Nodulation gene inducer flavonoids increase the overall production of autoinducers and the expression of *N*-acyl homoserine lactone synthesis genes in rhizobia

Running title: AHL production in rhizobia is induced by flavonoids.

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Nucleotide and amino acid sequence data are available in the EMBL database under accession numbers FJ515278, FJ516401, and FJ516402.

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Abstract

Legume-nodulating rhizobia use *N*-acyl homoserine lactones (AHLs) to regulate several physiological traits related to the symbiotic plant-microbe interaction. In this work we show that *Sinorhizobium fredii* SMH12, *Rhizobium etli* ISP42, and *Rhizobium sllae* IS123, three rhizobial strains with different nodulation ranges, produced a similar pattern of AHL molecules, sharing in all cases the production of *N*-octanoyl homoserine lactone and its 3-oxo and/or 3-hydroxy derivatives. Interestingly, the production of AHLs was enhanced when these three rhizobia were grown in the presence of their respective *nod* gene inducer flavonoid and a new molecule, C14-HSL, was produced by *S. fredii* SMH12 upon genistein induction. In addition, the expression of the AHL synthesis genes *traI* from *S. fredii* SMH12 and *cinI* and *rail* from *R. etli* ISP42 increased when induced with flavonoids as demonstrated by *qRT*-PCR analysis.

Keywords: *Rhizobium*; flavonoid; acyl-homoserine lactone

1. Introduction

Many bacteria regulate their gene expression in response to changes in their population density in a process known as quorum sensing (QS), which is mediated by small diffusible signal molecules called autoinducers (AI) (Fuqua et al., 1994; Miller et al., 2001). Quorum sensing-regulated genes are involved in adaptive changes in the physiology of the bacterial population, adapting and modifying their behaviour when a certain cell density is reached. Furthermore, quorum sensing-mediated gene expression can regulate complex interactions between bacteria of different species and even with some eukaryotic organisms with which they closely interact (Sánchez-Contreras et al., 2007).

In Gram-negative bacteria, the major autoinducers have been identified as *N*-acyl homoserine lactones (AHL), and their production has been detected in plant-associated bacteria belonging to the genera *Agrobacterium*, *Rhizobium*, *Sinorhizobium*, *Pantoea*, *Erwinia*, *Pseudomonas*, and *Xanthomonas* (Cha et al., 1998), among others. Different bacterial species can produce the same AHL or AHLs with similar structures and properties, suggesting that there is a crosstalk among populations and that quorum sensing via AHLs is more common among plant-associated bacteria than among the general population of soil bacteria (D'Angelo-Picard et al., 2005; Elasri et al., 2001).

Factors regulated by QS in rhizobia include plasmid transfer, production of surface polysaccharides, growth inhibition, stationary-phase adaptation, nodulation efficiency, symbiosome development, and nitrogen fixation, all of which are important for the establishment of a successful symbiosis. Several AHL synthesis and regulator genes have been described and vary from four in *Rhizobium leguminosarum* bv. *viciae* (*rhlR/rhlI*, *raiR/raiI*, *traR/traI*, and *cinR/cinI*), two in *R. etli* CNPAF512 (*cinR/cinI* and *raiR/raiI*) and possibly in *Sinorhizobium meliloti* Rm1021 (*sinR/sinI* and *mel*), to one (*traR/traI*) in *R. etli* CFN42 and *Rhizobium* sp. NGR234. *N*-octanoyl homoserine lactone (C8-HSL) and its 3-oxo

1 and 3-hydroxyl derivatives (3-oxo-C8-HSL and 3-OH-C8-HSL, respectively) have been
2 found in most of these strains. In addition, other AHLs with longer acyl chains (up to eighteen
3 carbon atoms in the case of *S. meliloti*) are also produced. In the case of *R. leguminosarum*
4 bv. *viciae* this large AHL is the 3-OH-C14, that acts as a bacteriocin (González and Marketon,
5 2003). *Agrobacterium tumefaciens*, which is closely related to rhizobia, also possesses
6 *traR/traI* genes and produces 3-oxo-C8-HSL (Sánchez-Contreras et al., 2007).

7 Rhizobia are characterized by their symbiosis with legume plants, which involves an
8 exchange of signal molecules from both partners. Flavonoids play a key role in the
9 organogenesis of nitrogen-fixing nodules (Cooper, 2007; Perret et al., 2000; Shaw et al.,
10 2006). Thus, flavonoids interact with the rhizobial protein NodD, which in turn activates the
11 transcription of the nodulation genes (*nod* genes) implicated in the biosynthesis of
12 lipochitooligosaccharidic Nod factors that elicit the deformation of plant root hairs and
13 support rhizobial entry via infection threads. Moreover, in some rhizobial strains, flavonoids
14 also induce the biosynthesis of proteins (Nops) secreted by the type III secretion system or
15 changes in surface polysaccharides (Le Queré et al., 2006; López-Baena et al., 2008).

16 In this work we show that three rhizobial strains with different nodulation ranges produce
17 a similar pattern of AHL molecules, mainly those with C8 chains, which are the most
18 common AHLs among rhizobia. Moreover, we show for the first time that the overall
19 production of QS molecules by these strains was enhanced when they were cultured in the
20 presence of flavonoids able to induce the expression of the *nod* genes. Interestingly, this effect
21 was not observed in the presence of non inducing flavonoids. In addition, the expression of
22 the AHL synthesis genes *traI* from *S. fredii* SMH12 and *rail* and *cinI* from *R. etli* ISP42
23 increased in the presence of inducing flavonoids.

2. Materials and methods

2.1 Strains and media

Sinorhizobium fredii SMH12 (Rodríguez-Navarro et al., 2003), *Rhizobium etli* ISP42 (Rodríguez-Navarro et al., 2000) and *R. sullivanii* IS123 (Espuny et al., 1987; Squartini et al., 2002), were grown at 28 °C in tryptone-yeast extract (TY) medium (Beringer, 1974) and yeast extract mannitol (YM) medium (Vincent, 1970) with a lower mannitol concentration (3 g l⁻¹).

S. fredii SMH12 (pMP240) (Dardanelli et al., 2010), *R. etli* ISP42 (pMP240) (Dardanelli et al., 2008), and *R. sullivanii* IS123 (pMP240) (constructed by conjugation in this work) were grown at 28 °C in YM medium supplemented with 1, 5, and 10 µg ml⁻¹ of tetracycline, respectively. Plasmid pMP240 (tetracycline resistance) carries the *nodA* gene promoter fused to *lacZ* (de Maagd et al., 1988). *Chromobacterium violaceum* CV026 (Latifi et al., 1995; Throup et al., 1995) was grown at 28 °C in LB medium (Sambrook et al., 1989) supplemented with kanamycin (30 µg ml⁻¹) and *A. tumefaciens* NT1 (pZLR4) was grown at 28 °C (Cha et al., 1998) in YM with carbenicillin (100 µg ml⁻¹) and gentamicin (30 µg ml⁻¹).

The flavones apigenin, quercetin, morin, and 7, 4'-dihydroxyflavone; the flavanone naringenin; the isoflavones daidzein and genistein; and other flavonoids as isoliquiritigenin and umbelliferone were dissolved in ethanol and used at 1 µg ml⁻¹. AHL standards were dissolved in methanol and used at different concentrations. Flavonoids and AHLs were purchased from Fluka (Sigma-Aldrich, USA).

To analyse the influence of flavonoids on bacterial growth, strains were grown in 5 ml of YM medium to early stationary phase and then diluted to OD₆₀₀ values near 0.03 in fresh YM medium with or without flavonoids. Growth was monitored by measuring the OD₆₀₀ for at least 29 h. Each experiment was performed at least twice in triplicate.

2.2. Well diffusion assay and thin layer chromatography analysis for detecting autoinducer production

A. tumefaciens NT1 (pZLR4) was used for the detection of AHLs in well diffusion assays in Petri dishes as described by McClean et al. (1997) and Cha et al. (1998).

Supernatants of the rhizobial strains grown for 6 days in 15 ml of YM medium were extracted with dichloromethane, evaporated to dryness and analyzed by thin layer chromatography (HPTLC plates RP-18 F_{254s} 1.13724 and 1.05559, Merck, Germany) using methanol:water (60:40 v/v) as eluent, dried and developed with *A. tumefaciens* NT1 (pZLR4) (Shaw et al., 1997).

2.3. HPLC analysis

Rhizobia were grown in 500 ml of YM medium, supplemented with flavonoids when necessary, for 6 days at 28 °C with shaking. Cultures were centrifuged and supernatants were extracted with dichloromethane and the organic layer was evaporated. The extracts were dissolved in 1 ml of methanol:water (1:1 v/v) containing 0.1 % (v/v) formic acid, microfiltered (0.2 µm), and 20 µl injected onto an HPLC system equipped with a Tracer Hypersyl ODS column (250 × 4.6 mm, 5 µm particle size) (Teknokroma, Spain). Elutions were carried out at room temperature with a flow rate of 400 µl min⁻¹ using a gradient of water and methanol, both acidified with 0.1% formic acid (Morin et al., 2003).

HPLC studies were performed on a Perkin Elmer Series 200 HPLC system (Waltham, USA) coupled to a mass spectrometer for MS/MS study, or a Waters Alliance 2695 HPLC system (Waters, USA) coupled to a Gilson FC203B fraction collector (Gilson, USA) for the β-galactosidase assays with *A. tumefaciens* NT1 (pZRL4) in microplates (see below). In this case, 1 ml fractions were collected.

2.4. Mass spectrometry analysis

Methods using liquid chromatography and tandem mass spectrometry have been applied to profile the bacterial quorum sensing molecules from different bacteria (Cataldi et al., 2009; Gould et al., 2006; Kumari et al., 2008; Ortori et al., 2007). Multiple-Reaction ion Monitoring (MRM) is a tandem MS/MS method in which user-selected specific ions are transmitted through the first analyzer (a quadrupole, Q1) and user-selected specific fragments arising from collision induced decomposition (CID) in Q2 are measured by the second analyzer (Q3). This technique has shown to be very suitable for the identification and study of components in very complex mixtures. Since both precursor (usually pseudomolecular ion, $[M+H]^+$) and product ions must be indicated before carrying out the analysis, the compound to identify must be known and has been well-characterized previously before this type of experiment is undertaken. The AHL family shows pseudomolecular peaks at $[M+H]^+$ when electrospray is performed in positive mode (precursor ion), and the CID fragmentation generates a common product ion at m/z 102, which formally corresponds to this ring plus the nitrogen atom as NH_3^+ , together with the product ions corresponding to $[M+H-101]^+$.

A second IDA method, called “ion precursor”, was also used in our analysis. This method was set to register those compounds that generate fragments at m/z 102 in Q3. This technique results complementary to MRM, as it can detect those compounds which are not included in the MRM Q1/Q3 list, such as adducts of AHL with ammonium or solvent molecules (Makemson et al., 2006), although the solvents were acidified and the declustering potential was optimized to minimize the formation of this kind of adducts (Ortori et al., 2007).

Besides IDA methods, Enhanced Product Ion (EPI) spectra were also recorded of the two more intense peaks (above 4000 counts per second) to verify the structure of the detected AHL by comparison with mass spectra of standards.

All MS experiments were conducted on a 2000 QTRAP hybrid triple-quadrupole-linear trap mass spectrometer (Applied Biosystem, USA) equipped with a Turbo Ion source used in positive ion electrospray mode. Mass spectrometric conditions were optimized by infusing solutions of standards dissolved in methanol ($100 \mu\text{g ml}^{-1}$) at a flow of $10\text{-}100 \mu\text{l min}^{-1}$: C4-HSL, C6-HSL, C8-HSL, C10-HSL, C12-HSL, C14-HSL, 3-oxo-C6-HSL, and 3-OH-C6-HSL. The probe capillary voltage was optimized at 5500 V. Desolvation temperature was set to 50°C . Pressures of curtain, nebulising and turbo spray gases were set to 35, 20 and 0 (arbitrary units), respectively. Nitrogen was used for CID. Ions were scanned from m/z 150 to m/z 500 at a scan rate of 4000 Th s^{-1} .

2.5. *β -galactosidase activity determination*

β -galactosidase assays were conducted in order to: **i**) know the capacity of flavonoids to induce *nod* genes in the three rhizobial strains, **ii**) compare the AI production by the three rhizobial strains in the presence and absence of flavonoids, and **iii**) determine how many different AI molecules were produced by the rhizobial strains.

To study the inducing capacity of different flavonoids in each strain, bacteria carrying plasmid pMP240 were grown in liquid YM medium, supplemented with the flavonoids mentioned above, and the β -galactosidase activity was determined (López-Baena et al., 2008). Briefly, $200 \mu\text{l}$ of bacterial cultures ($\text{OD}_{660 \text{ nm}} \approx 0.2$) were mixed with $800 \mu\text{l}$ of buffer Z, $20 \mu\text{l}$ of chloroform and $10 \mu\text{l}$ of SDS (0.1%), and the mixture was vortexed vigorously for ten seconds. $200 \mu\text{l}$ of ONPG (4 mg/ml in buffer Z) were added and vortexed again. The reaction was stopped by adding $500 \mu\text{l}$ of Na_2CO_3 1M. Finally, the OD at 420 nm was measured. Miller units were calculated as $\text{OD}_{420\text{nm}} \times 10^3 / \text{OD}_{660} \times \text{time of reaction in minutes} \times \text{volume of culture in millilitres}$. At least three independent experiments were performed, in triplicate, for each strain.

To determinate the overall AI production of the rhizobial strains using *A. tumefaciens* NT1 (pZLR4) as reporter strain, a modified β -galactosidase activity assay was used. Supernatants from bacterial cultures grown for 6 days in YM medium, supplemented with the appropriate flavonoid when required, were obtained and sterilized by microfiltration. Different volumes of supernatants were mixed with YM to obtain a final volume of 2.5 ml and reach supernatant concentrations between 1% (v/v) and 20% (v/v), and then tested to determine whether the signal was saturated. The mixtures with an optimal concentration of supernatant were inoculated with approximately 10^7 cells ml⁻¹ of the reporter strain, incubated with shaking for 12 h and assayed for β -galactosidase activity. The largest differences were obtained with supernatant concentrations of 5% (v/v) for ISP42 and IS123, and 1% (v/v) in the case of SMH12. As controls, 125 μ l of sterile distilled water or 125 μ l of *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) 5.5 μ M, were used. The experiment was repeated independently 5 times with 3 replicates in each condition.

β -galactosidase assay was also used to quantify AI production using HPLC fractions in microplates. Thus, 1 ml fractions from the HPLC separation were evaporated under air flow to remove methanol, and the volume readjusted to 1 ml with water. Experiments were carried out in 96-well U-bottom plastic microplates (Greiner Bio-One, Germany). 100 μ l aliquots of the HPLC fractions were added to each microtiter well containing 100 μ l of a log-culture of *A. tumefaciens* NT1 (pZLR4) (OD_{660} = 0.4-0.6). Wells containing the bioreporter culture with distilled water or standard AHLs at different concentrations were prepared as controls. After 4 days of incubation at 28 °C, the absorbance of each well was measured at 660 nm with an automatic OASYS UVM 340 well plate reader (Anthos Labtec Instruments, United Kingdom). Forty microlitres of culture of each well were placed in a new microplate and β -galactosidase activity was carried out adapting the Miller's protocol to the volume of the wells, and using toluene instead of chloroform. The experiments were repeated twice.

2.6. Molecular biology techniques

Genomic DNA extraction and agarose gel electrophoresis were performed as described by Sambrook et al. (1989). PCR amplifications were carried out as described elsewhere (Vinardell et al., 2004) using primers designed from conserved regions of published gene sequences of related microorganisms. Primers used were: *traI*-F (5'-CCAGAAGATTGGGATTGACA), *traI*-R (5'-TGTCCGCCTATCGGAAGCTCA), *raiI*-F (5'-CCCCGGGATATCGCCTCTCGA), *raiI*-R (5'-GCAACCATGGCGCGATGTGA), *cinI*-F (5'-CCTATCTCGTCTGGTGCA), *cinI*-R (5'-GCTGCCATCTCCAGGAA), *sinI*-F (5'-CTGCGTAAGCGCGTGTCCA), *sinI*-R (5'-GCGTGCCGTTTCAAGCGACA), *mellI*-F (5'-GCAGATGATCGTGCTGA), and *mellI*-R (5'-CCAGGGAAACGGAGGA). The PCR products were purified from the agarose gel, cloned into the pMBL1-T vector (Dominion MBL, Spain) and sequenced to confirm that they were AI synthesis related genes.

For bacterial RNA extraction, *R. etli* ISP42 and *S. fredii* SMH12 were incubated with shaking at 28 °C in YM medium supplemented with daidzein (3.9 µM), apigenin (3.7 µM) or umbelliferone (6.2 µM) when necessary. Isolation and quantification of RNA and cDNA synthesis were carried out as described by López-Baena et al. (2009).

To quantify the expression of the *R. etli* ISP42 *raiI* and *cinI* genes and the *S. fredii* SMH12 *traI* gene using quantitative RT-PCR, primers *rt-raiI*-F (5'-CCGATACGACGAGGCTGA), *rt-raiI*-R (5'-GCATGCGGATGAAGGCAA), *rt-cinI*-F (5'-GTCTGGTGCAACGACA), *rt-cinI*-R, (5'-GCATCGATGTTGGGGAA), *rt-traiI*-F (5'-CCCACCTACATCCTAGCCGTA), *rt-traiI*-R (5'-CAAGGAAGGTGTCCACGCAA), amplifying internal fragments of 198 bp (*raiI*), 197 bp (*cinI*), and 158 bp (*traI*), were used. Expression was calculated relative to bacteria not induced with flavonoids. The *R. etli* ISP42 and *S. fredii* SMH12 RNA *16S* genes were used as internal controls to normalize gene expression. RNA *16S* primers were designed from a conserved

1 sequence (nucleotides from 725 to 874 of the RNA *16S* gene) and the same pair of primers
2 was used to quantify the expression of the RNA *16S* gene in both bacteria (Crespo-Rivas et
3 al., 2009).

4 The expression data shown are the mean ($\pm$ standard deviation of the mean) for three
5 biological replicates. The fold change in the target gene, normalized to RNA *16S*, and the
6 relative to the gene expression in the control sample was calculated. PCR was conducted on
7 the iCycler IQTM (Bio-Rad Laboratories, USA) with the following conditions: 95 °C, 10 min;
8 95 °C, 30 s; 52 °C, 30 s; 72 °C 20 s; Forty cycles, followed by the melting curve profile from
9 60 to 95 °C to verify the specificity of the reaction. The threshold cycles (Ct) were determined
10 with the iCycler software and the individual values for each sample were generated by
11 averaging three technical replicates that varied less than 0.5 cycles.

3. Results

3.1. Extraction and analysis of autoinducer molecules produced by the rhizobial strains

Spent supernatants (bacterial cultures grown for 3 to 9 days) from *S. fredii* SMH12 (hereafter SMH12), *R. etli* ISP42 (hereafter ISP42), and *R. sultae* IS123 (hereafter IS123) were first assayed for AI production using a well diffusion assay. Results showed that these rhizobial strains produced higher quantities of AI when cultured in a mineral medium (YM) rather than in a rich medium (TY), and that SMH12 was the best producer (Fig. S1). The maximum AI production was observed after 6 days of growth (Fig. S1). When 6-day old supernatants were analyzed by thin layer chromatography (TLC) several spots appeared, usually overlapped. These results indicated that SMH12 produced at least five different autoinducers, whereas ISP42 and IS123 produced at least three (Fig. S2).

In order to unequivocally identify the molecules produced by these rhizobia, mass spectrometry analyses were carried out. Thus, C8-HSL and 3-oxo-C8-HSL were detected in the supernatant from SMH12; C8-HSL, 3-OH-C8-HSL, and *N*-(3-oxo-tetradecanoyl)-L-homoserine lactone (3-oxo-C14-HSL) were found in the supernatant of ISP42; and, in the case of IS123, C8-HSL, 3-OH-C8-HSL, and 3-oxo-C8-HSL were detected.

To elucidate whether there were other autoinducer molecules or analogues that could not be detected by our mass spectrometry methods, supernatant extracts were fractionated by HPLC and fractions (2.5 min per tube, 1 ml) were independently analyzed by their capacities to induce β -galactosidase activity using *A. tumefaciens* NT1 (pZLR4) as bioreporter in microtiter plates (Fig. 1). Peaks with β -galactosidase activity were detected in fractions 7-9, 11, 15-17 and 25 from SMH12; in fractions 7, 12, 16 and 25 from ISP42, and in fractions 5, 8-10, 12-13, 16, and 25 from IS123. Taking into consideration the retention times of the standards used in HPLC-MS/MS and doing the equivalence with the HPLC fractionation employed for the microtiter assays, peaks around fractions 7-9 correspond to 3-oxo-C8-HSL

and 3-OH-C8-HSL, those around 11-12 correspond to C8-HSL, and peaks around 15-17 correspond to C14-HSL and/or its OH- or oxo- derivatives. Most of them were found in the three strains assayed. Some peaks, especially around fraction 25, could not be detected as an AHL molecule in our spectrometric analysis.

3.2. Influence of flavonoids on the production of autoinducers and on the expression of AHL synthesis genes

Flavonoids that induced the expression of the *nod* genes in the rhizobial strains studied were determined prior to the studies of the influence of flavonoids on the production of AIs. Thus, a set of flavonoids was tested for their capacity to induce a *nodA* promoter of *R. leguminosarum* bv. *viciae* fused to the *lacZ* gene harboured in plasmid pMP240 (De Maagd et al., 1988) that was previously transferred by conjugation to each strain assayed. Results showed that the best inducing flavonoids were apigenin (7.9-fold induction with respect to non induced control cultures) and genistein (7.2-fold) for SMH12, apigenin and 4', 7-dihydroxyflavone, (more than 4-fold) for ISP42 and daidzein (more than 7-fold) and genistein (6-fold) for IS123 (Table 1). Therefore, for further experiments, genistein, apigenin, and daidzein were selected as inducing flavonoids for SMH12, ISP42, and IS123, respectively. On the other hand, the worst inducers were umbelliferone for SMH12 and ISP42, and apigenin for IS123. These flavonoids were selected as non inducing flavonoids (Table 1).

In order to elucidate whether any of the above studied *nod* gene inducer flavonoids produced qualitative changes in the production of AIs, supernatants of the rhizobial strains grown in the presence or absence of an inducing flavonoid were extracted and analyzed by TLC and mass spectrometry. In parallel, HPLC fractions were tested for the presence of AIs through bioassays in microtiter plates. No changes in the number or type of the AI molecules were detected by TLC in the presence of inducing flavonoids (Fig. S3). In the case of HPLC-

MS/MS analyses, a new molecule, C14-HSL, was detected in SMH12 when induced with genistein. This molecule could not be detected when this strain was cultured in the absence of this flavonoid. Microtiter bioassays using *A. tumefaciens* NT1 (pZLR4) of HPLC-fractionated supernatant extracts showed also higher levels of induction. Furthermore, an increase in β -galactosidase activity corresponding to C14-HSL appears in the case of SMH12 induced with genistein. As shown in Fig. 1(A), there was a general increase of the activity of the reporter strain with fractions 5, 7-9, 12, 14-15, 17, and 22-25 of SMH12 induced with genistein. In the case of ISP42 grown in the presence of apigenin, an increase in the activity on the reporter strain were detected in fractions 7-8, 11-12 and 14-16 and 24 (Fig. 1B). Finally, in the case of IS123 grown in the presence of daidzein, increased activity were detected in fractions 8-9, 12, 16, and 24-25 when compared with the activity of the fractions obtained in the absence of flavonoids (Fig. 1C).

In addition, the influence of flavonoids on the overall production of autoinducers was analyzed by adding the supernatants obtained in the presence/absence of different flavonoids in a final concentration of 5% (ISP42 and IS123) and 1% (SMH12) (v/v) to a fresh culture of *A. tumefaciens* NT1 (pZLR4) and studying their global capacities to induce β -galactosidase activity. As controls, water and different flavonoids were used as inducers of *A. tumefaciens* NT1 (pZLR4). The supernatants obtained without flavonoids induced the β -galactosidase activity of *A. tumefaciens* NT1 (pZLR4) at levels similar to those obtained in the presence of 3-oxo-C6-HSL (Table 2). Moreover, the supernatants of SMH12, IS123, and ISP42 obtained in the presence of genistein, daidzein, and apigenin, respectively, significantly increased ($\alpha = 10\%$) the β -galactosidase activity of *A. tumefaciens* NT1 (pZLR4) with respect to the control without flavonoids (Table 2). In all cases, the percentage of β -galactosidase activity induction decreased or did not change when the flavonoid used, umbelliferone for SMH12 (+12 %) and ISP42 (7 %) or apigenin for IS123 (-8 %), was not a *nod* gene inducer. In the case of IS123

1 induced with apigenin, this decrease was statistically significant at a level $\alpha = 10\%$. The
 2 values of activity of *A. tumefaciens* NT1 (pLZLR4) grown in the presence of these flavonoids
 3 were similar to those obtained in the presence of water.

4 It could be possible that the qualitative and quantitative changes in AHLs production
 5 observed could be due to differences in growth in the presence of flavonoids. Therefore, it
 6 was necessary to determine the effect of flavonoids on bacterial growth. Results obtained
 7 showed that the presence of flavonoids did not affect bacterial growth and even a slight
 8 increase could be observed in the presence of flavonoids (Fig. S4).

9 To find out whether gene transcription was correlated with the increase in AHL
 10 production upon flavonoids induction, quantitative real time RT-PCR assays carried out.
 11 Firstly, rhizobial AHL-synthesis genes were identified and cloned using primers designed
 12 from published sequences of genes from closely related rhizobial and *Agrobacterium* species.
 13 The selected genes were *traI* from *A. tumefaciens* C58 (accession number AF057718), *rail*
 14 and *cinI* (NC_0077661) from *R. etli* CFN42, and *sinI* and *mell* (AL591688) from *S. meliloti*
 15 Rm1021. PCR amplification of the SMH12, ISP42 and IS123 genomic DNAs using these
 16 primers and the further cloning and sequencing of the fragment, resulted in the identification
 17 of the *traI* gene in SMH12 (FJ515278), and the *rail* (FJ516401) and *cinI* (FJ516402) genes in
 18 ISP42. However, none of these genes could be amplified using these primers in the case of
 19 IS123.

20 TraI of SMH12 (ACM41876) was 98% and 74% identical to the deduced proteins of
 21 *Rhizobium* sp. NGR234 (AAB92427) and *A. tumefaciens* C58 (BAB47268), respectively.
 22 Rail (ACL67951) and CinI of ISP42 were 90% and 98% identical, respectively, to the
 23 corresponding proteins of *R. etli* CFN42 (ABC94330 and ABC91684).

24 qRT-PCR assays were used to quantify the expression of these genes in SMH12 and
 25 ISP42. Results showed that the expression of *traI*, *cinI* and *rail* significantly increased (7-

1 fold, 9-fold, and 6-fold, respectively) in the presence of their respective inducing flavonoid
2 (daidzein for SMH12 and apigenin in the case of ISP42) when compared to the control
3 without flavonoids or in the presence of the non inducing flavonoid umbelliferone (Fig. 2).

4

4. Discussion

There is a wide variety of AIs produced by rhizobia and even strains of the same species produce a different range of molecules (Sánchez-Contreras et al., 2007). In order to elucidate whether in rhizobia the production of autoinducers is modulated by plant flavonoids, we selected three strains that possess different host specificities: *S. fredii* SMH12 is a wide host-range strain that nodulates soybean and dozens of other legumes, *R. etli* ISP42 nodulates mainly beans, and *R. sulae* IS123 nodulates *Hedysarum coronarium* as almost its unique host plant.

Firstly, we analysed AIs production using diffusion bioassays to find out which strain produced larger quantities of AIs and when they were mostly produced and mass spectrometry analyses were then carried out to identify the AIs synthesized by these strains. These studies showed that these strains produced different AHLs, sharing in all cases the production of C8-HSL. However, the number of peaks showing increased β -galactosidase expression identified by microtiter assays using *A. tumefaciens* NT1 (pZLR4) as bioreporter (Fig. 1) indicated that possibly these strains produce more QS molecules when induced with flavonoids that can not be detected with our analytical spectrometric studies, as the used methodology is exclusively focused on AHLs. These molecules do not have an AHL nature or are produced at a very low concentration which is enough for its detection by the bacterial bioreporter. It has been reported that *A. tumefaciens* NT1 (pZLR4) is able to detect molecules other than AHLs (Holden et al., 1999), and the sensibility of this bioreporter strain depends on the structure and concentration of these molecules (Shaw et al., 1997; Steindler and Venturi, 2007).

To determine the role of flavonoids on AIs production, best inducer flavonoids for each strain were determined as well as flavonoids unable to induce *nod* gene expression by using plasmid pMP240, which carries the *nodA* gene promoter fused to *lacZ*. Most of the flavonoids

1 used could induce *nod* gene expression in SMH12. ISP42 was also responsive to most of the
2 flavonoids tested. However, in IS123 *nodA* was only induced by daidzein and genistein
3 (Table 1). These results could be explained by differences in their nodulation host-range.
4 Thus, it has been reported that the NodD protein from broad host-range rhizobia is usually
5 responsive to a wider range of flavonoids (Gyorgypal et al., 1991; Gyorgypal and Kondorosi,
6 1991).

7 Once selected the flavonoids for each strain (Table 1), we studied their effect on the
8 overall production of AIs. Thus, rhizobial supernatants from spent cultures obtained in the
9 presence of flavonoids were evaluated using diverse techniques. In one hand, a significant
10 increase in β -galactosidase activity using our adapted assay with *A. tumefaciens* NT1
11 (pZLR4) was observed when the rhizobial strains were cultured in the presence of a *nod* gene
12 inducing flavonoid with respect to those cultured without flavonoids or in the presence of a
13 non inducing flavonoid (Table 2). Using mass spectrometry, a new molecule that
14 corresponded to C14-HSL was observed in the supernatants of SMH12 induced with
15 genistein, but no other changes were observed in the cases of ISP42 and IS123. It was not
16 possible to determine changes in AHL concentration upon induction with flavonoids using
17 TLC (Fig. S3) or the mass spectrometry approach used in this work because they are not
18 quantitative, e. g. 5-fold increases in AHL concentrations are needed to observe changes in
19 the diameter of the spots in a TLC so this approach is not sensitive enough to study slight
20 differences in AHL production (Fig. S3). However, increases in the level of induction of the
21 fractions obtained by HPLC from cultures grown in the presence of inducing flavonoids were
22 detected when they were analyzed in microtiter bioassays with *A. tumefaciens* NT1 (pZLR4)
23 as reporter strain (Fig. 1). These higher levels of induction can not be attributed to the direct
24 influence of the flavonoids on *A. tumefaciens* NT1 (pZLR4), but to the influence of
25 flavonoids on the rhizobial strains. In fact, when the flavonoid used was not a *nod* gene

inducer, the β -galactosidase activity did not change or decreased (Table 2). Moreover, when flavonoids were used to directly induce *A. tumefaciens* NT1 (pZLR4), β -galactosidase activities observed were similar to those obtained with the control with water despite they were used at a concentration ($0.2 \mu\text{g ml}^{-1}$ of culture) higher than that used on the rhizobial cultures ($0.05 \mu\text{g ml}^{-1}$ of culture) (Table 2). Bacterial growth was not affected either by the presence of flavonoids in the culture medium (Fig. S4). Finally, quantitative RT-PCR analyses showed that only *nod* gene inducer flavonoids increased (6- to 9-fold) the expression of the three *luxI*-homologous genes detected (Fig. 2).

All these results indicate that flavonoids have a role in the production of AIs by rhizobia, and that this effect is flavonoid-specific. Therefore, leguminous plants could modulate the production of some of the rhizobial QS signals that regulate factors directly involved in the establishment of the symbiosis (Gao and Teplitski, 2008; Mathesius et al., 2003). In fact, the tested inducing flavonoids are produced by the respective host plants for each bacterium. In common bean, flavonoids quercetin, kaempferol, genistein, and naringenin were detected (Hungria et al., 1991a; 1991b), and soybean roots secrete apigenin, coumestrol, daidzein, genistein, isoliquiritigenin, naringenin, quercetin, 7, 4'-dihydroxyflavone, and umbelliferone (Dardanelli et al., 2010; Pueppke et al., 1998).

In rhizobia, QS-regulated genes identified are involved in the interactions with their host legumes (González and Marketon, 2003). QS regulation controls the expression of *nod* and rhizosphere-expressed (*rhi*) genes that influence nodulation in *B. japonicum* and *R. leguminosarum* bv. *viciae*, respectively (Cubo et al., 1992; Loh et al., 2002). Likewise, plants seem to respond to the AHLs. In this sense, von Rad et al. (2008) have shown that the contact of *Arabidopsis thaliana* roots with *N*-hexanoyl-homoserine lactone (C6-HSL) resulted in distinct transcriptional changes in roots and shoots, respectively. Accordingly, plants could regulate the expression of the molecules implied in QS communication through their root

exudates, i.e. flavonoids. There are numerous evidences that suggest that flavonoids induce the expression of many rhizobial genes other than *nod* genes; e.g. exposure of free-living cells of *Rhizobium sp.* NGR234 to daidzein enhanced the transcription of 147 open reading frames which were previously silent (Perret et al., 1999). Flavonoid-dependent induction extends to rhizobial genes that do not contain *nod* box motifs in their promoter regions. Such is the case of genes involved in type III-secretion and encoders of a type I and possibly also type IV secretion systems (Deakin and Broughton, 2009). Many other genes and proteins in rhizobia are flavonoid-inducible but their functions are unknown (Cooper, 2007). Proteome analyses of rhizobia have also detected new proteins whose expression requires the presence of flavonoids in the culture medium (Chen et al., 2000; Guerreiro et al., 1997; 1999). It is likely that plants control the activity of genes implied in the synthesis of quorum QS signals and, consequently, interfere with activities of the bacteria involved in the establishment of a successful symbiosis.

Acknowledgements

This work was supported by projects AGL2006-13758-C05 and AGL2009-13487-C04 from the Spanish Ministerio de Ciencia y Tecnología (MCyT). F. Perez-Montañó is recipient of a FPU fellowship from the MCyT.

References

Beringer, J.E., 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84, 188-198.

Cataldi, T.R.I., Bianco, G., Abate, S., Mattia, D., 2009. Analysis of *S*-adenosylmethionine and related sulfur metabolites in bacterial isolates of *Pseudomonas aeruginosa* (BAA-47) by liquid chromatography/electrospray ionization coupled to a hybrid linear quadrupole ion trap and Fourier transform ion cyclotron resonance mass spectrometry. Rapid Commun. Mass Sp. 23, 3465-3477.

Cha, C., Gao, P., Chen, Y.C., Shaw, P.D., Farrand, S.K., 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol. Plant Microbe Interact. 11, 1119-1129.

Chen, H., Higgins, J., Oresnik, I.J., Hynes, M.F., Natera, S., Djordjevic, M.A., Weinman, J.J., Rolfe, B.G., 2000. Proteome analysis demonstrates complex replicon and luteolin interactions in pSymA-cured derivatives of *Sinorhizobium meliloti* strain 2011. Electrophoresis 21, 3833-3842.

Cooper, J.E., 2007. Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. J. Appl. Microbiol. 103, 1355-1365.

Crespo-Rivas, J.C., Margaret, I., Hidalgo, A., Buendía-Clavería, A., Ollero, F.J., López-Baena, F.J., Murdoch, P.S., Rodríguez-Carvajal, M.A., Soria-Diaz, M.E., Reguera, M., Lloret, J., Sumpton, D.P., Mosely, J.A., Thomas-Oates, J., van Brussel, A.A.N., Gil-Serrano, A.,

1 Vinardell, J.M., Ruiz-Sainz, J.E., 2009. *Sinorhizobium fredii* HH103 *cgs* mutants are unable
2 to nodulate determinate- and indeterminate-nodule forming legumes and overproduce an
3 altered EPS. *Mol. Plant Microbe Interact.* 22, 575-588.

4
5 Cubo, M.T., Economou, A., Murphy, G., Johnston, A.W., Downie, J.A., 1992. Molecular
6 characterization and regulation of the rhizosphere-expressed genes *rhiABCR* that can
7 influence nodulation by *Rhizobium leguminosarum* biovar *viciae*. *J. Bacteriol.* 174, 4026-
8 4035.

9
10 D'Angelo-Picard, C., Faure, D., Penot, I., Dessaux, Y., 2005. Diversity of N-acyl homoserine
11 lactone-producing and -degrading bacteria in soil and tobacco rhizosphere. *Environ.*
12 *Microbiol.* 7, 1796-1808.

13
14 Dardanelli, M.S., Fernández de Córdoba, F.J., Espuny, M.R., Rodríguez-Carvajal, M.A.,
15 Soria-Díaz, M.E., Gil-Serrano, A.M., Okon, Y., Megías, M., 2008. Effect of *Azospirillum*
16 *brasilense* coinoculated with *Rhizobium* on *Phaseolus vulgaris* flavonoids and Nod factor
17 production under salt stress. *Soil Biol. Biochem.* 40, 2713-2721.

18
19 Dardanelli, M.S., Manyani, H., González-Barroso, S., Rodríguez-Carvajal, M.A., Gil-Serrano,
20 A., Espuny, M.R., López-Baena, F.J., Bellogín, R.A., Megías, M., Ollero, F.J., 2010. Effect of
21 the presence of the plant growth promoting rhizobacterium (PGPR) *Chryseobacterium*
22 *balustinum* Aur9 and salt stress in the pattern of flavonoids exuded by soybean roots. *Plant*
23 *Soil* 328, 483-493.

1 Deakin W.J., Broughton, W.J., 2009. Symbiotic use of pathogenic strategies: rhizobial protein
2 secretion systems. Nat. Rev. Microbiol. 7, 312-320.

3
4 De Maagd, R.A., Wijffelman, C.A., Pees, E., Lugtenberg, B.J.J., 1988. Detection and
5 subcellular localization of two Sym plasmid-dependent proteins of *Rhizobium leguminosarum*
6 biovar *viciae*. J. Bacteriol. 170, 4424-4427.

7
8 Elasri, M., Delorme, S., Lemanceau, P., Stewart, G., Laue, B., Glickmann, E., Oger, P.M.,
9 Dessaux, Y., 2001. Acyl-homoserine lactone production is more common among plant-
10 associated *Pseudomonas* spp. than among soilborne *Pseudomonas* spp. Appl. Environ.
11 Microbiol. 67, 1198-1209.

12
13 Espuny, M.R., Ollero, F.J., Bellogín, R.A., Ruiz-Sainz, J.E., Perez-Silva, J., 1987. Transfer of
14 the *Rhizobium leguminosarum* biovar *trifolii* symbiotic plasmid pRtr5a to a strain of
15 *Rhizobium* sp. that nodulates on *Hedysarum coronarium*. J. Appl. Microbiol. 63, 13-20.

16
17 Fuqua, W.C., Winans, S.C., Greenberg, E.P., 1994. Quorum sensing in bacteria: the LuxR-
18 LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. 176, 269-275.

19
20 Gao, M., Teplitski, M., 2008. RIVET-a tool for in vivo analysis of symbiotically relevant
21 gene expression in *Sinorhizobium meliloti*. Mol. Plant Microbe Interact. 21, 162-70.

22
23 González, J.E., Marketon, M.M., 2003. Quorum sensing in nitrogen-fixing rhizobia.
24 Microbiol. Mol. Biol. Rev. 67, 574-592.

25

- 1 Gould, T.A., Herman, J., Krank, J., Murphy, R.C., Churchill, M.E., 2006. Specificity of acyl-
2 homoserine lactone synthases examined by mass spectrometry. *J. Bacteriol.* 188, 773-783.
3
- 4 Guerreiro N., Djordjevic, M.A., Rolfe, B.G., 1999. Proteome analysis of the model
5 microsymbiont *Sinorhizobium meliloti*: isolation and characterisation of novel proteins.
6 *Electrophoresis* 20, 818-825.
7
- 8 Guerreiro N., Redmond, J.W., Rolfe, B.G., Djordjevic, M.A., 1997. New *Rhizobium*
9 *leguminosarum* flavonoid-induced proteins revealed by proteome analysis of differentially
10 displayed proteins. *Mol. Plant Microbe Interact.* 10, 506-516.
11
- 12 Gyorgypal, Z., Kiss, G.B., Kondorosi, A., 1991. Transduction of plant signal molecules by the
13 *Rhizobium* NodD proteins. *Bioessays* 13, 575-581.
14
- 15 Gyorgypal, Z., Kondorosi, A., 1991. Homology of the ligand-binding regions of *Rhizobium*
16 symbiotic regulatory protein NodD and vertebrate nuclear receptors. *Mol. Gen. Genet.* 226,
17 337-340.
18
- 19 Holden, M.T., Ram-Chhabra, S., Stead, R.N.P., Bainton, N.J., Hill, P.J., Manefield, M.,
20 Kumar, N., Labatte, M., England, D., Rice, S., Givskov, M., Salmond, G.P., Stewart, G.S.,
21 Bycroft, B.W., Kjelleberg, S., Williams, P., 1999. Quorum-sensing cross talk: isolation and
22 chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-
23 negative bacteria. *Mol. Microbiol.* 33, 1254-1266.
24

1 Hungría, M., Joseph, C.M., Phillips, D.A., 1991. Anthocyanidins and flavonols, major *nod*
2 gene inducers from seeds of a black-seeded common bean (*Phaseolus vulgaris* L.). Plant
3 Physiol. 97, 751-758.

4
5 Hungría, M., Joseph, C.M., Phillips, D.A., 1991. *Rhizobium nod* gene inducers exuded
6 naturally from roots of common bean (*Phaseolus vulgaris* L.). Plant Physiol. 97, 759-764.

7
8 Kumari, A., Pasini, P., Daunert, S., 2008. Detection of bacterial quorum sensing *N*-acyl
9 homoserine lactones in clinical samples. Anal. Bioanal. Chem. 391, 1619-1627.

10
11 Latifi, A., Winson, M.K., Foglino, M., Bycroft, B.W., Stewart, G.S., Lazdunski, A., Williams,
12 P., 1995. Multiple homologues of LuxR and LuxI control expression of virulence
13 determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa*
14 PAO1. Mol. Microbiol. 17, 333-343.

15
16 Le Queré, A.J., Deakin, W.J., Schmeisser, C., Carlson, R.W., Streit, W.R., Broughton, W.J.,
17 Forsberg, L.S., 2006. Structural characterization of a K-antigen capsular polysaccharide
18 essential for normal symbiotic infection in *Rhizobium* sp. NGR234: deletion of the *rkpMNO*
19 locus prevents synthesis of 5, 7-diacetamido-3, 5, 7, 9-tetradecoxy-non-2-ulosonic acid. J. Biol.
20 Chem. 281, 28981-28992.

21
22 Loh, J., Russell, W.C., William, S.Y., Stacey, G., 2002. Bradyoxetin, a unique chemical
23 signal involved in symbiotic gene regulation. Proc. Natl. Acad. Sci. USA 99, 14446-14451.

1 López-Baena, F.J., Monreal, J.A., Pérez-Montano, F., Guasch-Vidal, B., Bellogín, R.A.,
2 Vinardell, J.M., Ollero, F.J., 2009. The absence of Nops secretion in *Sinorhizobium fredii*
3 HH103 increases *GmPR1* expression in Williams soybean. Mol. Plant Microbe Interact. 22,
4 1445-1454.

5
6 López-Baena, F.J., Vinardell, J.M., Pérez-Montano, F., Crespo-Rivas, J.C., Bellogín, R.A.,
7 Espuny, M.R., Ollero, F.J., 2008. Regulation and symbiotic significance of nodulation outer
8 proteins secretion in *Sinorhizobium fredii* HH103. Microbiology 154, 1825-1836.

9
10 Makemson, J., Eberhard, A., Mathee, K., 2006. Simple electrospray mass spectrometry
11 detection of acylhomoserine lactones. Luminescence 21, 1-6.

12
13 Mathesius, U., Mulders, S., Gao, M., Teplitski, M., Caetano-Anolles, G., Rolfe, B.G., Bauer,
14 W.D., 2003. Extensive and specific responses of a eukaryote to bacterial quorum-sensing
15 signals. Proc. Natl. Acad. Sci. USA. 100, 1444–1449.

16
17 Miller, M.B., Bassler, B.L., 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. 55,
18 165-199.

19
20 Morin, D., Grasland, B., Vallee-Rehel, K., Dufau, C., Haras, D., 2003. On-line high-
21 performance liquid chromatography-mass spectrometric detection and quantification of *N*-
22 acylhomoserinelactones, quorum sensing signal molecules, in the presence of biological
23 matrices. J. Chromatogr. 1002, 79-92.

24

- 1 Ortori, C.A., Atkinson, S., Chhabra, S.R., Cámara, M., Williams, P., Barrett, D.A., 2007.
2 Comprehensive profiling of *N*-acylhomoserine lactones produced by *Yersinia*
3 *pseudotuberculosis* using liquid chromatography coupled to hybrid quadrupole-linear ion trap
4 mass spectrometry. *Anal. Bioanal. Chem.* 387, 497-511.
5
- 6 Perret, X., Freiberg, C., Rosenthal, A., Broughton, W.J., Fellay, R., 1999. High-resolution
7 transcriptional analysis of the symbiotic plasmid of *Rhizobium* sp. NGR234. *Mol. Microbiol.*
8 32, 415-425.
9
- 10 Perret, X., Staehelin, C., Broughton, W.J., 2000. Molecular basis of symbiotic promiscuity.
11 *Microbiol. Mol. Biol. Rev.* 64, 180-201.
12
- 13 Pueppke, S.G., Bolaños-Vasquez, M.C., Werner, D., Bec-Ferté, M.P., Promé, J.C., Krishnan,
14 H.B., 1998. Release of flavonoids by the soybean cultivars McCall and Peking and their
15 perception as signals by the nitrogen-fixing symbiont *Sinorhizobium fredii*. *Plant Physiol.*
16 117, 599-608.
17
- 18 Rodríguez-Navarro, D.N., Bellogín, R., Camacho, M., Daza, A., Medina, C., Ollero, F.J.,
19 Santamaria, C., Ruiz-Sainz, J.E., Vinardell, J.M., Temprano, F.J., 2003. Field assessment and
20 genetic stability of *Sinorhizobium fredii* strain SMH12 for commercial soybean inoculants.
21 *Eur. J. Agron.* 19, 299-309.
22
- 23 Rodríguez-Navarro, D.N., Buendía, A.M., Camacho, M., Lucas, M.M., Santamaría, C., 2000.
24 Characterization of *Rhizobium* spp. bean isolates from South-West Spain. *Soil Biol. Biochem.*
25 32, 1601-1613.

1
2 Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: a laboratory manual, 2nd
3 edn. Cold Spring Harbor NY: Cold Spring Harbor Laboratory.

4
5 Sánchez-Contreras, M., Bauer, W.D., Gao, M., Robinson, J.B., Downie, J.A., 2007. Quorum-
6 sensing regulation in rhizobia and its role in symbiotic interactions with legumes. Philos. T.
7 R. Soc. Lond. B. Biol. Sci. 362, 1149-1163.

8
9 Shaw, L.J., Morris, P., Hooker, J.E., 2006. Perception and modification of plant flavonoid
10 signals by rhizosphere microorganisms. Environ. Microbiol. 8, 1867-1880.

11
12 Shaw, P.D., Ping, G., Daly, S.L., Cha, C., Cronan, J.E., Rinehart, K.L., Farrand, S.K., 1997.
13 Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer
14 chromatography. Proc. Natl. Acad. Sci. USA. 94, 6036-6041.

15
16 Squartini, A., Struffi, P., Doring, H., Selenska-Pobell, S., Tola, E., Giacomini, A., Vendramin,
17 E., Velazquez, E., Mateos, P.F., Martínez-Molina, E., Dazzo, F.B., Casella, S., Nuti, M.P.,
18 2002. *Rhizobium sullae* sp. nov. (formerly '*Rhizobium hedysari*'), the root-nodule
19 microsymbiont of *Hedysarum coronarium* L. Int. J. Syst. Evol. Microbiol. 52, 1267-1276.

20
21 Steindler, L., Venturi, V., 2007. Detection of quorum-sensing *N*-acyl homoserine lactone
22 signal molecules by bacterial biosensors. FEMS Microbiol. Lett. 266, 1-9.

23
24 Throup, J.P., Cámara, M., Briggs, G.S., Winson, M.K., Chhabra, S.R., Bycroft, B.W.,
25 Williams, P., Stewart, G.S., 1995. Characterisation of the *yenI/yenR* locus from *Yersinia*

1 *enterocolitica* mediating the synthesis of two *N*-acylhomoserine lactone signal molecules.
2 Mol. Microbiol. 17, 345-356.

3
4 Vinardell, J.M., Ollero, F.J., Hidalgo, A., López-Baena, F.J., Medina, C., Ivanov-Vangelov,
5 K., Parada, M., Madinabeitia, N., Espuny, M.R., Bellogín, R.A., Camacho, M., Rodríguez-
6 Navarro, D.N., Soria-Díaz, M.E., Gil-Serrano, A.M., Ruiz-Sainz, J.E., 2004. NolR regulates
7 diverse symbiotic signals of *Sinorhizobium fredii* HH103. Mol. Plant. Microbe Interact. 17,
8 676-685.

9
10 Vincent, J.M., 1970. The modified Fahraeus slide technique. In *A manual for the practical*
11 *study of root nodule bacteria*, pp. 144-145. Edited by J. M. Vincent. Oxford, UK: Blackwell
12 Scientific Publications.

13
14 von Rad, U., Klein, I., Dobrev, P.I., Kottova, J., Zazimalova, E., Fekete, A., Hartmann, A.,
15 Schmitt-Kopplin, P., Durner, J., 2008. Response of *Arabidopsis thaliana* to *N*-hexanoyl-DL-
16 homoserine-lactone, a bacterial quorum sensing molecule produced in the rhizosphere. Planta
17 229, 73-85.

18
19 Williams, P., 2007. Quorum sensing, communication and cross-kingdom signalling in the
20 bacterial world. Microbiology 153, 3923-3938.

Legends to figures

Figure 1. β -galactosidase activity of the autoinducers contained in HPLC fractions obtained from supernatants of *S. fredii* SMH12 (a), *R. etli* ISP42 (b), and *R. sllae* IS123 (c), using an adapted assay with the reporter strain *A. tumefaciens* NT1 (pZLR4). The activity is expressed in Miller units (M.U.). Bacteria were grown in YM medium without (white square) or with their corresponding inducing flavonoids (black square): genistein for SMH12, apigenin for ISP42, and daidzein for IS123.

Figure 2. Quantitative RT-PCR analysis of the expression of autoinducer synthesis genes from *S. fredii* SMH12 (*traI*) and *R. etli* ISP42 (*rail* and *cinI*). Expression data shown are the mean ($\pm$ standard deviation of the mean) for three biological replicates. Expression was calculated relative to the expression without flavonoids (white bars). Gray bars: non inducing flavonoids (umbelliferone for SMH12 and daidzein for ISP42), and dotted bars: inducing flavonoids (genistein for SMH12 and apigenin for ISP42).

Supplementary figure 1. Well diffusion assays of bacterial supernatants obtained from *S. fredii* SMH12, *R. etli* ISP42, and *R. sllae* IS123 grown in **A**, TY and **B**, YM medium for 3, 6 and 9 days.

Supplementary figure 2. TLC analysis of autoinducers produced by *S. fredii* SMH12, *R. etli* ISP42, and *Rhizobium sllae* IS123. Migration of the different AHL standards is shown on the left of the figure.

Supplementary figure 3. TLC analysis of autoinducers produced by *S. fredii* SMH12 (**A**), *R. etli* ISP42 (**B**), and *R. sllae* IS123 (**C**) without flavonoids and in the presence of non

1 inducing flavonoids and inducing flavonoids. **D**, TLC analysis of standard HHL 500 µg/ml
2 and OHL 50 µg/ml used at different concentrations: 1, 1.2-fold, and 5-fold.

3
4 **Supplementary figure 4.** Effect of flavonoids on growth of *S. fredii* SMH12, *Rhizobium etli*
5 ISP42, and *Rhizobium sllae* IS123. The graphs represent the mean ($\pm$ standard deviation of
6 the mean) of at least two independent experiments performed in triplicate.

Table 1. *nodA* gene expression in the presence of different flavonoids.

	<i>S. fredii</i> SMH12	<i>R. etli</i> ISP42	<i>R. sultae</i> IS123
Without flavonoids	1.0 ± 0	1.0 ± 0	1.0 ± 0
Apigenin	7.9 ± 0.59	4.68 ± 1.05 ^a	1.1 ± 0.03 ^b
Daidzein	6.81 ± 0.52	1.37 ± 0.45	7.82 ± 0.75 ^a
7, 4'-dihydroxyflavone	5.84 ± 0.5	5.63 ± 1.21	1.24 ± 0.05
Genistein	7.2 ± 0.69 ^a	1.31 ± 0.23	6.12 ± 0.18
Isoliquiritigenin	6.0 ± 0.11	4.29 ± 0.84	1.25 ± 0.08
Morin	5.11 ± 1.65	1.27 ± 0.24	1.06 ± 0.04
Naringenin	6.18 ± 0.13	4.39 ± 0.11	0.9 ± 0.05
Quercetin	1.77 ± 0.14	1.04 ± 0.1	0.94 ± 0.06
Umbelliferone	1.02 ± 0.4 ^b	0.86 ± 0.18 ^b	1.94 ± 0.06

Data represent the mean (± standard deviation of the mean) of the fold β-galactosidase activity induced by different flavonoids compared with the control without flavonoids. All the strains carry plasmid pMP240, which harbours the *nodA* gene promoter fused to *lacZ*.

a: Flavonoid selected as inducer for each strain, and b: flavonoid selected as not inducer.

Results correspond to three different experiments performed in triplicate.

Table 2. β -galactosidase activity obtained using an adapted assay with *A. tumefaciens* NT1 (pZLR4) as bioreporter and grown in the presence of rhizobial supernatants.

	Miller units	n (%)
Control (YM)	58.2 $\pm$ 1.5	
3-oxo-C6-HSL (5.5 μ M)	351.4 $\pm$ 1.9	
SMH12	282.3 $\pm$ 2.1	100
SMH12 + umbelliferone (6.2 μ M)	318.2 $\pm$ 8.4	113
SMH12 + genistein (3.7 μ M)	370.8 $\pm$ 10.3	131 ^a
ISP42	337.1 $\pm$ 23.2	100
ISP42 + umbelliferone (6.2 μ M)	361.3 $\pm$ 3.4	107
ISP42 + apigenin (6.2 μ M)	398.4 $\pm$ 15.7	119 ^a
IS123	322.4 $\pm$ 3.2	100
IS123 + apigenin (6.2 μ M)	297.3 $\pm$ 12	92 ^a
IS123 + daidzein (3.9 μ M)	421.4 $\pm$ 4.9	130 ^a

Data are the mean $\pm$ SD of at least five independent experiments performed in triplicate.

n: percentage of induction of each rhizobial supernatant obtained in the presence of flavonoids with respect to the control without flavonoids and defined as 100%.

Each β -galactosidase activity using rhizobial supernatant induced with flavonoids was individually compared to that obtained without flavonoids by using the Mann-Whitney non-parametrical test. Numbers on the same column followed by the letter a are significantly different at the level $\alpha = 10\%$.

1 **Figure 1**

1 **Figure 2**

1 **Supplementary figure 1**

2

3

4

1 **Supplementary figure 2**

2

3

4

1 **Supplementary figure 3**

2

3

Supplementary figure 4