Rice and bean AHL-mimic quorum-sensing signals specifically interfere with the capacity to form biofilms by plant-associated bacteria.

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Abstract

Many bacteria regulate their gene expression in response to changes in their population density in a process called quorum sensing (QS), which involves communication between cells mediated by small diffusible signal molecules termed autoinducers. N-acyl-homoserine-lactones (AHLs) are the most common autoinducers in proteobacteria. QS-regulated genes are involved in complex interactions between bacteria of the same or different species and even with some eukaryotic organisms. Eukaryotes, including plants, can interfere with bacterial QS systems by synthesizing molecules that interfere with bacterial QS systems.

In this work, the presence of AHL-mimic QS molecules in diverse *Oryza sativa* (rice) and *Phaseolus vulgaris* (bean) plant-samples were detected employing three biosensor strains. A more intensive analysis using biosensors carrying the lactonase enzyme showed that bean and rice seed-extract contain molecules that lack the typical lactone ring of AHLs. Interestingly, these molecules specifically alter the QS-regulated biofilm formation of two plant-associated bacteria, *Sinorhizobium fredii* SMH12 and *Pantoea ananatis* AMG501, suggesting that plants are able to enhance or to inhibit the bacterial QS systems depending on the bacterial strain. Further studies would contribute to a better understanding of plant-bacteria relationships at the molecular level.

Keywords: AHL mimic; biosensor; lactonase; quorum sensing; quorum quenching; biofilm.

1. Introduction

Many Gram-positive and Gram-negative bacterial species sense their population density through a cell-to-cell communication system in which the expression of target genes is induced when cell density reaches a threshold in a process called quorum sensing (QS) (Fuqua et al., 1994). This coordinated gene expression is mediated by the production, release and detection of small signal molecules termed autoinducers (AI). N-acyl homoserine lactones (AHL) are the most widespread AI in proteobacterium QS systems. These QS systems are usually mediated by two proteins that belong to the LuxI-LuxR protein families. LuxI-type proteins synthesize AHLs that interact with LuxR-type proteins and once a threshold in AHL concentration is reached, this LuxR-AHL complex can then bind to target promoters, regulating the expression of QS-regulated genes (Miller and Bassler, 2001).

Genes under QS control modulate a broad variety of phenotypes, such as toxin production, biofilm formation, exopolysaccharide production, virulence, plasmid transfer, and motility, which are essential for the successful establishment of a symbiotic or pathogenic relationship with eukaryotic hosts (Marketon et al., 2003; Ohtani et al., 2002; Quiñones et al., 2005; Rice et al., 2005; Rinaudi and Giordano, 2010). In plant-associated bacteria, including species belonging to the genera *Agrobacterium*, *Rhizobium*, *Sinorhizobium*, *Pantoea*, *Erwinia*, *Pseudomonas* and *Xanthomonas*, QS coordinates the expression of genes involved in virulence, colonization and symbiosis (Cha et al., 1998).

Biofilm formation allows soil bacteria to colonize surrounding habitat, and to survive common environmental stresses such as desiccation and nutrient limitation. Biofilms are defined as bacterial communities surrounded by a self-produced polymeric matrix and is reversibly attached to an inert or a biotic surface (Costerton et al., 1995). After attachment to the surface, the bacteria multiply and the communities acquire a three-dimensional structure, in some cases permeated by channels, which act as the biofilm circulatory system (Costerton

et al., 1995; Stanley and Lazazzera, 2004). Biofilm formation is a highly regulated process in which bacterial surface components, especially exopolysaccharides, flagella, and lipopolysaccharides, in combination with the presence of bacterial QS signals, play an essential role in this process (Rinaudi and Giordano, 2010).

Rhizobia, soil bacteria that fix nitrogen in symbiosis with legumes, have been described as forming microcolonies or biofilms when they colonize legume roots in a process regulated by QS systems. This structure is mainly composed of water and bacterial cells. The three-dimensional structure of the biofilm is due to an extracellular matrix, which is formed by exopolysaccharides (EPS) (Sutherland, 2001), and Nod factors (in the case of *Sinorhizobium meliloti*) (Fujishige *et al.*, 2008). In bacteria belonging to the genus *Pantoea*, the QS systems govern biosynthesis of EPS, bacterial adhesion, biofilm development and host colonization (Koutsoudis et al., 2006; Morohoshi et al., 2007). Therefore, the QS-regulated biofilm mode of life, besides being crucial for bacterial survival, it is also important for a successfully colonization of the host root in plant-associated bacteria.

Eukaryotes, including plants, produce different molecules able to interfere with bacterial QS systems (Gao et al., 2003). These molecular signals, called AHL mimics, imitate AHL activities and interact with bacterial QS systems to inhibit or enhance the phenotypes regulated by this system, including the biofilm formation (Bauer and Mathesius, 2004; Teplitski et al., 2000; Zhang, 2003). The best and first characterized AHL mimics were halogenated furanones from the red alga *Delisea pulchra* (Manefield et al., 1999), which promote the degradation of the AHL-LuxR complex and therefore inhibiting the QS system that regulates swarming motility in *Serratia liquefaciens* (Manefield et al., 2002). Secretion of AHL-mimic molecules has also been reported in a variety of higher plants. In *Medicago sativa* (alfalfa), L-canavanine, an arginine analogue, inhibits EPS production in *Sinorhizobium meliloti*, a process regulated by QS (Keshavan et al., 2005). *Oryza sativa* (rice) plants secrete

AHL-mimic molecules that can activate different QS biosensors. These molecules are extremely sensitive to the lactonase enzyme but their biological origin is not clear (Degrassi et al., 2007). In the case of *Pisum sativum* (pea), plant seedlings and their exudates contain compounds that inhibit violacein production, a QS-regulated pigment of *Chromobacterium violaceum* (Teplitski et al., 2000). In *Medicago truncatula*, Gao et al. (2003) described the presence of almost 20 compounds in seeds and seed exudates able to inhibit or activate several LuxR-type biosensors. Nevertheless, the precise structure of QS-interfering molecules in rice, pea and *Medicago truncatula* is currently unknown. Recently, the flavonoid flavan-3-ol catechin exuded by *Combretum albiflorum*, has been described as one of the molecules responsible for the inhibition of the production of virulence factors regulated by QS in *Pseudomonas aeruginosa* PAO1 (Vandeputte et al., 2010).

The aim of this work was to study the production by *O. sativa* (rice) and *P. vulgaris* (bean) plants of molecules that mimic bacterial AHL activity and to determine their chemical structure and their involvement in a known QS-regulated process such as biofilm formation using two different bacteria, *Sinorhizobium fredii* SMH12, a broad host-range rhizobial species, and *Pantoea ananatis* AMG501, a plant growth promoting rhizobacterium isolated from rice paddy fields. Results obtained demonstrate that both plants produce compounds that did not have the typical lactone ring and therefore must be considered as non-AHL-type molecules. Interestingly, the biofilm formation was specifically altered depending on the type of bacterium in the presence of rice and bean AHL-mimic QS signals suggesting that rice and bean AHL-mimic compounds could have a key biological function during the first steps in plant-bacterium interaction.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

The phenotypes of the reporter strains and a list of molecules that activate each biosensor are described in Table 1. *Chromobacterim violaceum* CV026, a *C. violaceum* ATCC 31532 derivative unable to synthesize C6-HSL (McClean et al., 1997), was grown at 28 ºC in Luria-Bertani (LB) medium (Sambrook et al., 1989) supplemented with kanamycin at 30 μg ml^{-1}.

Plasmid pME6863, which harbours the *aiiA* gene that codes for a lactonase of *Bacillus cereus* strain A24 (Reimmann et al., 2002), was transferred by conjugation (Simon et al., 1986) to *Escherichia coli* JM109 (pSB536) (Swift et al., 1997), *Agrobacterim tumefaciens* NT1 (pZRL4) (Cha et al., 1998), *Sinorhizobium fredii* SMH12 (Rodríguez-Navarro et al., 1996), and *Pantoea ananatis* AMG501 (this work). As control, the broad-host-range plasmid pME6000 (Maurhofer et al., 1998), without the *aiiA* gene, was introduced by conjugation in *E. coli* JM109 (pSB536) and *A. tumefaciens* NT1 (pZRL4).

E. coli and *P. ananatis* strains were grown in LB medium at 37ºC and 28ºC, respectively. When required media were supplemented with tetracycline at 10 μg ml^{-1} for *E. coli* JM109 (pSB536) and *P. ananatis* AMG501 (pME6863) or at 200 μg ml^{-1} for *E. coli* JM109 (pSB536) (pME6863) and JM109 (pSB536) (pME6000). *A. tumefaciens* NT1 (pZLR4), which carries the AHL-responsive gene *traG* fused to *lacZ*, was grown at 28ºC in a modified yeast mannitolmedium (YM-3) (3 g l^{-1} of mannitol instead of the usual 10 g l^{-1}) (Pérez-Montaño et al., 2011) supplemented with gentamycine at 30 μg ml^{-1}. The derivative strains *A. tumefaciens* NT1 (pZLR4) (pME6863) and NT1 (pZLR4) (pME6000) were grown in YM-3 supplemented with tetracycline at 10 μg ml^{-1}.

Finally, *S. fredii* SMH12 was grown at 28ºC in YM-3 supplemented with ampicillin at 200 μg ml^{-1}. In the case of *S. fredii* SMH12 (pME6863), tetracycline was used at a concentration of 10 μg ml^{-1}.

2.2. Plant material and preparation of exudates and extracts

Oryza sativa cv. Puntal (provided by "Federación de Arroceros de Sevilla", Spain) and *Phaseolus vulgaris* cv. BBL (provided by Dr. D.N. Rodriguez-Navarro from "IFAPA", Spain) seeds were sterilized by soaking for one minute in ethanol 96% and 20 (rice) or 12 (bean) minutes in commercial bleach. Then, seeds were washed repeatedly with sterile distilled water, germinated and checked for sterility and correct disinfection. Rice and bean plants were grown under controlled hydroponic conditions in Rigaud-Puppo solution (Rigaud and Puppo, 1975) supplemented with KNO_3 (0,5 g l^{-1}) as nitrogen source. The photoperiod was set to 18 h of light and 6 h of darkness. Temperature and humidity were 25ºC and 60%, respectively, in light conditions; and 22ºC and 70% in dark conditions.

To obtain root exudates, 1 g of sterile seeds were soaked in 5 ml of sterile distilled water for 16 h at 4ºC. Seeds were germinated in plates containing water-agar (0.8%) for 40 hours, at 28ºC in darkness. Seedlings were then transferred aseptically to a grid inserted into a tube containing 15 ml Rigaud-Puppo sterile solution. Plants were grown for 10 days and then root exudates were collected.

To obtain seed extracts, seeds were disinfected as described above, fast frozen in liquid nitrogen and crushed. A volume of 5 ml of methanol:water (1:1 v/v) was added *per* gram of crushed seed and the suspension was incubated in agitation for 16 h at 4ºC. Finally, the supernatant was collected after centrifugation of the suspension.

Controls for contamination of the different samples were performed spreading 100 μl of each exudate or extract on Petri dishes of TY medium (Behringer, 1974) and incubated at

28ºC. In the case of whole roots, controls were performed placing roots in the same medium and incubated at the same temperature.

2.3. Well diffusion assays and thin-layer chromatography analysis for detecting molecules that mimic AHL activities

Well diffusion assays and thin-layer chromatography analysis were carried out as previously described (Pérez-Montaño et al., 2011). *C. violaceum* CV026, *E. coli* JM109 (pSB536) and *E. coli* JM109 (pSB536) (pME6863) were grown for 24 h with shaking and *A. tumefaciens* NT1 (pZLR4) and *A. tumefaciens* NT1 (pZLR4) (pME6863) were grown for at least 48 h. A volume of 200 µl of the bacterial cultures was resuspended in 4 ml of melted LB or YM-3 supplemented with 0.8% agar. Inhibition of violacein synthesis by roots was measured in reverse CV026 bioassays by adding 30 µl of C6-HSL (5 µg ml^{-1}), an AHL that strongly activates violacein production in *C. violaceum*, to the bacterial suspension in the soft agar. In the case of *A. tumefaciens* NT1 (pZLR4), 80 µl of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal; 20 µg ml^{-1}) were added to the mixture. A volume of 200 µl of the seed exudates, root exudates or seed extracts were poured into the wells. For plant roots, the soft agar with the bacteria were poured onto the surface of culture medium, and aseptically whole roots were placed over the layer of soft agar containing the bacteria. Time and temperature of incubation depended on the biosensor strain. Images of the luminescent bacteria, *E. coli* JM109 (pSB536) and *E. coli* JM109 (pSB536) (pME6863), were acquired with a FUJIFILM LAS-3000 intensified CCD camera (Fujifilm, Japan) and analysed with the Image Reader LAS-3000 software.

To check the sensibility to different AHLs of biosensors (wild-type strains, lactonase derivatives and strains containing the plasmid without the lactonase gene) well diffusion assays were carried out adding in each well AHL standards at different concentrations.

For TLC analysis, 40 µl of each seed extract were loaded in TLC plates (HPTLC plates RP-18 $_{F254s}$ 1.13724 and 1.05559, Merck, Germany) using methanol:water (60:40 v/v) as eluent, dried and overlaid with a soft agar culture of the biosensor *A. tumefaciens* NT1 (pZLR4) or *A. tumefaciens* NT1 (pZLR4) (pME6863).

2.4. Fractioning of seed extracts

Dichloromethane extracts of rice and bean seeds were filtered through glass wool and completely dried on a rotary vacuum evaporator at room temperature. In each case, the solid residue was solubilised in 50% methanol (5-6 ml) and fractionated using solid phase extraction (SPE-C18, Teknokroma, Spain, 1000 mg/6 ml). The cartridge was washed with water (3 x 5 ml) and methanol (3 x 5 ml), and conditioned with 50% methanol (3 x 5 ml). Then, the sample was passed through the cartridge and collected (control fraction, CF) To obtain the fractions A o F, the cartridge was rinsed three times with 5 ml of 50%, 60%, 70%, 80%, 90%, and 100% methanol, , respectively. One third of each fraction was dried on a rotary vacuum evaporator and dissolved in 500 µl of water for further quantification of QS mimic molecules by bioassay in microplates and for biofilm assays.

Quantification assays of the seed extract fractions were carried out using the biosensor strains *A. tumefaciens* NT1 (pZRL4) and *A. tumefaciens* NT1 (pZRL4) (pME6863). Experiments were performed on microtiter plates U form (Deltalab S.L., Spain). First, each well was filled with 100 µl of a master mix [20 ml of YM-3 1.2% agar, 10 ml of NT1 (pZRL4) or NT1 (pZRL4) (pME6863) in exponential phase (OD_{660} = 0.4-0.6) grown in YM-3 medium and 80 µl of X-gal at 20 mg ml^{-1}]. Once solidified, 50 µl of each seed extract fraction was added to each well. A volume of 50 µl of distilled water was used as a negative control and 49 µl of distilled water and 1 µl of 3-oxo-C6-HSL at different concentrations (ranging from 1.5 µg ml^{-1} to 100 µg ml^{-1}) were used as positive controls. Immediately, absorbance at

615 nm was measured with a microtiter automatic reader Synergy HT (BioTec, USA). The microtiter plate was incubated for 24 h at 28ºC and the absorbance was measured again. Biosensor induction levels were obtained measuring the increase of absorbance at 615 nm after the incubation. For each experiment six replicates were performed in three different experiments.

2.5. HPLC-Mass spectrometry analysis

Fractions Control, and A to F were dried, dissolved in 1 mL of 50% methanol containing 0.1% formic acid and microfiltered (0.2 μm).Then, 20 μL were injected into a Perkin Elmer Series 200 HPLC system (Waltham) coupled to a 2000 QTRAP hybrid triple-quadrupole-linear trap mass spectrometer (Applied Biosystem) equipped with a Turbo Ion source used in positive ion electrospray mode (Perez-Montaño *et al.*, 2011). Chromatography was carried out on a Zorbax Eclipse XDB-C18 column (2.1 x 150 mm, 3.5 μm particle size) at room temperature with a flow rate of 250 μL min^{-1} using the following elution profile of water and methanol, both acidified with 0.1% formic acid: starting from 50% methanol, isocratic for 5 min, lineal gradient up to 90% methanol (15 min), isocratic for 5 min, and isocratic for 5 min with 50% methanol.

Mass spectrometric conditions were optimized by infusing solutions of standards dissolved in methanol (100 μg mL^{-1}) at a flow of 10-100 μL min^{-1}: C4-HSL (98.7% purity), C6-HSL (98.7% purity), C7-HSL (97.6% purity), C8-HSL (99.5% purity), C10-HSL (99.3% purity), C12-HSL (97.3% purity), C14-HSL (99.1% purity), 3-oxo-C6-HSL (99.0% purity), 3-oxo-C8-HSL (99.0% purity), 3-oxo-C10-HSL (100% purity), 3-oxo-C12-HSL (99.0% purity), 3-oxo-C14-HSL (99.0% purity), 3-OH-C12-HSL (98.1% purity), and 3-OH-C14-HSL (96.8% purity) from Sigma-Aldrich (USA). The probe capillary voltage was optimized at 5500 V. Desolvation temperature was set to 50ºC. Pressures of curtain, nebulising and turbo

spray gases were set to 35, 20 and 0 (arbitrary units), respectively. Nitrogen was used for collisionally induced dissociation (CID). Ions were scanned from *m/z* 150 to *m/z* 500 at a scan rate of 4000 Th s^{-1}.

The mass spectrometer was set to use the information-dependent acquisition (IDA) function, in particular, Multiple Reactions Monitoring (MRM): Ions were monitored at Q1 (quadrupole) and, after CID, at Q3 (linear trap). Only those compounds that generated the previously selected ions at both detectors were registered.

2.6. Biofilm assays and thin-layer chromatography analysis for quantification of the AHLs production

First step of bacterial biofilm formation is the bacterial attachment to a biotic or an abiotic surface. The bacterial attachment to an abiotic surface (polystyrene) was measured in experiments with microtiter plates. For this purpose, bacterial strains were inoculated in 5 ml of the appropriate medium and grown for 48 h at 28ºC . Then, each culture was diluted to a final OD_{600} of approximately 0.2. Diluted cultures were added to polystyrene microtiter plates U form (Deltalab S.L., Spain) and incubated for 6 days in the case of *S. fredii* SMH12 or 2 days in the case of *P. ananatis* AMG501, at 28ºC with gentle shaking (100 r.p.m.). Then, bacterial cultures were carefully removed and the plate was dried, only the attached bacteria remaining, which is indicative of biofilm formation. Once dried, the microtiter plate was soaked three times with NaCl 0.9% and dried again. A volume of 100 µl crystal violet 0.1% (in water) was added to each well and after 20 minutes, the microtiter plate was soaked three times with distilled water. Finally, the plate was dried again, 100 µl of 96% ethanol were added to each well and the absorbance at 570 nm was measured with a microtiter automatic reader Synergy HT (BioTec, USA).

In the case of biofilm assays in the presence of seed extract fractions, the procedure was similar, but 50 µl of diluted cultures (OD_{600} = 0.4) were added to each well and mixed with 50 µl of each seed extract fraction. Control biofilm assays were conduced adding 50 µl of filtered supernatant from each wild-type bacterial cultures to 50 µl of diluted cultures (OD_{600} = 0.4) of wild-type or lactonase strains. For TLC analysis, cultures previously removed from each microtiter plate were extracted with the same volume of dichloromethane, evaporated to dryness and analyzed by thin-layer chromatography as described above. *A. tumefaciens* NT1 (pZRL4) was used as biosensor strain. Six replicates were performed in three different experiments. In both cases, to check that seed extract fractions do not alter the bacterial growth, cultures were removed from the microtiter plate and mixed. Then, the absorbance at 600 nm was measured.

3. Results

3.1. Detection of AHL-mimic QS signals from rice and bean plants

To elucidate whether rice and bean plants produce AHL-mimic QS signals, seed and root exudates, seed extracts, and plant roots were tested with three biosensor strains in well diffusion assays.

In rice, AHL-mimics were detected in root exudates, seed extracts, and plant roots using *E. coli* JM109 (pSB536) as biosensor (Fig. 1A). When the biosensor *A. tumefaciens* NT1 (pZLR4) was used, these molecules were detected in seed extracts and plant roots but not in seed and root exudates. Control assays with medium containing only X-gal were carried out to check β–galactosidase activity of the plant samples and as expected, no β–galactosidase activity was detected in any samples (data not shown). In the case of the biosensor *C. violaceum* CV026, AHL-mimics were detected only in reverse assays and unexpectedly, more production of violacein was observed around the roots, which means that these AHL-mimic compounds would function as agonist of the cognate AHL of *C. violaceum* (Fig. 1A).

Assays with bean samples showed that when *E. coli* JM109 (pSB536) was used as biosensor, AHL-mimics were detected in seed exudates, seed extracts and plant roots (Fig. 1B). When *A. tumefaciens* NT1 (pZLR4) was used as biosensor, AHL-mimic molecules were detected in seed exudates and seed extracts but not in plant roots. Control assays only with X-gal verified no β–galactosidase activity in bean samples. Finally, in reverse assays with *C. violaceum* CV026, molecules that inhibited violacein production surrounding the roots were detected (Fig. 1B).

3.2. AHL-mimic QS signals from rice and bean activate biosensors expressing the lactonase enzyme.

So far, plant AHL-mimic QS signals detected are not AHL-type molecules but they are able to induce several biosensors (Gonzalez and Keshavan, 2006). To discard that the AHL-mimic QS signals from rice and bean were AHL-type molecules, biosensors based on *A. tumefaciens* NT1 (pZRL4) and *E. coli* JM109 (pSB536) unable to detect AHL molecules were constructed. Thus, plasmid pME6863 (Reimmann et al., 2002), which carries the *aiiA* gene (lactonase enzyme) from *B. cereus* A24, was transferred by conjugation to both biosensors. The lactonase enzyme hydrolyzes the ester bond of the homoserine lactone ring of acylated homoserine lactones. This enzyme is extremely potent in context of different AHLs regardless of length and substitution of the acyl chain and shows considerable residual activity against nonacyl homoserine lactones (Wang et al., 2004). Thus, the sensitivity of the new biosensors in the presence of AHL standards is strongly decreased (Fig. 2).

Diffusion assays in agar plates with rice and bean seed extracts showed that the derivative biosensor strain *A. tumefaciens* NT1 (pZRL4) (pME6863) was inducible only by seed extracts and not by AHL standards (Fig. 2). This observation was confirmed in assays with roots from both plants using *E. coli* JM109 (pSB536) and *E. coli* JM109 (pSB536) (pME6863) (Fig. 2).

In both biosensors, the introduction of pME6000 (plasmid without the *aiiA* gene) did not alter the sensitivity to AHL standards (data not shown).

3.3. Separation and quantification of AHL-mimic QS signals.

Rice and bean seed extracts were separated by TLC and developed using biosensors *A. tumefaciens* NT1 (pZLR4) and *A. tumefaciens* NT1 (pZLR4) (pME6863). Only one spot that did not migrate in the chromatography plates was detected in each seed extract using these biosensors (Fig. 3A). In addition, a strong reduction in the diameter of the spots generated by AHL standards was observed in the presence of the lactonase biosensor (Fig. 3A). Namely,

from 15 to 5 mm in the case of C8-HSL, and from 13 to 7 mm with C6-HSL, when the biosensor expressing lactonase was used in comparison with the assay using the wild type biosensor strain. However, spot diameters of seed extracts showed no reduction when the biosensor expressing the lactonase was used with respect to the original biosensor *A. tumefaciens* NT1 (pZLR4) (Fig. 3B).

Rice and bean seed extracts were loaded in a SPE-C18 column to quantify and separate the AHL-mimic QS signals. The elution was carried out with different methanol concentrations. Seven fractions were obtained for both seed extracts, corresponding to the seed extract elution through the column [control fraction (CF)], and those that correspond to the elution of methanol 50% (A), 60% (B), 70% (C), 80% (D), 90% (E), and 100% (F) through the same column which has retained seed extract molecules. This fractionation allowed a separation of the AHL-mimic QS signals according to the affinity for the different eluents. Induction bioassays with all the seven different fractions, including the control fraction, were performed to quantify the AHL-mimic QS signals present in rice (Fig. 4A) and bean (Fig. 4B) extracts using the biosensors *A. tumefaciens* NT1 (pZLR4) and *A. tumefaciens* NT1 (pZLR4) (pME6863). A standard curve with the 3-oxo-C6-HSL at concentrations ranging from one to 100 μg ml^{-1} and using *A. tumefaciens* NT1 (pZLR4) and NT1 (pZLR4) (pME6863) as biosensor strains was also carried out. As expected, the biosensor expressing lactonase was not induced at any concentration. In contrast, *A. tumefaciens* NT1 (pZLR4) was linearly induced reaching saturation at 30 μg ml^{-1} (Fig. 4C).

Induction bioassays in microplates with *A. tumefaciens* NT1 (pZLR4) and its derivative strain that expressed the lactonase enzyme showed that the A, B, E and F fractions from rice seed extracts contain molecules which significantly induce *A. tumefaciens* NT1 (pZLR4) at induction values similar to the control fraction. However, only fractions A and B induced the lactonase biosensor, suggesting that molecules present in the E and F fractions

probably possess the typical lactone ring of the AHLs. On the other hand, molecules present in the first two fractions (A and B) maintained the induction values using both biosensors, suggesting that these molecules were not AHL-type (Fig. 4A). Supporting this observation, when these A and B fractions were analyzed by mass spectrometry analysis no AHL-type molecules were detected. Interestingly, in the case of E fraction, in which the plant mimic signal is susceptible to degradation by lactonase enzyme, traces of a putative 3-oxo-C4-HSL molecule were detected.

In the case of bean seed extracts, only fraction A significantly induced the biosensors *A. tumefaciens* NT1 (pZLR4) and *A. tumefaciens* NT1 (pZLR4) (pME6863) with induction values similar to the control (Fig. 4B). In this case, the level of induction of both biosensors was 4-fold the induction observed with rice seed extract fractions (Fig. 4). A slight induction (not statistically significant) of both biosensors was observed in the B fraction. Lastly, as expected, no AHL-type molecules were detected by mass spectrometry analysis in the A fraction of seed bean extract.

3.4. AHL-mimic QS signals specifically interfere with the capacity to form biofilms by two plant-associated bacteria by altering their AHL production.

Plasmid pME6863 was transferred by conjugation to *S. fredii* SMH12 and *P. ananatis* AMG501 (hereafter referred to as SMH12 and AMG501, respectively) to determine the role of QS systems in biofilm formation. Attachment assays on poliestirene surface showed a significantly reduction (more than 60 % of reduction) in the bacterial capacity to form biofilm in the presence of the lactonase enzyme, indicating that QS systems are involved in biofilm formation in both SMH12 and AMG501 (Fig. 5A). Biofilm experiments with each wild-type and lactonase strain grown in the presence of their own filtered supernatant cultures showed no difference in bacterial biofilm attachment. These results indicate that the addition of their

own AHL molecules does not enhance the biofilm formation either in wild type strains or in lactonase strains (data not shown).

Once the role of QS systems in the bacterial surface attachment was determined, the biofilm formation by SMH12 and AMG501 in the presence of rice and bean seed extracts was assessed to study the influence of AHL-mimic QS molecules on this process. Results showed that in SMH12, only fractions A, B and the control fraction (seed extract through the fractionating column) generated a statistically significant increase (at least 4 fold more) in biofilm formation in comparison to the values obtained in control cultures just with the bacteria. These results were obtained with either bean seed extracts or rice seed extracts (Fig. 5B). Interestingly, in all the seed extract fractions that altered the SMH12 biofilm formation rice and bean AHL-mimic QS signals had been detected previously (Fig. 4A and B). In the case of AMG501, attachment assays showed a statistically significant decrease (at least 5 fold less) on biofilm formation only in A fraction and the control fraction (CF) of the bean (Fig. 5C). As expected, fractions which altered biofilm formation in AMG501 were the same fractions in which bean AHL-mimic QS signals were detected (Fig. 4B) No differences were observed when rice fractions were used with respect to the values obtained in control cultures only with AMG501. As in the case of SMH12, bean fractions that altered biofilm formation in AMG501 were the same fractions in which AHL-mimic QS signals were previously detected (Fig. 4B). The presence of rice and bean fractions did not alter bacterial growth (data not shown).

Finally, due to the relation between biofilm formation and QS systems, the production of bacterial QS signals associated with the same biofilm cultures in microtiter wells was studied to elucidate whether the fractions that altered biofilm formation in both bacteria also changed AHL production. TLC assays showed that SMH12 produced at least 3 different AHLs. No changes were detected in the number of AHLs in the presence of fractions, but it

1 was observed that rice and bean fractions that previously increased biofilm formation (A, B
2 and control fraction in rice, A and control fraction in bean) (Fig. 5B) caused an increase in the
3 overall AHL production (Fig. 6A). In the case of AMG501, the AHL profile showed at least
4 one AHL, and only the A and the control bean fractions provoked an important reduction in
5 AHL production (Fig. 6B). These bean fractions are the same that reduced the biofilm
6 formation in AMG501 (Fig. 5C). In all the cases, the rice and bean fractions with a biological
7 function (interference of biofilm formation) are those in which we found AHL-mimic QS
8 signals.
9

4. Discussion

Although the production of AHL-mimic compounds by various plants has been reported for over a decade, little progress has been made with respect to their structure and/or biological function. This work proves previous reported results and contributes to knowledge of the possible nature and function of these compounds. In this sense, AHL-mimic QS molecules were differentially detected by the biosensors in most of the analyzed samples from rice or bean plants which would imply their different natures, especially in the case of reverse assays with CV026 using whole roots. Probably, while rice roots produce molecules that stimulate violacein production (agonist molecule), bean roots could produce molecules that reduce violacein production (antagonist) (Fig. 1).

Results indicate that rice plants produce AHL-mimic QS signals mainly by roots. Hard teguments of rice seeds could restrict diffusion of these signals. Once the plant root is developed, rice could interact with bacterial populations and therefore the production of these molecules would increase. On the other hand, the production of AHL-mimic QS signals in the common bean was elevated in seeds and roots. In the case of the symbiotic association with nitrogen-fixing rhizobia, this interference could be beneficial to the plant during all life stages in order to improve rhizobial colonization. Gao et al (2003) showed that *M. truncatula* produces QS active compounds, most of them agonists, at different times during seedling development, and the secreted compounds often differ from those present inside the plant tissues. AHL-mimic molecules from the rice and bean seed extracts as well as those from the roots of both plants without the typical lactone ring of the AHL molecules, maintain their capacity to induce the biosensors with lactonase activity (Fig. 2). The TLC assays detected a hydrophobic molecule from rice seed-extracts (Fig. 3A) that suffered a reduction in the intensity of the spot when the biosensor expressing lactonase was used, which would indicate the presence of at least two inducer molecules, one sensitive and the other insensitive to the

lactonase enzyme (Fig. 3B). The presence of two kinds of molecules in rice seed extracts was also demonstrated after the fractionation of the seed extracts, using a solid phase extraction, showing that A and B fractions induced the biosensor in the presence and in the absence of lactonase (Fig. 4A). By contrast, fractions E and F only induced the biosensor in the absence of lactonase (Fig. 4B). According to these results, mass spectrometry analysis did not show the presence of AHL-type molecules in A and B rice fractions. However, an AHL molecule (a putative 3-oxo-C4-HSL) was detected in the E fraction, where molecules sensitive to the lactonase enzyme were detected previously. All these results strongly support the idea that in rice seed extracts there are at least two different molecules, one of which would not possess an AHL-type structure, and the other could be 3-oxo-C4-HSL. Degrassi et al. (2007) showed that AHL-mimic QS signals from rice were sensitive to the lactonase enzyme, but they could not identify their chemical structure and discuss the possible bacterial origin of these AHL-type molecules. The presence of several endophytic bacteria in rice plants has been reported (Tan et al., 2001). Despite the low bacterial density in plants (less than 10^7c.f.u. per gram of plant tissue) it is not possible to discard the production of AHLs by these endophytic bacterial populations (You et al., 2005). Interestingly, a recent metagenomic report shows that bacterial endophytic communities present in rice possess a remarkable number of QS systems (Sessitsch et al., 2012). In bean seed extract, only in fraction A, an AHL-mimic QS signal with a chemical structure different to AHL-type molecules was detected, meanwhile the absence of AHL molecules was confirmed by mass spectrometry analysis. A possible approach to obtain the chemical structure of these molecules would consist of a spectrometric analysis of each active fraction and the analysis of each detected molecules to find out whether or not they behave as mimic QS signals. These processes would be expensive economically and especially would they be prohibitively time-consuming. In addition we cannot expect positive results with these experiments because it might be possible that the

final effect observed would be the result of the activity of a cocktail of molecules, which is probably what is really happening in natural environments. However, our results represent the first report, to our knowledge, demonstrating the presence of AHL-mimics QS signals on beans and the presence of molecules not sensitive to lactonase on rice.

Leaving the chemical structure aside, the interference of AHL-mimic QS molecules produced by rice and bean on the capacity to form biofilms, a QS-regulated essential trait for the successful establishment of a symbiotic or pathogenic relationship with the eukaryotic hosts was studied. Two plant-associated bacteria, *S. fredii* SMH12, a broad host-range rhizobial strain and *P. ananatis* AMG501, a plant growth-promoting rhizobacterium isolated from rice paddy fields were selected. In these strains, biofilm formation was demonstrated to be regulated by QS (Fig. 5A). In SMH12, only those rice and bean fractions in which we detected no AHL-type QS signals (A and B fractions) could induce an increase in biofilm formation (Fig. 5B). This bacterium produces C8-HSL, 3-OH-C8-HSL and C14-HSL (Pérez-Montaño et al., 2011); consequently, the 3-oxo-C4-HSL (detected in rice E fraction) did not alter the biofilm formation in SMH12 (Fig. 5A).probably due to this molecule not being recognized by the LuxR-type protein of this bacteria.

Biofilm formation enhancement in *S. fredii* SMH12 by both rice and bean AHL-mimic QS signals probably would be directed to promote a better root colonization, indicating that it is recognized as a beneficial microorganism. Likewise, rice fractions did not alter the capacity to form biofilm by *P. ananatis* AMG501, suggesting that it would be recognized as a potential plant growth promoting bacterium by rice plants (Fig. 5C). Contrarily, bean seed extract fractions containing AHL-mimic QS signals would block the colonization by AMG501 through the reduction of their ability to form biofilm, thus being recognized as not- beneficial. Finally, results obtained in control biofilm experiments with supernatant that contains the

respective AHL molecules of each bacterium (data not shown), suggest that the increase of the AHL concentration is not enough for bacterial biofilm enhancement.

These results indicate that AHL-mimic QS signals could have biological functions and strongly support the idea that plants have developed mechanisms to respond to or interfere with bacterial communication using these signals for their own benefit. However, the molecular mechanisms responsible for these interferences are currently unknown; consequently, more effort is needed to answer the questions proposed and to chemically identify these molecules in order to clarify their mode of action.

Acknowledgements

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Table 1. Biosensors, phenotypes and detected molecules.

Biosensor strain	Phenotype	Detected molecules
C. violaceum CV026	Violaceine production	C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, 3-oxo-C8-HSL, others non AHL-type molecules
C. violaceum CV026 (reverse)	Violaceine inhibition	C10-HSL, 3-oxo-C10-HSL, C12-HSL, 3-oxo-C12-HSL, others non AHL-type molecules
A. tumefaciens NT1 (pZRL4)	β-galactosidase activity	C4-HSL, 3-oxo-C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, 3-oxo-C8-HSL, C10-HSL, 3-oxo-C10-HSL, C12-HSL, 3-oxo-C12-HSL, others non AHL-type molecules
E. coli JM109 (pSB536)	Bioluminiscence	C6-HSL, C8-HSL, C10-HSL, C12-HSL, C14-HSL, oxo- and hidroxi-C6/C8/C10/C12/C14-HSL derivatives, others non AHL-type molecules

The lactonase derivatives *A. tumefaciens* NT1 (pZRL4) (pME6863) and *E. coli* JM109 (pSB536) (pME6863) drastically reduce their sensitivity to AHL compounds but continue being fully activated without AHL-type molecules. The control strains *A. tumefaciens* NT1 (pZRL4) (pME6000) and *E. coli* JM109 (pSB536) (pME6000) behave like the original biosensors.

Legends to figures

Figure 1. Diffusion assays in agar plates. Biosensor strains *A. tumefaciens* NT1 (pZRL4), *E. coli* JM109 (pSB536) and *C. violaceum* CV026 (direct and reverse assays) were assayed with seed and root exudates, seed extracts, and whole roots of **A**, rice and **B**, bean plants. The same assays were performed with standard AHLs and used as controls. Arrows indicate the halos of pigmentation or luminescence produced after induction of the biosensors.

Figure 2. Diffusion assays in agar plates with biosensors expressing the enzyme lactonase. Biosensor strains *A. tumefaciens* NT1 (pZRL4), *A. tumefaciens* NT1 (pZRL4) (pME6863), *E. coli* JM109 (pSB536), and *E. coli* JM109 (pSB536) (pME6863) were assayed with seed extracts and whole roots of rice and bean plants. The same assays were performed with standard AHLs and used as controls. Arrows indicate the halos of pigmentation or luminescence produced after induction of the biosensors.

Figure 3. Thin layer chromatography. Reverse phase C18 chromatoplaques were loaded with rice and bean seeds extracts. Commercial C6-HSL and C8-HSL were used as controls. Plates were developed with the biosensor strains (**A**) *A. tumefaciens* NT1 (pZRL4) and (**B**) *A. tumefaciens* NT1 (pZRL4) (pME6863), which expresses the enzyme lactonase.

Figure 4. Biosensor induction assays in microtiter plates. Biosensor strains *A. tumefaciens* NT1 (pZRL4) (dark grey columns or squares) and *A. tumefaciens* NT1 (pZRL4) (pME6863) (light grey columns or squares) were used as bioreporters for induction of the different fractions separated by SPE-C18 columns (control fraction (CF): seed extract through the column A to F correspond to fractions eluted with 50, 60, 70, 80, 90 and 100% of methanol, respectively). The absorbance was measured at 615 nm. **A**, assays with fractions obtained

from rice seeds extracts; **B**, assays with fractions obtained from bean seeds extracts; **C**, control assay using AHL standards. Data are the mean (± standard deviation of the mean) of 6 biological replicates. Data are the mean (± standard deviation of the mean) of 6 replicates. Each β-galactosidase activity was individually compared to that obtained without added fractions by using Mann-Whitney non-parametrical test. Each column marked with * is significantly different at the level α = 5% and with ** is significantly different at the level α = 10%.

Figure 5. Biofilm assays in microtiter plates. Biofilm formation was quantified by staining with crystal violet and measuring the absorbance at 615 nm. Represented values are relative to the *S. fredii* SMH12 and *P. ananatis* AMG501 biofilm formation in YM-3 and LB media, respectively. **A.** Biofilm formation by SMH12 (white columns) and AMG501 (black columns) and their lactonase derivatives (+ pME6863). **B.** Biofilm formation by SMH12 in the presence of the different rice (light grey columns) and bean (dark grey columns) fractions [A-F and control fraction (CF): seed extract through the column]. **C.** Biofilm formation by AMG501 in the presence of the different rice (light grey columns) and bean (dark grey columns) fractions [A-F and control fraction (CF): seed extract through the column]. Data are the mean (± standard deviation of the mean) of 6 biological replicates. Each value of biofilm attachment was individually compared to that obtained in the wild type strain (SMH12 or AMG501) growth without added fractions by using Mann-Whitney non-parametrical test. Each column marked with * is significantly different at level α = 5%. In all cases the values of absorbance at 600 nm before staining was similar.

Figure 6. Thin layer chromatography from biofilm cultures. Reverse phase C18 chromatoplaques were loaded with bacterial culture extracts after growing with different rice

and bean fractions [A-F and control fraction (CF)]. Commercial C6-HSL and C8-HSL were used as controls. Plates were developed with the biosensor strain *A. tumefaciens* NT1 (pZRL4). **A.** Extracts from *S. fredii* SMH12 cultures. **B.** Extract from *P.ananatis* AMG501 cultures.

Figures.

Figure 1

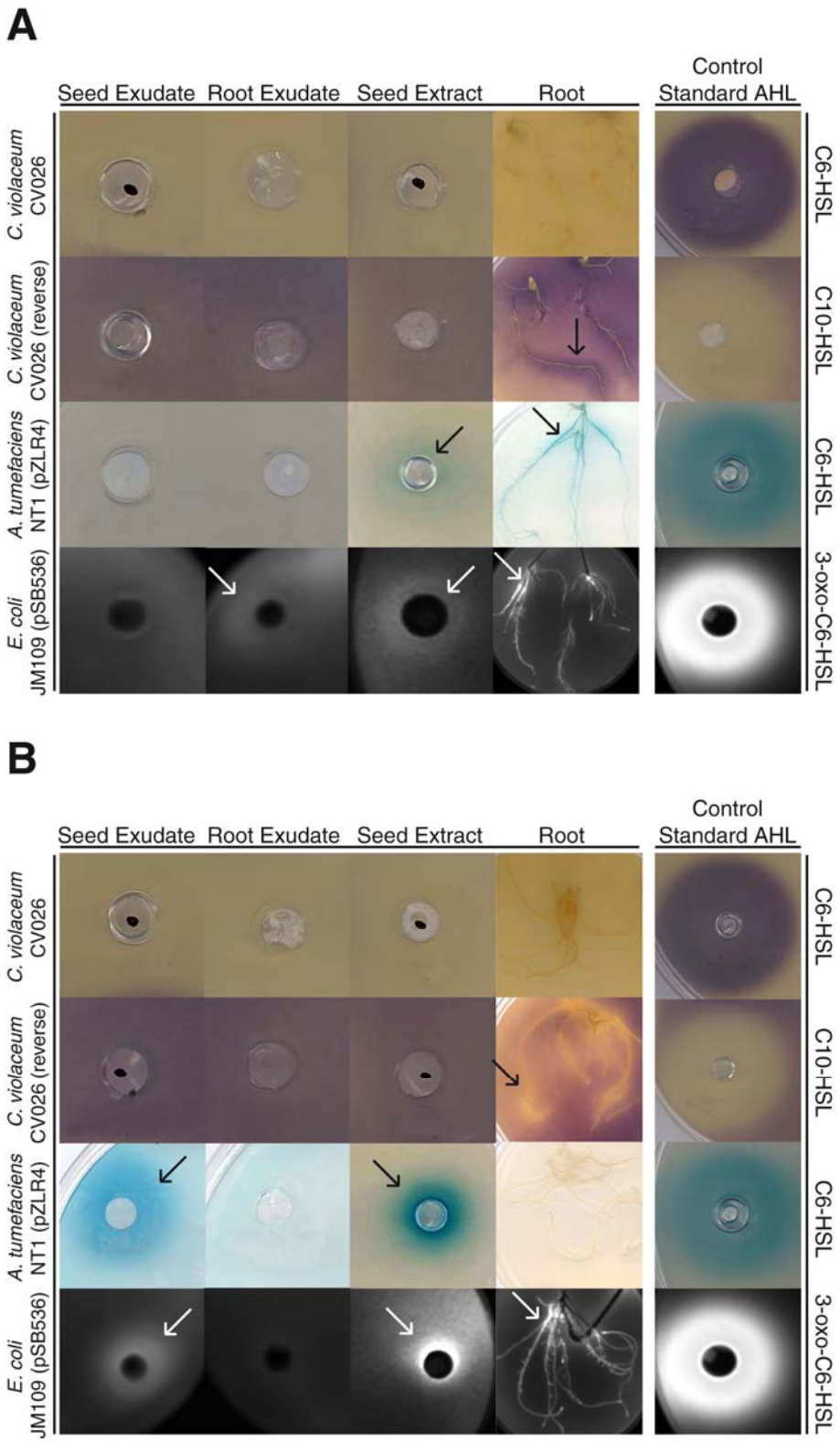


Figure 2

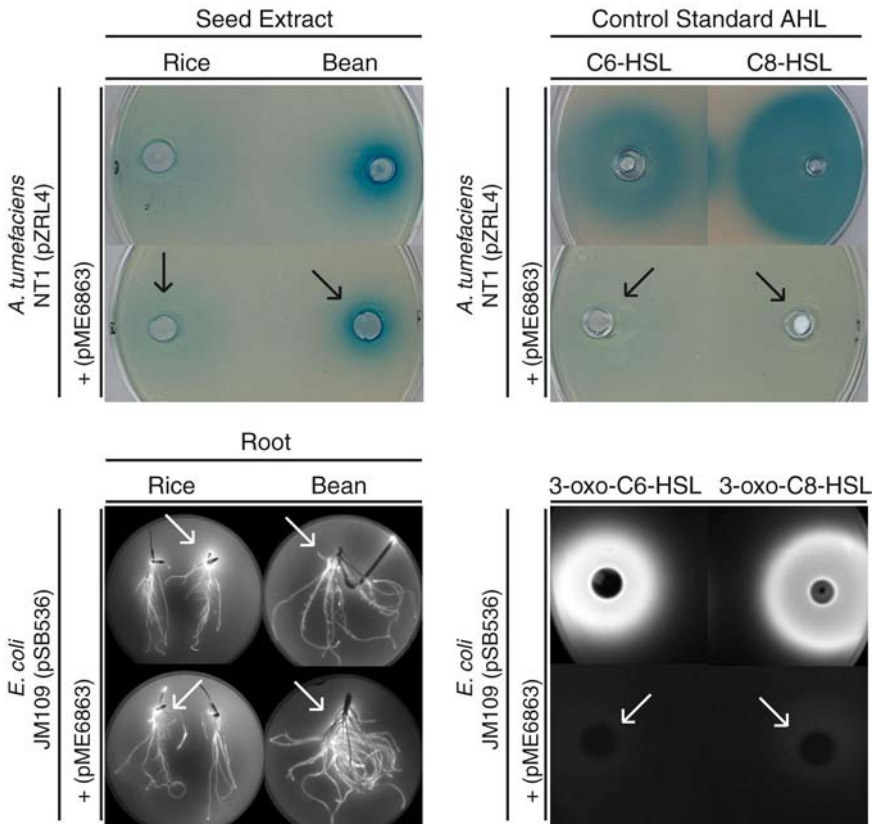

Seed Extract
Rice
Bean
Control Standard AHL
C6-HSL
C8-HSL
A. tumefaciens NT1 (pZRL4)
+ (pME6863)
Root
Rice
Bean
3-oxo-C6-HSL
3-oxo-C8-HSL
E. coli JM109 (pSB536)
+ (pME6863)

Figure 3

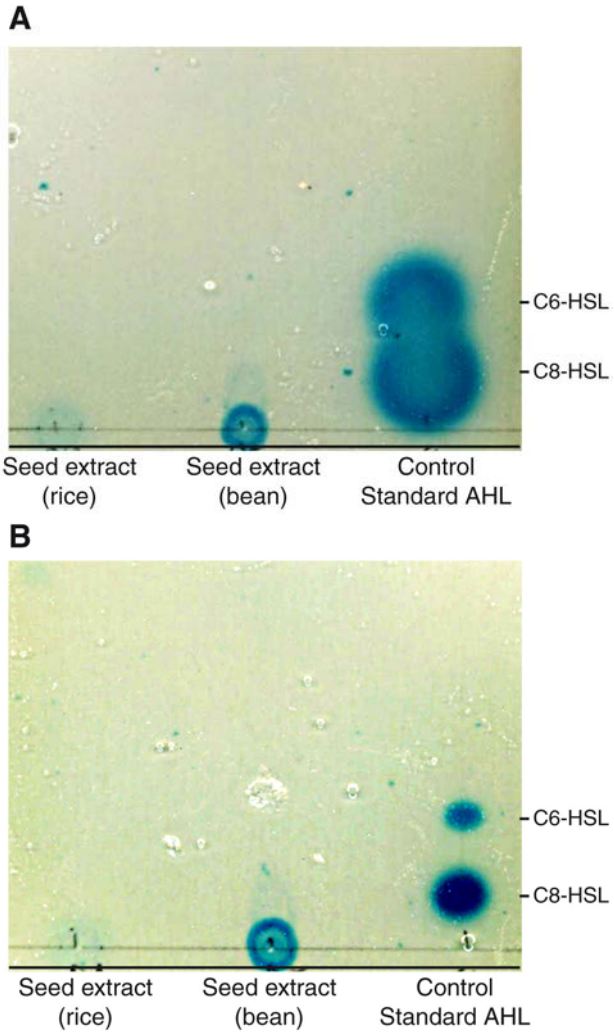

A
C6-HSL
C8-HSL
Seed extract (rice)
Seed extract (bean)
Control Standard AHL
B
C6-HSL
C8-HSL
Seed extract (rice)
Seed extract (bean)
Control Standard AHL

Figure 4

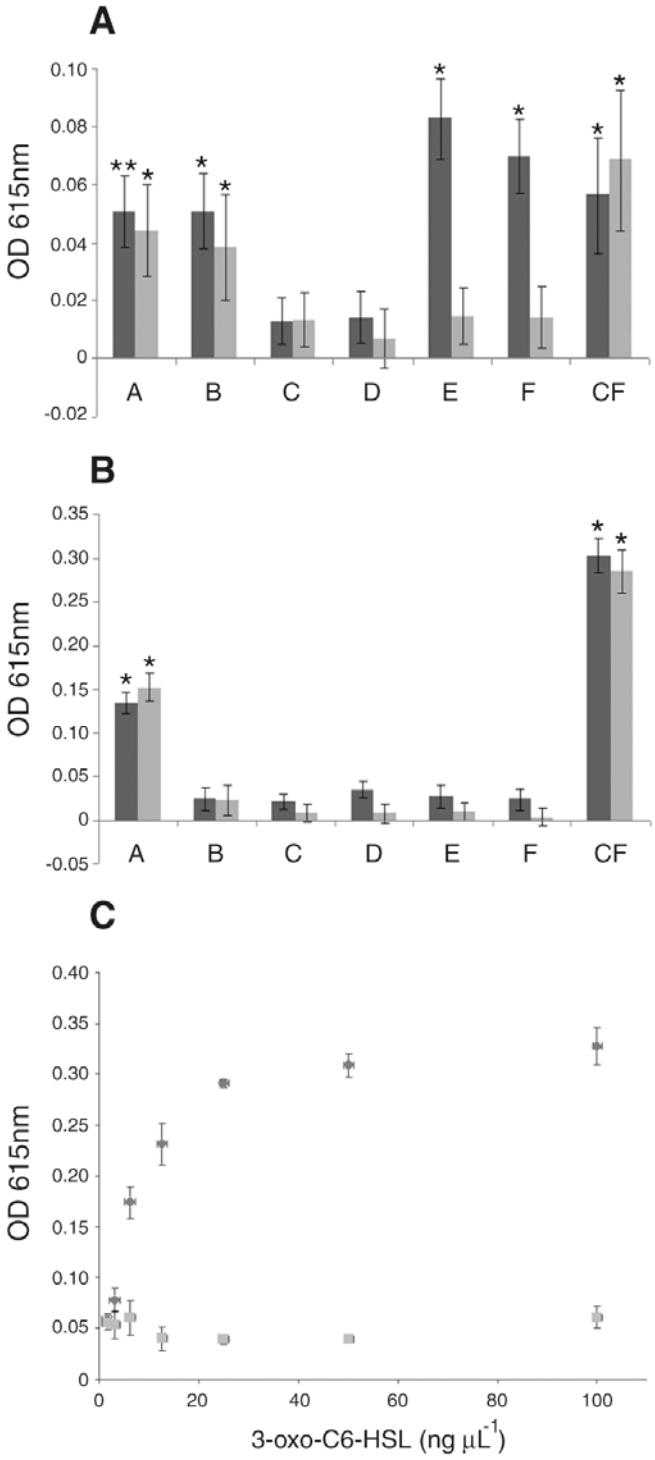

A
OD 615nm
0.10
0.08
0.06
0.04
0.02
0
-0.02
A
B
C
D
E
F
CF
B
OD 615nm
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
-0.05
A
B
C
D
E
F
CF
C
OD 615nm
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
0
20
40
60
80
100
3-oxo-C6-HSL (ng μL-1)

Figure 5

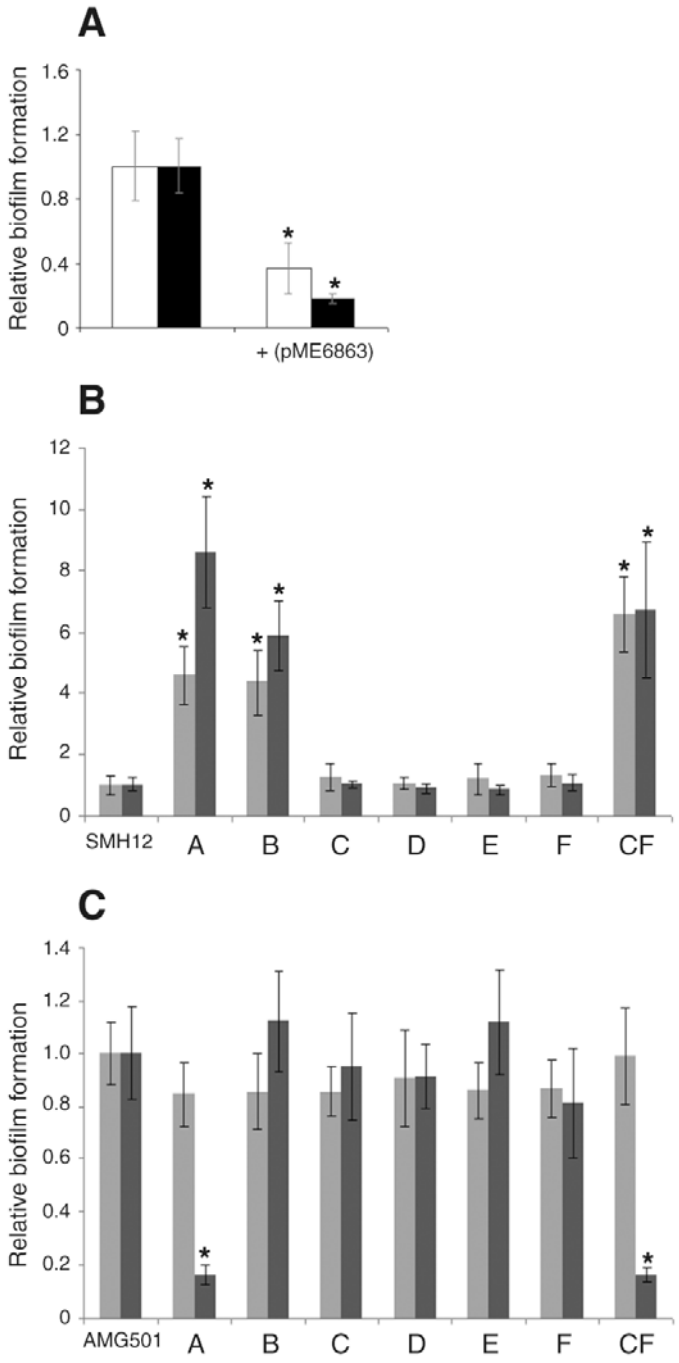
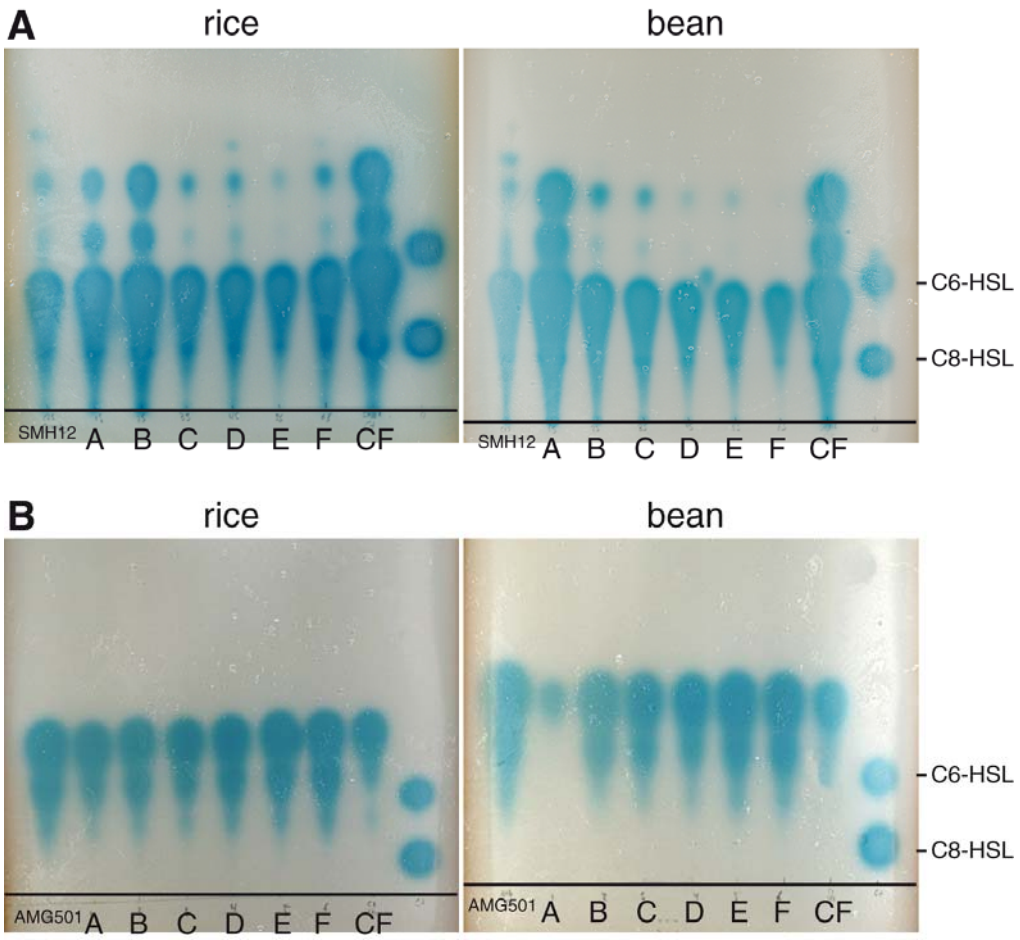

A
Relative biofilm formation
1.6
1.2
0.8
0.4
0
+ (pME6863)
B
Relative biofilm formation
12
10
8
6
4
2
0
SMH12
A
B
C
D
E
F
CF
C
Relative biofilm formation
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
AMG501
A
B
C
D
E
F
CF

Figure 6


A
rice
bean
C6-HSL
C8-HSL
SMH12 A B C D E F CF
SMH12 A B C D E F CF
B
rice
bean
C6-HSL
C8-HSL
AMG501 A B C D E F CF
AMG501 A B C D E F CF