permanently transformed cell line about 2 months from the beginning of the experiment. As in previous experiments (*3*), CBL treated with RSVE/EBV DNA alone were transiently growth-stimulated and died after 4 to 5

Molecular Biology Laboratory, Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68105.

*To whom correspondence should be addressed.