Flavonoids, NodD1, NodD2, and *Nod*-Box NB15 Modulate Expression of the *y4wEFG* Locus That Is Required for Indole-3-Acetic Acid Synthesis in *Rhizobium* sp. strain NGR234

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Flavonoids secreted by host plants activate, in conjunction with the transcriptional activator NodD, *nod* gene expression of rhizobia resulting in the synthesis of Nod factors, which trigger nodule organogenesis. Interestingly, addition of inducing flavonoids also stimulates the production of the phytohormone indole-3-acetic acid (IAA) in several rhizobia. Here, the molecular basis of IAA synthesis in *Rhizobium* sp. NGR234 was investigated. Mass spectrometric analysis of culture supernatants indicated that NGR234 is capable of synthesizing IAA via three different pathways. The production of IAA is increased strongly by exposure of NGR234 to daidzein in a NodD1-, NodD2-, and SyrM2-dependent manner. This suggests that the *y4wEFG* locus that is downstream of *nod*-box NB15 encodes proteins involved in IAA synthesis. Knockout mutations in *y4wE* and *y4wF* abolished flavonoid-inducible IAA synthesis and a functional *y4wF* was required for constitutive IAA production. The promoter activity of NB15 and IAA production both were enhanced by introduction of a multicopy plasmid carrying *nodD2* into NGR234. Surprisingly, the *y4wE* mutant still nodulated *Vigna unguiculata* and *Tephrosia vogelii*, although the nodules contained less IAA and IAA conjugates than those formed by the wild-type bacterium.

Additional keywords: auxin, nodulation, tryptophan.

Legume root-nodule bacteria (genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium*, collectively called rhizobia) provoke nitrogen-fixing symbioses in conjunction with an appropriate legume partner. Symbiosis is initiated when flavonoids, which accumulate in the rhizosphere of the host-plant, trigger expression of nodulation (*nod*) genes by interacting with NodD, one of a family of LysR-type transcriptional activators. Tetrameric NodD proteins bind to *cis*-regulatory elements (*nod*-boxes) that are found in the promoter regions of most *nod* genes, and activate transcription in the presence of compatible flavonoids (Feng et al. 2003; Fischer and Long 1993). Most of these nodulation genes (*nod*, *noe*, and *nol*) are involved in the syn-

thesis of host-specific lipo-chito-oligosaccharides (LCOs) called Nod factors that are essential for the initial infection of root hairs (Downie 1998; Perret et al. 2000).

Some plant-associated bacteria, including rhizobia, have the capacity to produce the plant hormone indole-3-acetic acid (IAA) (Badenoch-Jones et al. 1982; Basu and Ghosh 1998, 2001; Datta and Basu 1998, 2000; Ernstsens et al. 1987; Fukuhara et al. 1994; Kawaguchi et al. 1990; Minamisawa and Fukai 1991; Patten and Glick 1996). So far, three bacterial IAA biosynthesis pathways have been characterized: tryptophan can be converted to IAA via indole-3-acetamide, tryptamine, or indole-3-pyruvic acid (Fig. 1) (Costacurta and Vanderleyden 1995; Patten and Glick 1996). Phytohormone production is known to be involved in pathogenesis (Jameson 2000; Morris 1995), but the role of bacterial IAA in symbiosis remains unclear.

Interestingly, in *Rhizobium meliloti* and *R. leguminosarum*, the production of IAA is enhanced in the presence of *nod*-inducing flavonoids (Prinsen et al. 1991), suggesting that rhizobial genes involved in IAA production are regulated in a host-specific manner. *Rhizobium* sp. NGR234, which nodulates over 112 genera of legumes (Pueppke and Broughton 1999), has a symbiotic plasmid (pNGR234a) that encodes two *nodD* homologues (*nodD1* and *nodD2*) as well as 19 *nod*-boxes (NB1 through NB19) (Freiberg et al. 1997; Kobayashi et al. 2004; Perret et al. 1999). Daidzein can induce transcription of all *nod*-box-*lacZ* fusions, with the exception of NB3. The protein encoded by *nodD1* is the key activator of flavonoid-inducible loci (Kobayashi et al. 2004). NodD2 is involved in the delayed repression of several Nod-factor synthetic genes and is specifically required for optimal transcription of some *nod*-boxes (Fellay et al. 1998; Kobayashi et al. 2004). In turn, expression of *nodD2* requires proteins encoded by *syrM2* (another member of the LysR family), which is controlled by NB19. Thus, NodD1, SyrM2, and NodD2 form a regulatory cascade that coordinates the expression of at least 42 genes in a flavonoid- and host-specific manner. Here, we show that IAA production by NGR234 is stimulated by flavonoids, and that the *y4wEFG* locus, which is immediately downstream of NB15, is required for IAA synthesis. Although IAA levels in nodules infected with an *y4wE*-defective mutant were reduced significantly, the flavonoid-inducible IAA synthesis of NGR234 is not essential for establishment of symbiosis with the two host plants tested.

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RESULTS

IAA synthesis pathways in NGR234.

In the absence of tryptophan, several storage and breakdown products of the indole-3-pyruvic acid (IPyA) pathway (Fig. 1, number 3), including indole-3-lactic acid (ILA), indole-3-aldehyde (IAld), and indole-3-ethanol (IEt), were detected in the supernatants of NGR234 (Fig. 2, open bars). Upon addition of exogenous tryptophan to the cultures, NGR234 secreted substantial amounts of the intermediates indole-3-acetaldehyde (IAAld), IPyA, indole-3-acetamide (IAM), and tryptamine (Tra) (Fig. 2, black bars). Thus, NGR234 is capable of synthesizing IAA via the three pathways previously identified in

other bacteria. Nevertheless, the IPyA pathway (Fig. 1, number 3) is predominant when the supply of tryptophan is limited.

Flavonoids enhance IAA synthesis in a NodD-dependent manner.

In the presence of 0.2 μM daidzein, IAA levels in NGR234 supernatants were increased significantly compared with those found under noninduced conditions (Fig. 3). Luteolin, another flavonoid, also enhanced IAA production by NGR234 (data not shown). To determine the role of symbiotic transcription factors in flavonoid-induced IAA synthesis, IAA production was assessed in the mutants NGR Ω nodD1, NGR Ω nodD2, and

Fig. 1. Pathways of indole-3-acetic acid (IAA) synthesis starting from the precursor tryptophan. So far, three IAA biosynthetic pathways have been identified in rhizobia, via indole-3-acetamide (IAM; no. 1), tryptamine (Tra; no. 2), and indole-3-pyruvic acid (IPyA; no. 3). AA, antranilate; Trp, tryptophan; IPyA, indole-3-pyruvic acid; Tra, tryptamine; IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; ILA, indole-3-lactic acid; IAld, indole-3-aldehyde; IEt, indole-3-ethanol; IMe, indole-3-methanol. Breakdown and storage products are labeled by *.

Fig. 2. Intermediates of indole-3-acetic acid (IAA) synthesis found in the supernatants of *Rhizobium* sp. NGR234 grown in *Rhizobium* minimal medium containing succinate as the carbon source (RMS) (open bars) or RMS supplemented with tryptophan (5 $\mu\text{g ml}^{-1}$, gray bars). Samples were taken 36 h after the addition of tryptophan and analyzed by liquid chromatography electro-spray interface–tandem mass spectrometry. Results are presented on a logarithmic scale. AA, antranilate; Trp, tryptophan; IPyA, indole-3-pyruvic acid; Tra, tryptamine; IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; IAA, indole-3-acetic acid; ILA, indole-3-lactic acid; IAld, indole-3-aldehyde; IEt, indole-3-ethanol; IMe, indole-3-methanol.

NGR_{syrM2::uidA}. In the absence of daidzein, only basal, constitutive levels of IAA synthesis, similar to those found using the wild-type strain, were detected in the supernatants of the mutants (Fig. 3, open bars). Furthermore, addition of daidzein did not change the levels of IAA in the supernatants (Fig. 3, black bars), suggesting that flavonoid-inducible IAA synthesis is under control of the NodD1-SyrM2-NodD2 regulatory cascade.

y4wEFG is involved in IAA synthesis.

Flavonoid induction of IAA synthesis requires a functional *nodD2*; therefore, we speculated that one or more genes responsible for IAA synthesis are regulated by a NodD2 dependent *nod*-box. Of the 19 *nod*-boxes, four (NB6, NB15, NB16, and NB17) require NodD2 for their optimal activity (Kobayashi et al. 2004). Among them, NB15 is located upstream of three open reading frames (ORF): y4wE, y4wF, and y4wG (Kobayashi et al. 2004) (Fig. 4). y4wE and y4wF code for a class-2 aminotransferase that is similar to the putative histidinol aminotransferase HisC and a monooxygenase, respectively (similarities ranged from 82 to 85% for HisC and 76 to 80% for monooxygenases). Although none of these homologues have been shown to be involved in IAA synthesis, similar enzymes, including an aminotransferase as well as an indole acetaldehyde oxidase, are involved in the IPyA pathway (Fig. 1, number 3). Most probably, y4wG is a cryptic ORF, because it possesses neither conserved domains nor homologues. To examine the involvement of y4wEFG in IAA production, y4wE and y4wF were mutated ("knocked-out") by inserting an Ω interposon in their internal *XhoI* and *BglIII* sites, respectively (Table 1; Fig. 4). The resulting mutant NGR Ω wE showed a reduced level of constitutive IAA production (160 pmol optical density [OD]⁻¹ ml⁻¹), which is less than 30% of the level produced by the wild type (550 pmol OD⁻¹ ml⁻¹), and was not inducible by flavonoids (220 pmol OD⁻¹ ml⁻¹) (Fig. 5). Surprisingly, the mutant NGR Ω wF was unable to produce IAA in both a constitutive and flavonoid-inducible manner (0 to 6 and 0 to 10 pmol OD⁻¹ ml⁻¹, respectively) (Fig. 5). Introduction of the plasmid pwEFG, which carries NB15 and y4wEFG in a 5.8-kb *BamHI* fragment (Fig. 4) into both mutants, rescued constitutive and flavonoid-inducible IAA synthesis (Fig. 5), confirming that y4wE and y4wF are necessary for auxin synthesis in NGR234.

Fig. 3. Indole-3-acetic acid (IAA) concentrations present in the growth media of 1, *Rhizobium* sp. NGR234 and its mutants 2, NGR Ω nodD1; 3, NGR Ω nodD2; and 4, NGR_{syrM2::uidA} grown in *Rhizobium* minimal medium containing succinate as the carbon source in the absence (open bars) or presence (black bars) of the flavonoid daidzein (0.2 μ M). Samples were taken 36 h after the addition of daidzein and analyzed by gas chromatography-mass spectrometry. Results are presented on a logarithmic scale.

y4wE and y4wF encode for enzymes of the IPyA pathway.

The effect of daidzein on intermediates of IAA synthesis were also analyzed in supernatants of NGR234 (Fig. 6, open bars), NGR Ω wE (gray bars), and NGR Ω wF (black bars). Comparison of concentration of intermediates in the absence (Fig. 6A) or in the presence (Fig. 6B) of daidzein shows that daidzein induces the accumulation of IAla, IAAld, ILA, and IET in wild-type NGR234, indicating that the IPyA pathway is responsible for the flavonoid-inducible IAA synthesis. Levels of these intermediates in both NGR Ω wE and NGR Ω wF were reduced significantly and were not increased in the presence of daidzein, indicating that y4wE and y4wF encode enzymes involved in the IPyA pathway.

Regulation of the y4wEFG cluster.

To confirm the role of NodD2 in the regulation of IAA production, pRAF130 which contains multiple copies of *nodD2* (Fellay et al. 1998) was mobilized into NGR234, NGR(pMP-NB8), NGR(pMP-NB15), and NGR234(pMP-*nodD1*) (Kobayashi et al. 2004). Introduction of *nodD2* on a multiple copy-number plasmid totally abolished the basal activity of *nodD1* and the induction of the activity of NB8 (the *nod*-box which regulates synthesis of the Nod-factor core) (Table 2), confirming the suppressor role of NodD2 (Fellay et al. 1998). In contrast, NB15 showed strong constitutive promoter activity even in the absence of daidzein, which was manifested by high levels of constitutive IAA production (25 nmol OD⁻¹ ml⁻¹) in the NGR234(pRAF130) transconjugant (Fig. 5), suggesting that NodD2 can activate transcription from NB15 in the absence of an inducer.

To examine whether NB15 regulates transcription of both y4wE and y4wF, we constructed pwE(F Ω)G by inserting an Ω interposon into the *BglIII* site of y4wF of pwEFG (Fig. 4). Assuming that the Ω interposon in y4wE has polar effects on the downstream ORF y4wF (i.e., that both y4wE and y4wF are co-transcribed from NB15 following flavonoid induction), mobilization of pwE(F Ω)G into NGR Ω wE did not restore flavonoid-inducible IAA synthesis even if the transconjugant also carried a functional y4wE. Only basal levels of IAA (500 pmol OD⁻¹ ml⁻¹) were found in daidzein-treated cultures of NGR Ω wE (pwE(F Ω)G) (Fig. 5); therefore, it is likely that transcription of both y4wE and y4wF is enhanced by NB15 in the presence of flavonoids.

Because residual amounts of IAA were found in the supernatants of the NGR Ω wE but not the NGR Ω wF mutants (Fig. 5), we studied the intergenic region between the two ORF for the presence of possible supplementary promoters. The ATG of y4wF is 0.4 kb downstream of the y4wE termination codon. For this reason, a 0.9-kb *XbaI*-*NsiI* fragment containing the

Fig. 4. Physical map of the y4wEFG locus of pNGR234a. A total of 407 bp separate wE and wF, whereas there are 38 bp between wF and wG. Restriction sites are as follows: *BamHI* (Ba), *BglIII* (Bg), and *XhoI* (X). y4wE was mutated by inserting a spectinomycin-resistant Ω cassette into the internal *XhoI* site. y4wF was mutated by inserting a kanamycin-resistant Ω cassette into the internal *BglIII* site. Locations of the Ω insertions are shown by Ω . The 952-bp fragment containing the intergenic region between y4wE and y4wF was fused to a promoterless *lacZ* gene for analysis of its promoter activity.

complete intergenic region was cloned by polymerase chain reaction (PCR) and fused to a promoterless *lacZ* gene giving pMP-wF5' (Fig. 4; Table 1). When mobilized into NGR234 and its derivatives, weak constitutive activity that was independent of inducers and symbiotic regulators (75 ± 4 Miller's units) was found, suggesting that a second promoter of y4wF lies in the intergenic region.

y4wEFG-dependent IAA synthesis in planta.

To test the role of the flavonoid-inducible IAA synthesis in nodulation, *Vigna unguiculata* and *Tephrosia vogelii* were inoculated with NGR234 or NGRΩwE. NGR234 forms determinate and indeterminate nodules, respectively, on these hosts.

Although no significant differences were found in nodule number, plant growth, or intracellular structures of the nodules induced by the wild-type strain or the mutant on both plants (data not shown), the concentrations of both IAA and IAA conjugates were reduced significantly in nodules formed by NGRΩwE (Fig. 7). This suggests that y4wEFG is also involved in IAA synthesis within nodules. However, nodules produced by NGRΩwE transconjugants carrying pwEFG contained only mutant levels of IAA (Fig. 7). Possibly this was due to the instability of NGRΩwE(pwEFG) transconjugants within nodules, although random isolations of bacteria from nodules induced by NGRΩwE(pwEFG) showed them all to be tetracycline resistant, implying that pwEFG had been main-

Table 1. Strains and plasmids used in this study

	Relevant characteristics ^a	Reference
Strains		
NGR234	Rif ^r derivative of wild-type NGR234	Lewin et al. 1990
NGRΩnodD1	NGR234 derivative containing an Ω insertion in the <i>Bam</i> HI site of <i>nodD1</i> , Sp ^r	Relić et al. 1993
NGRΩnodD2	NGR234 derivative containing an Ω insertion in the <i>Bam</i> HI site of <i>nodD2</i> , Km ^r	Fellay et al. 1995
NGR _{syrM2::uidA}	NGR234 derivatives containing the <i>uidA-aph</i> insertion in the <i>Sac</i> I site of <i>syrM2</i> , Km ^r	Kobayashi et al. 2004
NGRΩwE	NGR234 derivative containing an Ω insertion in the <i>Xho</i> I site of y4wE, Sp ^r	This work
NGRΩwF	NGR234 derivative containing an Ω insertion in the <i>Bgl</i> III site of y4wF, Km ^r	This work
NGR(pwEFG)	NGR234 containing pwEFG	This work
NGRΩwE(pwEFG)	NGRΩwE containing pwEFG	This work
NGRΩwF(pwEFG)	NGRΩwF containing pwEFG	This work
NGR(pwE(FΩ)G)	NGR234 containing pwE(FΩ)G	This work
NGRΩwE(pwE(FΩ)G)	NGRΩwE containing pwE(FΩ)G	This work
Plasmids		
pBluescriptII KS+	Phage f1, <i>lacZ</i> , Ap ^r	Stratagene
pBBR-MCS5	Broad host range cloning vector, Gm ^r	Kovach et al. 1995
pLAFR-6	Broad host range vector containing transcriptional terminators flanking the cloning site, Tc ^r	D. Dahlbech and B. Staskawicz, unpublished
pMP220	IncP expression vector containing a promoterless <i>lacZ</i> gene, Tc ^r	Spaink et al. 1987
pwEFG	NB15 and y4wEFG cloned into pLAFR-6 as a 5.8-kb <i>Bam</i> HI fragment	This work
pwE(FΩ)G	pwEFG derivative containing an Ω insertion in the <i>Bgl</i> III site of y4wF	This work
pMP-NB8	NB8 cloned into pMP220 as a 0.3-kb <i>Kpn</i> I- <i>Xba</i> I fragment	Kobayashi et al. 2004
pMP-NB15	NB15 cloned into pMP220 as a 0.7-kb <i>Spe</i> I- <i>Pst</i> I fragment	Kobayashi et al. 2004
pMP- <i>nodD1</i> p	Promoter of <i>nodD1</i> cloned into pMP220 as a 0.7-kb <i>Pst</i> I fragment	Kobayashi et al. 2004
pRAF130	A 2.1-kb <i>Pst</i> II- <i>Bgl</i> III fragment containing <i>nodD2</i> cloned into pBBR1MCS-5	Fellay et al. 1998
pMP-wF5'	The upstream region of y4wF cloned into pMP220 as a 0.9-kb <i>Xba</i> I- <i>Nsi</i> I fragment	This work

^a Resistant to rifampicin (Rif^r), spectinomycin (Sp^r), kanamycin (Km^r), ampicillin (Ap^r), gentamycin (Gm^r), and tetracycline (Tc^r).

Fig. 5. Indole-3-acetic acid (IAA) concentrations in the supernatants of NGR234 and its derivatives grown in *Rhizobium* minimal medium containing succinate as the carbon source medium in the presence (black bars) or absence (open bars) of daidzein (0.2 μM). Samples were taken 36 h after the addition of daidzein and analyzed by gas chromatography-mass spectrometry.

tained in the bacteroids. A more likely explanation is that a different promoter, possibly the *nifA*- σ^{54} promoter that is immediately upstream of *y4wA* (and approximately 6 kb from *y4wE*) (Freiberg et al. 1997), and which is active within nodules (Perret et al. 1999), is necessary for IAA synthesis within the plant. This *nifA*- σ^{54} promoter is not present on the 5.8-kb *Bam*H1 fragment carried on pWEFG.

DISCUSSION

Interestingly, many plant-associated bacteria, including rhizobia, have the capacity to produce the essential plant hormone IAA. As far as is known, IAA biosynthesis in symbiotic bacteria occurs via the IAM, IPyA, and, to a lesser extent, Tra pathways (Fig. 1) (Costacurta and Vanderleyden 1995; Patten and Glick 1996). Biochemical activity of the IAM pathway (Fig. 1, number 1) has been demonstrated in *Bradyrhizobium japonicum* and *R. leguminosarum* bv. *viciae* (Kawaguchi et al. 1990; Sekine et al. 1989); whereas, in *B. elkanii*, the IPyA-pathway (Fig. 1, number 3) is prominent (Minamisawa et al. 1996). Several genes involved in bacterial IAA synthesis also have been identified. In *Azospirillum brasilense*, the gene encoding the enzyme indole-3-pyruvate decarboxylase has been isolated (Costacurta et al. 1994). In *B. japonicum*, the *bam* gene, a homologue of *tms-2* and *iaaH*, was shown to encode indole-3-acetamide aminohydrolase that converts IAM to IAA (Sekine et al. 1989).

In this report, we have identified two new genes involved in bacterial IAA synthesis: *y4wE* and *y4wF*, which are under the control of NB15 and the NodD1-SyrM2-NodD2 regulatory cascade. Close homologues of *y4wE* and *y4wF* have been

found in *Bradyrhizobium* sp. WM9 (ORF 380 and 342) (Stepkowski et al. 2003), *Mesorhizobium loti* MAFF303099 (*mlr5786* and *mlr5784*) (Kaneko et al. 2000), R7A (*msi396* and *msi395*) (Sullivan et al. 2002), and *R. etli* CE3 (*hisC* and *yp108*) (Hernandez-Gonzalez et al. 2003). In *Bradyrhizobium* sp. WM9 and both the *M. loti* strains, homologues of *y4wE* and *y4wF* are arranged in the same order as in NGR234, but without the *nod*-box. In *R. etli* CE3, the two genes are separated by approximately 80 kb, and a *nod*-box is upstream of *yp108*, the *y4wF* homologue. However, none of these homologues is linked to an *y4wG*-like ORF, supporting our conten-

Table 2. β -Galactosidase activity of promoter-*lacZ* fusions in NGR234 carrying *nodD2* on the multicopy plasmid pRAF130^a

Promoter, time (hpi)	β -Galactosidase activity (Miller's units) ^b	
	Noninduced	Daidzein-induced
NB8		
1	317 $\pm$ 19	318 $\pm$ 16
24	302 $\pm$ 22	253 $\pm$ 19
NB15		
1	10,300 $\pm$ 800	10,300 $\pm$ 500
24	10,700 $\pm$ 800	11,100 $\pm$ 1,100
<i>nodD1p</i>		
1	12 $\pm$ 0.4	12 $\pm$ 0.6
24	11 $\pm$ 0.2	11 $\pm$ 0.3

^a β -Galactosidase assays were used to assess the flavonoid-dependent activities of the *nod*-boxes NB8 and NB15 as well as the *nodD1*-promoter (*nodD1p*) in NGR234 harboring pRAF130. β -Galactosidase activities are given in Miller's units and were measured 1 and 24 h postinduction (hpi).

^b Values = mean $\pm$ standard error ($n = 3$).

Fig. 6. Intermediates of indole-3-acetic acid (IAA) synthesis found in the supernatants of NGR234 (open bars), and its derivatives NGRQwE (gray bars) and NGRQwF (black bars) grown in *Rhizobium* minimal medium containing succinate as the carbon source medium for 36 h. **A**, Bacteria grown without inducer, **B**, bacteria induced with 0.2 μ M daidzein. Samples were taken 36 h after the addition of daidzein and analyzed by liquid chromatography electrospray interface–tandem mass spectrometry.

tion that y4wG is cryptic. Nevertheless, this widespread distribution of y4wE and y4wF suggests that they play important roles under natural conditions.

Mutation of either y4wE or y4wF abolished flavonoid-induced stimulation of IAA production and diminished constitutive IAA production. Storage and breakdown products of the Tra, IAM, and IPyA pathways were detected, implying that NGR234 is capable of synthesizing IAA by three different pathways. Interestingly, flavonoid induction only stimulated increases in the levels of intermediates of the IPyA pathway, an effect that was absent in the mutants NGR Ω wE and NGR Ω wF (Fig. 6). These data strongly suggest that y4wE and y4wF encode enzymes involved in the IPyA pathway (Fig. 1, number 3). Conserved domain searches of public databases suggest that y4wE encodes a tryptophan transferase and y4wF an indole-3-acetaldehyde oxidase. Because indole-3-acetaldehyde oxidase plays a role in both the IPyA and Tra pathways (Fig. 1, number 2), this observation helps to explain why mutation of y4wF has more drastic effects on constitutive IAA production than the Ω insertion into y4wE. Furthermore, although the y4wE mutant is polar (with attendant effects on downstream ORF including y4wF), y4wF still may be transcribed from the weak constitutive promoter in the intergenic region, thus maintaining activity of the Tra pathway at reduced levels.

It is noteworthy that extra copies of *nodD2* converted NB15 into a constitutive promoter, suggesting that NodD2 does not require co-inducers. Similar effects of extra copies of *nodD2* on *nodC* expression were reported in *R. fredii* USDA191 (a close relative of NGR234), while extra copies of *nodD1* had no effect (Machado and Krishnan 2003). The response of NB15 to flavonoids is significantly delayed compared with *nod*-boxes that do not depend on NodD2 (e.g., *nod*-boxes controlling Nod-factor synthesis) (Kobayashi et al. 2004). Furthermore, mutation of y4wE strongly reduced IAA levels in nodules, indicating that the y4wEFG operon is

transcribed in later stages of the symbiosis. Both lines of evidence suggest that IAA production occurs after Nod-factor synthesis, and that bacterial IAA synthesis plays a role when more intimate contact between bacteria and their hosts has been established. Nod factors inhibit the transport of endogenous IAA, resulting in cortical-cell division, a necessary prelude to the formation of nodule primordia (Mathesius et al. 1998). Kondorosi and associates (1993) observed that IAA-sensitive lines of *Medicago varia* formed significantly more nodules than less sensitive variants. Our results are less clear cut. Although the y4wEFG locus is expressed in planta and produces significant quantities of IAA, disruption of the locus does not affect nodulation of the two hosts tested. Perhaps flavonoid-inducible IAA synthesis is needed for symbiosis only with certain hosts. Perhaps, too, bacterial IAA synthesis stimulates differentiation of the vascular system enhancing the connections between nodules and roots and, in this way, facilitates the exchange of carbon and nitrogen sources between the symbionts. Alternatively, IAA might act synergistically with other rhizobial signals (e.g., Nod factors, secreted proteins, or polysaccharides). More detailed experiments are planned with a greater number of hosts.

MATERIALS AND METHODS

Microbiological techniques.

Escherichia coli recombinants were grown at 37°C on or in Luria-Bertani media (Sambrook et al. 1989). NGR234 and its derivatives were raised at 27°C in or on *Rhizobium* minimal medium containing succinate as the carbon source (RMS) (Broughton et al. 1986) or TY (Beringer 1974). Ampicillin, gentamycin (Gm), kanamycin (Km), rifampicin (Rif), spectinomycin, and tetracycline were added at concentrations of 100, 20, 50, 50, 50, and 15 $\mu\text{g ml}^{-1}$, respectively. Tryptophan-supplemented cultures contained L-tryptophan at 1 or 5 $\mu\text{g ml}^{-1}$. Plasmid constructs were mobilized into NGR234 and its derivatives by triparental matings using pRK2013 as the helper plasmid (Ditta et al. 1980). Flavonoid induction was performed as follows: rhizobial cultures were grown to an optical density at 600 nm ($^{1\text{cm}}\text{OD}_{600}$) of 0.5 to 0.6, then diluted to $^{1\text{cm}}\text{OD}_{600} = 0.1$ in RMS medium and induced with 0.2 μM daidzein (Sigma-Aldrich, St. Louis) or 5 μM luteolin (Sigma-Aldrich). β -Galactosidase activity was assayed 1 and 24 h postinduction (hpi) as described by Miller (Miller 1972). For analysis of IAA and related compounds, 5-ml cultures were harvested 36 hpi when induction of IAA synthesis was maximal. The culture medium (supernatant) and bacterial cells (pellet) were separated by centrifugation (10 s at $3,000 \times g$, room temperature) and stored at -20°C until needed for extraction.

Extraction of IAA and metabolites for analysis by liquid chromatography–tandem mass spectrometry.

The following were added to 5 ml of the supernatants prepared as described above: 101 ng of ^{13}C -IAA (Cambridge Isotope Laboratory, Inc., Andover, MA, U.S.A.), 100 ng of d5-Trp (CON Isotopes Inc., Utrecht, The Netherlands), 150 ng of ^{15}N -anthranilic acid (Cambridge Isotope Labs), and 14.4 ng of ^{13}C -IAM (Van Onckelen et al. 1985) as internal tracers and 5 ml of 0.1 M HCl. The supernatant was concentrated on RP-C18 cartridges (Bond-Elut, Varian, Middelburg, The Netherlands). Following rinsing twice with 10 ml of 0.05 M HCl, all indole compounds were eluted from the cartridges with 1.5 ml of acetonitrile (two times). Acetonitrile was evaporated in a Speed Vac (RVC2-25, Christ Freeze Dryers, Osterode, Germany). Before liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis, the samples

Fig. 7. Concentrations of **A**, free indole-3-acetic acid (IAA) and **B**, IAA conjugates in nodules of *Vigna unguiculata* and *Tephrosia vogelii* produced by wild-type NGR234 (open bars), the mutant NGR Ω wE (gray bars), or the transconjugant NGR Ω wE(pwEFG) (black bars); * = not tested.

were dissolved in 100 μ l of acidified methanol and methylated with ethereal diazomethane following the procedure of Schlenk and Gellerman (1960), dried under nitrogen (TurboVap, LV evaporator; Zymark Corporation, Hopkinton, MA, U.S.A.), and dissolved in 100 μ l of methanol. After transfer to vials, the samples were dried under nitrogen and redissolved in 20% methanol.

Bacterial cells were resuspended in 200 μ l of acidified methanol and sonicated in a Vibracell VCX 400 (with stepped microtip; Sonics & Materials Inc., Danbury, CT, U.S.A.) for 3 s at 0°C (amplitude of the microtip 40, 400 W, pulse 5 s followed by a 5-s cool down period). Afterward, 10 ml of 0.05 M HCl was added as well as the internal tracers: 101 ng of ^{13}C -IAA, 100 ng of d5-Trp, 150 ng of ^{15}N -AA, and 14.4 ng of ^{13}C -IAM. Cell debris was removed by centrifugation (10 s at $3,000 \times g$, room temperature), and the supernatants concentrated on an RP-C18 cartridge, as described above.

Nodules were separated from the roots, frozen in liquid nitrogen, and kept at -70°C until extraction. Still-frozen nodules were crushed with mortar and pestle that was kept cold with liquid nitrogen. Indole compounds were extracted from the plant debris in 80% methanol at -20°C (overnight). Preparations were divided into two equal aliquots for the analysis of free and conjugated IAA. The free IAA fraction was diluted with distilled water to 50% (vol/vol) methanol and purified on a DEAE-Sephadex cartridge (DEAE-Sephadex A25, formate conditions; Pharmacia, Amersham Bioscience, Uppsala, Sweden). After loading and rinsing the DEAE with two additional column volumes of 50% (vol/vol) methanol, an RP-C18 cartridge was attached beneath the column and eluted with 6% formic acid. Under these conditions, IAA was concentrated on the RP-C18-cartridge underneath, from which it was eluted with diethyl ether and dried under nitrogen. The IAA conjugates were hydrolyzed for 3 h in 7 M NaOH under the flow of water-saturated nitrogen (Bialek and Cohen 1989). After hydrolysis, the samples were desalted on an RP-C18 cartridge at pH 2 and IAA conjugates were extracted as described for free IAA.

Extraction of IAA for measurement by gas chromatography-MS.

The extraction procedure for supernatants as well as for bacterial pellets prior to gas chromatography (GC)-MS analysis was the same as that described above for measurement by LC-MS/MS. ^{13}C -IAA (101 ng) was added as an internal tracer and samples were derivatized as described by Epstein and Cohen (1981) using pentafluorobenzylbromide (PFBB; Sigma-Aldrich) and ethylpiperidine (Sigma-Aldrich). IAA also was converted into its pentafluorobenzyl (PFB)-ester using this procedure.

LC/(+)ES-MS/MS conditions.

Samples containing indole compounds were analyzed using a micro-high-performance liquid chromatography (HPLC) system linked to a Quattro II mass spectrometer equipped with an electro-spray interface (LC/(+)ES-MS/MS; Waters, Milford, MA, U.S.A.) Samples (25 μ l) were injected onto an RP-C18 reversed-phase column (5 μ l of ODS(3) 100A; 100 by 1.00 mm; Phenomenex, Torrance, CA, U.S.A.) and eluted with a methanol–0.01 M ammonium acetate gradient (1 min at 20% MeOH; linear gradient from 20 to 90% MeOH in 2 min; 3.5 min at 90% MeOH, 5 min at 20% MeOH, flow 0.06 ml min $^{-1}$) (HPLC pump: BIO-TEK Instruments, system 522; Waters) (Prinsen et al. 1998). Quantification was performed by multiple reactant monitoring of the $[\text{MH}^+]$ ion and the appropriate product ion (Prinsen et al.

1997). AA, Trp, IPyA, Tra, IAAld, IAM, ILA, IAld, IET, IMe, and IAA can be detected with this system. All data were processed by Masslynx software (Waters).

GC-MS conditions.

Samples extracted for IAA measurement were analyzed by GC-MS. IAA-pentafluorobromide was analyzed by negative chemical ionization GC-MS. The sample (1 μ l) was injected on a Varian DB-XLB capillary column (15 m, 0.25-mm inside diameter, 0.25- μ m film thickness) on a Hewlett Packard 5890 series II gas chromatograph with an HP 6890 automatic injector (temperature 325°C), He flow 1.5 ml min $^{-1}$. Temperature gradient was 2 min at 175°C , from 175 to 300°C at $15^\circ\text{C min}^{-1}$, and 300°C for 2 min. Quantification was performed on a VG Trio 2000 mass spectrometer in the negative chemical ionization mode (Waters, Micromass) using methane as the ionization gas. Detection was performed in the ion-monitoring mode, using m/z 180 and 174 for the quantification of ^{13}C -IAA-PFB and IAA-PFB, respectively. Data were processed by Masslynx software (Waters).

Construction of y4wE and y4wF mutants and plasmids.

To mutate y4wE, cosmid pXB368 (Perret et al. 1991) was digested with *Bgl*II, and the 3.4-kb fragment containing y4wE was purified and cloned into the *Bam*HI site of pJQ200-mp18 (Quandt and Hynes 1993). A *Bam*HI fragment from pHP45 Ω (Fellay et al. 1987) containing the spectinomycin-resistant Ω interposon was inserted into the unique *Xho*I site within y4wE after treatment with the Klenow fragment. To mutate y4wF, cosmid pXB368 was digested with *Bam*HI, and the 5.8-kb fragment containing NB15 as well as y4wEFG was purified and cloned into the *Bam*HI site of pJQ200-mp18 (this same *Bam*HI fragment also was cloned into the *Bam*HI site of pLAFR-6, giving rise to pwEFG). A *Bam*HI fragment from pHP45 Ω -Km containing the kanamycin-resistant Ω interposon was inserted into the unique *Bgl*III site within y4wF. The *Bam*HI fragment containing the interposon-inserted y4wF was cloned into the *Bam*HI site of pLAFR-6 giving pwE(F Ω)G. The upstream region of y4wF was amplified by PCR from pwEFG using wFpF (5'-GCGTCTAGATAACCGCTATTCG-3') and wFpR (5'-TGCCAATGCATTACCGTCCTCG-3') as primers and *pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). This 952-bp fragment then was cloned into the *Eco*RV site of pBluescriptKS+ (Stratagene) and later as an *Xba*I-*Nsi*I insert into the *Xba*I-*Pst*I sites of pMP220 (resulting in plasmid pMP-wF5'). Plasmids were mobilized into NGR234 by triparental matings. For constructs to knock out y4wF and y4wE, transconjugants first were selected on TY plates containing Rif and Gm, then on TY plates containing Rif, Km, and 5% (wt/vol) sucrose to select for double crossovers. Marker exchange leading to NGR Ω wE and NGR Ω wF was confirmed by Southern hybridizations.

Plant assays.

Nodulation tests were performed in Magenta jars (Magenta Corp., Chicago) (Lewin et al. 1990). All plants were grown at a day temperature of 26°C , a night temperature of 20°C , and a photoperiod of 16 h. Each plant was inoculated with 10^8 bacteria and harvested 6 to 8 weeks after inoculation.

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