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The plant pathogen *Pseudomonas fuscovaginae* contains two conserved quorum sensing systems involved in virulence and negatively regulated by RsaL and the novel regulator RsaM

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Summary

Pseudomonas fuscovaginae **is a Gram-negative fluorescent pseudomonad pathogenic towards several plant species. Despite its importance as a plant pathogen, no molecular studies of virulence have thus far been reported. In this study we show that *P. fuscovaginae* possesses two conserved *N*-acyl homoserine lactone (AHL) quorum sensing (QS) systems which we designated PfsI/R and PfvI/R. The PfsI/R system is homologous to the BviI/R system of *Burkholderia vietnamiensis* and produces and responds to C10-HSL and C12-HSL whereas PfvI/R is homologous to the LasI/R system of *Pseudomonas aeruginosa* and produces several long-chain 3-oxo-HSLs and responds to 3-oxo-C10-HSL and 3-oxo-C12-HSL and at high AHL concentrations can also respond to structurally different long-chain AHLs. Both systems were found to be negatively regulated by a repressor protein which was encoded by a gene located intergenically between the AHL synthase and LuxR-family response regulator. The *pfsI/R* system was regulated by a novel repressor designated RsaM while the *pfvI/R* system was regulated by both the RsaL repressor and by RsaM. The two systems are not transcriptionally hierarchically organized but share a common AHL response and both are required for plant virulence. *Pseudomonas fuscovaginae* has therefore a unique complex regulatory network composed of at least two different repressors which directly regulate the AHL QS systems and pathogenicity.**

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Introduction

Pseudomonas fuscovaginae is a Gram-negative fluorescent pseudomonad first identified and reported as a pathogen of rice (*Oryza sativa*) in Japan in 1976 (Tanii *et al*., 1976; Miyajima *et al*., 1983). Typical symptoms on mature rice plants are characterized by brown-black, water-soaked spots on the adaxial side of flag leaf sheath, with grain discoloration, poor spike emergence and sterility in severe cases (Miyajima *et al*., 1983; Zeigler and Alvarez, 1987; Duveiller *et al*., 1989). *Pseudomonas fuscovaginae* is now regarded as a plant pathogen that causes bacterial brown sheath rot on several cereals including maize (*Zea mays*), sorghum (*Sorghum bicolor*) (Duveiller *et al*., 1989) and wheat (*Triticum aestivum*) (Duveiller, 1990). *Pseudomonas fuscovaginae* belongs to the authentic rRNA group I of pseudomonads, being one of the 18 validly described *Pseudomonas* plant pathogenic species part of the oxidase positive cluster (Anzai *et al*., 2000; Hofte and De Vos, 2006).

Pseudomonas fuscovaginae produces three different types of phytotoxic metabolites, syringotoxin, fuscopeptin A (FP-A) and fuscopeptin B (FP-B) (Ballio *et al*., 1996; Flamand *et al*., 1996), that have been shown to be involved in generating the symptoms. Syringotoxin belongs to a group of anti-fungal metabolites known as lipodepsipeptides (LDPs) acting at the level of plasma membrane forming ion channels and consequently increasing membrane permeability (Hutchison and Gross, 1997; Batoko *et al*., 1998). FP-A and FP-B, equally characterized as LDPs (Ballio *et al*., 1996), display similar toxic properties to syringotoxins and are structurally related to syringopeptins produced by plant pathogenic *Pseudomonas syringae* pv. *syringae* strains (Ballio *et al*., 1991). FP-A and FP-B have the same quantitative amino

acid composition differing only for fatty acid moieties (Ballio *et al*., 1996).

Despite the importance of *P. fuscovaginae* as plant pathogen on several plant hosts around the world, no molecular studies of virulence have thus far to our knowledge been reported. In this study we investigated the role of the quorum sensing (QS) gene regulatory system in causing sheath rot by *P. fuscovaginae* in rice. QS is an intercellular communication system that couples bacterial cell density to gene expression via the production and detection of signal molecules (for reviews, see references: Bassler, 2002; Fuqua and Greenberg, 2002; Von Bodman *et al*., 2003; Lazdunski *et al*., 2004; Camilli and Bassler, 2006). In Gram-negative bacteria, *N*-acyl homoserine lactones (AHL) signal molecules are most commonly used; they are produced by an AHL synthase which in most cases belongs to the LuxI-protein family. A transcriptional regulator belonging to the LuxR family then forms a complex with the cognate AHL at threshold ('quorum') concentration and affects the transcriptional status of target genes (Fuqua *et al*., 2001). QS-dependent regulation in bacteria is most often involved in the coordinated community action of bacteria like biofilm formation, conjugation, bioluminescence, production of extracellular enzymes, virulence factors and pigment formation (Whitehead *et al*., 2001; Bassler, 2002; Fuqua and Greenberg, 2002; Von Bodman *et al*., 2003). Well-characterized examples of QS-dependent regulation include extracellular enzyme production in *Erwinia carotovora*, conjugation in *Agrobacterium tumefaciens* and toxin production in *Burkholderia glumae* (Von Bodman *et al*., 2003; Kim *et al*., 2004).

In this study we report the identification and characterization of AHL QS of the rice sheath rot pathogen *P. fuscovaginae*. We studied 11 *P. fuscovaginae* strains isolated from diseased rice from various parts of the world for AHL production. These strains were shown to possess two conserved AHL QS systems, designated PfvI/R, which is homologous to the LasI/R and PpuI/R systems of *Pseudomonas aeruginosa* and *P. putida*, respectively, and PfsI/R which is homologous to the BviI/R system of *Burkholderia vietnamiensis*. The two systems were found to be regulated by a repressor protein located intergenically between the AHL synthase and LuxR-family response regulator. PfvI/R was negatively regulated by the RsaL repressor whereas PfsI/R by a novel repressor designated RsaM. The two regulatory systems are not transcriptionally hierarchically organized and are both important for rice sheath rot in rice and in virulence of *Chenopodium quinoa*. This is the first report of a molecular study of virulence in this important plant pathogen and of the novel RsaM repressor of AHL QS systems.

Results

Pseudomonas fuscovaginae *possesses two AHL QS systems*

It was established that 11 *P. fuscovaginae* strains isolated from various parts of the world all produced a similar set of several AHL molecules (data not shown). All strains however produced very little quantities of AHLs since in order to detect them on TLC plates using three different AHL bacterial biosensors (which can detect a wide range of structurally different AHLs), purification from large volumes (100 ml) of spent supernatants had to be performed (see *Experimental procedures*). Using synthetic AHLs as markers in TLC analysis it was postulated that *P. fuscovaginae* synthesized a large number of AHLs including 3-oxo-C12-HSL, 3-oxo-C10-HSL, C12-HSL and C10-HSL (see below). In order to unequivocally determine the AHLs produced by *P. fuscovaginae* UPB0736 we used C_{18} reverse-phase high-performance liquid chromatography (HPLC) and mass spectrometry (MS) and were able to clearly identity the production of 3-oxo-C12-HSL, 3-oxo-C10-HSL, C12-HSL and C10-HSL (Fig. S3). It was therefore concluded that *P. fuscovaginae* under the growth conditions we used produced small quantities of four types of long-chain AHL and it was hypothesized that the AHL QS system(s) were themselves regulated. In order to identify the AHL QS system(s) of *P. fuscovaginae*, a cosmid gene bank of *P. fuscovaginae* UPB0736 was constructed and conjugated *en masse* in an AHL biosensor. Using this approach, two different cosmids, designated pCOS14 and pCOS16, were isolated which could induce *gfp* expression in the AHL biosensor *P. putida* F117 (pKRC12) which is based on LasR. Cosmid pCOS14 contained an AHL QS system consisting of a *luxI* homologue designated *pfvI*, and a *luxR* homologue designated *pfvR* (Fig. 1A). The PfvI/R system displays significant homology to the LasI/R QS system of *P. aeruginosa* (Passador *et al*., 1993) having 57% similarity with LasI and 40% with LasR and to the PpuI/R system of *P. putida* (Bertani and Venturi, 2004) having 63% similarity with PpuI and 45% with PpuR. In addition, similarly to the *lasI/R* and *ppuI/R* systems, it contained the *rsaL* repressor located intergenically between the *luxI* and *luxR* homologue (Fig. 1A). Cosmid pCOS16 also contained an AHL QS system consisting of a *luxI* homologue designated *pfsI*, and a *luxR* homologue designated *pfsR* (Fig. 1B). The PfsI/R system displayed significant homology to the BviI/R QS system of *B. vietnamiensis* (Conway and Greenberg, 2002; Malott and Sokol, 2007) having 77% similarity with BviI and 75% with BviR. It was therefore determined that *P. fuscovaginae* UPB0736 contained at least two AHL QS systems.

In order to establish if the *pfvI/R* and *pfsI/R* systems are conserved in other *P. fuscovaginae* isolates, Southern analysis under high-stringency conditions using *pfvI* and

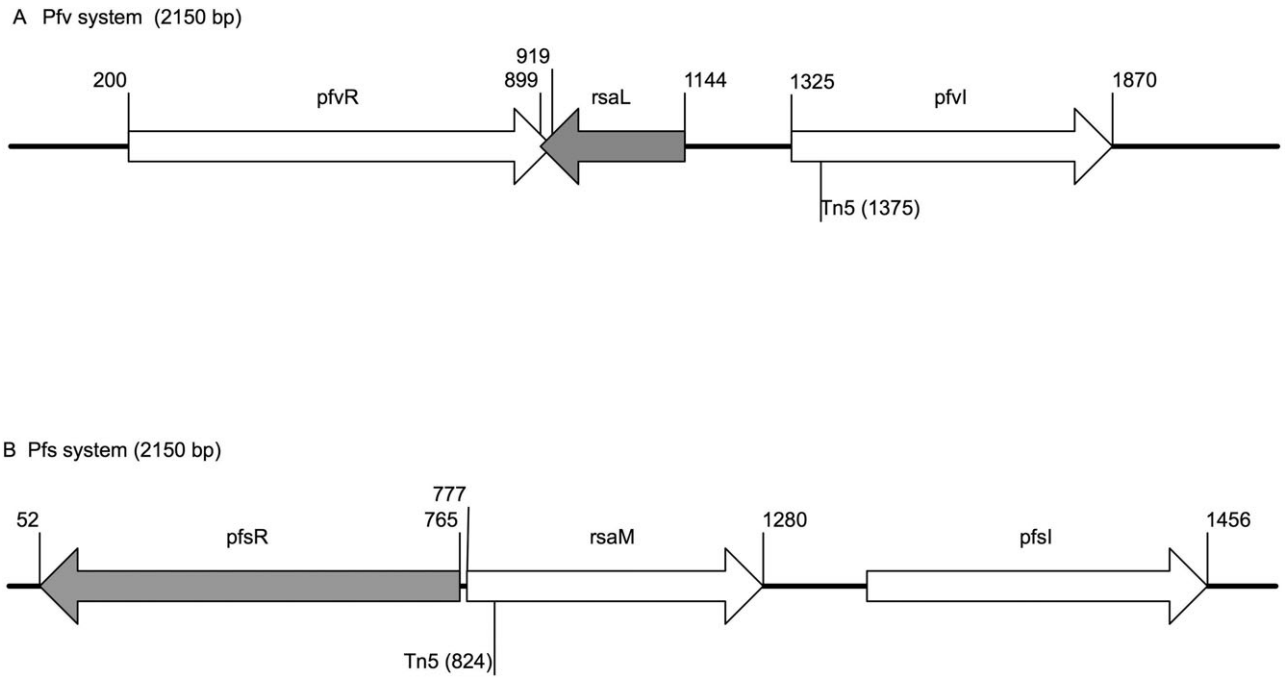

Fig. 1. Gene map of the two quorum sensing loci identified in *P. fuscovaginae* UPB0736.
A. Map of the 2.15 kb region from cosmid pCOS14 carrying the *pvfI/R* system.
B. Map of the 2.15 kb region from pCOS16 carrying the *pfsI/R* system.
In both maps genes are drawn to scale and the Tn*5* insertion positions are indicated. The nucleotides sequences of both of these two loci are deposited in data banks under Accession No. AM943857 and FN598970 respectively.

pfsI as DNA probes against 10 chromosomal DNAs of different *P. fuscovaginae* isolates was performed. It was determined that other 10 strains isolated from various parts of the world (Table 1) contained the *pfs* and *pfv* AHL QS systems showing that the two systems are well conserved within the species (data not shown).

PfsR responds to C12-HSL and C10-HSL whereas PfvR responds to 3-oxo-C12-HSL and 3-oxo-C10-HSL and at high AHL concentration also to C12-HSL and C10-HSL

The functioning of *pfvI/R* and *pfsI/R* systems will depend on the AHL specificity of both PfvR and PfsR proteins. In order to determine this, the two proteins were expressed in *Escherichia coli* in the presence of different AHL molecules and the cognate *pfvI* and *pfsI* promoter activities were determined. The reason for this experimental set-up is that in most AHL QS systems, the *luxI*-family synthase is positively regulated by the cognate LuxR-family protein in the presence of quorum levels of AHL. The two synthase gene promoters were cloned in the broad host range low-copy-number β-galactosidase promoter probe vector pMP220, yielding pPFVI220 and pPFSI220 respectively. These two transcriptional fusions were transformed into *E. coli* containing pQEPfvR or pQEPfsR; these plasmids express the PfvR and PfsR proteins, respectively, generating *E. coli* M15 (pPFVI220)(pQEPfvR) and *E. coli* M15 (pPFSI220)(pQEPfsR). Testing promoter activities in the presence of many different AHLs at 10 nM showed that the activity of the *pfvI* promoter increased significantly only in the presence of PfvR and of two long-chain AHLs, 3-oxo-C12-HSL and 3-oxo-C10-HSL (Fig. 2B). Interestingly if this assay was performed using 1 μM AHLs, PfvR responded well also to 3-oxo-C8-HSL, C12-HSL, C10-HSL, 3-OH-C10-HSL and 3-OH-C12-HSL (Fig. 2A). The activity of the *pfsI* promoter, increases only in the presence of PfsR and of C10-HSL and C12-HSL even at 1 μM (Fig. 2C). In all experiments no increase of promoter activity was observed when the pQE30 empty vector and AHLs were used (Fig. 2A–C). It was concluded that PfvR displayed specificity towards 3-oxo-C12-HSL and 3-oxo-C10-HSL and a relaxed specificity towards different types of long-chain AHLs at high concentration. PfsR on the other hand, displayed a specific response to C10- and C12-HSLs; importantly PfvR at high AHL concentration also responded well to these two AHLs indicating that PfvR and PfsR have under certain circumstances a similar AHL response.

In order to learn the identity of AHLs produced by PfvI and PfsI, AHLs were extracted from spent supernatants of *P. fuscovaginae* strains of several genetic backgrounds and analysed by TLC. The double *pfvI/pfsI* mutant (0736PFIDM) did not produce any AHLs indicating that

Table 1. *Pseudomonas fuscovaginae* strains, plasmids and primers used.

Strains/plasmids/primer	Relevant characteristics	Reference or source
P. fuscovaginae strains		
P. fuscovaginae LMG 2158^T	Wild-type strain isolated from diseased rice in Japan	LMG Collection – Gent-B
P. fuscovaginae LMG 2192	Wild-type strain isolated from diseased rice in Japan	LMG Collection – Gent-B
P. fuscovaginae LMG 5097	Wild-type strain isolated from diseased rice in Japan	LMG Collection – Gent-B
P. fuscovaginae LMG 5742	Wild-type strain isolated from diseased rice in Burundi	LMG Collection – Gent-B
P. fuscovaginae LMG 12424	Wild-type strain isolated from diseased rice in unknown location	LMG Collection – Gent-B
P. fuscovaginae LMG 12425	Wild-type strain isolated from diseased rice in unknown location	LMG Collection – Gent-B
P. fuscovaginae UPB 0304	Wild-type strain isolated from diseased rice in Japan	H. Maraite
P. fuscovaginae UPB 0305	Wild-type strain isolated from diseased rice in Japan	H. Maraite
P. fuscovaginae UPB 0306	Wild-type strain isolated from diseased rice in Japan	H. Maraite
P. fuscovaginae UPB 0306 b	Wild-type strain isolated from diseased rice in Japan	H. Maraite
P. fuscovaginae UPB 0736	Wild-type strain isolated from diseased rice in Madagascar	H. Maraite
P. fuscovaginae 0736PFVI	*pfvI*::Tn*5* of *P. fuscovaginae* UPB0736; Km^R	This study
P. fuscovaginae 0736PFVR	*pfvR*::Km of *P. fuscovaginae* UPB0736; Km^R	This study
P. fuscovaginae 0736RSAL	*rsaL*::Km of *P. fuscovaginae* UPB0736; Km^R	This study
P. fuscovaginae 0736PFSI	*pfsI*::Km of *P. fuscovaginae* UPB0736; Km^R	This study
P. fuscovaginae 0736PFSR	*pfsR*::Km of *P. fuscovaginae* UPB0736; Km^R	This study
P. fuscovaginae 0736RSAM	*rsaM*::Tn*5* of *P. fuscovaginae* UPB0736; Km^R	This study
P. fuscovaginae 0736PFIDM	*pfvI*::Tn*5*, *pfsI*::Tc of *P. fuscovaginae* UPB0736; Km^R Tc^R	This study
Plasmids		
pRK2013	Tra$^+$ Mob$^+$ ColE1 replicon; Km^R	Figurski and Helinski (1979)
pMOSBlue	Cloning vector; Amp^R	Amersham-Pharmacia
pMP220	Promoter probe vector, IncP; Tet^R	Spaink *et al.* (1987)
pQE30	Expression vector, Amp^R	Qiagen, Hilden, D.
pBBRmcs5	Broad-host-range vector, Gm^R	Kovach *et al.* (1995)
pLAFR3	Broad-host-range cloning vector, IncP1; Tet^R	Staskawicz *et al.* (1987)
pKNOCK-Km	Conjugative suicide vector; Km^R	Alexeyev (1999)
pKNOCK-Tc	Conjugative suicide vector; Tc^R	Alexeyev (1999)
pCOS14	pLAFR3 containing UPB0736 genomic DNA	This study
pCOS16	pLAFR3 containing UPB0736 genomic DNA	This study
pPFSI220	*pfsI* promoter cloned into pMP220, Tet^R	This study
pPFVI220	*pfvI* promoter cloned into pMP220, Tet^R	This study
pPFSR220	*pfsR* promoter cloned into pMP220, Tet^R	This study
pPFVR220	*pfvR* promoter cloned into pMP220, Tet^R	This study
pQEPfvR	*pfvR* cloned in pQE30	This study
pQEPfsR	*pfsR* cloned in pQE30	This study
pKNOCK-PfvR	Internal *pfvR* fragment cloned in pKNOCK-Km	This study
pKNOCK-rsaL	Internal *rsaL* fragment cloned in pKNOCK-Km	This study
pKNOCK-PfsI	Internal *pfsI* fragment cloned in pKNOCK-Km	This study
pKNOCK-PfsR	Internal *pfsR* fragment cloned in pKNOCK-Km	This study
pKNOCK-rsaM	Internal *rsaM* fragment cloned in pKNOCK-Km	This study
pKNOCKTc-pfs2	Internal *pfsI* fragment cloned in pKNOCK-Tc	This study
pBBRsaL	*rsaL* cloned in pBBRmcs5 vector	This study
pBBRsaM	*rsaM* cloned in pBBRmcs5 vector	This study
pBBPfvI	*pfvI* cloned in pBBRmcs5	This study
pBBPfsI	*pfsI* cloned in pBBRmcs5	This study
Primers		
220PvIX	5′-AATCTAGATATGCATCTTTTGCAG-3′	This study
220PvIK	5′-TTGGTACCTGATCAAACTTCGCTC-3′	This study
220PsIK	5′-AAGGTACCATATCCAGGCAGGGTTTG-3′	This study
220PsIP	5′-CACTGCAGAAATAACCCGAGCCTC-3′	This study
QEPvRB	5′-TTGGATCCCTATTCATGGATGAATGTG-3′	This study
QEPvRH	5′-TTAAGCTTTCAGGGACTGATGAGGTGT-3′	This study
QEPsRB	5′-AAGGATCCTCTGATTGGCGTGAACAC-3′	This study
QEPsRP	5′-ACCTGCAGTCATTGAAGCATGCCCAAG-3′	This study
RsaLFW	5′-GTCTGGATAATTGACAGCCTA-3′	This study
RsaLREV	5′-TTGAAAGTCACCTCCAGACG-3′	This study
RsaMFW	5′-AATTCCTCTTCCGTTTTCGC-3′	This study
RsaMREV	5′-GGGATGTTCTGTCGTTCTGAT-3′	This study
KnfvRB	5′-AAGGATCCATTATCGACCCAACCG-3′	This study
KnfvRX	5′-AATCTAGATTGAAGCTGCTGGTCAC-3′	This study
KnvsLX	5′-AATCTAGAGCCAGGGTGCGTTCT-3′	This study
KnvsLB	5′-AAGGATCCACATACAGACCGAGCA-3′	This study
KnPsRB	5′-CAGGATCCAACTATTCTCCCGCCT-3′	This study
KnPsRX	5′-CATCTAGACCACTTCAAGGTCTCCAC-3′	This study
KnfsIX	5′-GGTCTAGACATGGAGATCGATGGCT-3′	This study

Table 1. *cont.*

Strains/plasmids/primer	Relevant characteristics	Reference or source
KnfsIB	5′-AAGGATCCGTGACGAACTGTTTCAC-3′	This study
KnfsIB	5′-AAGGATCCGTGACGAACTGTTTCAC-3′	This study
BBRIK	5′-TTGGTACCACCTACCAGATTG-3′	This study
BBRI1X	5′-TTTCTAGATGAAAGCGTGATG-3′	This study
BBRI2K	5′-TTGGTACCTTTGGGCAGCTA-3′	This study
BBRI2X	5′-TTTCTAGATAGAGAATCATGG-3′	This study
220fvrX	5′-TTTCTAGAATAGCATCGAGATATCCG-3′	This study
220fvrK	5′-TTGGTACCGGCTTCGAACGCAGATA-3′	This study
220fsrFW	5′-CAGGCGGGAGAATAGTTGGA-3′	This study
220fsrRV	5′-GGCAGCCCGAATTCTTTT AA-3′	This study

most likely PfsI and PfvI are the only AHL synthases present in *P. fuscovaginae* 0736 (Fig. 3). We next introduced in the 0736PFIDM mutant a plasmid expressing either *pfvI* or *pfsI* and then the AHLs produced were extracted and analysed. As shown in Fig. 3, PfvI was responsible for synthesizing C12-3oxo-HSL, C10-3oxo-HSL, C8-3oxo-HSL and C6-3oxo-HSL, whereas PfsI for C12-HSL, C10-HSL and C8-HSL. In order to confirm unequivocally the data obtained by TLC analysis of the AHLs produced we used C_{18} reverse-phase HPLC and MS and were able to clearly identity the production of 3-oxo-C12-HSL and 3-oxo-C10-HSL by the PfvI/R system and C12-HSL and C10-HSL by the PfsI/R system (Fig. S3). This analysis was performed from (i) supernatants of the wild-type strain UPB0736, (ii) supernatants of the 0736PFIDM double *pfsI–pfvI* mutant harbouring a plasmid expressing either *pfvI* or *pfsI* and (iii) supernatants of *pfsI* mutant 0736PFSI and *pfvI* mutant 0736PFVI. In all these experiments it was clearly established that PfvI drove the synthesis of 3-oxo-C12-HSL and 3-oxo-C10-HSL whereas PfsI of C12-HSL, C10-HSL (Fig. S3). It was therefore concluded that PfvI and PfsI directed synthesis of AHLs to which PfvR and PfsR, respectively, best responded to; in addition, as expected, PfvI resembles LasI and PpuI as it is responsible for production of similar AHLs.

We were interested to determine whether there was any hierarchical organization between the two AHL QS systems of *P. fuscovaginae*. An experiment was therefore set up in order to determine whether the PfvI/R system was regulating *pfsI/R* and vice versa. We introduced the transcriptional fusions pFVI220 and pFSI220, carrying the *pfvI* and *pfsI* gene promoters, respectively, in *E. coli* harbouring pQEPfsR or PQEPfvR respectively. The resulting transformants, *E. coli* (pQEPfsR)(pFVI220) and *E. coli* (pQEPfvR)(pFSI220), were grown in the presence of the cognate AHL for each of the LuxR-family protein (C10-HSL for PfsR and 3-oxo-C12-HSL for PfvR) and β-galactosidase activities were determined. Results showed that PfsR/C10-HSL does not activate the *pfvI* promoter and PfvR/3-oxo-C12-HSL does not activate the *pfsI* promoter (Fig. 4). The *pfvR* and *pfsR* promoters were also tested in a similar way and results showed that PfsR/C10-HSL does not activate the *pfvR* promoter and PfvR/3-oxo-C12-HSL does not activate the *pfsR* promoter (data not shown). In addition both PfvR and PfsR do not regulate their own promoter (data not shown). It was therefore concluded that the two systems do not transcriptionally regulate each other.

Isolation and characterization of AHL hyper-producing mutants of P. fuscovaginae

As *P. fuscovaginae* was producing very low quantities of AHLs, it was decided to screen for AHL over-producing mutants in order to get insights into possible regulation of the AHL QS systems. Screening 23 000 *P. fuscovaginae* UPB0736::Tn*5* mutants for hyperproduction of AHLs, as described in *Experimental procedures*, resulted in the identification of two different mutants, designated 0736LON and 0736RSAM, which produced considerably larger quantities of AHLs (see below; Fig. 5). The AHLs produced by the two mutants were quantified from spent supernatants, as described in *Experimental procedures*, showing that mutant 0736LON produced threefold more AHLs than the wild-type strain and 0736RSAM fourfold more (Fig. 5).

Mutant *P. fuscovaginae* 0736LON had a Tn*5* insertion in the *lon* protease gene; this was determined by cloning and sequencing a 567 bp fragment containing adjacent DNA to the Tn*5* transposon. It has already been previously reported that inactivation of the *lon* protease in *Pseudomonas* can result in higher production of AHLs (Bertani *et al*., 2007; Takaya *et al*., 2008). The other hyper-producer mutant, 0736RSAM, had a Tn*5* insertion located in the intergenic DNA region between the *pfsI* and *pfsR* genes (Fig. 1B). This intergenic region is rather large being 692 bp and the Tn*5* was located in a putative ORF which we designated *rsaM* encoding a protein of 167 amino acids. RsaM was therefore postulated to be a negative regulator of AHL production (see below). RsaM displays highest homology (approximately 33%) to a hypothetical protein in *Burkholderia pseudomallei*

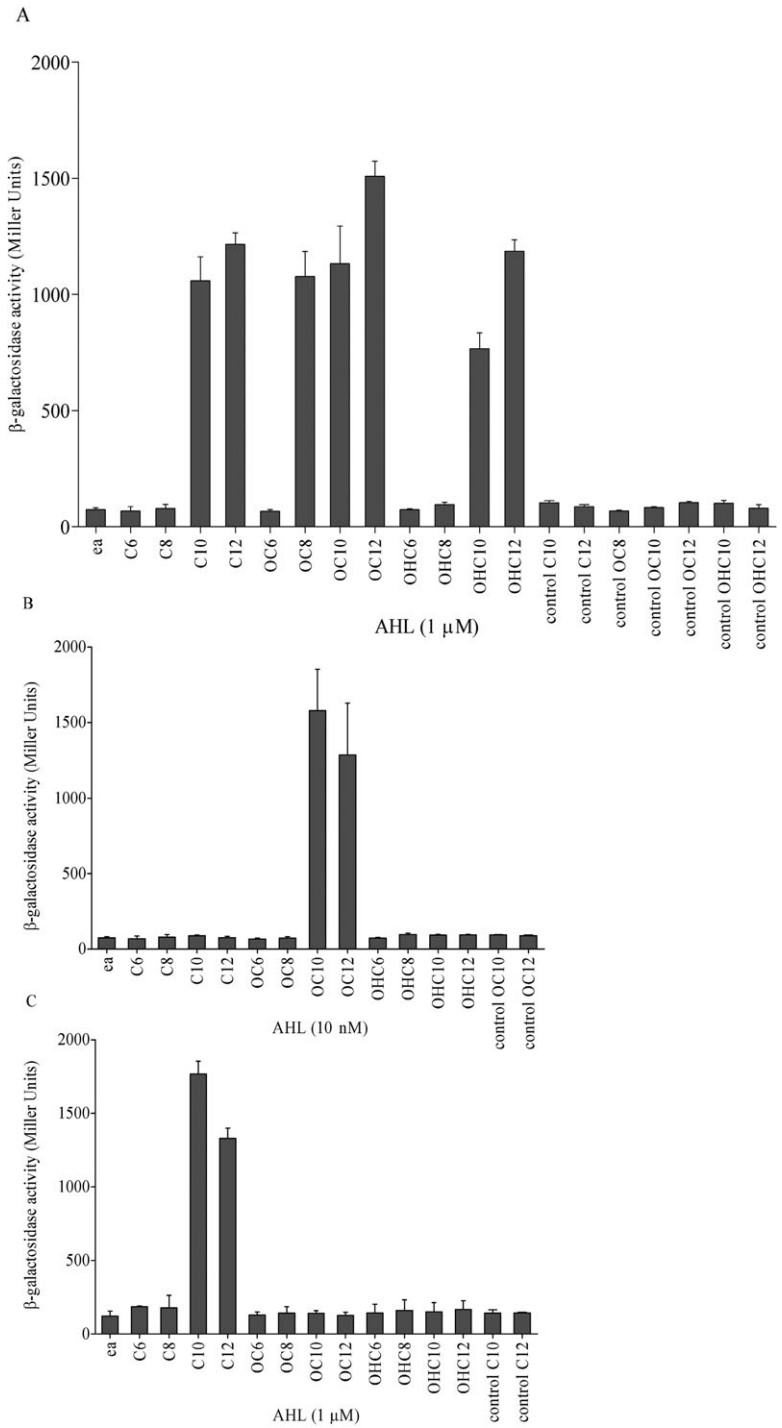

Fig. 2. Determination of the biologically active AHLs of the two AHL QS systems.
A. PfvR-AHL response: *E. coli* DH5α (pPFVI220, pQEPfvR) was grown for 4 h in the presence of 1 μM concentration of the indicated AHLs and β-galactosidase activities were determined. The control experiments consisted of using the empty vector pQE30, the pPFVI220 and all the AHLs which showed a response when using pQEPfvR.
B. PfvR-AHL response: *E. coli* DH5α (pPFVI220, pQEPfvR) was grown for 4 h in the presence of 10 nM concentration of the indicated AHLs and β-galactosidase activities were determined. The control experiments consisted of using the empty vector pQE30, the pPFVI220 and the two AHLs which showed a response when using pQEPfvR.
C. PfsR-AHL response: *E. coli* DH5α (pPFSI220, pQEPfsR) was grown for 4 h in the presence of 1 μM concentration of the indicated AHLs and β-galactosidase activities were determined.
In all three cases the growth was carried out in LB, an equivalent volume of ethyl acetate (ea) devoid of AHLs was used to measure baseline level of transcription. The control experiments consisted of using the empty vector pQE30, the pPFSI220 and the two AHLs which showed a response when using pQEPfsR. In all experiments of (A), (B) and (C), the means of biological triplicate experiments are given and the standard deviations are shown.

(BURPS1710b_A0144; BPSS1179; BURPS668_A1657) and of *Burkholderia thailandensis* (BTH_II1228): interestingly in both of these species the gene encoding for this hypothetical protein is located immediately upstream of the system 2 AHL *luxI* family synthase gene (*luxI2*). In fact, *B. thailandensis* and *B. pseudomallei* contain three AHL QS systems (system R1/I1, R2/I2 and R3/I3) and the R2/I2 system has recently been implicated in regulation of antibiotic synthesis (Duerkop *et al*., 2009). RsaM also displayed homology (approximately 30%) to a putative ORF present in the intergenic region of the CepI/CepR QS system of the members of the *Burkholderia cepacia* complex (BCC) as for example BCAM1869. The role of this ORF in BCC bacteria is currently unknown.

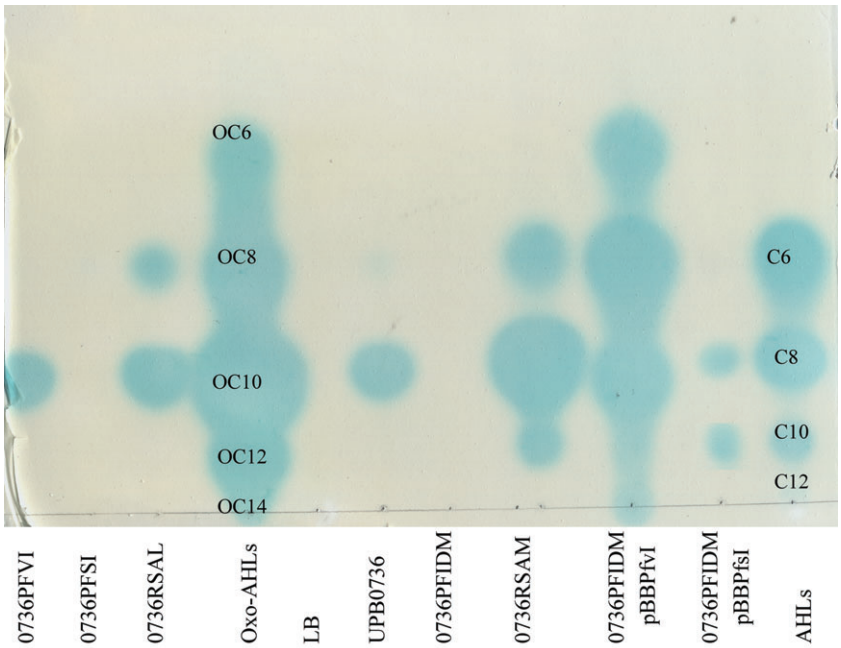

Fig. 3. TLC analysis of the AHLs produced by the *P. fuscovaginae* UPB0736 and mutants derivatives. AHLs extraction was performed as described in *Experimental procedures* and TLCs were run in methanol 60% for 4 h; for each strain the equivalent of 1.5×10^{10} cells was loaded. *Agrobacterium tumefaciens* (pNTL4) was used to detect the AHLs signals, standards were loaded as follows: OC14 (C14-3oxo-HSL), 4 nmol; OC12 (C12-3oxo-HSL), 2 nmol; OC10 (C10-3oxo-HSL), 4 nmol; OC8 (C8-3oxo-HSL), 0.2 nmol; OC6 (C6-3oxo-HSL), 0.3 pmol and C14 (C14-HSL), 24 nmol; C12 (C12-HSL), 18 nmol; C10 (C10-HSL), 3 nmol; C8 (C8-HSL), 7.5 pmol; C6 (C6-HSL), 1 nmol.

Role of RsaL and RsaM in regulating pfvI/R *and* pfsI/R

As mentioned above, *P. fuscovaginae* 0736, just like all the other *P. fuscovaginae* strains we tested, produced very little quantities of AHLs under the conditions we tested; the reason for this low production indicated that most probably AHL production was under the control of other regulators. The *pfvI/R* system contains in its intergenic region the *rsaL* repressor which has been shown in *P. aeruginosa* and *P. putida* to be directly negatively regulating transcription of the genetically linked *lasI* and *ppuI* AHL synthase gene respectively (Bertani and Venturi, 2004; Rampioni *et al.*, 2006). The *pfsI/R* system (as reported above) contains a putative gene of 501 bp,

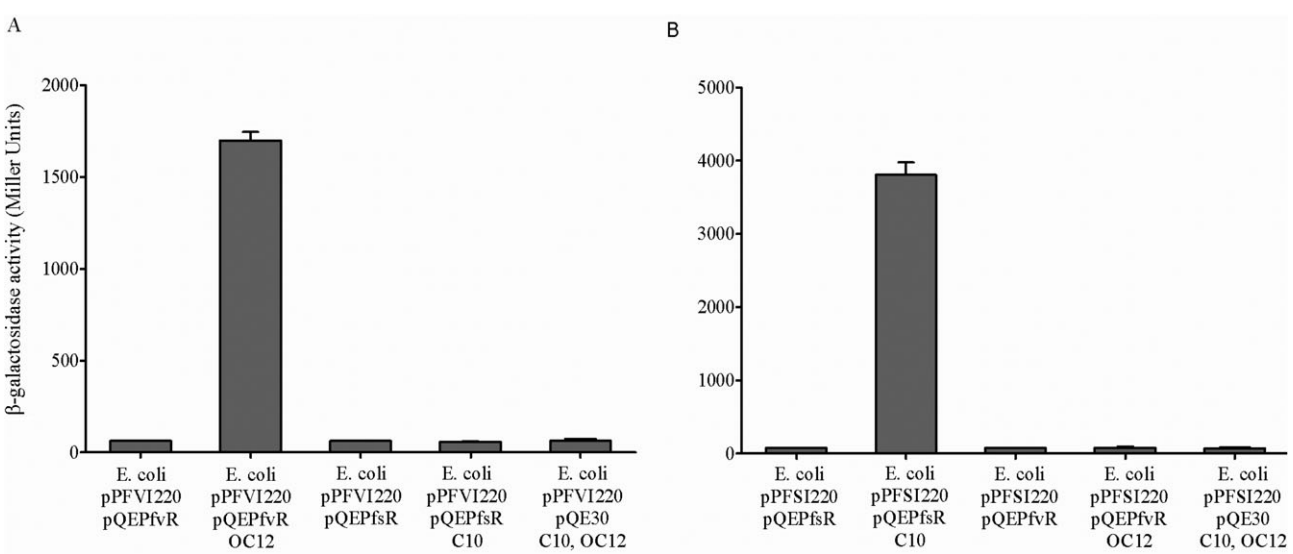

Fig. 4. Cross-regulation between the two AHL quorum sensing systems of *P. fuscovaginae* UPB0736: the ability of the regulators PfvR and PfsR to activate transcription of both AHL *luxI* family synthases was analysed.
A. *Escherichia coli* (pPFVI220, pQEPfvR) and *E. coli* (pPFVI220, pQEPfsR) were grown in the absence or presence of the respective cognate AHL.
B. *Escherichia coli* (pPFSI220, pQEPfsR) and *E. coli* (pPFSI220, pQEPfvR) were grown in the absence or presence of the respective cognate AHL.
The control experiments consisted in using the empty vector pQE30 in the presence of the AHLs to which PfvR and PfsR respond to. The means of biological triplicate experiments are given and the standard deviation is shown.

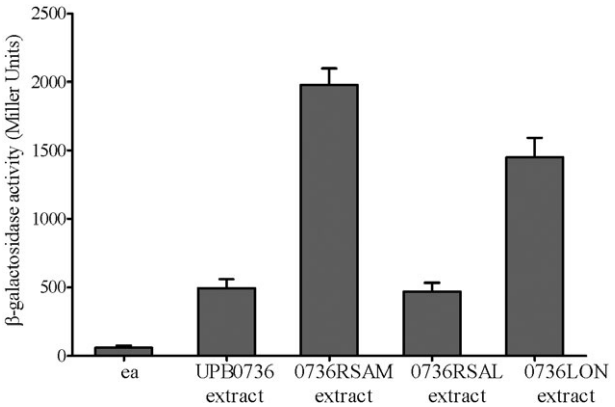

Fig. 5. Quantification of AHL levels of *P. fuscovaginae* UPB0736 and derivative mutants. *Escherichia coli* (pPFVI220) (pQEPfvR) was grown for 4 h in the presence of AHLs extracts coming of *P. fuscovaginae* UPB0736, 0736RSAM, 0736RSAL and 0736LON. Extracts used correspond to 5×10^{10} cfu and the corresponding volume of ethyl acetate (ea) was used to measure baseline levels. The means of biological triplicate experiments are given and the standard deviation is shown.

which we designated *rsaM*, in its intergenic region that when disrupted results in the strain being able to synthesize considerably larger quantities of AHLs (see above). It was of interest therefore to determine the role of RsaL and the newly identified RsaM in regulating the two AHL QS systems of *P. fuscovaginae*.

In order to establish if RsaM and/or RsaL regulate the *pfvI* and *pfsI* promoters we firstly set up the following experiment in *P. fuscovaginae*. We introduced the pPFSI220 and pPFVI220 transcriptional fusions in *P. fuscovaginae* UPB0736 and *rsaM* and *rsaL* mutant derivatives. These experiments showed that the *pfsI* promoter was strongly de-repressed in the *rsaM* mutant; promoter activity could be restored to almost the activity displayed in the wild type by introducing a plasmid expressing the *rsaM* gene (Fig. 6A). The *pfsI* promoter showed no expression in the *rsaL* mutant (Fig. 6A). This result indicated that the *pfsI* AHL synthase is stringently regulated by RsaM. On the other hand the *pfvI* promoter displayed no activity in the wild type as well as in both repressor mutants (Fig. 6B). In addition no increase of AHL production was detected in the *rsaL* mutant further indicating that RsaL alone has no major role in AHL regulation (Fig. 5); however, an AHL migrating at the same position as 3-oxo-C6-HSL was detected in the *rsaL* mutant which was not present in the spent supernatant of the wild type. As no *pfvI* promoter activity was detected in *P. fuscovaginae* and thus no putative role for the RsaL and RsaM repressors in controlling this promoter was established, we set up the following experiment in *E. coli*. Three different and compatible plasmids were introduced in *E. coli*: (i) pPFVI220, the plasmid containing the *pfvI* promoter-transcriptional fusion, (ii) pQEPfvR, the plasmid expressing PfvR, and (iii) pBBRsaL or pBBRsaM, the plasmids expressing RsaL or RsaM respectively. In the presence of 3-oxo-C12-HSL, the cognate AHL for PfvR, it was observed that RsaL could repress the *pfvI* promoter by almost 70%, and, importantly, the promoter could also be repressed by RsaM by 30% (Fig. 6C). The same experiment was carried out also for the PfsI/R system confirming the results obtained in *P. fuscovaginae* (data not shown). It was therefore concluded that in the *E. coli* heterologous system, the RsaM repressor could repress both the *pfsI* and *pfvI* promoters, whereas the RsaL repressor could only repress the *pfvI* promoter.

In conclusion it was established that RsaM is a novel repressor in *P. fuscovaginae* that stringently regulated the *pfsI* promoter. As observed from studies in *E. coli,* RsaM could also repress the transcription of the *pfvI g*ene. On the contrary RsaL is able to repress the *pfvI* synthase transcription and has no effect on the transcription of the *pfsI* gene. Unfortunately all our attempts to construct a double *rsaM/rsaL* mutant failed indicating that the absence of both regulators might be lethal to the cell.

The PfsI/R and PfvI/R are involved in P. fuscovaginae *pathogenicity* in planta

To examine whether the PfvI/R and PfsI/R AHL QS systems of *P. fuscovaginae* were involved in pathogenicity and in disease development, *P. fuscovaginae* UPB0736 parental strain and several derivative knock-out mutants were inoculated on *C. quinoa* and *O. sativa* host plants by the stem puncture method and disease development was evaluated. The ability of *P. fuscovaginae* to cause virulence on *C. quinoa* was previously reported (Maraite and Weyns, 1997); here we demonstrate for the first time the use of *C. quinoa* as a plant model to assess *P. fuscovaginae* pathogenicity (Fig. S1). Five days after infection (see *Experimental procedures* for details) disease development on *C. quinoa* and rice was evaluated with a 0–5 disease severity index as depicted in Figs S2 and S3. *Pseudomonas fuscovaginae* UPB0736 parental strain was very pathogenic on *C. quinoa* and towards rice plants.

In order to assess the role in plants of the PfsI/R and PfvI/R, several mutants were generated in this study, as described in *Experimental procedures*, and were infected in both *C. quinoa* and rice. More precisely, the *pfsI, pfsR, rsaM, pfvI, pfvR, rsaL* and double *pfsI–pfvI* knock mutants were infected in both *C. quinoa* and rice and results are presented in Fig. 7. Results have shown that with respect to the PfvI/R system, the *pfvR* mutant 0763PFVR was attenuated in virulence in both *C. quinoa* and rice models whereas the *pfvI* mutant displayed significant attenuation only in the *Chenopodium* model when compared with the wild type; the *rsaL* mutant (0736RSAL) did not display significant difference on both virulence models. The

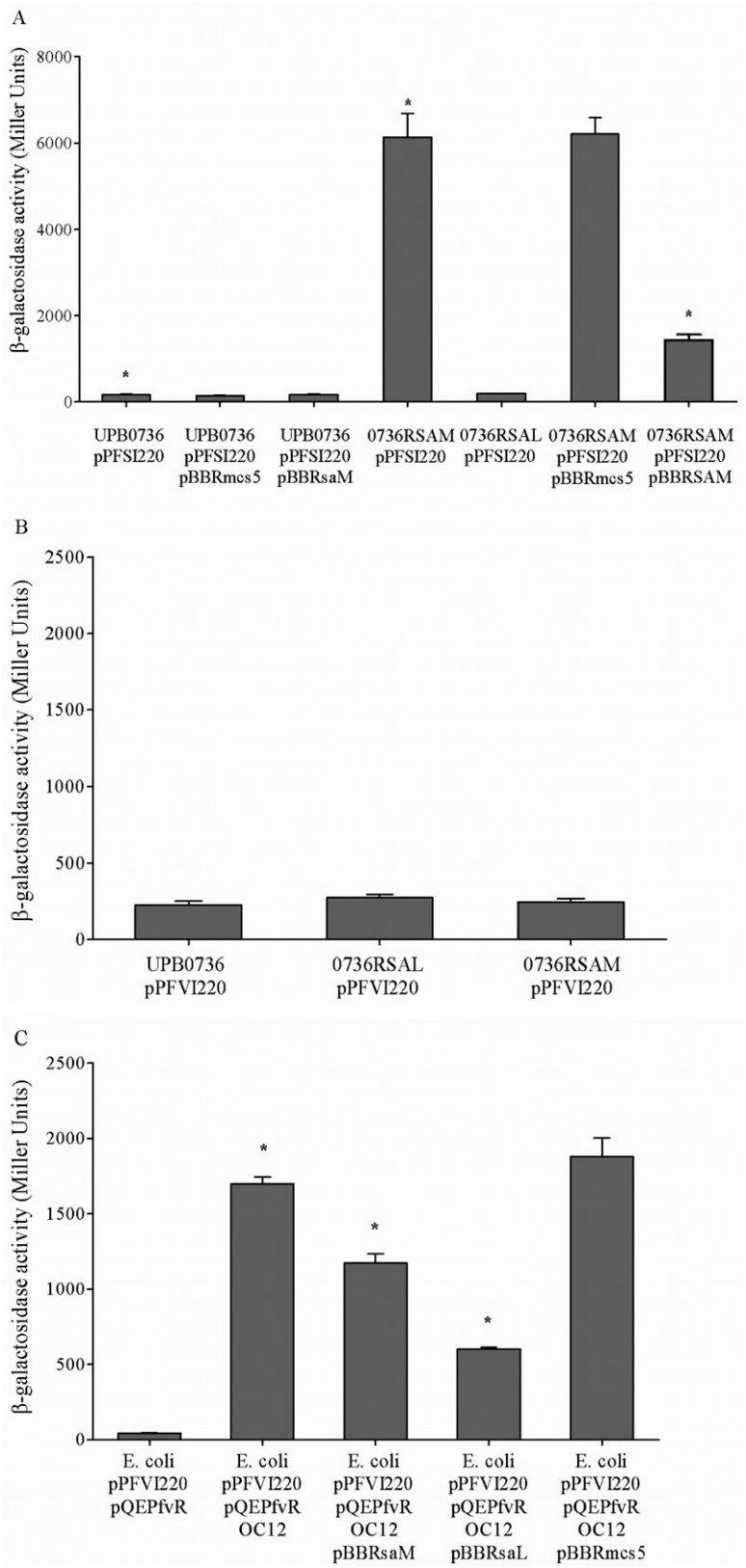

Fig. 6. Role of the RsaM and RsaL repressors in the regulation of *pfvI* and *pfsI*.
A. *pfsI* promoter activity was determined in *P. fuscovaginae* UPB0736, 0736RSAL and in 0736RSAM in the absence and presence RsaM via pBBRsaM.
B. *pfvI* promoter activity was determined in *P. fuscovaginae* UPB0736, in 0736RSAL and 0736RSAM.
C. β-Galactosidase activity of *E. coli* (pPFVI220, pQEPvfR) in the presence of 3-oxo-C12-HSL (OC12; the AHL cognate for PfvR) and in the presence of RsaL (pBBRsaL) and RsaM (pBBRsaM) was measured.
In all the experiments controls with the empty vector pBBRmcs5 were carried out; the means of biological triplicate experiments are given and the standard deviations are shown. The asterisk (*) indicates treatments with a $P < 0.0001$ (one-way ANOVA $P < 0.001$) compared with the control.

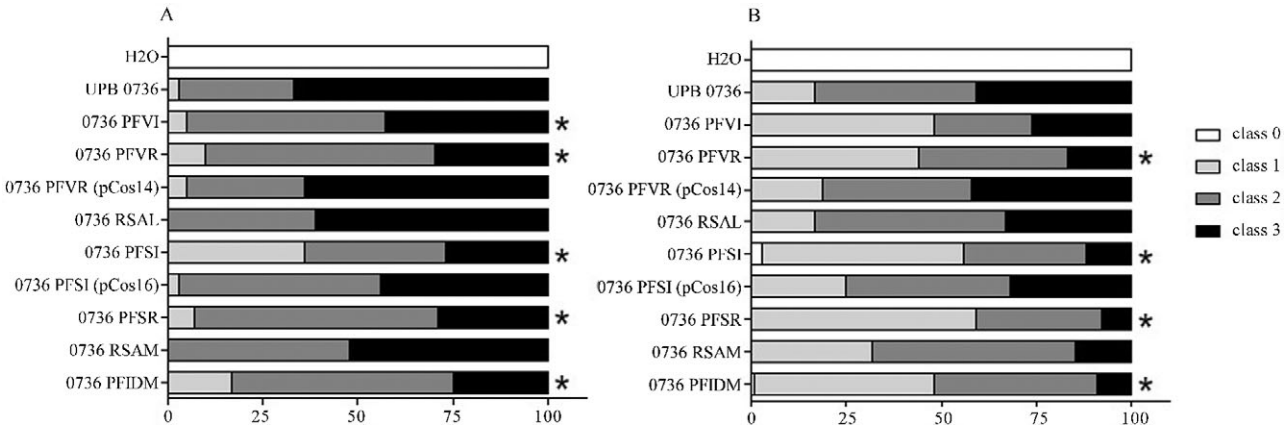

Fig. 7. Plant pathogenicity assays of *P. fuscovaginae* UPB0736 and mutants derivatives.
A. Pathogenicity assay on *Chenopodium quinoa*: plants were infected as described in *Experimental procedures* and the disease evaluation was performed 5 days after inoculation, using a 0–5 disease severity scale (Fig. S1). The figure depicts the scores grouped into four classes as follows: class 0, scores 0; class 1, scores 1 and 2; class 2, scores 3 and 4; class 3, score 5. Score ratings in the four groups were analysed statistically by SPSS 15.0 software using the Kruskal–Wallis multiple comparison test ($\chi^2 = 47.448$, $P < 0.0001$ for the group including the WILD TYPE and mutants of the PfvI/R system, $\chi^2 = 12.353$, $P < 0.05$ for the group including WILD TYPE and mutants of the PfsI/R system) and then each group pair has been tested by the Mann–Whitney comparison test with the minimum level of statistical significance set at $P = 0.05$. The asterisk (*) indicates treatments with $P < 0.0001$ compared with the wild-type strain UPB0736.
B. Pathogenicity assay on *Oryza sativa*: plants were infected as described in *Experimental procedures* and the disease evaluation was performed 5 days after inoculation, using a 0–5 disease severity scale (Fig. S2). The figure depicts the scores grouped into four classes as follows: class 0, scores 0; class 1, scores 1 and 2; class 2, scores 3 and 4; class 3, score 5. Score ratings in the four groups were analysed statistically by SPSS 15.0 software using the Kruskal–Wallis multiple comparison test ($\chi^2 = 33.423$, $P < 0.0001$ for the group including the wild type and mutants of the PfvI/R system, $\chi^2 = 30.758$, $P < 0.0001$ for the group including wild type and mutants of the PfsI/R system) and then each group pair has been tested by the Mann–Whitney comparison test with the minimum level of statistical significance set at $P = 0.05$. The asterisk (*) indicates treatments with a $P < 0.0001$ compared with the wild-type strain UPB0736.

attenuation of virulence displayed by the *pfvR* mutant could be restored to wild-type levels by providing *pfvR* when complemented *in trans* with pCOS14. In the PfsI/R system, both the *pfsI* and *pfsR* mutants (0736PFSI and 0736PFVI respectively) were attenuated in plant virulence models and could be complemented for their virulence by introducing pCOS16. The *rsaM* mutant (0736RSAM) did not display attenuation in the *C. quinoa* model whereas it displayed attenuation when infected in rice. Finally the double *pfvI–pfsI* mutant (0737PFIDM) was also attenuated in virulence indicating that AHL production in *P. fuscovaginae* is important for plant pathogenicity. Plant colonization assays were performed for all the *in vivo* infections and results have demonstrated that the colonization potential of all bacterial mutants was comparable to the wild type since the cfu per gram of plant material was comparable (data not shown).

In addition to plant pathogenicity, several phenotypes were tested for their regulation by the two AHL QS systems. The ability to produce extracellular lipolytic and proteolytic activities was not altered in any of the mutants which were generated in this study when compared with the behaviour of the wild type. Similarly, the ability to swarm and swim was also not altered in any of the mutants. Finally since it is known that *P. fuscovaginae* produces lipodepsipeptides which have anti-microbial activity, we tested the ability of all the mutants to inhibit growth of five fungal species. All the mutants were not altered in this ability when compared with the UPB0736 wild-type strain.

Discussion

This study describes the isolation and characterization of two AHL QS systems of the plant pathogen *P. fuscovaginae*. Major conclusions are that (i) all strains studied here contain two very well-conserved systems designated *pfvI/R* and *pfsI/R*, (ii) the two systems are transcriptionally expressed at very low levels and hence synthesize low amounts of AHLs, (iii) both systems are necessary for plant pathogenicity, and (iv) both systems are regulated by repressor genes located intergenically, one of which (RsaM) has never been described before and hence it is a novel regulator in bacteria.

The PfsI/R system produces and responds to C12-HSL and C10-HSL and is highly similar to the BviI/R system of *B. vietnamiensis* which also produces and responds to the same AHLs (Conway and Greenberg, 2002). *Burkholderia vietnamiensis* possesses another AHL QS system called CepI/R which is found in all members of the BCC (Venturi *et al*., 2004; Eberl, 2006) and unlike what occurs in *P. fuscovaginae*, the two systems are intimately transcriptionally connected since CepI/R regulates *bviI/R* (Malott and Sokol, 2007). The *rsaM* repressor gene is not present

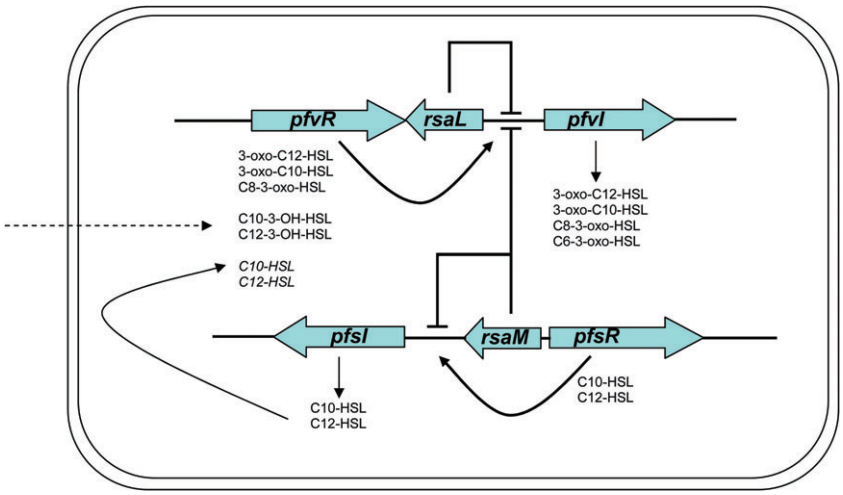

Fig. 8. Working model for the organization and function of the two AHL QS systems of *P. fuscovaginae*. The AHLs produced and the AHLs to which the LuxR-family sensor/response regulators are shown; note that PfvR (at high concentration) and PfsR respond to C12-HSL and C10-HSL which are produced by PfsI and PfvR responds to cognate C12-3-OH- and C10-3-OH-HSLs. The response of PfvR to a wide range of long-chain AHLs at high concentration could indicate a response to exogenously AHLs produced by other bacteria. The roles of the RsaM and RsaL repressors are also depicted.

intergenically between the *bviI/R* genes; however, a putative ORF encoding for a protein having 30% similarity with RsaM is located intergenically in between the *cepI/R* genes in *B. vietnamiensis*. The role of this ORF is currently unknown and following the results presented in this study, the possible involvement of this protein in regulating *bvi/R* expression is hypothesized. This ORF is however located intergenically between the *cepI/R* genes in all members of the BCC complex all of which, with the exception of *B. vietnamiensis*, do not possess the *bviI/R* system. This ORF if functional in BCC might therefore be involved in the regulation of the *cepI/R* system and/or other gene(s). A putative ORF similar to *rsaM* is also found located adjacent to AHL synthase genes in *B. pseudomallei* and *B. thailandensis*; both of these species contain multiples AHL QS systems and the role of the RsaM-like repressor in regulating these systems in currently unknown.

The PfvI/R system produces and responds well to several 3-oxo substituted AHLs. Importantly it also responds to C12-HSL and C10-HSL, the two AHLs produced by the PfsI/R system indicating that the two systems are connected since PfvR will most probably regulate gene(s), as well as activating the *pfvI/R* system, in response to PfsI synthesized AHLs. The two systems do not transcriptionally regulate each other since the PfsI/R system does not regulate pfvI/R gene promoters and *vice versa*. The two systems are however linked since at high AHL concentrations they have a level of coordinated organization via the AHL response. This is because the PfsI/R system can switch on the PfvI/R system (at high AHL concentration it can respond C10- and C12-HSLs) but not vice versa since the Pfv synthesized AHLs are not recognized by PfsR. The PfvI/R system is highly similar to the LasI/R system of *P. aeruginosa* (Passador *et al*., 1993), the PpuI/R system of *P. putida* (Steidle *et al*., 2002; Bertani and Venturi, 2004) and the BraI/R system of *Burkholderia kururiensis* (Suarez-Moreno *et al*., 2008). All these systems produce and respond specifically to 3-oxo-C12-HSL; PfvR at high AHL concentration has a more relaxed specificity being able to respond to C10-3oxo-HSL, C8-3oxo-HSL as well as C10- and C12- with unsubstituted or hydroxylated at position 3. This feature could allow PfvR to react to a wide range of structural different AHLs which indicates that in addition to responding to endogenous PfvI/R- and PfsI/R-produced AHLs (see above) it could also respond to exogenous AHLs produce by other neighbouring bacteria (Fig. 8).

All 10 *P. fuscovaginae* strains tested here produce very low amounts of AHLs, as determined by TLC analysis (Fig. 3) and AHL quantification (Fig. 5), indicating that the *pfv* and *pfs* QS systems might be under regulation. In fact, in our experiments, both *pfvI* and *pfsI* AHL synthase gene promoters displayed very low levels of transcription in strain UPB0736 (Fig. 6). This was in contrast to what observed in the *E. coli* heterologous system; here both synthase gene promoters had strong activities when the cognate 'R' protein and AHL signal was provided (Fig. 3). It can therefore be hypothesized that in *P. fuscovaginae* both promoters are under stringent regulation via a regulator(s) which result in a controlled production of AHLs. By performing a thorough screen of a Tn*5* genomic mutant bank for AHL hyper-producers, only two different mutants were isolated which had higher AHL production. One mutant has a Tn*5* insertion in the *lon* protease which has been previously implicated in negative regulation of QS systems (Bertani *et al*., 2007; Takaya *et al*., 2008) and the other mutant was located in an ORF designated as *rsaM* which was found in between the *pfsI/R* genes. The *rsaM* gene is divergently transcribed from *pfsR* and the two translation start codons are separated by only 12 bp (Fig. 2) indicating that most likely the *rsaM* gene promoter is located in the *pfsR* ORF. It must be noted that the screening used here to isolate AHL hyper-producing

mutants would not identify positive regulators which could activate transcription of the AHL synthase genes under particular environmental conditions.

The *pfv* system also possesses repressor gene (i.e. *rsaL*) located intergenically between the synthase and regulatory genes (Fig. 1). The *rsaL* repressor in *P. aeruginosa*, *P. putida* and *B. kururiensis* has been reported to directly regulate the transcription of the adjacent AHL synthase gene (Bertani and Venturi, 2004; Rampioni *et al*., 2006; Suarez-Moreno *et al*., 2008). The *rsaM* repressor is a novel regulator which when inactivated resulted in dramatic higher levels of *pfsI* transcription and much higher levels of AHL production. Constructing a *rsaL* knock-out mutant on the other hand it did not result in increase of *pfvI* transcription or AHL levels; this was surprising since in the other three homologous systems studied (i.e. in *P. aeruginosa*, *P. putida* and *B. kururiensis*), inactivation of *rsaL* leads to sudden and dramatic increase of transcription of the adjacent gene which also results in very high levels of AHL production (Bertani and Venturi, 2004; Rampioni *et al*., 2006; Suarez-Moreno *et al*., 2008). It is postulated that in *P. fuscovaginae pfvI* transcription is repressed by both RsaL and RsaM (i.e. they are redundant for *pfvI* expression) thus inactivating either RsaL or RsaM will not result in activation of *pfvI* transcription. In order to further elucidate the regulation by RsaM and RsaL of the *pfs* and *pfv* systems it was decided to set up experiments also in the *E. coli* heterologous system; this allowed assessing the direct role of RsaM and RsaL towards *pfs* and *pfv* gene promoters. It was established that RsaL represses the *pfvI* promoter and the RsaM represses both the *pfsI* and *pfvI* promoters meaning that the most likely reason for not observing an increase in *pfvI* promoter activity in the *rsaL* mutant of *P. fuscovaginae* is because the promoter is still repressed by RsaM. This could have been verified by constructing a double *rsaL–rsaM* mutant; unfortunately several attempts failed indicating that this mutant might not be viable in *P. fuscovaginae*. It cannot be excluded that other regulators in addition to RsaL and RsaM are also involved in the regulation of the *pfvI/R* system. In the future it will be of interest to determine the mode of action of RsaM and RsaL repression of the *pfvI* promoter.

The two AHL systems are involved in virulence in two plant models tested indicating that some of the targets are virulence associated factors. Importantly all AHL QS mutants were able to grow and colonize the plant just like the wild-type strain. All phenotypes that were tested *in vitro* were however not regulated by the Pfv and/or the Pfs system. Since this study has highlighted the complex regulatory organization of AHL QS in *P. fuscovaginae*, it cannot be excluded that under the conditions that the phenotypes were tested, the PfvI/R and PfsI/R systems were not functional and were switched off or being expressed at very low levels. It is likely that the regulation of the two systems is dependent on other cellular factors or environmental conditions highlighting that QS in *P. fuscovaginae* is not solely dependent on cell density but a more complex regulatory network could ensure that additional stimuli are required. Figure 8 summarizes the findings in this study depicting the two AHL QS systems and the information acquired in this study. QS via AHLs in *P. fuscovaginae* indicates that these systems are part of more multifarious regulatory networks as it has emerged following the larger number of studies on QS in *P. aeruginosa* (Schuster and Greenberg, 2006; Venturi, 2006; Williams and Camara, 2009).

Experimental procedures

Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas fuscovaginae* strains were isolated from diseased rice grown in different geographical regions. This strains were grown at 30°C in either M9 minimal medium (Sambrook *et al*., 1989) supplemented with 0.2% glucose and 0.3% casamino acids or Luria–Bertani (LB) medium (Miller, 1972). *Escherichia coli* DH5α (Sambrook *et al*., 1989), *E. coli* DH5α (pRK2013) (Figurski and Helinski, 1979), *E. coli* HB101 (Magazin *et al*., 1986) and *E. coli* M15 (Qiagen) were grown at 37°C in LB medium. AHL bacterial biosensors used for AHL detection were *Chromobacterium violaceum* strain CV026 (McClean *et al*., 1997), *P. putida* F117 (pKRC12) (Riedel *et al*., 2001) and *A. tumefaciens* NTL4 (pZLR4) (Cha *et al*., 1998). *Chromobacterium* and *Pseudomonas* AHL detector strains were grown at 30°C in LB medium, while *Agrobacterium* was grown at 28°C in AB minimal medium (Chilton *et al*., 1974).

When required, antibiotics were added at the following concentrations: ampicillin 100 μg ml^{-1}, gentamicin 20 μg ml^{-1} (*E. coli*) or 40 μg ml^{-1} (*Pseudomonas*), kanamycin 100 μg ml^{-1}, nalidixic acid 25 μg ml^{-1} and tetracycline 15 μg ml^{-1} (*E. coli*) or 40 μg ml^{-1} (*Pseudomonas*). 5-Bromo-4-cloro-3-indoyl-β-D-galactopyranoside (X-gal) was used at 80 μg ml^{-1} when necessary.

Gene transcriptional fusion plasmids, based on pMP220 promoter probe vector, for the *pfvI* and *pfsI* promoters were constructed as follows. Primers 220PvIX and 220PvIK, 220PsIK and 220PsIP were used to amplify, using *P. fuscovaginae* UPB0736 chromosomal DNA as template, the promoter regions of *pfvI* (215 nt) and *pfsI* (292 nt) respectively. The amplified fragments were cloned in pMOSBlue (Amersham Biosciences, UK) to yield pMprpfvI and pMprpfsI. The gene promoters were then removed as a KpnI/XbaI or KpnI/PstI fragments and cloned in the corresponding sites pMP220 yielding pPFVI220 and pPFSI220.

The promoter regions of *pfvR* and *pfsR* were cloned in pMP220 as follows. Primers 220fvrX and 220fvrK and 220fsrFW and 220fsrRV were used to amplify the promoter region of *pfvR* and *pfsR*, respectively, using UPB0736 chromosomal DNA as template. The amplified fragments were cloned in pMosBlue (Amersham Biosciences, UK) to yield

pMprpfvR and pMprpfsR. The gene promoters were then removed as XbaI/KpnI and HindIII/KpnI fragments and cloned in the corresponding sites of pMP220 yielding pPFVR220 and pPFSR220.

The *pfvR* and *pfsR* genes were amplified from chromosomal DNA of the strain UPB0736 using primers pairs QEPvRB/QEPvRH and QEPsRB/QEPsRP respectively. The amplified fragments were cloned as BamHI/HindIII or BamHI/PstI fragments in the pQE30 expression vector (Qiagen) yielding pQEPfvR and pQEPfsR.

The repressors *rsaL* and *rsaM* genes were cloned in the pBBRmcs5 vector generating pBBRsaL and pBBRsaM as follows: *rsaL* and *rsaM* were PCR amplified from the chromosomal DNA of the strain UPB0736 by using the primer pairs RsaLFW/RsaLREV and RsaMFW/RsaMREV respectively. The PCR products were cloned as KpnI/XbaI or BamHI/PstI fragments in pBBRmcs5. The AHL synthases genes *pvfI* and *pfsI* were amplified from UPB0736 genomic DNA using the primers BBRI1K and BBRI1X and primers BBRI2K and BBRI2X respectively. The amplified fragments were cloned in pBBRmcs5 generating pBBPfvI and pBBPfsI. All the constructs described above were verified by DNA sequencing.

Recombinant DNA techniques

DNA manipulations, including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 ligase and transformation of *E. coli*, were performed as described previously (Sambrook *et al*., 1989). Southern hybridizations were performed by using N+Hybond membranes (Amersham Biosciences). Digoxigenin (DIG) labelling, DNA hybridization and detection were performed as recommended by the supplier (Roche Applied Science). Plasmids were purified using Jet star columns (Genomed GmbH, Löhne, Germany) or by the alkaline lysis method (Birnboim, 1983) and total DNA from *Pseudomonas* was isolated by Sarkosyl-pronase lysis as described previously (Better *et al*., 1983). Triparental matings from *E. coli* to *P. fuscovaginae* were carried out with the helper strain *E. coli* (pRK2013) (Figurski and Helinski, 1979). The DNA sequences flanking the transposon insertions in genomic mutants were determined by using an arbitrary PCR technique as previously described (O'Toole and Kolter, 1998).

Isolation of the two AHL QS systems of P. fuscovaginae *UPB0736*

A pLAFR3 cosmid library of partially digested EcoRI of *P. fuscovaginae* strain UPB0736 genomic DNA was constructed using Gigapack III XL-4 packaging kit as recommended by the supplier (Stratagene). In order to identify the cosmid containing the QS genes, the *E. coli* HB101 harbouring the cosmid library were used as donors in a triparental conjugation with, as acceptors the AHL biosensor *P. putida* F117 (pKRC12). After overnight incubation at 30°C, the conjugations were plated on LB containing gentamicin and tetracycline for selection of transconjugants and ampicillin, kanamycin and nalidixic acid for counter-selection of *E. coli*. The transconjugants which displayed GFP expression were further studied. Two different cosmids, named pCOS14 and pCOS16, inducing stronger GFP expression, were isolated and were completed sequenced: (i) pCOS14 was found to contain an AHL QS locus designated PfvI/R consisting of three genes, *pfvI–rsaL–pfvR*, and (ii) pCOS16 was found to contain an AHL QS locus designated PfsI/R consisting of three genes, *pfsI–rsaM–pfsR*.

In order to identify *P. fuscovaginae* UPB0736 mutants overproducing AHLs, a Tn*5* genomic mutant library was constructed using pSUP2021, as previously described (Simon *et al*., 1983; 1986). The mutant bank was screened against the AHL biosensor strain *C. violaceum* CVO26 as previously described (Yao *et al*., 2002; Bertani and Venturi, 2004). Briefly, we spread in one plate 1000–2000 cfu of AHL biosensor *C. violaceum* CVO26 and 300–500 *P. fuscovaginae* Tn*5* mutant cells and screened for strong purple loci. If colonies induced strong pigmentation they were AHL over-producer mutants since *P. fuscovaginae* UPB0736 induced only very slight pigmentation when streaked in close proximity to strain *C. violaceum* CVO26. Approximately 23 000 Tn*5* genomic mutants were screened. This led to the identification of two different Tn*5* mutants (designated *P. fuscovaginae* M2C and M5A), in which the Tn*5* located in two different genes could induce strongly pigmentation of CVO26. The positions of the Tn*5* were mapped through arbitrary PCR as previously described (Bertani and Venturi, 2004). The M2C mutant presented the Tn*5* in the *lon* protease gene (at position 702) and was named 0736LON; the mutant 5A presented the Tn*5* in the *rsaM* gene at position 1327 (Fig. 1B) and was named 0736RSAM.

Construction of P. fuscovaginae *UPB0736 knock-out mutants*

Most of the QS genomic null mutants were created utilizing the conjugative suicide vectors of the pKNOCK series (Alexeyev, 1999). The *pfvR* was inactivated by amplifying via PCR an internal fragment (281 bp) of this gene using oligonucleotides KnfvRB and KnfvRX and cloned as a XbaI–BamHI fragment in pKNOCK-Km generating pKNOCK-PfvR. This latter plasmid was then used as a suicide delivery system in order to create a *pfvR* knock-out mutant through homologous recombination generating *P. fuscovaginae* 0736PFVR. Similarly an internal fragment (358 bp) of *pfsR* was amplified using oligonucleotides KnfsRB and KnfsRX and cloned as a XbaI–BamHI fragment into pKNOCK-Km to yield pKNOCK-PfsR2 which was used to generate *P. fuscovaginae* PFSR. To generate the *pfsI* mutant, primers KnfsIX and KnfsIB were used to amplify an internal fragment (270 bp) and cloned as a BamHI–XbaI in pKNOCK-Km to yield pKNOCK-Km-PfsI which was used to generate *P. fuscovaginae* 0736PFSI.

To generate the *pfvI* mutant first cosmid pCOS14 was mutagenized using Tn*5* as previously described (Bertani and Venturi, 2004), then the cosmids containing the Tn*5* were conjugated in the AHL biosensor *P. putida* F117 (pKRC12). Colonies that did not express the reporter gene were selected, and the position of the Tn*5* in the *pfvI* gene was mapped through arbitrary PCR as previously described. The cosmid pCOS14::Tn*5* carrying a Tn*5* insertion in the *pfvI* gene, 50 nucleotides downstream the ATG codon, was homogenitized with the corresponding target region of the

genome of *P. fuscovaginae* UPB0736 by a marker exchange procedure (Corbin *et al*., 1982); pPH1JI was used as the incoming IncP1 incompatible plasmid. The generated mutant was designated *P. fuscovaginae* 0736PFVI.

The *pfvI–pfsI* double mutant was created by amplifying part of the *pfsI* gene (270 bp) using oligonucleotides KnfsIX and KnfsIB and cloning it as a BamHI–XbaI fragment into pKNOCK-Tc to yield pKNOCKTc-pfs2; the latter was then conjugated into *P. fuscovaginae* PFVI to generate *P. fuscovaginae* 0736PFIDM. Finally, the *rsaL* mutant was created by amplifying part of the *rsaL* gene (114 bp) using oligonucleotides KnvsLX and KnvsLB and cloning it as a BamHI–XbaI fragment into pKNOCK-Km to yield pKNOCK-rsaL; the latter was then conjugated into *P. fuscovaginae* wild-type strain to yield the mutant *P. fuscovaginae* RSAL. The fidelities of all marker exchange events were confirmed by Southern analysis (data not shown).

Reporter gene fusion assays

β-Galactosidase activities were determined during growth in LB medium essentially as described by Miller (1972), with the modifications of Stachel and colleagues (1985). All experiments were performed in triplicate.

Purification, detection, characterization and quantification of AHLs

Pseudomonas strains were grown in M9 minimal medium supplemented with glucose and casaminoacids and the supernatant of the culture was extracted with an equal volume of ethyl acetate acidified with 0.1% acetic acid. The preparation was centrifuged (5000 rpm × 5 min) and the ethyl acetate phase collected. The extract was then dried and resuspended in a small volume of ethyl acetate and was run on C_{18} reverse-phase chromatography plates besides synthetic AHLs used as standards (which were purchased either from Fluka-Sigma-Aldrich or from P. Williams, University of Nottingham, UK), using 60% (v/v) methanol in water as the mobile phase. The plates were overlaid with a thin layer of LB agar seeded with either *C. violaceum* CVO26, *E. coli* (pSB1075) or AB seeded with *A. tumefaciens* NTL4/pZLR4 as AHL biosensors as previously described (Shaw *et al*., 1997).

AHL levels produced by wild-type strain UPB0736 and mutants 0736RSAM and 0736LON were determined using a sensor constructed in this study. The sensor used was *E. coli* containing two plasmids: pQEPfvR overexpressing PfvR and pPFVI220 containing the *pfvI* promoter fused to a promoterless *lacZ* gene. Upon supply of exogenous AHLs to *E. coli* (pQEPfvR)(pPVFI220), PfvR will responds to all the AHLs produced by *P. fuscovaginae* (see *Results*) and this will result in the activation of the *pfvI* promoter which can be detected via β-galactosidase activity. Overnight cultures of *E. coli* (pQEPfvR)(pPFVI220) were diluted in 10 ml of LB medium to an A_{660} of 0.1; AHL extract from 20 ml of an overnight *P. fuscovaginae* culture (OD_{600} of 2 in M9 glucose and casaminoacids) was then added and after 4 h of growth, β-galactosidase was determined.

AHLs detection and identification by HPLC and MS

The AHLs produced by *P. fuscovaginae* were identified by LC/MS/MS in a multiple reaction monitoring (MRM) experiment as previously described (Gould *et al*., 2006). Monitoring was performed on the transition from the parent ion to both the acyl and lactone moiety peaks. The peaks were compared with known standards and evaluated also by chromatographic retention time analysis. Two hundred millilitres of cell-free culture supernatants were extracted by using the same volume of ethyl acetate, after the addition of 0.1% acetic acid. The organic phases were separated and dried under a chemical hood. The extracted samples for LC/MS/MS were resuspended in 100 μl of acetonitrile, filtered through a 0.2 μm filter (Millex LCR4, Millipore, Billerica, MA, USA), and diluted to 300 μl with MilliQ water containing 0.1% trifluoroacetic acid (TFA). One hundred microlitres of this solution was injected onto a 2.0 mm by 150 mm Gemini C_{18} column (Phenomenex, Torrance, CA, USA) operated at a flow rate of 200 μl min^{-1} with the effluent flowing directly into the mass spectrometer. Solvent A consisted of water containing 0.05% TFA, and solvent B was acetonitrile containing 0.05% TFA. The column was equilibrated in 20% B for 15 min, the sample injected and the column washed for additional 15 min. A gradient elution method from 20% B to 95%B in 40 min was then used for the separation of the AHLs and the column finally washed in 95% B for additional 10 min before re-equilibration. Mass spectrometric analyses were performed on a Bruker Esquire 4000 ion Trap. The ions monitored for each AHL are shown in Table S1.

Plant inoculations

The infection with the wild-type strain and the knockout mutants of the two systems was performed on 4-week-old *C. quinoa* plants, choosing the fifth, the sixth and the seventh leaves from the apex. *Chenopodium quinoa* was grown in a greenhouse at 28°C, humidity 70% with a photoperiod of 16 h light and 8 h dark. *Pseudomonas fuscovaginae* UPB0736 and the derivative mutants were grown on LB agar plates adding antibiotics when required, bacteria were collected with a pin and the petiole was then pierced passing through all the tissues. The control experiment was performed by piercing the petiole with a clean sterile pin. Each treatment was performed on at least 16 plants and three leaves per plant were inoculated. After the infection, the plants were sprinkled with water and placed into a humid chamber for 4 days, at 28°C, 100% humidity and with a photoperiod of 16 h light and 8 h dark. Infected plants were placed for 1 day outside of the humid chamber before the evaluation. After 5 days, disease index was performed on a 0–5 severity scale; 0: no symptoms, 1: necrosis on less than 2 mm around the puncture, 2: necrosis from 2 to 10 mm around the puncture, 3: necrosis from 2 to 10 mm around the puncture and bending of the petiole, 4: collapse of the petiole, and 5: wilting of the leaf (Fig. S1).

The infection on rice (Fig. S2; *O. sativa*, cv. IR24; provided by IRRI, Philippines) was performed on 3-week-old plants grown in the greenhouse at 28°C, humidity 70% with a photoperiod of 16 h light and 8 h dark. The stem was pierced with a syringe full of water paying attention not to pass through the

entire stem but maintaining the tip of the needle in the centre of the shaft. The water was pushed inside the plant to wet and saturate all the leaves. *Pseudomonas fuscovaginae* UPB and mutants were grown on LB plates, bacteria were then collected from the plate with a pin which was used to pierce in the same point where the water was injected, passing the pin through the entire stem. The control experiment was performed by injecting the water into the stem and, with the same needle, passing through the entire shaft. Each treatment was performed on at least 60 leaves. After the infection, the plants were sprinkled with water and placed into a humid chamber for 4 days, at 28°C, 100% of humidity and with a photoperiod of 16 h of light and 8 h of dark. The evaluation was performed 5 days after infection, the plants were placed for 1 day outside of the humid chamber before disease evaluation. The disease index was on a 0–5 disease severity scale; 0: no symptoms, only the sign of the puncture, 1: necrosis around the puncture till 1 cm, 2: necrosis around the puncture and chlorosis from 1 to 3 cm on the stem, 3: necrosis around the puncture till 5 cm on the stem, 4: necrosis around the puncture for the two-thirds of the new leaf, and 5: necrosis around the puncture throughout the new leaf (Fig. S2).

The statistical significance of the differences between wild type and mutant strains in *C. quinoa* and rice infections was tested with SPSS 15.0 software. Score ratings between the groups were analysed statistically using the Kruskal–Wallis multiple comparison test. Then the groups were tested two by two by the Mann–Whitney comparison test. The minimum level of statistical significance was set at $P = 0.05$.

Chenopodium quinoa colonization assays were performed with the following strains: the wild-type *P. fuscovaginae* UPB0736, the *pfvI* mutant (0736PFVI), the *pfvR* mutant (0736PFVR), the *pfsI* mutant (0736PFSI), the *pfsR* mutant (0736PFSR) and the double mutant *pfvI–pfsI* (0736PFIDM). Before the plant infection the plasmid pBBRmcs5 vector (Gm^R) was conjugated in each strain to allow cfu count on LB gentamicin plates. The infection on 4-week-old *C. quinoa* plants was performed as described previously. After 5 days, the disease index of infected leaves was performed on a 0–5 severity scale. Each leaf was then weighed and ground with a pestle and mortar in 5 ml physiological solution (0.85% NaCl). Serial dilutions were plated on LB gentamicin, and cfu were counted on the following day after overnight incubation at 30°C. Values of chenopodium colonization are given as cfu g^{-1} leaves and the experiment was performed in triplicate; in each experiment 10 plants were used and on each three leaves were infected. The statistical significance of the differences between WILD TYPE and mutant strains in *C. quinoa* colonization ability was tested with SPSS 15.0 software as described above.

Exoenzyme production, motility assays and anti-fungal activities

Both proteolytic and lipolytic activity were determined on KB agar plates supplemented with 2% w/v powder skim milk and 1% v/v tributyrin, respectively, as previously described (Huber *et al*., 2001). After inoculation, plates were incubated at 30°C for 3 days. Swimming assays were performed on 0.3% KB agar plates while swarming assays were performed on 0.8% KB agar plates. The inoculation was performed spotting 0.5 µl of each culture grown at OD_{600} 2.0 in the plate. The swimming zone was measured after 24 h incubation at 30°C, while swarming plates were incubated at 30°C overnight, and then at room temperature for additional 48 h. Screening for anti-fungal activity was performed on PDA medium. Pathogens used were *Fusarium graminearum*, *F. verticillioides*, *Bipolaris oryzae*, *Pyricularia grisea* and *Rhizoctonia solani* (obtained from Professor Favaron, Department of Agriculture, University of Padova, Italy). A water suspension of the fungal conidia was inoculated in the melted PDA. The bacterial strains were grown to an OD_{600} of 2, and then 0.5 µl were spotted on the PDA plate containing the fungi. The plates were then grown at 25°C for approximately 1 week during which the fungal mycelia completely covered the agar surface unless mycelial growth was inhibited by the bacteria, in which case we observed an inhibition halo surrounding the bacterial spot.

DNA sequencing and nucleotide sequence accession numbers

DNA sequencing was performed by Macrogen (http://www.dna.macrogen.com). The nucleotide sequences of 2150 bp fragment harbouring *pfvI*, *rsaL* and *pfvR* of strain and the 2150 bp fragment harbouring *pfsI*, *rsaM* and *pfsR* of strain UPB0736 have been deposited in GenBank/EMBL/DDBJ under the following accession numbers, respectively, AM943857 and FN598970.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Severity scale used to evaluate disease caused by *P. fuscovaginae* infection on *Chenopodium quinoa*: 0, no symptoms; 1, necrosis on less than 2 mm around the puncture; 2, necrosis on 2–10 mm around the puncture; 3, necrosis on 2–10 mm around the puncture and bending of the petiole; 4, collapse of the petiole; and 5, wilting of the leaf.

Fig. S2. Severity scale used to evaluate disease caused by *P. fuscovaginae* infection on rice: 0, no symptoms; 1, necrosis around the puncture till 1 cm; 2, necrosis around the puncture and chlorosis 1–3 cm on the new leaf; 3, necrosis around the puncture and chlorosis till 5 cm on the new leaf; 4, necrosis around the puncture and chlorosis for the two-thirds of the new leaf; and 5, necrosis around the puncture and chlorosis of all the new leaf.

Fig. S3. LC/MS/MS analysis of AHL synthetic standards and ethyl acetate extracted AHLs from *P. fuscovaginae*. The top two rows of every section refer to the reverse-phase chromatographic profile. AHLs were identified by retention time analysis and by comparison to synthetic standards. The third rows are the MS/MS fragmentation patterns. (A) C10-HSL synthetic standard; (B) C12-HSL synthetic standard; (C) 3-oxo-C10-HSL synthetic standard; (D) 3-oxo-C12-HSL synthetic standard; (E) UPB0736 wild-type extract, identification of C10-HSL; (F) UPB0736 wild-type extract, identification of C12-HSL; (G) UPB0736 wild-type extract, identification of 3-oxo-C10-HSL; (H) UPB0736 wild-type extract, identification of 3-oxo-C12-HSL; (I) extract of 0736IDM double *pfvI* and *pfsI* mutant harbouring pBBPfvI expressing *pfvI*, identification of 3-oxo-C10-HSL; (J) extract of 0736IDM double *pfvI* and *pfsI* mutant harbouring pBBPfvI expressing *pfvI*, identification of 3-oxo-C12-HSL; (K) extract of 0736IDM double *pfvI* and *pfsI* mutant harbouring pBBPfsI expressing *pfsI*, identification of C10-HSL; (L) extract of 0736IDM double *pfvI* and *pfsI* mutant harbouring pBBPfsI expressing *pfsI*, identification of C12-HSL; (M) extract of 0736PFVI single *pfvI* mutant, identification

of C10-HSL; (N) extract of 0736PFVI single *pfvI* mutant, identification of C12-HSL; (O) extract of 0736PFSI single *pfsI* mutant, identification of 3-oxo-C10-HSL; (P) extract of 0736PFSI single *pfsI* mutant, identification of 3-oxo-C12-HSL.
Table S1. In this table, the ions monitored for each AHL in the mass spectrometric analyses are provided.