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The *Rhizobium* Strain NGR234 *nodD*1 Gene Product Responds to Activation by the Simple Phenolic Compounds Vanillin and Isovanillin Present in Wheat Seedling Extracts

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Vanillin and isovanillin are present in extracts of wheat seedlings and interact with the nodulation (*nod*) gene *nodD*1 from *Rhizobium* strain NGR234 to induce expression of *R. leguminosarum* bv. *trifolii nod* genes. Seven varieties of Australian wheat were examined. Vanillin, isovanillin, or both were present in five of the varieties tested. Assays of a wide range of authentic flavonoid and other phenolic compounds for transcriptional induction of the same *nodA*::*lacZ* fusion revealed that a hydroxyl group para to an electron-withdrawing group and/or the presence of a cluster of oxygen functions are the prime structural requisites for transcriptional activation of NodD1-activated *nod* genes.

Additional keywords: *nodD*, *nod* gene induction, phenolic inducers, wheat extract.

Extensive studies have shown that the nodulation (*nod*) gene *nodD* is a crucial gene involved in the earliest stages of host recognition between various rhizobia and legumes (Spaink *et al.* 1987b). The product of *nodD* is believed to be a transcriptional activator protein (Henikoff *et al.* 1988) that binds to the promoters of inducible *nod* genes (Hong *et al.* 1987; Fisher *et al.* 1988) in a region closely corresponding to the *nod*-box (Rostas *et al.* 1986; Long 1989). Plant-synthesized compounds, in concert with the *nodD* gene product, activate inducible *nod* genes, which then initiate the early processes involved in nodulating a plant host (Mulligan and Long 1985; Innes *et al.* 1985; Rossen *et al.* 1985; Shearman *et al.* 1986; Spaink *et al.* 1987b; Bassam *et al.* 1988; Spaink *et al.* 1989).

Flavonoid compounds that interact with *nodD* have been identified in many narrow host range *Rhizobium*-legume symbioses. Examples include luteolin, isolated from alfalfa (Peters *et al.* 1986), and 7,4′-dihydroxyflavone (DHF), isolated from clover (Redmond *et al.* 1986). Anti-inducers such as coumarin, flavonol, and some isoflavones (Redmond *et al.* 1986; Firmin *et al.* 1986; Djordjevic *et al.* 1987) appear to competitively inhibit *nodD*-dependent induction (Djordjevic *et al.* 1987). In contrast, the isoflavone daidzein has been isolated from soybean and shown to induce *nodD*-dependent *nod* genes of *Bradyrhizobium japonicum* (Buchanan) Jordan (Kosslak *et al.* 1987).

Rhizobium strain NGR234 (Trinick 1980) is a broad host range *Rhizobium* and is one of the few *Rhizobium* strains capable of forming nodules with a nonlegume host, the woody tree *Parasponia* from the Ulmaceae family (Trinick and Galbraith 1980). Molecular characterization of the *nodD*1 gene of strain NGR234 shows it to be highly conserved at the DNA sequence level with the *nodD* genes of other rhizobia (J. J. Weinman, unpublished data). Bender *et al.* (1988) prepared a range of plant extracts from various legume hosts, from *Parasponia*, and from nonhost plants such as the nonlegumes *Trema* (a close relative of *Parasponia*), *Casuarina* (which forms symbioses with *Frankia*), and the cereals rice, maize, and wheat. These extracts are capable of inducing transcriptional activation of *nod* genes in the presence of the *nodD*1 gene from strain NGR234. Despite the high level of molecular conservation with other *nodD* alleles, the *nodD*1 gene of strain NGR234 is less specific in host recognition than the *nodD* genes from more widely characterized, narrow host range rhizobia (Bender *et al.* 1988; Bassam *et al.* 1988).

Flavonoid compounds are widespread throughout plant families. A broad range of these compounds induce *nodD*-dependent activation in strain NGR234, including some which have been identified as anti-inducers in other rhizobia (Bassam *et al.* 1988). While flavonoids and isoflavonoids induce expression of *Rhizobium nod* genes (Djordjevic *et al.* 1987; Horvath *et al.* 1987) and simple phenolic compounds induce expression of *Agrobacterium tumefaciens* (Smith and Townsend) Conn *vir* genes (Stachel *et al.* 1985a, 1985b), simple phenolic compounds have not been previously shown to induce *Rhizobium nod* genes.

To determine the characteristics of compounds capable of inducing *nod* gene transcription in conjunction with the *nodD*1 gene from *Rhizobium* strain NGR234, a large number of authentic plant-derived phenolic compounds were assayed, and the common structural features of the inducing compounds were analyzed. Unlike *nodD* alleles from other *Rhizobium* strains, simple compounds also activated *nod* gene transcription. Simple compounds were identified from fractionated wheat seedling extracts that similarly activated *nod* gene transcription.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are detailed in Table 1.

Plant materials. The principal wheat (*Triticum aestivum* L.) used in this investigation was Australian Standard White (ASW), the chief grade of Australian export wheat containing a mixture of hard wheat varieties. Seven varieties of wheat, grown under differing Australian conditions, were examined for the presence of inducing compounds following the identification of such compounds in extracts of ASW. Varieties were obtained as a gift from the CSIRO Bread Research Institute, North Ryde, Australia.

Media. BMM (Bergersen 1961), GMM, and GMY (Bender *et al.* 1988) media for the growth of *Rhizobium* have been described elsewhere. The vitamins thiamine (100 μg/L) and biotin (25 μg/L) were added for the culture of *R. leguminosarum* bv. *trifolii* Jordan strains. The antibiotic tetracycline was used for the selection and maintenance of *Rhizobium* strains harboring plasmid pMD1 in solid media at a concentration of 4 μg/ml.

Construction of pMD1. A 0.7-kilobase (kb) *Bam*HI DNA fragment containing the *nodD*/*nodA* intergenic region of *R. l.* bv. *trifolii* ANU843 was cloned into the pBS+ vector obtained from Stratagene (La Jolla, CA). This DNA fragment was derived from ANU843 containing an insertion of Mu*d*II1734 in the 5′ end of *nodA* and included 116 base pairs (bp) of the Mu*d*II1734 DNA (McIver *et al.* 1989). Using a *Pst*I site located 207 bp 3′ to the initiation codon for *nodD*, and lying within the *nodD* coding region of this fragment (Schofield and Watson 1986), and the *Pst*I site from the polylinker sequence of the pBS+ vector, a 0.7-kb *Pst*I fragment was excised and cloned (see Fig. 1) into the *Pst*I site of the plasmid pMP220 (Spaink *et al.* 1987a). This resulted in the fusion of the *nodA* promoter with the promoterless *lacZ* gene of pMP220. This plasmid was isolated and linearized with *Eco*RI, and a 2.9-kb *Eco*RI DNA fragment containing the NGR234 *nodD*1 gene (Bassam *et al.* 1988) was cloned into the *Eco*RI site (Fig. 1). The ANU843 *nodD* fragment (207 bp) is unlikely to interfere with the NGR234 *nodD* gene since deletions in ANU843 *nodD* near this site result in an inactive *nodD* (unpublished data). Furthermore, intact ANU843 *nodD* in a similar construct does not activate *nod* genes in response to the simple phenolic compounds discussed (unpublished data).

The patch mating procedure of Sinclair and Holloway (1982) was used to transfer the constructed plasmid pMD1 into strain ANU265 by triparental mating using plasmid pRK2013 (Ditta *et al.* 1980) as the helper plasmid.

β-Galactosidase assays of gene induction and expression. The biological activity of samples was determined by an *in vitro* assay measuring β-galactosidase activity from the *nodA*::*lacZ* fusion of pMD1, indicative of the induction of *Rhizobium* strain NGR234 *nodD*1-dependent transcription. Aliquots of test compounds or preparations were dried under vacuum, resuspended in water, and assayed as described in Miller (1972) with the modifications of Bender *et al.* (1988) and McIver *et al.* (1989) using *o*-nitrophenyl-β-D-galactopyranoside, obtained from Sigma Chemical Co. (St. Louis, MO), as the substrate. Inductions of the *nodA*::*lacZ* construct were measured after a 2-hr exposure to test compounds or wheat extracts. Maximal induction (i_{max}) levels are indicated as units of β-galactosidase activity (Miller 1972) with comparisons to levels for both DHF and background (H_2O).

Chemical materials and techniques. Phenolic compounds were obtained from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. Methanolic solutions were stored at −18° C. Matrex Silica C18 (90–130 μm) was from

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference
NGR234	Wild-type, fast-growing, broad host range *Rhizobium* strain infecting tropical legumes	Trinick 1980
ANU265	Sym plasmid-cured derivative of strain NGR234; Sm^r, Sp^r, and Nod$^-$	Morrison *et al.* 1983
ANU843	Wild-type *R. leguminosarum* bv. *trifolii* strain	Rolfe *et al.* 1980
pMP220	Broad host range IncP1 promoter cloning vector, stably maintained in *Rhizobium* strain ANU265; Tc^r, promoterless *Escherichia coli lacZ* gene	Spaink *et al.* 1987a
pMD1	pMP220 carrying a 2.9-kb *Eco*RI fragment containing the NGR234 *nodD*1 gene and a 0.7-kb *Pst*I fragment containing the *nodA* promoter of *R. l.* bv. *trifolii* ANU843	This study
pRK2013	Helper plasmid for mobilization into rhizobia, unable to be maintained in *Rhizobium*	Ditta *et al.* 1980

[a] Nod$^-$, the inability to form nodules on the hosts of strain NGR234; Sm^r, resistance to streptomycin; Sp^r, resistance to spectinomycin; Tc^r, resistance to tetracycline; and kb, kilobase.

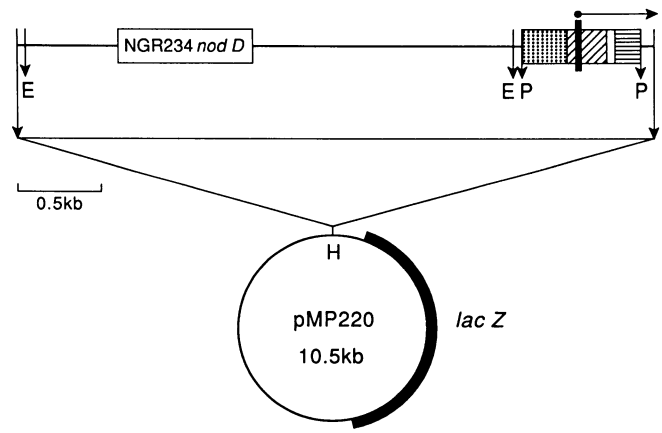

Fig. 1. Map of plasmid pMD1. The locations of the 2.9-kilobase (kb) *Eco*RI fragment containing the *nodD*1 gene from *Rhizobium* strain NGR234 (Bassam *et al.* 1988) and the 0.7-kb *Pst*I fragment containing the *nodA* promoter region from strain ANU843 (McIver *et al.* 1989) inserted into the multilinker of plasmid pMP220 (Spaink *et al.* 1987a) are shown. The *nodA* promoter region is positioned to drive transcription of the promoterless *lacZ* gene in the vector (arrow). The *nodA* promoter region contains 207 base pairs (bp) of the 5′ region of the ANU843 *nodD* gene (stippled area); the *nodD*/*nodA* intergenic region (crosshatched area); the *nod*-box (black vertical bar); the *nodA* coding region (unshaded area); and 116 bp of Mu*d*II1734 DNA (horizontal shaded area) inserted in the 5′ end of the *nodA* coding region. Plasmid pMP220 is not drawn to the same scale as the inserted fragments. Other sites in the multilinker are not shown. E = *Eco*RI; P = *Pst*I; and H = *Hin*dIII.

Amicon Corporation (Danvers, MA), and silicic acid (Keiselgel 60, 70–230 mesh) was from E. Merck (Darmstadt, Federal Republic of Germany).

Reverse-phase high performance liquid chromatography (HPLC) was conducted using an RP-8 column (MPL analytical cartridge, 100 × 4.6 mm, 5 μm) from Brownlee Labs (Santa Clara, CA), eluted with a gradient generated from solvent A (0.2% trifluoroacetic acid in water) and solvent B (methanol), and monitored by a Waters 490 multiwavelength detector; data were acquired with a Shimadzu Chromatopak CR-4. Peaks were characterized by retention time and ultraviolet (UV) profiles in the stop-scan mode.

Isolation and characterization of simple phenolic compounds from wheat seedlings. Wheat (ASW) was sterilized by soaking in 70% ethanol for 5 min, followed by thorough washing in sterile distilled water. After imbibing for 15 min, the wheat seeds were grown hydroponically in sterile distilled water on metal grids at 25° C for 2–4 days (Canter-Cremers *et al.* 1986). The seedlings were drained and snap-frozen in liquid nitrogen.

Batches (500 g) of frozen seedlings were homogenized in boiling distilled water (100 ml). Ethanol (500 ml) was added, and the mixture was boiled for 15 min, cooled, and filtered through Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, U.K.). The filtrate was evaporated to dryness under reduced pressure, and the residue was triturated thoroughly in methanol (100 ml). The dispersion was diluted with chloroform (3 volumes), mixed thoroughly, allowed to stand for 2 hr, and filtered. The filtrate was evaporated under reduced pressure, and the residue was reconstituted in methanol.

Combined extracts from approximately 85,000 ASW seedlings were preadsorbed onto silicic acid (25 g) and applied to the top of a column (200 × 30 mm) of silicic acid packed in 10% methanol/chloroform. Elution was conducted with the same solvent (450 ml), 40% methanol/chloroform (450 ml) and methanol (450 ml), and 12 fractions of 110 ml were collected. Fractions were assayed for NGR234 *nodD*1-dependent *nod*-gene-inducing activity using strain ANU265(pMD1) in β-galactosidase assays as described above. Activity was detected in fractions A2, A6, and A12. Earlier experiments (data not shown) indicated that DHF elutes at approximately fraction A6 and that highly hydroxylated and glycosylated flavones require methanol for elution (fraction A12). Thus, fraction A2 appeared to contain a new class of active compound and was selected for further study.

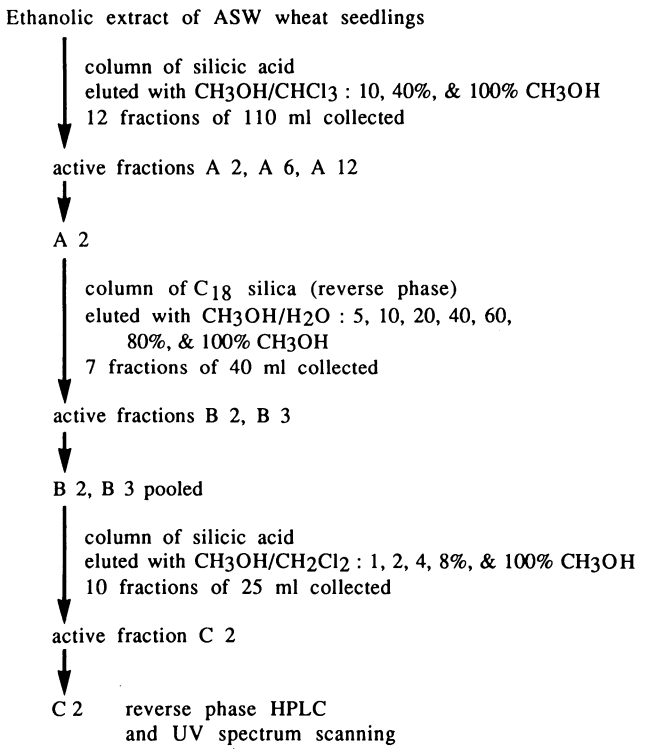

Fig. 2. Summary of the fractionation procedure for Australian Standard White (ASW) wheat seedling extracts.

Fraction A2 was dried down and dissolved in a small volume of methanol with water being added until the solution was 5% methanol/water. This was then applied to a column (100 × 10 mm) of Matrex Silica C18 (reverse-phase material) and eluted with methanol/water mixtures from 5 to 100% methanol. Seven fractions of approximately 45 ml were collected. Fractions B2 and B3 contained inducing activity (collected from 55 to 135 ml) and were combined, rechromatographed on a column of silicic acid, and eluted with methanol/methylene chloride (1 to 100%). Ten fractions of 25 ml were collected. The resultant active fraction C2 (25 to 50 ml) was evaporated carefully in a nitrogen stream and subjected to analysis by reverse-phase HPLC as described above. (See Fig. 2 for a summary of the wheat seedling extract fractionation procedure.)

Samples of different wheat varieties were processed on a smaller scale than ASW wheat (approximately 2,000 seedlings of each). An active fraction equivalent to fraction A2 of ASW was identified from extracts of each variety, and these fractions were then analyzed by reverse-phase HPLC and UV spectrum analysis as described above.

RESULTS

Phenolic compounds inducing *nodD*-dependent transcription. Some 70 phenolic compounds were examined for their capacity to induce the *nodA*::*lacZ* fusion of the *nodD*1-dependent construct pMD1 in *Rhizobium* strain ANU265. Assays were conducted over a range of concentrations from 10^{-3} to 10^{-9} M (Table 2). Inhibition of bacterial cell growth was detected for a number of compounds and is also indicated in Table 2. The concentration at which half-maximal induction occurs (A_{50}) was determined for approximately 20 representative active compounds, and the results are shown in Table 3. The comparison of A_{50} gives a clearer picture of potent and weak inducers than does the comparison of i_{max}.

The most potent inducers were daidzein, genistein, DHF, and apigenin, all with A_{50} values of 10^{-8} or lower (Table 3). Other compounds able to induce activity at low concentrations (A_{50} below 10^{-6}) included coumestrol, formononetin, biochanin A, quercetin, kaempferol, naringenin, hesperetin, and 7-hydroxyflavone. Those inducing at higher concentrations (A_{50} above 10^{-6}) included vanillin, isovanillin, syringaldehyde, 7-hydroxychromone, and umbelliferone. Those unable to induce detectable *nodA*

expression included 3,4-dimethoxybenzoic acid, 3,4-dimethoxybenzaldehyde, 4-methoxyacetophenone, *o*-hydroxyacetophenone, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), and flavone (Table 2).

The key features of compounds able to induce expression of *nodD*1-dependent *nod* genes in strain NGR234 were determined by analysis of the characteristics of potent inducers, weak inducers, and noninducing compounds. These are presented in Figure 3 and can be summarized as follows:

1) The presence of an hydroxyl (—OH) group para to an electron-withdrawing function increases biological activity, for example —OH para to a carbonyl function as in 4-hydroxybenzoic acid or in 7-hydroxyflavone.

2) The presence of a cluster of oxygen functions increases biological activity, for example pyrogallol.

3) Hydroxylation of the B-ring increases the potency of flavones (that is, decreases A_{50}).

Chromatographic analysis of wheat seedling extracts. Vanillin and isovanillin were characterized from fraction C2 of ASW wheat seedling extract by retention times in reverse-phase HPLC analysis and confirmed by UV spectrum analysis of the identified peaks in the stop-scan mode (Fig. 4).

Vanillin and isovanillin are structural isomers and have almost identical retention times under the HPLC conditions used. Close inspection of the UV spectra of eluates indicated a mixture of the two compounds present in ASW wheat seedling extract. The mixture could not be further separated under the conditions available, but comparison with standard preparations and their UV spectra under the same conditions indicated that the peak consisted predominately of vanillin (data not shown).

Whole extracts of all varieties induced the *nodA* promoter of ANU265(pMD1). After analysis of comparable active fractions, vanillin and/or isovanillin could be unequivocally shown in the following varieties: ASW, Halberd, Suneca, Rosella, Hartog, and Vulcan. In the remaining two varieties, Matong and Eradu, no clear identification of vanillin or isovanillin could be made at the levels of detection available because of unidentified overlapping peaks. Comparison of vanillin peak heights from HPLC data to known quantities of standard vanillin under identical conditions allowed approximate calculation of the amount of vanillin present in wheat variety preparations. Table 4 gives details of varietal characteristics, i_{max}, and vanillin concentration.

DISCUSSION

This study reports the first case of simple plant phenolic compounds contributing to transcriptional activation of the *nodD*-dependent *nod* genes of *Rhizobium*. Vanillin and isovanillin were identified in extracts of wheat seedlings, and authentic samples of these compounds in the presence of *nodD*1 from strain NGR234 induced expression of the *nodA* promoter of *R. l.* bv. *trifolii* ANU843. Vanillin and isovanillin did not induce expression of the same promoter in the presence of the *nodD* gene of strain ANU843. These findings extend the work of Bender *et al.* (1988) who found that wheat extracts are capable of inducing *nodD*-dependent *nod* gene expression only with the *nodD*1 gene from strain NGR234.

The fractionation procedure for wheat extract was optimized for extraction of simple phenolic compounds capable of inducing *Rhizobium nodD*-dependent *nod* genes. Other fractions of wheat extract also induced *nodD*-dependent expression in strain ANU265(pMD1) and, on the basis of

Table 2. Phenolic compounds assessed for biological activity[a]

Compound class	Active compounds (range 10^{-3} to 10^{-9} M)	Inactive compounds (range 10^{-3} to 10^{-6} M)
Acids	4-OH benzoic acid (A), 2,3-diOH benzoic acid (B, I), 2,4-diOH benzoic acid (B, I), 2,6-diOH benzoic acid (B, I), 3,4-diOH benzoic acid (A, I), pyrogallic acid (B), acetyl salicylic acid (A)	Benzoic acid (I), salicylic acid (I), 3-OH benzoic acid, 3,5-diOH benzoic acid, 3,4-diOMe benzoic acid, cinnamic acid (I), 2-coumaric acid, 3-coumaric acid, 4-coumaric acid (I), sinapinic acid
Phenols	Catechol (C), resorcinol (A), vanillin (C, I), isovanillin (C), quinol (A)	Anisole
Aldehydes	Benzaldehyde (A), 3-OH benzaldehyde (B), 3,4-diOH benzaldehyde (C), 3,4-diOMe benzaldehyde (A), syringaldehyde (C)	Salicylaldehyde, 2,4-diOMe benzaldehyde, cinnamaldehyde (I), piperonal
Ketones	3-OH acetophenone (C), 4-OH acetophenone (C), 2′4′-diOH acetophenone (C), 2′6′-diOH acetophenone (B), 2,3,4-triOH acetophenone (C), 4-OH,3′-OMe acetophenone (B), 4-OH,3-Me acetophenone (C), acetovanillone (C), 2-OH,4-OMe acetophenone (A), acetosyringone (B), 2,4,4,-triOH chalcone (B)	Acetophenone, 2-OH acetophenone, 4-OMe acetophenone
Chromone	7-OH chromone (A)	
Coumarin	Coumarin (A), umbelliferone (C)	Fraxetin
Coumestan	Coumestrol (C)	
Isoflavone	Daidzein (C), biochanin A (C), formononetin (C), genistein (C)	
Flavonol	Quercetin (B), kaempferol (C)	
Flavanone	7,4′-diOHflavanone (A), hesperetin (C), naringenin (C)	Bayin, taxifolin, naringin, 4′-OH flavanone
Flavone	7,4′-diOH flavone (C), morin (C), 7-OH flavone (C), chrysin (C), apigenin (C)	Flavone, 3-OH flavone, 7,4′-diOMe flavone, 5-OH,7-OMe flavone, diosmin

[a] OH, hydroxy; diOH, dihydroxy; triOh, trihydroxy; OMe, methoxy; diOMe, dimethoxy; and Me, methyl. I = inhibition of bacterial cell growth noted at concentrations of 10^{-3} or 10^{-4} M. i_{max} = maximal levels of induction: A, low level of induction (approximately 400—700 units); B, intermediate level of induction (700—1,500 units); and C, high level of induction (1,500—4,500 units) (background, [H_2O] 300 units).

Table 3. Relative induction levels of compounds inducing *Rhizobium* strain NGR234 *nodD*1-dependent *nod* gene expression (indicated by β-galactosidase activity)

Compound class	Compound	log A_{50}[a]
Acids	4-Hydroxybenzoic acid	−4.3
Phenols	Vanillin	−6.3
	Isovanillin	−5.5
Aldehyde	Syringaldehyde	−5.3
Ketone	Acetophenone	−4.3
Chromone	7-Hydroxychromone	−3.5
Coumarin	Umbelliferone	−5.2
Coumestan	Coumestrol	−7.3
Isoflavone	Daidzein	−8
	Genistein	−8
	Formononetin	−6.3
	Biochanin A	−6.3
Flavanol	Quercetin	−7
	Kaempferol	−7.5
Flavanone	Naringenin	−7.3
	Hesperetin	−7
Flavone	7-Hydroxyflavone	−7.3
	7,4′-Dihydroxyflavone	−8
	Apigenin	−8.3

[a] A_{50}, concentration at which induction is half-maximal for each compound.

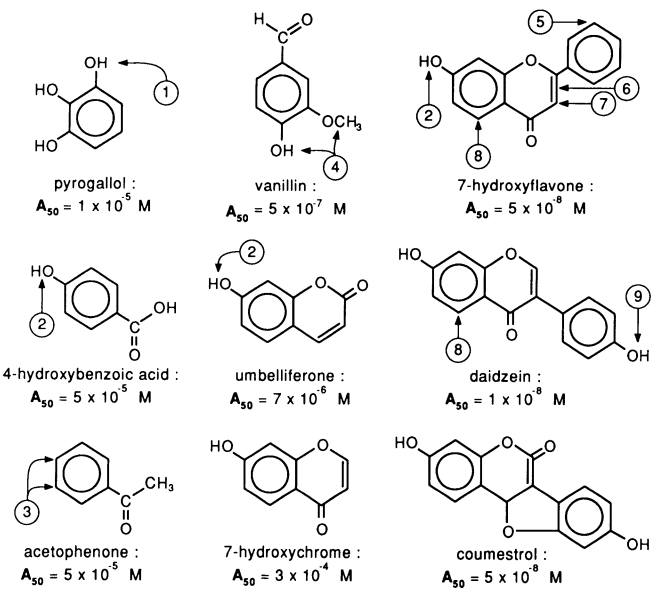

Fig. 3. Representative substances that induce *Rhizobium* strain NGR234 *nodD*1-dependent β-galactosidase activity in strain ANU265(pMD1). Modifications to the basic structures are listed numerically as follows, with the effect on *nodA* induction also being provided (A_{50} = concentration at which induction is half-maximal): at circle position (1), removal of the OH group increases A_{50}; (2), the OH group is essential for activity; (3), an OH group is necessary in at least one of the positions; (4), both functional groups are required for activity, but may be interchanged; (5), an OH group at this site decreases A_{50}; (6), reduction of the double bond does not significantly affect activity; (7), an OH group increases A_{50}; (8), an OH at this site marginally lowers A_{50}; and (9), a methoxy (CH_3O) group at this site increases A_{50}.

the separation system used, these probably contained more complex flavonoid compounds.

A variety of simple and complex phenolic compounds have been identified previously in wheat including tricin (Anderson 1932), vanillin glucosides, *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde in graminaceous cell walls (Hartley and Keene 1984), *p*-coumaric acid in association with lignin and ferulic acid from nonlignified tissue (Scalbert *et al.* 1985), and apigenin glycosides from wheat bran (Feng *et al.* 1988). Of these, the aglycones syringaldehyde, vanillin, and apigenin have been found to induce *nodA* expression in strain ANU265(pMD1) (Tables 2 and 3). Neither vanillin nor isovanillin has been specifically reported to occur in host plants of NGR234. Analysis of root extracts of *Parasponia* was initiated but abandoned due to lack of available plant material.

Plant substances capable of inducing optimal expression of *nodD*-dependent *nod* genes in other rhizobia have specific structural characteristics, such as hydroxylation of the flavonoid skeleton at the 7 and 4′ positions of both flavones and flavanones (Firmin *et al.* 1986; Redmond *et al.* 1986; Kapulnik *et al.* 1987; Gyorgypal *et al.* 1988). Induction of the *nod* genes of *R. leguminosarum* biovars also occurs with compounds hydroxylated at the 3′ position, and activity is further enhanced if a methoxy group is present at the 4′ position. Any substitution at the 3 position results in inactive compounds (Firmin *et al.* 1986; Zaat *et al.* 1987).

Induction in *R. l.* bv. *trifolii* and *R. meliloti* Dangeard is also observed with compounds hydroxylated at either or both the 5 and 3′ positions (Peters *et al.* 1986). Antagonists of activation in these species are usually isoflavonoids (Djordjevic *et al.* 1987). In contrast *B. japonicum*, a slow-

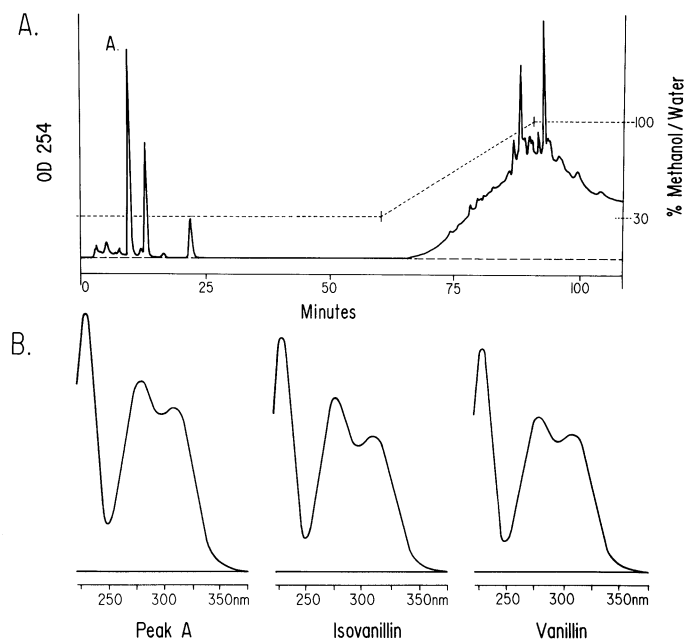

Fig. 4. HPLC and UV spectrum analysis of Australian Standard White (ASW) wheat fraction C2. (**A**) HPLC profile of fraction C2 of the ASW wheat seedling extract. The relative retention time, occurrence of peaks as detected at 254 nm, and concentration of methanol in the eluting solvent are shown. (**B**) UV profile of peak A, over the range of 225 to 400 nm, recorded in the stop-scan mode is compared to UV profiles of authentic vanillin and isovanillin recorded under the same conditions.

growing species, is activated by flavonoids similar to those described above and also by isoflavonoid compounds conforming to the same general parameters; however, compounds hydroxylated at either or both the 3 and 5′ positions are inactive (Kosslak *et al.* 1987; Gottfert *et al.* 1988).

These characteristics specify a subset of compounds capable of inducing *nod* genes in the presence of the *nodD*1 gene from *Rhizobium* strain NGR234. However, the range of compounds capable of interaction with this *nodD*1 gene extends to include a greater variety of complex, multiringed phenolic compounds, in addition to the simple phenolic compounds reported here.

The *nodD*1 gene from strain NGR234 may thus represent a less specialized *nodD* allele, perhaps retaining some aspects of an ancestral plant recognition gene. Alternatively, the NGR234 *nodD*1 gene may be a more versatile gene, incorporating the highly specialized interactive capacity of the narrow host range rhizobia along with the capacity to respond to simple phenolic compounds.

Several recent findings indicate the second alternative as being unlikely. Chemically induced single base substitution mutations to *R. l.* bv. *trifolii nodD* result in the extension of host range to *Parasponia*, the nonlegume host of strain NGR234 (McIver *et al.* 1989). Clearly *R. l.* bv. *trifolii* has most of the genes required to infect this illegitimate host, since only a point mutation of the *nodD* gene confers extended nodulation host range. Furthermore, a significant relationship at the genetic level has recently been established between the *nodD* genes of several *Rhizobium* species and the *nahR* gene of *Pseudomonas putida* (Trevisan) Migula (Schell and Sukordhaman 1989). NahR is a transcriptional activator of the *nah* and *sal* operons, activating transcription of the *sal* promoter only in the presence of salicylate (a salt or ester of salicylic acid). It is interesting to note that the NGR234 *nodD*1 gene does not interact with salicylic acid.

The expression of *A. tumefaciens vir* genes (required for plant cell transformation and crown gall formation) is dependent upon a two-component regulatory system. The *virA* gene product interacts with simple phenolic compounds to activate *virG*, and the resultant VirG protein induces expression of the remaining *vir* genes (Winans *et al.* 1986). Inducers of the *vir* genes include acetosyringone, α-hydroxyacetosyringone, acetovanillone, syringaldehyde, and sinapinic acid (Stachel *et al.* 1985a), all of which are single-ring, simple phenolic compounds. Acetosyringone production is significantly increased in wounded tissue (Stachel *et al.* 1985b), and other active compounds are found at sites of lignin production such as root elongation zones and tissue damage repair sites. *Rhizobium nod* genes are also induced by acetosyringone, acetovanillone, and syringaldehyde in the presence of the NGR234 *nodD*1 gene but not by sinapinic acid. Thus, some wound-induced compounds, lignin production intermediates, and degradation products will induce NGR234 *nodD*1-dependent *nod* genes. Interestingly, *vir* gene activating compounds have also been found in extracts of oats and of wheat seeds (Usami *et al.* 1988). These are apparently different from acetosyringone, possibly being a phenolic structure conjugated to a hydrophobic molecule. Usami *et al.* (1988) also postulate the existence of inhibitors of *vir* gene induction in monocotyledonous tissue.

There has been no reported DNA or protein homology between *virA* and either *nodD* or *nahR*, yet all have a common ability to interact with simple phenolic-inducing compounds. It is interesting to speculate on the possible evolutionary relationship between these genes; perhaps *virA* development diverged early on from an ancestral gene, and *nahR* and *nodD* developed subsequently. Alternatively, the genes may have converged from separate ancestral genes to perform a similar role.

Identification of simple phenolic compounds as inducers of *nodD*-dependent *nod* genes from *Rhizobium* has allowed a clearer definition of the chemical nature of the inducer-*nodD* interaction that may contribute to the further development of a model for the mechanism of action of the *nodD*-encoded protein. Further investigation of the nature of this interaction involving the mutation of the NGR234 *nodD*1 gene to alter the affinity of the *nodD*-encoded protein for phenolic compounds and the precise characterization of such mutations will enable definition of the critical domains of the protein involved in the inducer-*nodD* interaction.

Table 4. Characteristics of Australian wheat varieties examined

Variety	Characteristics	Induction levels[a] (i_{max})	Vanillin/ isovanillin[b]	Vanillin concentration[c]
ASW	Australian Standard White	850	+	1.3×10^{-8}
Halberd	Hard, dry-land variety; low boron intake	900	+	4.2×10^{-8}
Suneca	Hard, high-yield variety, will not germinate if wet during harvest	1,100	+	4.0×10^{-8}
Hartog	Hard, high-yield variety	980	+	4.4×10^{-8}
Vulcan	Hard, high-yield variety	1,280	+	2.8×10^{-8}
Rosella	Soft, long-season variety	1,010	+	1.0×10^{-8}
Eradu	Soft, rapid-maturing variety	720	–	
Matong	Soft, late-maturing, lower protein variety	990	–	

[a] i_{max}; maximal levels of induction of fraction A2 equivalents expressed in units of β-galactosidase activity (Miller 1972) for comparison, DHF at 10^{-6} M gives 700 units, background of H_2O gives 110 units.
[b] Presence or absence of vanillin/isovanillin determined by reverse-phase HPLC analysis and UV spectrum scanning as described in the text.
[c] Estimate of the amount of vanillin/isovanillin in wheat variety extracts, expressed as moles per 100 g of seeds.

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