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# Photosynthetic *Bradyrhizobium* Sp. Strain ORS285 Synthesizes 2-*O*-Methylfucosylated Lipochitooligosaccharides for *nod* Gene–Dependent Interaction with *Aeschynomene* Plants

Adeline Renier,<sup>1</sup> Fabienne Maillet,<sup>2</sup> Joel Fardoux,<sup>1</sup> Véréna Poinot,<sup>3</sup> Eric Giraud,<sup>1</sup> and Nico Nouwen<sup>1</sup>

<sup>1</sup>Laboratoire des Symbioses Tropicales et Méditerranéennes, UMR113 IRD, Laboratoire des Symbioses Tropicales et Méditerranéennes, UMR IRD/SupAgro/INRA/UM2/CIRAD, F-34398 Montpellier, France; <sup>2</sup>Laboratoire des Interactions plantes/microorganismes LIPM, UMR441/2594 INRA/CNRS 441/2594, 24 chemin de Borde Rouge, 31326 Castanet-Tolosan, France; <sup>3</sup>Laboratoire des Interactions Moléculaires et Réactivité Chimique et Photochimique, UMR 5623 CNRS/Université Paul Sabatier, 118 Route de Narbonne, F-31062 Toulouse, France

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*Bradyrhizobium* sp. strain ORS285 is a photosynthetic bacterium that forms nitrogen-fixing nodules on the roots and stems of tropical aquatic legumes of the *Aeschynomene* genus. The symbiotic interaction of *Bradyrhizobium* sp. strain ORS285 with certain *Aeschynomene* spp. depends on the presence of nodulation (*nod*) genes whereas the interaction with other species is *nod* gene independent. To study the *nod* gene-dependent molecular dialogue between *Bradyrhizobium* sp. strain ORS285 and *Aeschynomene* spp., we used a *nodB-lacZ* reporter strain to monitor the *nod* gene expression with various flavonoids. The flavanones liquiritigenin and naringenin were found to be the strongest inducers of *nod* gene expression. Chemical analysis of the culture supernatant of cells grown in the presence of naringenin showed that the major Nod factor produced by *Bradyrhizobium* sp. strain ORS285 is a modified chitin pentasaccharide molecule with a terminal N-C<sub>18:1</sub>-glucosamine and with a 2-*O*-methyl fucose linked to C-6 of the reducing glucosamine. In this respect, the *Bradyrhizobium* sp. strain ORS285 Nod factor is the same as the major Nod factor produced by the nonphotosynthetic *Bradyrhizobium japonicum* USDA110 that nodulates the roots of soybean. This suggests a classic *nod* gene-dependent molecular dialogue between *Bradyrhizobium* sp. strain ORS285 and certain *Aeschynomene* spp. This is supported by the fact that *B. japonicum* USDA110 is able to form N<sub>2</sub>-fixing nodules on both the roots and stems of *Aeschynomene afraspera*.

Rhizobia have the ability to establish a symbiotic relationship with leguminous plants. In this association, the bacteria induce the plant to develop a new organ, the nodule, in which the symbiont resides and fixes atmospheric nitrogen. This complex interorganismal relationship is mediated by signal mole-

cules produced by both bacteria and plants (Spaink 1992). Leguminous plants synthesize and exude a diverse array of phenolic compounds such as flavonoids into the rhizosphere. These compounds serve as chemoattractants for rhizobia, influence bacterial growth, and selectively induce the expression of bacterial nodulation (*nod*) genes, leading to the biosynthesis of the bacterial signal molecules, the Nod factors (NF) (Caetano-Anolles et al. 1988; Dharmatilake and Bauer 1992). NF are composed of three to five 1-4  $\beta$ -linked *N*-acetyl glucosamine units (GlcNac) with the *N*-acetyl group of the terminal nonreducing sugar replaced by an acyl chain with 16 or 18 carbons and with varying degrees of saturation. Various modifications at both reducing and nonreducing termini of the chitin backbone are possible, such as methylation, acetylation, carbamoylation, fycosylation, or sulfation (D’Haeze and Holsters 2002; Perret et al. 2000). The structure of the fatty acyl chain, the number of GlcNac residues, and the presence of extra substituents determine the host specificity of the bacterium (Denarie et al. 1996). Thus, it is not surprising that broad host range symbionts such as *Rhizobium* sp. strain NGR234, *Rhizobium fredii*, *R. tropici*, and certain *Bradyrhizobium* spp. produce a range of NF (Bec-Ferte et al. 1996; Carlson et al. 1993; Poupot et al. 1993; Price et al. 1992).

Photosynthetic *Bradyrhizobium* spp. establish a symbiotic interaction with tropical aquatic legumes of the *Aeschynomene* genus (Giraud and Fleischman 2004). In addition to being photosynthetic, these bacteria and the interaction with *Aeschynomene* have other atypical characteristics compared with the classical and well-studied rhizobia–legume interaction. First, photosynthetic *Bradyrhizobium* spp. are able to fix nitrogen in the free living state (Alazard 1990). Second, in addition to nodulating roots, photosynthetic *Bradyrhizobium* spp. are also able to nodulate the stems of *Aeschynomene* plants (Alazard 1985). Third, the infection process of the plant does not involve root hair curling but bacteria enter the plant via epidermal fissures (“cracks”) generated by the emergence of lateral or adventive roots (Sprent 2008). Fourth, certain photosynthetic *Bradyrhizobium* spp. (ORS278 and BTAi1) lack the genes that encode the enzymes that synthesize the core structure of NF (*nodABC*) (Giraud et al. 2007). This indicates that some photosynthetic *Bradyrhizobium* spp. likely use a novel mechanism, independent of the long-time considered universal NF to interact with their legume hosts.

Corresponding author: N. Nouwen; E-mail: nico.nouwen@ird.fr

\*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure is published online and Figure 5 appears in color online.

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Photosynthetic *Bradyrhizobium* sp. strain ORS285 has a broad host range and interacts with many plants in the *Aeschynomene* genus (Molouba et al. 1999). In contrast to the recently (completely) sequenced photosynthetic *Bradyrhizobium* sp. strains ORS278 and BTAi1 (Giraud et al. 2007), strain ORS285 contains the *nodABC* genes (Chaintreuil et al. 2001). However, whereas a deletion of the *nodBC* genes in *Bradyrhizobium* sp. strain ORS285 results in the inhibition of nodulation of certain plants in the *Aeschynomene* genus, the mutant was still able to interact with a subgroup of plants that are nodulated by photosynthetic *Bradyrhizobium* spp. that do not contain *nodABC* genes (Giraud et al. 2007). This indicates that *Bradyrhizobium* sp. strain ORS285 interacts with certain *Aeschynomene* plants in a *nod* gene-dependent manner whereas, with others, it is using (or can use) a *nod* gene-independent mechanism.

In many aspects, the interaction between photosynthetic *Bradyrhizobium* spp. and *Aeschynomene* spp. is different from the classical rhizobia–legume interaction; therefore, the aim of this study was to determine which signal molecules are involved in the *nod* gene-dependent molecular dialogue between photosynthetic *Bradyrhizobium* sp. strain ORS285 and *Aeschynomene* spp. (i.e., the flavonoids that induce the expression of the *nodABC* operon and the determination of the NF structures produced by the bacterium).

## RESULTS

### The flavanones liquiritigenin and naringenin are strong inducers of *nodB* expression.

The photosynthetic *Bradyrhizobium* sp. strain ORS285 contains an operon containing the common *nod* genes (*nodABC*) (Chaintreuil et al. 2001) that are involved in the synthesis of the core structure of NF in other rhizobia. To determine which flavonoids induce the expression of genes in the *nodABC* operon, we used a *Bradyrhizobium* sp. strain ORS285 reporter strain which contained a *nodB-lacZ* transcriptional fusion on the chromosome. Growth of this re-

porter strain in the presence of flavonoids of different functional classes (Fig. 1) showed that the flavanones liquiritigenin and naringenin result in the highest induction of  $\beta$ -galactosidase activity (Table 1). Because both activities were not significantly different, C5 hydroxylation (as found in naringenin) seems not to be very important for inducing capacity. C3' hydroxylation of flavanones leads to a twofold reduction in  $\beta$ -galactosidase activity, whereas flavanones with a methoxy or glucoside at the C7 position (naringenin-7-*O*-glucoside, sakuranetin, and sakuranin) are inactive. Flavones are approximately twofold less active in induction of *nodB-lacZ* expression compared with flavanones. As with flavanones, attachment of a glucoside at the C7 position (apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside) results in a com-

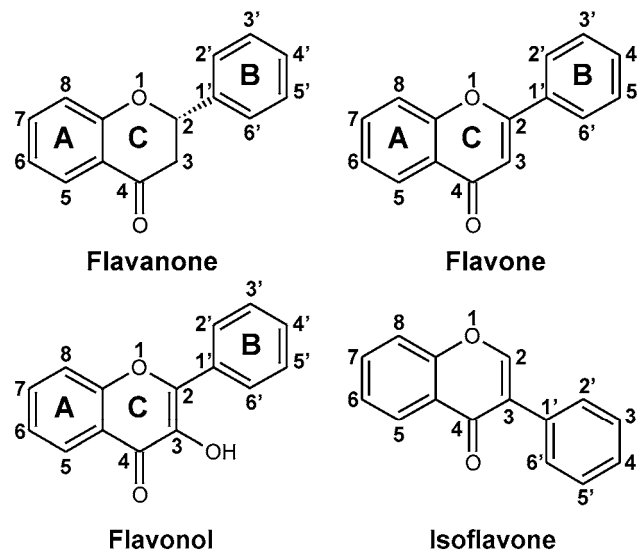


Fig. 1. Basic chemical structures of a flavanone, flavone, flavonol, and isoflavone.

Table 1. Induction of the *Bradyrhizobium* sp. strain ORS285 *nodB-lacZ* fusion by flavonoids

Putative inducer	Hydroxylation pattern					Miller units
	3	5	7	3'	4'	
Flavanones						
Liquiritigenin	...		OH	...	OH	3,676 ± 486
Naringenin	...	OH	OH	...	OH	4,265 ± 274
Naringenin-7- <i>O</i> -glucoside	...	OH	Glu	...	OH	945 ± 67
Eriodictyol	...	OH	OH	OH	OH	1,709 ± 205
Hesperetin	...	OH	OH	OH	Metx	1,108 ± 124
Sakuranetin	...	OH	Metx	...	OH	799 ± 100
Sakuranin	...	Glu	Metx	...	OH	801 ± 66
Flavones						
7,4'-dihydroxyflavone	...		OH	...	OH	2,758 ± 251
Apigenin	...	OH	OH	...	OH	2,290 ± 270
Apigenin-7- <i>O</i> -glucoside	...	OH	Glu	...	OH	869 ± 142
Acacetin	...	OH	OH	...	Metx	1,093 ± 111
Luteolin	...	OH	OH	OH	OH	2,227 ± 293
Luteolin-7- <i>O</i> -glucoside	...	OH	Glu	OH	OH	839 ± 57
Flavonols						
Kaempferol	OH	OH	OH	...	OH	1,483 ± 204
Kaempferol-3- <i>O</i> -glucoside	Glu	OH	OH	...	OH	744 ± 85
Kaempferide	Glu	OH	OH	...	Metx	816 ± 35
Quercetin	OH	OH	OH	OH	OH	833 ± 95
Isoquercitrin	Glu	OH	OH	OH	OH	826 ± 33
Isoflavones						
Daidzein	...	...	OH	...	OH	837 ± 51
Daidzin	...	...	Glu	...	OH	770 ± 63
Formononetin	...	...	OH	...	Metx	810 ± 36
Genistein	...	OH	OH	...	OH	904 ± 109
Control	...	...	...	...	...	805 ± 34

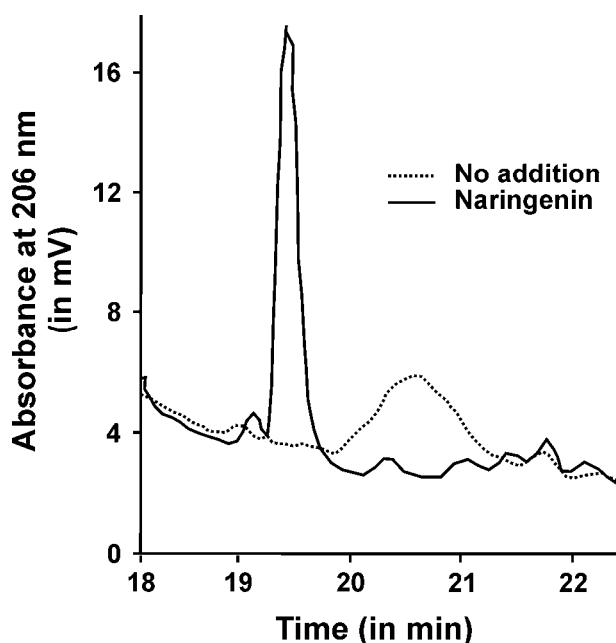
plete inhibition of inducing capacity. Except for kaempferol, all tested flavonols were not able to induce the expression of *nodB-lacZ* in *Bradyrhizobium* sp. strain ORS285.

The isoflavones differ from flavones and flavanones in that the B ring is attached to C3 instead of C2. Because all tested isoflavones (daidzein, daidzin, formononetin, and genistein) are unable to induce expression of *nodB-lacZ*, it can be concluded that the attachment of the B-ring to C2 as found in flavanones and flavones is crucial for the induction of genes in the *nodABC* operon of *Bradyrhizobium* sp. strain ORS285.

### The major NF of *Bradyrhizobium* sp. strain ORS285 is a pentameric LCO

#### with a 2-*O*-methylfucose at the reducing end.

Naringenin was the most active inducer of *nodB-lacZ* expression; therefore, this flavonoid was used to obtain maximum *nod* gene expression and to isolate synthesized NF from the culture supernatant of *Bradyrhizobium* sp. strain ORS285. HPLC analysis of the butanol extract of uninduced (– naringenin) and induced (+ naringenin) cells showed one major peak that was present in the induced culture but not in the uninduced culture (Fig. 2). Fractions corresponding to this peak in the HPLC profile were collected, pooled, and analyzed by electrospray ionization (ESI)-MS. The positive ion spectrum of compounds in the peak fraction shows that it consists of a mixture of at least four molecules (Fig. 3A; M<sub>1</sub> to M<sub>4</sub>). [M+Na]<sup>+</sup> ions at m/z 1235.7 and 1438.8 are compatible with *O*-methyl-fucosylated chito-oligomers bearing an *N*-octadecanoyl substituent. The difference in 203.1 atomic mass units (amu) between [M+Na]<sup>+</sup> ions at m/z 1235.7 and 1438.8 indicates that the smallest molecule lacks one *N*-acetylglucosaminosyl residue (203.1 amu) compared with the heaviest one and can be assigned as Nod-IV(C18:1,MeFuc) and Nod-V(C18:1,MeFuc) respectively. The mass difference of 43 amu between [M+Na]<sup>+</sup> ions at m/z 1438.8 and 1481.8 and between 1235.7 and 1278.7 suggest that the two chito-tetra and -pentamers contains an additional carbamoyl group and likely corresponds to Nod-IV(Cb, C18:1, MeFuc) and Nod-V(Cb,



**Fig. 2.** Elution profile of reverse-phase high-performance liquid chromatography purification of nodulation factors from *Bradyrhizobium* sp. strain ORS285. The culture supernatant of *Bradyrhizobium* sp. strain ORS285 cells grown in the presence and absence of naringenin was extracted with *n*-butanol, whereafter the concentrated extract was separated using a C18 reverse-phase column. Only the relevant part of the chromatogram is shown.

C18:1, MeFuc), respectively. Closer inspection of different spectra of the peak fraction showed that it also contained a very small fraction of Nod-V(C16:0, MeFuc) (data not shown). Based on the ESI-MS experiments, it can be concluded that *Bradyrhizobium* sp. strain ORS285 synthesizes at least five different LCO, of which Nod-V(C18:1, MeFuc) is the most abundant one (69% of the total) when the bacterium is grown in the presence of naringenin (Table 2).

To confirm the structure of the most abundant LCO in the peak fraction, an additional MS/MS experiment was performed (Fig. 3B). With the collision energy set at 30 V, a classical B fragmentation pattern was observed with the major LCO. This fragmentation is due to disruption of the osidic junction between the glucosamine subunits and produces a regular profile with a mass difference of 203.1 amu between each ion corresponding to GlcNac subunits (221.1 amu for the unmodified reducing end). Fragmentation of the molecular ion [M+H]<sup>+</sup> of the most abundant LCO produced a neutral loss of 381 amu directly from the molecular species, indicating that the reducing end is modified (381 = 221 + 160). This mass of 160 amu corresponds to a methyl fucose group. To our knowledge, only 2-*O*-methyl-fucose borne by the 6-*O* of the reducing end has been published as NF modification. The other losses were equivalents of 203.1 amu and the profile ended with a B<sub>1</sub> ion at m/z 426, indicating that the nonreducing end is substituted by a C18:1 acyl. Based on the MS/MS experiment, it can be concluded that the major LCO synthesized by *Bradyrhizobium* sp. strain ORS285 is Nod-V(C18:1, MeFuc) (Fig. 3C).

### *Bradyrhizobium* sp. strain ORS285 genes encoding enzymes and regulators involved in NF synthesis.

The synthesis of NF involves many different enzymes whose expression involves a complex regulation mechanism. To analyze whether the synthesized LCO correspond with the *nod* genes present in the genome and how the expression of these genes might be regulated, we searched published and unpublished DNA sequences of *Bradyrhizobium* sp. strain ORS285 for known genes involved in NF synthesis, secretion, and regulation. In total, we identified 10 putative genes involved in NF synthesis (*nodABCSUZ*, *noeI*, and *noIL*) and secretion (*nodIJ*) and 3 putative genes encoding proteins that regulate *nod* gene expression (*nodD1*, *nodD2*, and *nola*) (Table 3). The 13 genes, in total, are scattered over six different contigs in the genomic draft of *Bradyrhizobium* sp. strain ORS285. The largest contig (8 kb) contains the *nodABCSUIJ* genes that exhibit a perfect microsynthony in *B. japonicum* USDA110 (Supplementary Fig. S1). Comparison of genes involved in the regulation and synthesis of NF between these two strains showed

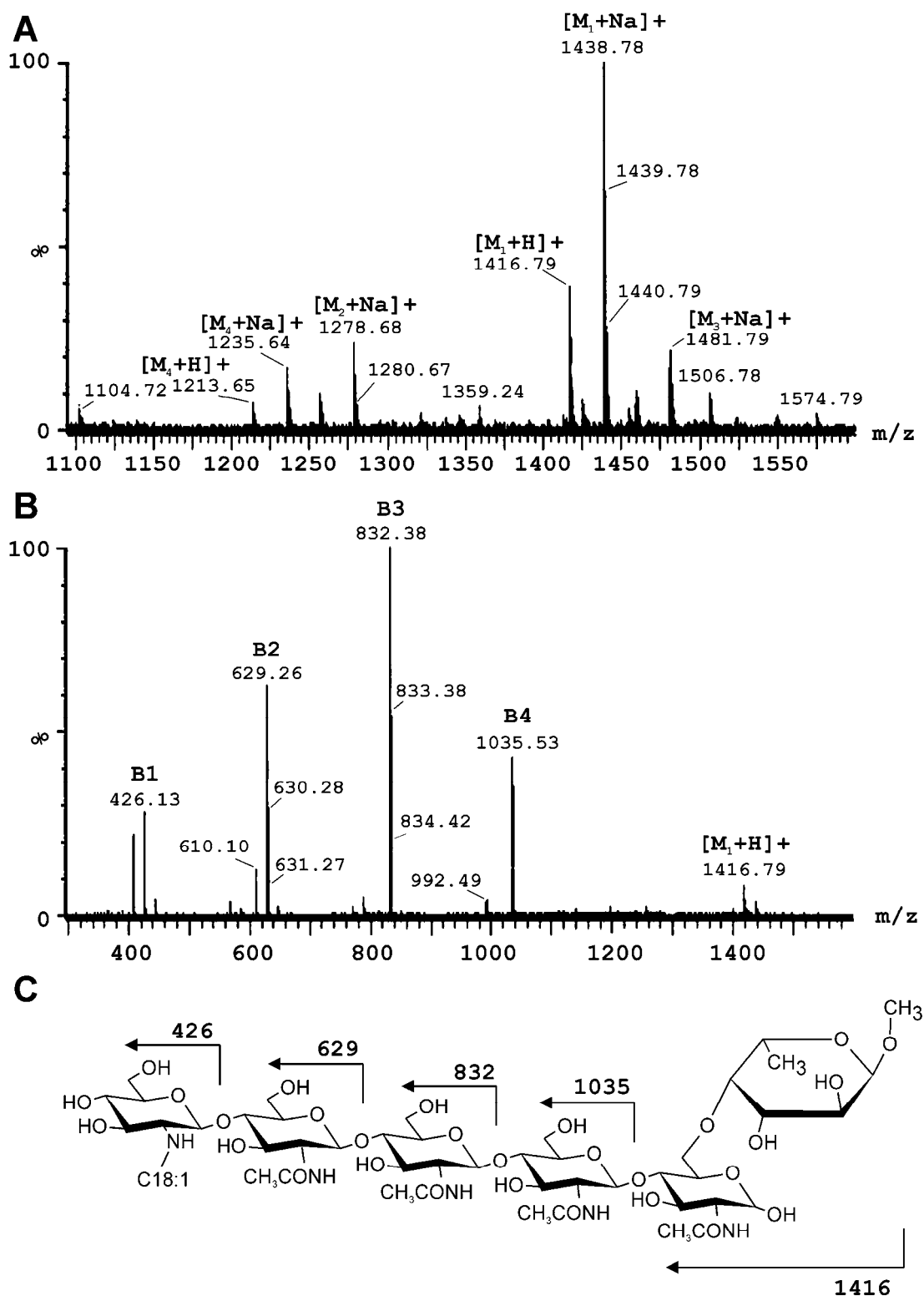
**Table 2.** Nodulation factors synthesized by *Bradyrhizobium* sp. strain ORS285<sup>a</sup>

Structure	Molecular weight	ESI-MS	Percentage (%)
V, C18:1, MeFuc	1,416	M <sub>1</sub>	69
V, C18:1	1,256	M <sub>2</sub>	12
V, C18:1, MeFuc, Cb	1,459	M <sub>3</sub>	9
IV, C18:1, MeFuc	1,213	M <sub>4</sub>	9
V, C16:0, MeFuc	1,390	...	1

<sup>a</sup> Based on the electrospray ionization mass spectrometry (ESI-MS) and tandem MS (MS/MS) spectra, we could identify five different lipochito-oligosaccharides (LCO) that are present in the isolated peak fraction from the reverse phase column. The molecular weight and the indication used in the ESI-MS spectrum are given. In the case of carbamoylation (Cb), the location of the carbamoyl group is on the C6 atom of the terminal *N*-octadecanoyl-glucosamine residue. The percentage of the different LCO in the peak fraction is based on the different ions found for the corresponding molecule in the ESI-MS spectrum.

that *Bradyrhizobium* sp. strain ORS285 does not contain the two-component regulatory system NodV/NodW and also lacks the carbamoyltransferase NodO. In contrast, *Bradyrhizobium* sp. strain ORS285 possesses a *nolL* gene that encodes for an acetyl transferase and which is absent in the *B. japonicum* USDA110 genome. Taken together, in the available DNA sequences of *Bradyrhizobium* sp. strain ORS285, we could iden-

tify all known genes that encode for enzymes that are responsible for the different modifications as found in the synthesized LCO. Moreover, the regulation of *nod* gene expression involves, as in all other rhizobia, the “classical” regulator NodD, but is likely more complex than a simple induction by flavonoids because a putative gene encoding the cell-density-dependent regulator NodA is also found.



**Fig. 3.** Mass spectrometric analysis of the C18 reverse-phase fraction induced by the flavonoid naringenin. **A**, Electrospray ionization mass spectrometry spectrum of the C18 reverse-phase fraction that is induced by naringenin. **B**, Tandem mass spectrometry spectrum of the major nodulation (Nod) factor present in the reverse-phase fraction. **C**, Structure and expected B fragmentation pattern of the major Nod factor of *Bradyrhizobium* sp. strain ORS285.

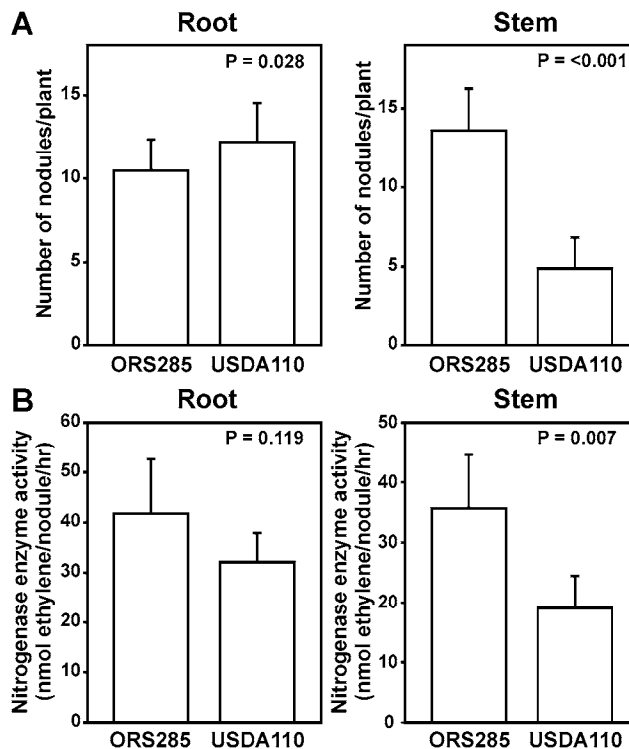
### ***B. japonicum* USDA110 forms stem and root nodules on the tropical aquatic legume *A. afraspera*.**

Photosynthetic *Bradyrhizobium* sp. strain ORS285 forms *nod* gene-dependent nodules on both the roots and stems of *A. afraspera* plants. Because the major LCO synthesized by *Bradyrhizobium* sp. strain ORS285, Nod-V(C18:1, MeFuc), is exactly the same as the major LCO synthesized by *B. japonicum* USDA110 (Sanjuan et al. 1992), we wondered whether the nonphotosynthetic *B. japonicum* USDA110 is able to stem and root nodulate *A. afraspera*. *B. japonicum* USDA110 NF are induced by the isoflavones genistein and daidzein (Kosslak et al. 1987) whereas isoflavones do not induce *nod* gene expression in *Bradyrhizobium* sp. strain ORS285 (Table 1). HPLC and spectral analysis of the root exudate of *A. afraspera* plants showed that, in addition to “naringenin-like” flavonoids, the isoflavone daidzein was a major compound in the root exudate (data not shown). This indicates that the synthesis of NF by *B. japonicum* USDA110 is likely induced upon contact with *A. afraspera* plants. To analyze whether this leads to the formation of nodules, we inoculated the stems and roots of 1-week-old *A. afraspera* seedlings with *B. japonicum* USDA110 and compared this with the nodule formation by *Bradyrhizobium* sp. strain ORS285. Two weeks after inoculation, the number of nodules formed on the root of *A. afraspera* plants infected with *B. japonicum* USDA110 was slightly higher compared with plants infected with *Bradyrhizobium* sp. strain ORS285 (Fig. 4A). In contrast, the number of nodules on the stems of *A. afraspera* plants infected with *B. japonicum* USDA110 was approximately two- to threefold lower compared with plants infected with *Bradyrhizobium* sp. strain ORS285 (Fig. 4A). For stem nodules containing *B. japonicum* USDA110, the nitrogenase enzyme activity per nodule as measured via the ARA was lower compared with nodules containing *Bradyrhizobium* sp. strain ORS285 (Fig. 4B) Macroscopic analysis of both stems and roots inoculated by *B. japonicum* USDA110 showed that the formed nodules have a similar size and shape compared with stem and root nodules produced by *Bradyrhizobium* sp. strain ORS285 (Fig. 5). Moreover, as with *Bradyrhizobium* sp. strain ORS285, root nodules induced by *B. japonicum* USDA110 are found only at the base of lateral roots. Taken together, these results indicate that *B. japonicum* USDA110 is able to form nodules on the tropical aquatic legume *A. afraspera* but that the nitrogenase activity per stem nodule is less compared with stem nodules formed by the original symbiotic partner, *Bradyrhizobium* sp. strain ORS285. However, because *A. afraspera* plants infected with *B. japonicum* USDA110 do not show any signs of nitrogen limitation,

the amount of N<sub>2</sub> fixation by the stem nodules seem to be sufficient to stimulate the growth of the plant.

## DISCUSSION

*Aeschynomene* spp. that form nodules on both the roots and stems are, in general, found to be nodulated by photosynthetic *Bradyrhizobium* spp. In contrast to many other rhizobia-leg-



**Fig. 4.** *Bradyrhizobium japonicum* USDA110 nodulates the root and stem of the tropical aquatic legume *Aeschynomene afraspera*. **A**, Roots or stems from *A. afraspera* were inoculated with *Bradyrhizobium* sp. strain ORS285 and *B. japonicum* USDA 110, respectively, and the number of nodules was determined 15 days postinfection. **B**, *A. afraspera* roots or stems were inoculated with *Bradyrhizobium* sp. strain ORS285 and *B. japonicum* USDA 110, respectively, and the nitrogenase enzyme activity per nodule was determined by the acetylene reduction assay. The amount of nodules and nitrogenase activity is the average of 10 infected plants. The *P* value of Student's *t* test on the experimental data is given in the upper right hand corner of the bar diagrams.

**Table 3.** *Bradyrhizobium* sp. strain ORS285 genes encoding enzymes and regulators involved in the synthesis of nodulation (Nod) factors<sup>a</sup>

Gene	Enzyme, transporter, regulator	Accession number	Reference
Enzyme			
<b><i>nodA</i></b>	N-acyltransferase	AAK50870.1	Chaintreuil et al. (2001)
<b><i>nodB</i></b>	Chitoooligosaccharide deacetylase	AAK50871.1	Chaintreuil et al. (2001)
<b><i>nodC</i></b>	N-acetylglucosaminyl transferase	AAK50872.1	Chaintreuil et al. (2001)
<i>nodS</i>	N-methyl transferase	FQ790398	This study
<i>nodU</i>	6- <i>O</i> -carbamoyl transferase	FQ790399	This study
<i>nodZ</i>	$\alpha$ -1,6-Fucose transferase	FQ790403	This study
<i>noeI</i>	2- <i>O</i> -methyltransferase (fragment)	FQ790402	This study
<i>noIL</i>	4- <i>O</i> -acetyl transferase	AAK50867.1	Chaintreuil et al. (2001)
Transporter			
<b><i>nodI</i></b>	ABC transporter, ATPase domain	FQ790400	This study
<b><i>nodJ</i></b>	ABC transporter, membrane domain	FQ790401	This study
Regulator			
<b><i>nodD1</i></b>	Transcriptional regulator NodD1	FQ790406	This study
<b><i>nodD2</i></b>	Transcriptional regulator NodD2	FQ790405	This study
<i>noIA</i>	Transcriptional regulator Nola	FQ790397	This study

<sup>a</sup> Common (bold) and specific genes encoding enzymes, transporters, and regulators involved in the synthesis and secretion of Nod factors as found in published and unpublished DNA sequences of *Bradyrhizobium* sp. strain ORS285.



ume interactions, relatively little is known about the symbiotic signal molecules involved in the molecular dialogue between photosynthetic *Bradyrhizobium* spp. and *Aeschynomene* plants. In this study, we analyzed the *nod* gene expression of photosynthetic *Bradyrhizobium* sp. strain ORS285 and the signal molecules that are synthesized upon their induction.

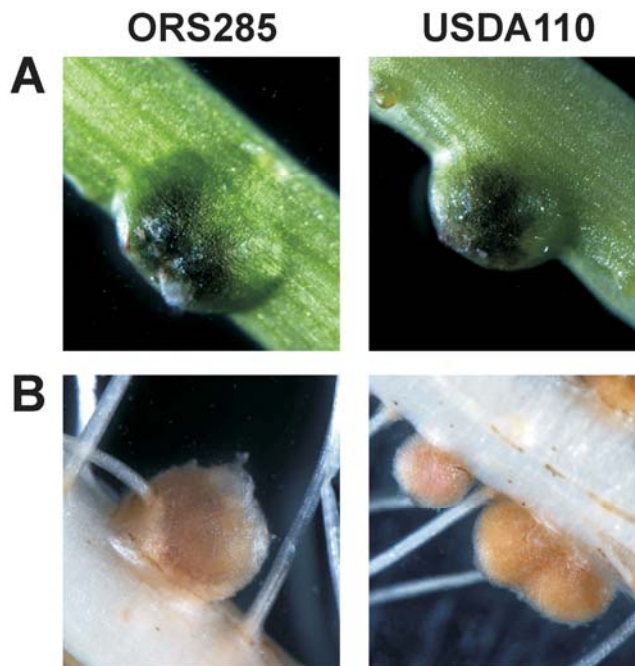
Plant roots secrete a lot of molecules into the rhizosphere, of which flavonoids have been shown to be strong inducers of *nod* gene expression in many rhizobia. Growth of a *nodB-lacZ* reporter strain in the presence of different flavonoids showed that the flavanones liquiritigenin and naringenin result in the highest induction of  $\beta$ -galactosidase activity (Table 1). Spectral and mass spectrometric analysis of the root exudate of *A. afraspera*—a species that is nodulated by *Bradyrhizobium* sp. strain ORS285 in a *nod* gene-dependent manner—showed the presence of many “naringenin”-like flavonoids and the isoflavonoid daidzein (A. Renier, unpublished results). The latter flavonoid did not induce *nod* gene expression in vitro; therefore, flavanones of the naringenin type are likely the natural inducers of *Bradyrhizobium* sp. strain ORS285 *nod* gene expression when interacting with *A. afraspera* in the rhizosphere.

Nodulation genes can be classified into two categories: the common *nod* genes (*nodABC*) that are involved in the formation of the *N*-acylated chitin oligomer NF core and specific *nod* genes (*nodZ*, *nodH*, *nodS*, and so on) that encode enzymes involved in the synthesis and transfer of additional modifying groups on the NF core. Analysis of published (Chaintreuil et al. 2001) and unpublished DNA sequences showed that *Bradyrhizobium* sp. strain ORS285 contains the common *nodABC* genes involved in the synthesis of the NF core and the specific *nodSUZ*, *noeI*, and *noII* genes involved in *N*-methylation (*nodS*) (Geelen et al. 1995), and carbamoylation (*nodU*) (Jabbouri et al. 1995), fucosylation (*nodZ*) (Stacey et al. 1994) of the NF core structure and methylation (*noeI*) (Jabbouri et al. 1998) or 4-*O*-acetylation of the fucosyl residue (*noII*) (Berck et al. 1999). Except for *N*-methylation and 4-*O*-acetylation, NF with all other above-mentioned modifications have been found in the culture supernatant isolated from *Bradyrhizobium* sp. strain ORS285 cells grown in the presence of naringenin (Table 2). This indicates that the genes encoding for enzymes involved in these modifications are expressed in the presence of naringenin but, in addition, that *Bradyrhizobium* sp. strain ORS285 might be capable of synthesizing even a larger collection of NF.

Despite the low amino acid identities of Nod proteins from *Bradyrhizobium* sp. strain ORS285 and *B. japonicum* strains such as USDA110, the chemical structure of the major LCO synthesized by *Bradyrhizobium* sp. strain ORS285 (V, C18:1, MeFuc) is exactly the same as the major LCO synthesized by *B. japonicum* strains (Carlson et al. 1993; Sanjuan et al. 1992). The important sequence divergence from other Nod proteins as found for *Bradyrhizobium* sp. strain ORS285 seems to be a general characteristic of photosynthetic stem-nodulating *Bradyrhizobium* spp. that are capable of nodulating *Aeschynomene* spp. in a *nod* gene-dependent and *nod* gene-independent manner (Miche et al. 2010). It has been suggested that, for these photosynthetic *Bradyrhizobium* spp., the *nod* gene-dependent association with host plants is an alternative ecological lifestyle and that a relaxation of selection pressure for this lifestyle has resulted in an important sequence divergence of Nod proteins (Miche et al. 2010). In *Bradyrhizobium* sp. strain ORS285, this divergence has led to the synthesis of NF being induced by flavanones, flavones, and flavonoles whereas, in *B. japonicum* strains, these are induced by isoflavones (genistein and daidzein) (Kosslak et al. 1987). Moreover, *B. japonicum* strains contain a two-component flavonoid recognition system (NodV/W) that positively regulates *nod* gene expression and that is absent in

*Bradyrhizobium* sp. strain ORS285. Thus, except for a different induction of *nod* gene expression in photosynthetic *Bradyrhizobium* sp. strain ORS285, the rest of the molecular dialogue between plant and bacteria might be similar to that of nonphotosynthetic *B. japonicum* strains. Indeed, *B. japonicum* USDA110 was able to form nodules on both the root and stem of *A. afraspera* (Figs. 4 and 5). In contrast to root nodules, the number and nitrogenase enzyme activity of stem nodules induced by *B. japonicum* USDA110 was less compared with *Bradyrhizobium* sp. strain ORS285 (Fig. 4B and D). However, this is likely due to the nonphotosynthetic characteristic of *B. japonicum* USDA110 because, for photosynthetic *Bradyrhizobium* spp., it has been shown that the ability to photosynthesize contributes to the efficiency to form nodules on the stem (Giraud et al. 2000) and provides energy for nitrogenase activity inside stem nodules (Evans et al. 1990). Interestingly, *B. japonicum* USDA110 forms nodules at the base of lateral roots and on the stem of *A. afraspera*, whereas it does not form these types of nodules on its original host plants, such as soybean and sirato. This indicates that the ability to stem nodulate and to form nodules at the base of lateral roots is primarily determined by the plant and not by the bacterium.

Taken together, here we have shown that the *nod* gene-dependent molecular dialogue between photosynthetic *Bradyrhizobium* sp. strain ORS285 and tropical aquatic legumes of the *Aeschynomene* genus is similar to the classic rhizobium–legume interaction (i.e., induction by host plant flavonoids and subsequent synthesis of LCO). However, *Bradyrhizobium* sp. strain ORS285 is also able to establish a symbiotic interaction with certain species of the *Aeschynomene* genus in a *nod* gene-independent manner. Therefore, future studies should address whether, during the interaction with these “*nod*-independent” *Aeschynomene* spp., the bacterial *nod* genes are expressed, whether NF-like molecules are synthesized, and whether regulators known to play a role in *nod* gene-dependent symbiosis (NodD) are important. These stud-



**Fig. 5.** Nodules induced on *Aeschynomene afraspera* by *Bradyrhizobium* sp. strain ORS285 and *Bradyrhizobium japonicum* USDA110 have the same morphology. **A**, Stem nodules and **B**, root nodules of *A. afraspera* 17 days postinfection formed by *Bradyrhizobium* sp. strain ORS285 and *B. japonicum* USDA110.

ies will enhance our understanding of the symbiosis between photosynthetic *Bradyrhizobium* and *Aeschynomene* spp. and, in general, may shine a light on how the symbiosis between rhizobia and legumes has evolved.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions.

*Bradyrhizobium* sp. strain ORS285 (Molouba et al. 1999), its derivative *Bradyrhizobium* sp. strain ORS285::*nodB-lacZ-kan* (Giraud et al. 2007), and *Bradyrhizobium japonicum* USDA110 (gift of M. J. Sadowsky, University of Minnesota, Minneapolis, MN, U.S.A.) were grown in modified YM medium (Giraud et al. 2000) or a minimal growth medium (buffered nodulation medium [BNM-B]; pH 6.8) at 37°C. BNM-B is a synthetic plant growth medium (Ehrhardt et al. 1992) that has been supplemented with succinate (10 mM), glutamate (6 mM), and a cocktail of vitamins (riboflavin at 0.2 µg/ml, biotin at 0.12 µg/ml, thiamine-HCl at 0.8 µg/ml, myo-inositol at 0.5 µg/ml, p-aminobenzoic acid at 0.1 µg/ml, nicotinic acid at 0.5 µg/ml, calcium pantothenate at 0.8 µg/ml, and cyanocobalamin at 1 ng/ml) to support growth of *Bradyrhizobium* strains.

### Plant growth.

Seed of *Aeschynomene afraspera* was surface sterilized in concentrated sulfuric acid for 45 min. After five washes with 50 ml of sterile water, the seed was incubated overnight in sterile water at room temperature. After one wash with sterile water, the seed was spread on 1% water agar plates and germinated upside down overnight at 37°C in the dark. The germinated seedlings were transferred to sterile Falcon tubes that had been closed by a sterile aluminum cover and which contained 55 ml of nitrogen-free BNM medium (Ehrhardt et al. 1992). To allow passage of the radicle, a small hole in the aluminum cover was made using a sterile toothpick. To protect the roots from light exposure, the body of the Falcon tube was covered with aluminum foil. The Falcon tubes containing the seedlings were placed in a small greenhouse and grown in a growth chamber with a 16-h light regime and 70% humidity at 28°C. To obtain a high humidity inside the small greenhouse, it contained 1 liter of sterile water. After 1 week of growth, the roots were inoculated with 0.5 ml of *Bradyrhizobium* sp. strain ORS285 or *B. japonicum* USDA110 cells with an optical density at 600 nm ( $OD_{600}$ ) of 1.0 and the growth of the plants was continued for 2 to 3 weeks. Stem nodulation was performed by careful "painting" of the stem using a Buccal swab that had been soaked in a bacterial culture with an  $OD$  of 1.0.

### Nodule number and nitrogenase activity in nodules.

Two weeks after inoculation, the number of nodules on the roots and stems were counted and, after 17 days, the acetylene reduction assay (ARA) was used to measure the nitrogenase enzyme activity. The ARA is a very sensitive assay to quantify the relative nitrogenase enzyme activity in  $N_2$ -fixing samples. For each experimental condition, 10 plants were used and the experiments were repeated at least two times. For this, one plant was placed into a 150-ml glass bottle containing 1 ml of water and closed with a screw cap containing an airtight rubber septum. Air in the glass bottle was replaced with 10%  $CH_4$  in air, following by overnight incubation at 28°C in the dark. After incubation, 1-ml gas samples from the bottles were sampled, and the ethylene concentration was assayed using a gas chromatograph (Agilent 6850 series GC system; Agilent Technologies, Santa Clara, CA, U.S.A.). Operating conditions for the gas chromatograph were as follows: column, Inventory AB001 (50 m by 530 µm by 15 µm); nitrogen as carrier gas at

a flow rate of 12.8 ml/min; temperatures: column, 60°C; injector port, 120°C; flame ionization detector, 250°C.

### *nodB* gene inducing capabilities of flavonoids.

The *Bradyrhizobium* sp. strain ORS285 *nodB-lacZ* reporter strain was used to assess the capacities of flavonoids to induce the expression of the *nodB-lacZ* transcriptional fusion. Purified flavonoids were purchased from Extrasynthese (Genay, France) or Sigma-Aldrich (St. Louis). *Bradyrhizobium* sp. strain ORS285::*nodB-lacZ* was grown in BNM-B medium until an  $OD_{600}$  of approximately 0.4. Subsequently, the bacterial culture was diluted into fresh BNM-B medium ( $OD_{600} = 0.1$ ) and supplemented with flavonoid. To be able to detect the induction of NodB expression by less-efficient flavonoids, a relatively high flavonoid concentration was used in the assay (20 µM final concentration). After 24 h of growth at 28°C,  $\beta$ -galactosidase activities were measured according to the method of Miller (Miller 1972).

### NF extraction and purification.

*Bradyrhizobium* sp. strain ORS285 was grown in modified YM medium until the culture reached an absorbance at 600 nm of 0.05. Then, the cells were induced to produce NF by the addition of naringenin (20 µM final concentration) and grown for 72 h at 37°C. NF were extracted from the filtered culture supernatant by partition with *n*-butanol as described by Roche and associates (1991). Purification of different NF was performed on a high-performance liquid chromatography (HPLC) system (Waters 2690 separation module; Waters, Milford, MA, U.S.A.) equipped with a semipreparative  $C_{18}$  reverse-phase column (5 µm, 46 by 200 mm; Equisorb ODS2 CIL-Cluzeau) using an isocratic gradient of solvent A (water-acetonitrile, 80:20 [vol/vol]) for 10 min, followed by a 20-min linear gradient from solvent A to solvent B (100% acetonitrile), and finally an isocratic gradient of solvent B for 10 min, at a flow rate of 1 ml  $min^{-1}$ . The eluent of the HPLC column was monitored at 206 nm. Acetonitrile present in the collected fractions was removed by two successive freeze-drying steps.

### Mass spectrometry.

HPLC fractions containing putative lipochitooligosaccharides (LCO) were analyzed using an ESI-QqToF Ultima apparatus (Waters) by direct injection. Spectra were recorded in both the positive and the negative mode and peaks detected in the expected range ( $m/z$  1,000 to 1,500 for the simple-charged species or 600 to 700 for the double-charged ones) were submitted to tandem mass spectrometry (MS/MS) to confirm their LCO nature.

Energies were the following: probe, 3 kV; cone, 100 V; Rf, 70 V; collision cell, 15 V for MS and 30 V for MS/MS. collision gas, argon. Direct inlet: solvent, AcCN/ $H_2O$  1:1, 1% acetic acid; rate, 10 µl  $min^{-1}$ . Concentrations were approximately  $10^{-4}$  M.

### Genome sequencing and construction of a genomic draft of *Bradyrhizobium* sp. strain ORS285.

Genomic DNA from *Bradyrhizobium* sp. strain ORS285 was isolated from a 5-day-old culture grown in modified YM medium at 37°C. Genomic DNA was sequenced by the GenomeScope using the pyrosequencing technology developed by Roche (Basel, Switzerland). In total, 943,814 GSflx reads, corresponding to 26-fold coverage, were assembled with Newbler (version 1.1.03.24). Contigs of more than 500 nucleotides (301; total size 7.6 Mb) were integrated into the Microscope Platform and manually annotated. DNA sequences of genes of *Bradyrhizobium* sp. strain ORS285 discussed in this article are deposited in GenBank (Table 3 lists accession numbers).



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