The flavanone naringenin reduces the production of quorum sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1

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Preliminary screening of the Malagasy plant *Combretum albiflorum* for compounds attenuating the production of quorum sensing (QS)-controlled virulence factors in bacteria led to the identification of active fractions containing flavonoids. In the present study, several flavonoids belonging to the flavone, flavanone, flavonol and chalcone structural groups were screened for their capacity to reduce the production of QS-controlled factors in the opportunistic pathogen *Pseudomonas aeruginosa* (strain PAO1). Flavanones (i.e. naringenin, eriodictyol and taxifolin) significantly reduced the production of pyocyanin and elastase in *P. aeruginosa* without affecting bacterial growth. Consistently, naringenin and taxifolin reduced the expression of several QS-controlled genes (i.e. *lasI*, *lasR*, *rhlI*, *rhlR*, *lasA*, *lasB*, *phzA1* and *rhlA*) in *P. aeruginosa* PAO1. Naringenin also dramatically reduced the production of the acylhomoserine lactones *N*-(3-oxododecanoyl)-*L*-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-*L*-homoserine lactone (C4-HSL), which is driven by the *lasI* and *rhlI* gene products, respectively. In addition, using mutant strains deficient for autoinduction (*DlasI* and *DrhlI*) and LasR- and RhlR-based biosensors, it was shown that QS inhibition by naringenin not only is the consequence of a reduced production of autoinduction compounds but also results from a defect in the proper functioning of the RhlR–C4-HSL complex.

Widely distributed in the plant kingdom, flavonoids are known for their numerous and determinant roles in plant physiology, plant development and in the success of plant–rhizobia interactions, but, as shown here, some of them also have a role as inhibitors of the virulence of pathogenic bacteria by interfering with QS mechanisms.

**INTRODUCTION**

In many pathogenic bacteria the production of virulence factors is triggered in a population density-dependent manner through quorum sensing (QS), a cell-to-cell communication mechanism that enables bacteria to coordinate virulence factor production by means of the synthesis, release and perception of small diffusible molecules called autoinducers (Antunes et al., 2010; Bjarnsholt et al., 2010; Case et al., 2008; Ng & Bassler, 2009). For instance, in the plant and mammal opportunistic pathogen *Pseudomonas aeruginosa*, two main QS systems (*lasI*/*R* and *rhlI*/*R*), responsible for the synthesis and perception of the acylhomoserine lactones (AHLs) *N*-(3-oxododecanoyl)-*L*-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-*L*-homoserine lactone (C4-HSL), respectively (Pesci & Iglewski, 1997), control the expression

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Received 25 February 2011
Revised 2 May 2011
Accepted 3 May 2011

Abbreviations: AHL, acylhomoserine lactone; C4-HSL, *N*-butanoyl-*L*-homoserine lactone; ESI-MS, electrospray ionization-MS; HHL, *N*-hexanoyl-*L*-homoserine lactone; 3-oxo-C12-HSL, *N*-(3-oxododecanoyl)-*L*-homoserine lactone; PI, propidium iodide; QS, quorum sensing.

†These authors contributed equally to this work.
of an arsenal of virulence factors. The transcription factors LasR and RhlR interact with and are activated by 3-oxo-C12-HSL and C4-HSL, respectively, triggering the production of biofilms, LasB elastase, LasA protease, Apr alkaline protease, exotoxin A, rhamnolipids, pyocyanin, hydrogen cyanide and cytotoxic lecithins, among other virulence factors (Davies et al., 1998; Gambello & Iglewski, 1991; Gambello et al., 1993; Pesci et al., 1997; Seed et al., 1995; Toder et al., 1991, 1994). In conjunction with a third QS system, using quinolone signalling, the las and rhl systems are tightly co-regulated in a hierarchical manner (Pesci & Iglewski, 1997; Pesci et al., 1999; Wade et al., 2005).

Considering its central role in regulating the production of virulence factors by pathogenic bacteria, QS is considered an attractive target for the development of strategies to attenuate the virulence of these bacteria (Bjarsholt et al., 2010; González & Keshavan, 2006; McDougald et al., 2007; Njoroge & Sperandio, 2009). In that context, plants have been widely screened for compounds that interfere with QS mechanisms (González & Keshavan, 2006; Janssens et al., 2008; Teplitski et al., 2011). Recently, we have shown that the Malagasy plant Combretum albilorum (Tul.) Jongkind contains phenolic compounds that affect the production of QS-regulated factors in P. aeruginosa PAO1 (Vandeputte et al., 2010). The characterization of one of the active chromatographic fractions led to the identification of several flavonoid-related structures and to the identification of catechin as one of the active compounds (Vandeputte et al., 2010). Flavonoids are widely produced by plants and have important physiological functions, acting as signals in the legume–rhizobia symbiosis; they present a large structural diversity and display many pharmacological activities (Buer et al., 2010; Dixon & Steele, 1999; Dixon & Pasinetti, 2010; Mandal et al., 2010).

Based on the screening performed on Combretum albilorum (Vandeputte et al., 2010), several commercially available flavonoids belonging to the flavone, flavanone, flavonol and chalcone groups have been selected to assess their capacity to interfere with QS mechanisms in P. aeruginosa PAO1 and Chromobacterium violaceum CV026. These structurally related compounds differ in their structures by the oxidation and hydroxylation of their central C-ring and by the substitution pattern of their A- and B-rings (Buer et al., 2010; Dixon & Steele, 1999; Dixon & Pasinetti, 2010) (Fig. 1). Among the tested flavonoids, those of the flavanone group (naringenin, eriodictyol and taxifolin) are shown to dramatically interfere with QS mechanisms with limited/or no effect on bacterial growth.

METHODS

Bacterial strains, plasmids and culture conditions. C. violaceum CV026 was grown in liquid Luria–Bertani (LB) medium at 28 °C (McClean et al., 1997) and tested as described previously (Vandeputte et al., 2010). P. aeruginosa PAO1 wild-type strain was obtained from the Pseudomonas Genetic Stock Center (strain PAO0001; http://www.pseudomonas.med.ecu.edu/). P. aeruginosa PAO1 mutant strains were obtained from the Transposon Mutant Collection (Department of Genome Sciences, University of Washington; http://www.gs.washington.edu/labs/manoil/libraryindex.htm) and include mutants 11174 (Δpa1432, ΔlasI), 17281 (Δpa1430, ΔlasR), 32454 (Δpa3476, ΔrhlI) and 3452 (Δpa3477, ΔrhlR) (Jacobs et al., 2003). Plasmids pJU1 and pJU2 (Ishida et al., 2007), pPCS223 and pLPR1 (Van Delden et al., 1998), pPSC1001 and pPSC1002 (Pesci et al., 1997) and pTB4124 (Kretschmar et al., 2008) (see Table 1) were introduced into P. aeruginosa PAO1 as described by Smith & Iglewski (1989). All P. aeruginosa strains were grown at 37 °C with agitation (175 r.p.m.) for 18 h in liquid LB (5 ml) supplemented with 50 mM MOPS at pH 7.0 and with the appropriate antibiotics (tetracycline at 60 μg ml⁻¹ and carbenicillin at 300 μg ml⁻¹) (Vandeputte et al., 2010). All strains were grown and incubated with the different flavonoids as previously described (Vandeputte et al., 2010).

Escherichia coli JLD271 biosensor strains harbouring LasR- and RhlR-based plasmids pAL105 and pAL101 and control plasmids pAL106 (LasR⁻) and pAL102 (RhlR⁻) (see Table 1) were grown in LB medium supplemented with tetracycline (10 μg ml⁻¹) and chloramphenicol (25 μg ml⁻¹) as previously described (Lindsay & Ahmer, 2005; Vandeputte et al., 2010). When required, the medium was supplemented with 0, 1, 10 or 100 μM (final) 3-oxo-C12-HSL or C4-HSL.

Chemicals. All flavonoids tested in this study were purchased from Sigma-Aldrich, dissolved in 100 % DMSO and used at 4 mM (final concentration, resulting in 1 % DMSO). These flavonoids included: apigenin (4',5,7-trihydroxyflavone), eriodictyol (3',4',5,7-tetrahydroxyflavone), kaempferol (3',5,7-trihydroxyflavone), luteolin (3',4',5,7-tetrahydroxyflavone), myricetin (3',3',5,5',7-pentahydroxyflavone), naringenin (4',5,7-trihydroxyflavone), naringin (4',5,7-trihydroxyflavone 7-rhamnoglucoside), quercetin (3',3',4',5',7-pentahydroxyflavone), taxifolin (3',3',5,7-pentahydroxyflavone) and chalcone (trans-benzylidenecacetophenone). The AHLS N-hexanoyl-L-homoserine lactone (HHL), 3-oxo-C12-HSL and C4-HSL were purchased from Sigma-Aldrich and dissolved in 100 % DMSO.

β-Galactosidase measurements. β-Galactosidase measurements were performed as previously described (Vandeputte et al., 2010). After growth in liquid LB-MOPS-carbenicillin at 37 °C and 175 r.p.m. for 18 h, PAO1 reporter strains were washed twice in fresh LB medium and resuspended in liquid LB-MOPS-carbenicillin as described previously (Vandeputte et al., 2010). Fifty microlitres of washed PAO1 reporter strains were added to 1 ml LB-MOPS-carbenicillin in order to obtain a starting OD₆₀₀ of 0.020–0.025, and 10 μl of the flavonoid to be tested (4 mM final) or 10 μl DMSO was added before incubation for 8 or 18 h at 37 °C with agitation (175 r.p.m.). After incubation, cell density was assessed by spectrophotometry (OD₆₀₀) and the β-galactosidase activity was measured using ONPG, as described elsewhere (Zhang & Bremer, 1995). All experiments were performed in triplicate and repeated in three independent experiments. The data were statistically analysed by Student’s t test (i.e. each test was compared with the DMSO condition) and P ≤ 0.01 was considered significant.

Quantitative analysis of violacein production in C. violaceum CV026. Inhibition of violacein production in C. violaceum CV026 by authentic flavonoid standards was tested by using a liquid assay according to reported protocols (Blosser & Gray, 2000; McClean et al., 1997). Violacein production was induced in C. violaceum CV026 by adding HHL (3 μM final). After 18 h and assessment of bacterial growth (OD₆₀₀), violacein contents were quantified according to Blosser & Gray (2000). Violacein absorbance was measured at 575 nm and violacein production was calculated as the ratio between A₅₇₅ and OD₆₀₀. The experiments were performed in triplicate and the data were statistically analysed by Student’s t test (i.e. each test was compared with the DMSO condition) and P ≤ 0.01 was considered significant.
Quantitative analysis of pyocyanin and elastase production in *P. aeruginosa* PAO1 and *P. aeruginosa* mutants. Inhibition of pyocyanin and elastase production was assessed according to described procedures (Ishida et al., 2007; Müh et al., 2006). *P. aeruginosa* PAO1 wild-type or mutant strains were grown for 18 h in liquid LB-MOPS supplemented with 60 μg tetracycline ml⁻¹ (mutant

**Table 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tr>
<td><strong>Strains</strong>&lt;br/&gt;C. violaceum CV026</td>
<td>Violacein-negative, cviI mini-Tn5 mutant of <em>C. violaceum</em></td>
<td>McClean et al. (1997)</td>
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<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>Wild-type (strain PAO0001; <a href="http://www.pseudomonas.med.ecu.edu/">http://www.pseudomonas.med.ecu.edu/</a>)</td>
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<tr>
<td><em>P. aeruginosa</em> ΔPA1430</td>
<td><em>P. aeruginosa</em> transposon mutant ID 17281; lasR::ISlacZ/hah; TetR&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Jacobs et al. (2003)</td>
</tr>
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<td><em>P. aeruginosa</em> ΔPA1432</td>
<td><em>P. aeruginosa</em> transposon mutant ID 11174; lasI::ISlacZ/hah; TetR&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Jacobs et al. (2003)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ΔPA3476</td>
<td><em>P. aeruginosa</em> transposon mutant ID 32454; rhlI::ISphoA/hah; TetR&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Jacobs et al. (2003)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ΔPA3477</td>
<td><em>P. aeruginosa</em> transposon mutant ID 3452; rhlR::ISlacZ/hah; TetR&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Jacobs et al. (2003)</td>
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<td><em>E. coli</em> JLD271</td>
<td><em>E. coli</em> K-12 ΔlacX74 sdiA271::Cam</td>
<td>Lindsay &amp; Ahmer (2005)</td>
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<td><strong>Plasmids</strong>&lt;br/&gt;p&lt;sub&gt;p&lt;/sub&gt;101</td>
<td>pQ50 derivative containing <em>Pseudomonas</em>-lacZ transcriptional fusion</td>
<td>Ishida et al. (2007)</td>
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<td>p&lt;sub&gt;p&lt;/sub&gt;102</td>
<td>pQ50 derivative containing <em>Pseudomonas</em>-lacZ transcriptional fusion</td>
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<td>p&lt;sub&gt;p&lt;/sub&gt;C5223</td>
<td>pLP170 derivative containing <em>Pseudomonas</em>-lacZ transcriptional fusion</td>
<td>Van Delden et al. (1998)</td>
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<td>pLP170 derivative containing <em>Pseudomonas</em>-lacZ transcriptional fusion</td>
<td>Pesci et al. (1997)</td>
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<td>p&lt;sub&gt;p&lt;/sub&gt;LR101</td>
<td>pLP170 derivative containing <em>Pseudomonas</em>-lacZ transcriptional fusion</td>
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<td>pLP170 derivative containing <em>Pseudomonas</em>-lacZ transcriptional fusion</td>
<td>Pesci et al. (1997)</td>
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<td>p&lt;sub&gt;p&lt;/sub&gt;AL101</td>
<td>pSB401 derivative containing rhl&lt;sup&gt;+&lt;/sup&gt; rhl&lt;sup&gt;.&lt;/sup&gt;luxCDABE; Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Lindsay &amp; Ahmer (2005)</td>
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<td>p&lt;sub&gt;p&lt;/sub&gt;AL102</td>
<td>pSB401 derivative containing rhl&lt;sup&gt;+&lt;/sup&gt;luxCDABE; Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Lindsay &amp; Ahmer (2005)</td>
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<td>Lindsay &amp; Ahmer (2005)</td>
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<td>p&lt;sub&gt;p&lt;/sub&gt;AL106</td>
<td>pSB401 derivative containing lasI&lt;sup&gt;.&lt;/sup&gt;luxCDABE; Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Lindsay &amp; Ahmer (2005)</td>
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<tr>
<td>p&lt;sub&gt;p&lt;/sub&gt;TB4124</td>
<td>pQ50 derivative containing <em>Pseudomonas</em>-lacZ transcriptional fusion</td>
<td>Kretzschmar et al. (2008)</td>
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strains) when appropriate at 37 °C and 175 r.p.m. *P. aeruginosa* cells were washed twice in fresh LB-MOPS medium and the cell suspension (50 μl), diluted appropriately, was added to 1 ml LB-MOPS (starting OD$_{600}$ 0.020–0.025) supplemented with 10 μl of authentic flavonoid standards (4 mM final) or DMSO. After 8 h (pyocyanin) or 18 h (elastase) of growth with or without flavonoids, samples were taken to assess bacterial growth (OD$_{600}$), to extract and quantify pyocyanin through absorbance measurements at 380 nm (Ishida et al., 2007; Müh et al., 2006), and to assess elastase production through the measurement of elastase activity using elasin–Congo red (Ishida et al., 2007; Müh et al., 2006). The experiments were performed in four replicates and the data were statistically analysed by Student’s t test (i.e. each test was compared with the DMSO condition) or one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test, and $P \leq 0.01$ was considered significant.

**Homoserine lactone quantification.** AHLs (C4-HSL and 3-oxo-C12-HSL) were extracted from PAO1 cultures and quantified by direct infusion in an electrospray mass spectrometer (electrospray ionization-MS; ESI-MS) as described by Makemson et al. (2006). *P. aeruginosa* PAO1 was grown at 37 °C with agitation at 175 r.p.m. for 8 and 18 h in 5 ml LB-MOPS medium supplemented with naringin or naringenin (4 mM final concentration) or DMSO (0.1%, v/v). Bacterial cultures were centrifuged (3200 g, room temperature, 5 min) and supernatants (4 ml) were acidified with 80 μl glacial acetic acid prior to being extracted three times with ethyl acetate (4 ml). Ethyl acetate extracts were combined, evaporated to dryness and dissolved in 1 ml acidified ethyl acetate (0.1%, v/v, glacial acetic acid). For background measurement, supernatants (4 ml) were alkalized with 80 μl M NaOH before ethyl acetate extraction, hydrolysed lactone rings being too polar to be extracted.

ESI-MS quantification was done by direct injection into a Finnigan LCQ DUO mass spectrometer under soft ionization conditions (positive ionization mode; nebulizer tip set at 250 mV) and scans were averaged over 1 min. The peak intensities for C4-HSL (pseudomolecular ion $m/z = 172$; ammonium adduct $m/z = 189$; sodium adduct $m/z = 194$; solvent adduct $m/z = 260$) and 3-oxo-C12-HSL (pseudomolecular ion $m/z = 298$; ammonium adduct $m/z = 315$; sodium adduct $m/z = 320$; solvent adduct $m/z = 386$) were combined and converted to concentrations by using a standard curve generated from the pure compounds. Background readings from hydrolysed samples extracted with ethyl acetate were subtracted from those of the acid-extracted bacterial cultures before conversion. The data ($n = 6$) were statistically analysed by Student’s $t$ test (i.e. each test was compared with the DMSO condition) and $P \leq 0.01$ was considered significant.

**Gene expression analysis by RT-PCR.** After incubation with naringenin (4 mM final) as described above, *P. aeruginosa* PAO1 cells were frozen in liquid nitrogen, disrupted with acid-washed glass beads (425–600 μm) from Sigma using a MagNA Lyser instrument (Roche Diagnostics) and homogenized in TRI Reagent (Sigma). After UV quantification, DNA contamination was eliminated by 1 h DNase treatment (Amplification Grade DNase I from Sigma) according to the manufacturer’s instructions. RNA quality and quantity were assessed at DNAvision (http://www.dnavision.com) with a Bioanalyzer 2100 (Agilent).

cDNAs were synthesized from 250 ng total RNA using the Reverse Transcription System (Promega) and random hexamers according to Promega’s instructions. Primers (5 μM each) were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) (Rozen & Skaltsky, 2000) and are as follows: lasB-left, 5'-ATAGCAGAAGTTGATGATCGAATCTGCCGATCC-3'; lasA-left, 5'-AAGGCGAATACCTGAGACCTG-3'; lasK-right, 5'-TGGATTTCCGGACCTGCC-3'; rhlA-left, 5'-AGACCGAAGTGTGACCTGC-3'; rhlB-right, 5'-AGTCGGATCCGATGTG-3'; phzA1-left, 5'-CGAACCACCTTCTGGTGGTGCATGC-3'; phzA1-right, 5'-GGGATATCGCATGGTTATTTGGC-3'.

Intensities of PCR products after SYBR Gold (Invitrogen) staining were quantified using the ImageQuant TL software (GE Healthcare Life Sciences) and normalized against the values obtained for the 16S rRNA amplification. Normalized values were used to calculate the gene expression ratios between expression in DMSO-treated and naringenin-treated *P. aeruginosa* PAO1. The data ($n = 3$) were statistically analysed by Student’s $t$ test (i.e. each test was compared with the DMSO condition) and $P \leq 0.01$ was considered significant.

**Assessment of *P. aeruginosa* PAO1 viability and growth kinetics.** *P. aeruginosa* PAO1 cells were incubated with DMSO or naringenin (4 mM final) as described above for 8 and 18 h and stained with SYTO-9 (Molecular Probes, Invitrogen) and propidium iodide (PI) according to the Molecular Probes instructions. Briefly, *P. aeruginosa* PAO1 cultures were diluted to an OD$_{600}$ 0.10–0.15, and 100 μl of a mix of SYTO-9 and PI was added to 100 μl diluted bacteria. Cells were transferred to a 96-well OptiPlate-96 F microplate (PerkinElmer) and fluorescence intensities were determined [excitation, 485 nm; emission, 530 nm (SYTO-9) and 630 nm (PI)] using a SpectraMax M2 device (Molecular Devices). Ratios between the green and the red fluorescence (530 nm/630 nm) were compared to assess the cytotoxicity of naringenin. DMSO-treated cultures were used as controls. The data ($n = 6$) were statistically analysed by Student’s $t$ test and $P \leq 0.01$ was considered significant. The effect of naringenin on the growth kinetics of *P. aeruginosa* PAO1 was evaluated by measuring the OD$_{600}$ of PAO1 cells grown under the same conditions over a 24 h period.

**RESULTS**

**Flavonoids affect the production of QS-dependent factors in *P. aeruginosa* PAO1 and *C. violaceum* CV026**

Several commercially available flavonoids belonging to the structural classes flavones (apigenin and luteolin), flavonols (kaempferol, quercetin and myricetin), flavanones (naringenin, naringin, eriodictyol and taxifolin) and chalcones (*trans*-benzylideneacetophenone) were screened for their capacity to interfere with QS in *P. aeruginosa* PAO1 and *C. violaceum* CV026. These flavonoids (Fig. 1) were tested at a final concentration of 4 mM, since, at this concentration, catechin has been shown to inhibit the production of QS-dependent factors by both species (Vandeputte et al., 2010). As shown in Fig. 2, several of the flavonoids had an effect on the production of pyocyanin and violacin. Indeed, with the exception of naringenin, all tested flavonoids inhibited the production of pyocyanin (Fig. 2a). Moreover, as shown in Fig. 2(b), the negative impact of the flavones (apigenin and luteolin) and the flavonoids (kaempferol, quercetin and myricetin) on pyocyanin production was correlated with a significantly reduced cell density of *P. aeruginosa* PAO1. Notably, the flavanones (i.e. naringenin, eriodictyol and taxifolin) that had a limited impact on PAO1 final cell density led to significant reductions in...
pyocyanin production by 86.8 ± 1.4%, 73.2 ± 5.2% and 55.8 ± 8.1%, respectively (n=4; Fig. 2a). Naringin, which is a 7-O-rhamnoglucoside derivative of naringenin (Fig. 1), did not reduce the production of pyocyanin (Fig. 2a), suggesting that steric effects or the presence of particular chemical functions are important factors in the capacity of naringenin to reduce pyocyanin production. An open chain flavonoid (i.e. trans-benzylideneacetophenone) had a negative impact on PAO1 pyocyanin production [68.3 ± 3.7% reduction (n=4); Fig. 2a], with no effect on the final bacterial density (Fig. 2b). QS mechanisms are also involved in the production of a purple pigment (violacein) in C. violaceum (McClean et al., 1997), a bacterium with a low incidence in human disease but with severe consequences upon infection (Teoh et al., 2006) that is widely used as a reporter strain in QS screening (Chu et al., 2011). Assays were carried out using the C. violaceum CV026 reporter strain, which is mutated in the AHL synthase gene cviI, and consequently this strain is able to produce violacein only when the natural inducer HHL is supplied in the growth medium (McClean et al., 1997). As shown in Fig. 2(c), several of the selected flavonoids had a negative effect on violacein production, although the flavones and the flavonols had an effect on CV026 growth (data not shown). The tested flavonones (naringenin, eriodictyol and taxifolin) reduced violacein production by 69 ± 2, 78 ± 1 and 67 ± 10% (n=4), while naringin and trans-benzylideneacetophenone had no significant effect on violacein production (Fig. 2c). Based on these preliminary screenings, flavones and flavonols were not further considered, and the effects of flavanones (which had no effect on the cell density of either P. aeruginosa PAO1 or C. violaceum CV026) on QS regulation in P. aeruginosa PAO1 were further analysed.

**Fig. 2.** Effect of flavonoids on (a) pyocyanin production by P. aeruginosa PAO1, (b) the final cell density of P. aeruginosa PAO1 and (c) violacein production by C. violaceum CV026. The cell density of the bacteria was assessed at 600 nm, and violacein and pyocyanin were extracted as described in Methods and quantified by absorbance measurements at 380 nm (pyocyanin) and 575 nm (violacein). Violacein production was calculated as the ratio between A575 and OD600. All flavonoids were used at 4 mM. DMSO-treated cultures were used as controls and the statistical significance of each test (n=4) was evaluated by conducting Student’s t test (i.e. each test was compared with the DMSO condition), and a P value of ≤ 0.01 was considered significant. Abbreviations: Api, apigenin; Lut, luteolin; Kae, kaempferol; Que, quercetin; Myr, myricetin; Nar, naringenin; Nin, naringin; Eri, eriodictyol; Tax, taxifolin; Ben, trans-benzylideneacetophenone. Asterisks indicate data that are statistically different (P<0.01). I, flavones; II, flavonols; III, flavanones.

**Flavanones inhibit the production of elastase by P. aeruginosa PAO1**

In order to assess the effect of flavanones on elastase production, PAO1 cells were incubated overnight in the presence of naringenin, naringin, eriodictyol and taxifolin (4 mM final). Elastase production was quantified by an elastolysis assay (Ishida et al., 2007; Müh et al., 2006), and as shown in Fig. 3, naringenin, eriodictyol and taxifolin reduced elastase production by 46 ± 1, 62 ± 6 and 47 ± 2%, respectively (n=4), although, as for pyocyanin production, naringin had no effect. The open chain flavonoid trans-benzylideneacetophenone was also tested and reduced elastase production by the same amount (51 ± 5%), further supporting the supposition that this particular flavonoid might also interfere with QS mechanisms.

**Flavanones reduce the expression of QS genes in P. aeruginosa**

If flavanones interfere with QS mechanisms, this should be reflected in the transcription level of QS-controlling and QS-regulated genes. The effect of flavanones on QS systems was therefore characterized by measuring the expression of
the AHL synthetase genes lasI and rhlI and the QS regulator genes lasR and rhlR in P. aeruginosa PAO1 grown with or without these flavanones. Due to its limited commercial availability, eriodictyol was not included in these further experiments. As shown in Table 2, after 8 h, naringenin significantly reduced the expression of all QS systems (lasRI and rhlRI) and downstream genes (lasB and rhlA), while naringin did not reduce the expression of any of the selected QS genes. This effect was more marked after 18 h, although naringin also had a negative effect on lasR and rhlR, possibly through partial hydrolysis of the rhamnogalactoside into the naringenin aglycone. After 8 h, taxifolin significantly reduced the expression of the synthase genes lasI and rhlI, although none of the other QS-related genes (i.e. lasB, lasR, rhlR and rhlA) seemed affected (Table 2). After 18 h, taxifolin significantly reduced the expression of all QS-genes with the exception of rhlA. trans-Benzylideneacetophenone reduced the expression of all QS-related (except lasI) genes after 8 h but had a limited impact after 18 h. Indeed, only the lasR, lasB and rhlA genes were affected, while the others had an expression equivalent to that of DMSO-treated PAO1 cells. To verify that the drop in β-galactosidase activity was indeed associated with a reduction in QS-related gene expression and was not due to a general effect on transcription/translation mechanisms, the activity of the aceA promoter (regulating the expression of the isocitrate lyase gene PA2634) from P. aeruginosa (Kretzschmar et al., 2008) was studied in strain PAO1 grown in the presence or absence of the selected flavanones. As shown in Table 2, the addition of the flavanoids did not have a negative impact on the transcription of the aceA gene (although naringenin had a significant positive impact), indicating that they affect the expression of QS-related genes without affecting the transcription machinery of P. aeruginosa PAO1.

Though these flavanones have closely related structures, they apparently have different effects on the expression of genes involved in QS, and naringenin seems to be the molecule with the widest and most persistent action on QS systems in P. aeruginosa PAO1. Therefore, and in order to provide more evidence that QS mechanisms in PAO1 are affected by naringenin, we analysed the expression of four QS-related genes by semiquantitative RT-PCR: those involved in the production of LasA protease (lasA), LasB elastase (lasB), pyocyanin (phzA1) and rhamnolipids (rhlA). PAO1 cells were incubated in triplicate for 18 h with or without naringenin (4 mM final). As shown in Fig. 4, the expression of these genes was downregulated by the application of naringenin, further confirming the observed reduction of pyocyanin and elastase production and the decreased expression of QS-related genes (Figs 2 and 3, Table 2). As shown in Fig. 4(c), none of the effects of naringenin could be attributed to a diminution of PAO1 growth or viability.

**Naringenin, but not naringin, inhibits the production of AHLs in P. aeruginosa PAO1**

To determine whether the QS inhibitory effect of naringenin was linked to a defect in 3-oxo-C12-HSL or C4-HSL synthesis, concentrations of 3-oxo-C12-HSL and C4-HSL were determined in the growth media of PAO1 cells grown for 8 or 18 h in the presence of DMSO, naringenin (4 mM final) or naringin (4 mM final). As shown in Fig. 5, naringenin significantly reduced the concentration of both autoinducers after 8 h (Fig. 5a, c) and 18 h (Fig. 5b, d). After 8 h, concentrations of 3-oxo-C12-HSL and C4-HSL were reduced three- and 10-fold, respectively. Eighteen hours after the addition of naringenin (Fig. 5c, d), two- and fourfold reductions in autoinducer concentrations were observed, respectively. Naringin, in contrast, had no significant effect on the production of either AHL, at 8 or 18 h.

**Exogenously supplied AHLs do not compensate the effect of naringenin on pyocyanin production by P. aeruginosa PAO1**

Since AHL production is impaired by naringenin, 3-oxo-C12-HSL and C4-HSL were added exogenously to naringenin-treated PAO1 cells. As shown in Fig. 6, the addition of 10 μM 3-oxo-C12-HSL or C4-HSL did not restore the production of pyocyanin in naringenin-treated wild-type PAO1 cells (Fig. 6a); while, as already observed, naringin had no effect (Fig. 6b). The same experiment was performed with the ΔPA1432 and ΔPA3476 mutant strains (which lack functional lasI and rhl synthetase genes, respectively). As shown in Fig. 6(c), the exogenous addition of 3-oxo-C12-HSL to the ΔPA1432 strain significantly increased pyocyanin production, but adding naringenin reduced this production (Fig. 6c; +Nar), indicating that the exogenous supply of AHLs is not sufficient to compensate...
Table 2. Effect of flavanones that inhibit pyocyanin and elastase production in P. aeruginosa PAO1 on the expression of QS-regulated genes after 8 and 18 h of incubation

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>lasI</th>
<th>lasR</th>
<th>lasB</th>
<th>rhlI</th>
<th>rhlR</th>
<th>rhlA</th>
<th>aceA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>532 ± 12</td>
<td>4621 ± 283</td>
<td>3248 ± 436</td>
<td>9075 ± 835</td>
<td>4154 ± 94</td>
<td>2848 ± 74</td>
<td>2615 ± 418</td>
</tr>
<tr>
<td>Naringenin</td>
<td>464 ± 14</td>
<td>3392 ± 326</td>
<td>2440 ± 124</td>
<td>4424 ± 503</td>
<td>2771 ± 250</td>
<td>2260 ± 107</td>
<td>2679 ± 129</td>
</tr>
<tr>
<td>Naringin</td>
<td>1602 ± 139</td>
<td>4579 ± 586</td>
<td>3607 ± 101</td>
<td>11826 ± 898</td>
<td>4701 ± 332</td>
<td>3414 ± 281</td>
<td>3480 ± 421†</td>
</tr>
<tr>
<td>Taxifolin</td>
<td>207 ± 19</td>
<td>5413 ± 650</td>
<td>2859 ± 39</td>
<td>5890 ± 746</td>
<td>3610 ± 526</td>
<td>2845 ± 201</td>
<td>2592 ± 161</td>
</tr>
<tr>
<td>trans-Benzylideneacetonaphone</td>
<td>558 ± 36</td>
<td>3026 ± 436</td>
<td>2676 ± 69†</td>
<td>11583 ± 428†</td>
<td>2305 ± 265†</td>
<td>2085 ± 252†</td>
<td>2841 ± 161†</td>
</tr>
<tr>
<td>18 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>968 ± 98</td>
<td>4814 ± 577</td>
<td>1116 ± 121</td>
<td>4046 ± 373</td>
<td>2206 ± 492</td>
<td>8455 ± 330</td>
<td>2424 ± 84</td>
</tr>
<tr>
<td>Naringenin</td>
<td>392 ± 62</td>
<td>1675 ± 103†</td>
<td>592 ± 32†</td>
<td>1675 ± 405†</td>
<td>1261 ± 316†</td>
<td>5274 ± 532†</td>
<td>2178 ± 177</td>
</tr>
<tr>
<td>Naringin</td>
<td>1363 ± 674</td>
<td>2993 ± 271†</td>
<td>848 ± 395</td>
<td>3107 ± 581</td>
<td>3174 ± 736</td>
<td>6742 ± 1192</td>
<td>2609 ± 350</td>
</tr>
<tr>
<td>Taxifolin</td>
<td>329 ± 49†</td>
<td>1951 ± 660†</td>
<td>885 ± 76†</td>
<td>938 ± 305†</td>
<td>1692 ± 227†</td>
<td>9134 ± 628</td>
<td>2845 ± 294</td>
</tr>
<tr>
<td>trans-Benzylideneacetonaphone</td>
<td>1168 ± 122</td>
<td>3457 ± 555†</td>
<td>655 ± 191†</td>
<td>3387 ± 477</td>
<td>3421 ± 409</td>
<td>5189 ± 98†</td>
<td>2477 ± 206</td>
</tr>
</tbody>
</table>

*Gene expression was measured as the β-galactosidase activity of the lacZ gene fusions expressed in Miller units.
†Expression of aceA (isocitrate lyase) was used as a QS-independent control.
§Significant at P<0.01.

The effect of naringenin. As shown in Fig. 6(e), the ΔPA3476 mutant was unable to produce pyocyanin unless C4-HSL was exogenously supplied (while 3-oxo-C12-HSL had no effect). Similarly to strain ΔPA1432, when naringenin was added to C4-HSL-induced ΔPA3476 cells, the level of pyocyanin production was lower than that observed when C4-HSL alone was added (Fig. 6e; +Nar). Adding naringenin to both strains had no effect on their capacity to produce pyocyanin when the appropriate autoinducer was added to the growth medium (Fig. 6d, f; +Nin), further highlighting that a steric non-hindrance of the A-ring is important for the activity of naringenin. For comparison, pyocyanin production in the ΔlasR (ΔPA1430) and ΔrhlR (ΔPA3477) mutants grown under the various conditions tested was also quantified, and only basal pyocyanin levels were detected (data not shown). As shown in Fig. 6(a, c, e), adding naringenin to PAO1 cells reduced pyocyanin production to levels between those detected in the ΔlasI and ΔrhlI mutant strains, demonstrating the potency of naringenin as an efficient QS inhibitor. In addition, pyocyanin and elastase production (Figs 2 and 3) was reduced to a greater extent by naringenin (80–90 and 50% reduction, respectively) than by catechin (50 and 30% reduction, respectively) (Vandeputte et al., 2010). The stronger effect of naringenin was also obvious in the expression of QS-related genes (Table 2). Indeed, naringenin clearly had a greater effect on these genes after 18 h than catechin or salicylic acid, while all molecules affected QS gene expression to the same extent after 8 h (Vandeputte et al., 2010).

**Naringenin interferes with the perception of AHLs by P. aeruginosa PAO1**

Since the addition of AHLs to naringenin-treated P. aeruginosa PAO1 did not completely restore its capacity to produce pyocyanin (Fig. 6), we determined whether the LasR and RhlR transcription factors were impaired in their capacity to perceive their respective QS signals (3-oxo-C12-HSL or C4-HLS). This was achieved by using E. coli biosensors carrying the pAL plasmids (Lindsay & Ahmer, 2005). These constructs allow the determination of whether the perception of 3-oxo-C12-HSL by LasR (pAL105) or C4-HSL by RhlR (pAL101) is affected. These biosensor strains were grown overnight and induced by adding the appropriate molecule directly to the cultures at various concentrations (0, 1, 10 and 100 μM). As shown in Fig. 7, adding increasing concentrations of C4-HSL to the pAL101 biosensor strain induced the expression of the lux operon and the consequent production of luminescence (while only background levels of luminescence were detected in the control pAL102 strain, which lacks the rhlR gene; data not shown). Naringenin and naringin were added to these biosensor strains at a concentration of 2 mM (final), a concentration lower than that used for PAO1 cells because of the toxicity of naringenin towards E. coli (data not shown). As shown in Fig. 7, the pAL101 biosensor strain produced less luminescence when naringenin was added to the growth medium as compared with DMSO- and naringenin-treated biosensor cells, indicating that the functioning of the RhlR protein is impaired. No significant difference was observed with the pAL105 biosensor strain, suggesting that the LasR protein is less or not affected (data not shown).

**DISCUSSION**

In order to cope with bacterial infections, the search for QS quenchers or inhibitors is an emerging strategy aimed at developing new compounds to attenuate or abolish the...
Naringenin inhibits quorum sensing in P. aeruginosa

Fig. 4. Analysis of the expression of the QS-regulated genes lasA (protease), lasB (elastase), phzA1 (pyocyanin) and rhlA (rhamnolipids) in P. aeruginosa PAO1 treated with DMSO or 4 mM naringenin. (a) Agarose gels of RT-PCRs stained with SYBR Gold. (b) Relative expression of the lasA, lasB, phzA1 and rhlA genes under control conditions (DMSO) and following addition of naringenin (Nar). Intensities of PCR products after SYBR Gold staining were quantified using ImageQuant TL software and normalized against the values obtained for 16S rRNA amplification. Normalized values were used to calculate the gene expression ratios between expression in DMSO-treated P. aeruginosa PAO1 and naringenin-treated P. aeruginosa PAO1. (c) Growth kinetics of P. aeruginosa PAO1 in the presence of DMSO (x) or 4 mM naringenin (○). PAO1 cell viability was assessed after 18 h using SYTO-9 and PI. All flavonoids were used at 4 mM. The statistical significance of each test (n = 3) was evaluated by Student's t test (i.e. each test was compared with the DMSO condition), and a P value of \( < 0.01 \) was considered significant.

production of virulence factors by pathogenic bacteria (Bjarnsholt et al., 2010). Various types of screening have been carried out to find candidate molecules, for example: (i) in libraries resulting from combinatorial chemistry or containing AHL analogues (Ishida et al., 2007; Janssens et al., 2008; Mühl et al., 2006); (ii) via virtual screening based on the structure of the LuxR homologues bound to their cognate signal molecules (Persson et al., 2005; Taha et al., 2006; Yang et al., 2009; Zeng et al., 2008); or (iii) in eukaryotes, including marine organisms (Givskov et al., 1996; Hentzer et al., 2002; Skindersoe et al., 2008) and plants (Adonizio et al., 2008; Bjarnsholt et al., 2005; Gao et al., 2003; Keshavan et al., 2005; Rasmussen et al., 2005; Teplitski et al., 2011).

We recently showed that extracts from Combretum albiflorum, a plant native to Madagascar and belonging to the family Combretaceae, negatively affect QS mechanisms in C. violaceum and P. aeruginosa PAO1 (Vandeputte et al., 2010). Catechin was identified as one of the flavonoids present in Combretum bark extract that reduces the production of QS-controlled virulence factors in P. aeruginosa PAO1 but, based on spectral characteristics, other active fractions were shown to contain flavonoid-like structures. Consequently, several commercially available flavonoids were screened here for their capacity to interfere with QS mechanisms in P. aeruginosa PAO1. Flavonoids derive from the phenylpropanoid pathway and are found ubiquitously in plants, where they fulfil different ecological and physiological functions (Buer et al., 2008; Dixon & Pasinetti, 2010). Flavonoids are also known for their useful medicinal properties, and have been shown to possess anti-inflammatory, cytotoxic antitumour, antiallergic, antioxidant and antimicrobial activities, among others (Crespo et al., 2008; Cushnie & Lamb, 2005; Lee et al., 2009; Singh et al., 2008; Williams et al., 2004). As antimicrobials, some flavonoids have been shown to inhibit gyrase activity, nucleic acid synthesis, type-IV topoisomerase, cytoplasmic membrane function and energy metabolism (reviewed by Cushnie & Lamb, 2005). Flavonoids are also known for their implication in cell-to-cell communication mechanisms involved in the establishment of the symbiosis between rhizobia bacteria and their respective legume hosts (Buer et al., 2010; Subramanian et al., 2007; Zhang et al., 2009).

From the data presented here and elsewhere (Vikram et al., 2010; Zeng et al., 2008), an additional function might be ascribed to certain groups of flavonoids as inhibitors of QS mechanisms. Indeed, the flavone baicalein has been shown to inhibit biofilm formation, which is QS dependent (Davies et al., 1998), in P. aeruginosa PAO1 (at micromolar concentrations) as well as to promote the proteolysis of the Agrobacterium tumefaciens QS-signal receptor TraR in E. coli cells at millimolar concentrations (Zeng et al., 2008). More recently, several flavonoids commonly found in Citrus plants were screened for their ability to interfere with QS-dependent bioluminescence mechanisms and biofilm formation (Vikram et al., 2010). Those authors
showed that naringenin, among other flavonoids, reduces
the induction of bioluminescence by the QS signals HAI-1 and
AI-2 in *Vibrio harveyi* reporter strains as well as the
production of biofilm by *V. harveyi* BB120 and *E. coli*
0157 : H7. It was also shown that the expression of three type
III secretion system genes, suggested to be controlled by cell-
to-cell signalling, is downregulated by naringenin (Vikram
et al., 2010); the mechanism(s) by which naringenin affects
QS in these biological systems was, however, not investi-
gated. From the present screening, naringenin also emerges
as a potential inhibitor of QS mechanisms in *P. aeruginosa*
PAO1, and we set out to better understand the effect of
naringenin on the regulation of these mechanisms. The data
collected here suggest that the action of naringenin most
probably results from a combination of the reduction of the
production of both AHL molecules (which could be the
major effect, and which is corroborated by the down-
regulation of the expression of the *lasI* and *rhlI* genes) and
of the capacity of the LuxR-type transcription factors to
perceive their cognate molecules, with a consequent
reduction of the expression of QS-related genes. It is widely
known that *lasI* and *rhlI* mutants deficient in AHL synthesis
are indeed impaired in their capacity to express a wide range
of so-called QS genes, among which are *lasB* (encoding LasB
elastase), *rhlA* (encoding the first protein involved in the
production of rhamnolipids) and the phz operon involved in
the production of pyocyanin (Brint & Ohman, 1995;
Gambello & Iglewski, 1991; Gambello et al., 1993; Latifi
et al., 1995; Pearson et al., 1997; Pesci et al., 1997; Schuster
et al., 2003; Toder et al., 1994; Wagner et al., 2004).

Flavonoids are a large class of phenylpropanoid-derived
plant metabolites that are classified according to the degree
of oxidation of their C-ring and whose structural diversity
results from substitutions of their carbon skeleton through
hydroxylation, glycosylation, methylation, acylation and
prenylation (Buer et al., 2010; Dixon & Steele, 1999; Dixon
& Pasinetti, 2010). Although all these molecules are
structurally similar, the selected flavanones (eriodictyol,
naringenin, taxifolin) demonstrated a strong QS-inhibition
activity, while, at the same concentration, the three flavonols
(kaempferol, myricetin, quercetin) and the two flavones
(apigenin, luteolin) showed no QS modulation, but a
bactericidal or bacteriostatic activity, as already reported
for other bacteria (Ulanowska et al., 2006; Xu & Lee, 2001).
This highlights the possible involvement of the hydroxyl
substitutions and/or the double bond in the central pyran C-
ring in the opposing activities (bacteriostatic/bactericidal
versus anti-QS) of these flavonoids. In the case of naringenin,
the hydroxyl in position 7 seems important for the anti-QS
activity, since naringin (a glycoside of naringenin in which
the hydroxyl group at position 7 is substituted by an
hydroxylation, methoxylation and glycosylation status of
flavonoids is well known to affect many of their biological
activities, underlining the importance of the relationship
between the precise structure and their biological properties
(Halbwirth, 2010; Jiang et al., 2010; Liu et al., 2010; Pourcel
et al., 2007; Shimada et al., 2010).

Finally, a more detailed investigation of the importance of
the various radicals and rings required for the anti-QS
activity of flavanones via quantitative structure–activity

Fig. 5. Naringenin, but not naringin, inhibits the
production of the AHLs 3-oxo-C12-HSL and
C4-HSL by *P. aeruginosa* PAO1. (a, b)
Quantification of 3-oxo-C12-HSL (3OC12-
HSL) produced by PAO1 cells in the presence
of DMSO, naringenin (Nar) or naringin (Nin)
after 8 h (a) and 18 h (b) of growth. (c, d)
Quantification of C4-HSL produced by PAO1
cells in the presence of DMSO, naringenin (Nar)
or naringin (Nin) after 8 h (c) and 18 h (d) of
growth. AHLs were extracted and quantified by
MS as described in Methods. All flavanones
were used at 4 mM. DMSO-treated cultures
were used as controls and the statistical
significance of each test (*n = 6*) was evaluated
by Student’s *t*-test (i.e. each test was compared
with the DMSO condition), and a *P* value of
*≤ 0.01* was considered significant. Asterisks
indicate data that are statistically different.
Naringenin inhibits quorum sensing in *P. aeruginosa*

**Fig. 6.** Effect of naringenin and naringin on the production of pyocyanin after exogenous supply of AHLs to wild-type and mutant strains of *P. aeruginosa*. Production of pyocyanin by the wild-type strain PAO1 (a, b), the ΔlasI (ΔPA1432, mutant ID 11174) (c, d) and the ΔrhlI (ΔPA3476, mutant ID 32454) (e, f) mutant strains was quantified as described in Methods and is expressed as the ratio between $A_{380}$ and $OD_{600}$. In each case, bacteria were incubated with DMSO, naringenin (Nar), naringin (Nin) or the appropriate AHL. Bacteria were also induced with the appropriate AHL and simultaneously treated with naringenin (+Nar) or naringin (+Nin). C4-HSL and 3-oxo-C12-HSL (3OC12-HSL) were added at 10 μM final concentration. DMSO-treated cultures were used as controls, the statistical significance of each test ($n=4$) was evaluated by conducting one-way ANOVA with Tukey's multiple comparison tests, and a $P$ value of $\leq 0.01$ was considered significant. All flavanones were used at 4 mM. Different letters (a, b, c, ab) above the bars indicate data that are statistically different from each other according to the one-way ANOVA with Tukey's multiple comparison test ($P \leq 0.01$).

relationship (QSAR) analysis might shed light on the chemical positions that can be modified in flavonoids that have anti-QS activities at high concentrations only, to confer anti-QS activity upon antimicrobial flavonoids or to modify promising QS inhibitors to obtain more potent compounds. This QSAR analysis could also serve for the rational design of anti-QS compounds through combinatorial chemistry. In addition, by using their natural
Fig. 7. Naringenin affects the functioning of the RhlR–C4-HSL complex. The effect of naringenin and naringin on the pAL101 E. coli biosensor was assessed by adding increasing concentrations of C4-HSL (0, 1, 10 and 100 μM) plus DMSO, naringenin (Nar) or naringin (Nin) to overnight cultures for 2 h, and samples were taken to record the luminescence of the culture in relative light units (RLU) and measure OD_{600}. The pAL102 E. coli biosensor was used for background measurements. All flavonoids were used at 2 mM. DMSO-treated cultures were used as controls, the statistical significance of each test (n=6) was evaluated by Student’s t test (i.e. each test was compared with the DMSO condition), and a P value of ≤0.01 was considered significant. Asterisks indicate data that are statistically different.

chemical diversity and versatility, flavonoids and flavanones in particular might become important lead compounds for the development of anti-QS compounds that could be used at lower concentrations.

ACKNOWLEDGEMENTS

O. M. V. and M. B. are respectively Post-doctoral Researcher and Senior Research Associate of the F.R.S.-F.N.R.S. (Fonds de la Recherche Scientifique, Belgium). S. R. is indebted to the A.U.F. (Agence Universitaire de la Francophonie, Madagascar - Belgium), the C.U.D. (Commission Universitaire pour le Développement, Belgium) and the Fondation David et Alice Van Buuren (Belgium) for pre-doctoral fellowships. M. K. is indebted to the C.U.D. (C.U.I. Université de Ouagadougou, Burkina Faso) for post-doctoral fellowships. This research is supported by the C.U.D. (program PIC 2009, Madagascar). We would like to thank Professor Barbara Iglewski, Rochester University (School of Medicine and Dentistry), USA, for kindly providing plasmids pPCS223, pPCS1001, pLPR1 and pPCS1002; Professor Junichi Kato, Hiroshima University (Department of Molecular Biotechnology), Japan, for kindly providing plasmids pQFS0, pJ01 and pJ02; and Professor Helmut Görisch, Technische Universität Berlin (Fachgebiet Technische Biochemie, Institut für Biotechnologie), Germany, for providing plasmid pTB4124.

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