Virulence and *in planta* movement of *Xanthomonas hortorum* pv. *pelargonii* are affected by the diffusible signal factor (DSF)-dependent quorum sensing system

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SUMMARY

Xanthomonas hortorum pv. *pelargonii* (*Xhp*), the causal agent of bacterial blight in pelargonium, is the most threatening bacterial disease of this ornamental worldwide. To gain an insight into the regulation of virulence in *Xhp*, we have disrupted the quorum sensing (QS) genes, which mediate the biosynthesis and sensing of the diffusible signal factor (DSF). Mutations in *rpfF* (encoding the DSF synthase) and *rpfC* (encoding the histidine sensor kinase of the two-component system RfpC/RpfG) and overexpression of *rpfF* showed a significant reduction in incidence and severity of the disease on pelargonium. Confocal laser scanning microscopy images of inoculated plants with a green fluorescent protein (GFP)-labelled wild-type strain showed that the pathogen is homogeneously dispersed in the lumen of xylem vessels, reaching the apex and invading the intercellular spaces of the leaf mesophyll tissue within 21 days. In contrast, the *rpfF* and *rpfC* knockout mutants, as well as the *rpfF*-overexpressing strain, remained confined to the vicinity of the inoculation site. The *rpfF* and *rpfC* mutants formed large incoherent aggregates in the xylem vessels that might interfere with upward movement of the bacterium within the plant. Both mutants also formed extended aggregates under *in vitro* conditions, whereas the wild-type strain formed microcolonies. Expression levels of putative virulence genes *in planta* were substantially reduced within 48 h after inoculation with the QS mutants when compared with the wild-type. The results presented indicate that an optimal DSF concentration is crucial for successful colonization and virulence of *Xhp* in pelargonium.

Keywords: aggregate formation, DSF, *Pelargonium hortorum*, *rpfC*, *rpfF*.

INTRODUCTION

Bacterial blight caused by the plant-pathogenic bacterium *Xanthomonas hortorum* pv. *pelargonii* (*Xhp*; previously *X. campestris* pv. *pelargonii*) is the most destructive disease of all cultivated pelargonium varieties worldwide (Dougherty *et al.*, 1974; Taylor *et al.*, 1989). The disease is widespread in various states of the USA, Europe, Australia and Israel, and may cause heavy economic losses. Warm and wet conditions favour infection and disease development. Normally, *Xhp* penetrates the plant via natural openings (e.g. hydathodes, stomata) or wounds, and spreads systemically through the vascular system. Symptoms are characterized by wilting of the plant, localized water-soaked lesions that often become necrotic and rotted cuttings (Dougherty *et al.*, 1974; Nameth *et al.*, 1999). V-shaped yellow areas in leaf margins are frequently observed. All commercial cultivars of *Pelargonium × hortorum* are susceptible to *Xhp* (Zhang *et al.*, 2009). Cuttings taken from infected mother-plant stock are the main source of inoculum. Therefore, the use of certified disease-free transplant seedlings generated from tissue culture is a critical control measure of *Xhp* (Manulis *et al.*, 1994).

Xanthomonas species and pathovars within species show a high degree of host plant specificity and may also exhibit tissue specificity by invading either the vascular system or the mesophyll tissue of the host (Ryan *et al.*, 2011). To date, the complete and draft genome sequences of at least seven species and nine pathovars of *Xanthomonas* have been determined, all of which contain the cluster of pathogenicity-associated genes designated as *rpf* (regulation of pathogenicity factors) (Ryan *et al.*, 2011). In contrast with the extensive molecular work published on the virulence of various *Xanthomonas* spp. (reviewed in Buttner and Bonas, 2010; Ryan *et al.*, 2011), none has been reported on *Xhp*.

Quorum sensing (QS) is a form of cell-to-cell communication that regulates many activities in bacteria, including virulence, antibiotic production, biofilm formation, bioluminescence, plasmid transfer and surface motility (Barnard *et al.*, 2007). Extensive studies on QS during the last three decades have led to the

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identification of several types of QS signals and different mechanisms of signal transduction (Bassler and Losick, 2006; von Bodman *et al.*, 2003; Williams *et al.*, 2007). Two major classes of QS signals used by Gram-negative bacteria are acylated homoserine lactones (AHLs) and diffusible signal factor (DSF) composed of α,β -unsaturated fatty acids (Barber *et al.*, 1997; von Bodman *et al.*, 2003; Rai *et al.*, 2012). The *X. campestris* pv. *campestris* (Xcc) DSF, which is *cis*-11-methyl-2-dodecenoic acid (Wang *et al.*, 2004), is synthesized by RpfF, a crotonase family enzyme (Barber *et al.*, 1997; Bi *et al.*, 2012). DSFs act as the main QS signal in *Xanthomonas* spp. and in other genera of the Xanthomonadaceae family, such as *Xylella fastidiosa* (Xf) and *Stenotrophomonas* spp., and are found together with AHLs in bacteria belonging to other families, such as *Burkholderia cenocepacia* or *Pseudomonas aeruginosa* (Ryan and Dow, 2011). Distinct bacterial species can synthesize single or numerous α,β -unsaturated fatty acid-DSF signals.

The DSF sensing mechanism involves RpfC, a two-component histidine kinase (HK) that contains two additional domains compared with typical HK proteins (Slater *et al.*, 2000). The RpfC REC domain resembles the receiver domains of response regulators. Following phosphorylation from the HK domain, the REC domain transfers the phosphoryl group to the histidine phosphotransfer (HPT) domain, which, in turn, relays the phosphoryl group to the cognate response regulator RpfG. Thus, RpfC controls two independent pathways, one via the HPT domain and the other through the REC domain (He *et al.*, 2006a). Although the entire phosphorelay system, consisting of the sensor kinase, the REC domain and HPT, are essential for the activation of virulence gene expression, only the REC domain is required to repress DSF biosynthesis. Interestingly, the downstream target of RpfC in DSF biosynthesis is the DSF synthase RpfF via direct protein–protein interaction, and phosphorylation of RpfC presumably releases RpfF (He *et al.*, 2006a). Transcription of *rpfF* is not regulated by DSF; thus, the autoregulation of DSF production is post-translational. The RpfG regulator contains the receiver domain and the HD-GYP domain, which is required for cyclic di-guanosine monophosphate (cyclic di-GMP) phosphodiesterase activity (Fouhy *et al.*, 2006).

DSF accumulates in the early stationary phase and, after being sensed by RpfC and RpfG, leads to the degradation of cyclic di-GMP (Ryan *et al.*, 2006; Slater *et al.*, 2000), which acts as a secondary messenger (Romling and Amikam, 2006) to influence the activity of the transcriptional regulator Clp, which, in turn, affects the expression of further transcriptional regulators, such as Zur and FhrR (He *et al.*, 2007). Genetic and gene expression analyses have shown that the DSF QS signalling pathway regulates diverse biological functions related to virulence, including the biosynthesis of polysaccharides (xanthan), extracellular enzymes, dispersal of the biofilm and other determinants involved in ecological competence (He and Zhang, 2008).

The present study was undertaken to investigate the effect of DSF signalling on the movement, colonization and virulence of *Xhp* in pelargonium by employing disruptive and overexpression mutants of the QS genes, as well as following green fluorescent protein (GFP)-labelled *Xhp* in planta through confocal microscopy.

RESULTS

Isolation and characterization of the genes involved in the QS system of *Xhp*

Initial indication that the wild-type strain *Xhp*305 produces the DSF compound was obtained using the Xcc DSF reporter strain Xcc8523/pKLN55, which expresses GFP in the presence of DSF (Newman *et al.*, 2004). When *Xhp*305 was co-cultured with the reporter strain, a strong fluorescence was detected on agar plates after 48 h. The isolation of the DSF-dependent QS genes of *Xhp*305 was carried out by employing primers based on homologous genes in the genomes of Xcc strain 8004 and Xf Temecula, as described in Experimental procedures. The cluster contains the following genes: *rpfF*, encoding the key enzyme for DSF biosynthesis; *rpfC/rpfG*, encoding the two-component system proteins; *rpfH*, encoding a protein that is structurally related to the sensory input domain of RpfC with no apparent regulatory role; and *rpfB*, encoding a long-chain fatty acyl CoA ligase (Fig. S1, see Supporting Information).

The sequence of the cluster was submitted to GenBank under the accession numbers JN165103 (for *rpfB*, *rpfF* and *rpfC*), JN656970 (for *rpfH*) and JN656971 (for *rpfG*). Sequence comparison of this cluster with those from the genomes of Xcc 8004, Xcc ATCC 33913 and *Xanthomonas campestris* pv. *vesicatoria* (Xcv) strain 85-10 showed similar organization and the same orientation of the coding regions. BLAST analysis revealed that RpfF_{Xhp305} is highly conserved and shares a high similarity of 92% in predicted amino acids with the corresponding proteins of Xcc and Xcv (Fig. S2, see Supporting Information). The deduced proteins of RpfC_{Xhp305} and RpfG_{Xhp305} also have a high degree of amino acid sequence similarity with the corresponding proteins of Xcc and Xcv (Fig. S3, see Supporting Information). RpfC_{Xhp305} is ~88% similar and RpfG_{Xhp305} 98% similar to the corresponding proteins from Xcc and Xcv.

Mutation in *rpfF* and *rpfC* of *Xhp* reduces disease symptoms

To investigate the role of QS in the virulence of *Xhp*, marker-exchanged mutants in *rpfF* and *rpfC* (*rpfF*⁻ and *rpfC*⁻, Table 1) were generated by insertion of a kanamycin (Km) resistance gene cassette, as described in Experimental procedures. The *rpfF* mutant did not produce detectable levels of the DSF signal when tested with the reporter strain Xcc8523/pKLN55. Complementation of

Table 1 Bacterial strains and plasmids used in this study.

Strains/plasmids	Relevant characteristics	Reference or source
Strain		
<i>Escherichia coli</i>		
DH5 α	<i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>gyrA96</i>	Invitrogen, San Diego, CA, USA
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1</i> Δ (<i>lac-proAB</i>) <i>mcrA</i> [F' <i>traD36 proAB lacIq lacZ</i> Δ M15]	Winson <i>et al.</i> (1998)
S17-1/ λ pir	RK2 <i>tra</i> regulon, <i>pir</i> , host for <i>pir</i> -dependent plasmid (pVIK165)	Simon <i>et al.</i> (1983)
<i>Xanthomonas hortorum</i> pv. <i>perlagonii</i>		
<i>Xhp305</i>	Pathogenic strain, with spontaneous rifampicin resistance	Manulis <i>et al.</i> (1994)
<i>rpff</i> ⁻	Rif ^r , Km ^r , <i>Xhp305</i> marker exchange mutant with a kanamycin cassette disrupting <i>rpff</i>	This study
<i>rpfc</i> ⁻	Rif ^r , Km ^r , <i>Xhp305</i> marker exchange mutant with a kanamycin cassette disrupting <i>rpfc</i>	This study
<i>rpff</i> ⁻ /pBBR1- <i>rpff</i>	Rif ^r , Km ^r , Gm ^r , marker exchange mutant of <i>rpff</i> harbouring pBBR1/ <i>rpff</i> for complementation	This study
<i>rpfc</i> ⁻ /pBBR1- <i>rpfc</i>	Rif ^r , Km ^r , Gm ^r , marker exchange mutant of <i>rpfc</i> harbouring pBBR1- <i>rpfc</i> for complementation	This study
<i>Xhp305</i> /pBBR1- <i>rpff</i>	Rif ^r , Gm ^r , wild-type <i>Xhp305</i> harbouring pBBR1- <i>rpff</i> for overexpression of <i>rpff</i>	This study
<i>Xhp305</i> /pBBR1	Rif ^r , Gm ^r , wild-type <i>Xhp305</i> harbouring pBBR1 for complementation control	This study
<i>Xhp305</i> -GFP	Rif ^r , Km ^r , Gm ^r , wild-type <i>Xhp305</i> containing pKT-Cm expressing green fluorescent protein (GFP)	This study
<i>rpff</i> -GFP	Rif ^r , Km ^r , Cm ^r , marker exchange mutant of <i>rpff</i> containing pKT-Cm expressing GFP	This study
<i>rpfc</i> -GFP	Rif ^r , Km ^r , Cm ^r , marker exchange mutant of <i>rpfc</i> containing pKT-Cm expressing GFP	This study
<i>Xanthomonas campestris</i> pv. <i>campestris</i>		
Xcc8523 (pKLN55)	Rif ^r , Spec ^r , <i>engXCA</i> promoter- <i>gfp</i>	Newman <i>et al.</i> (2004)
Plasmids		
pGEM-T-Easy	Amp ^r , cloning vector for PCR products	Promega, Madison, WI, USA
pJET	Amp ^r , cloning vector for PCR products	Fermentas, Vilnius, Lithuania
pUC4K	Km ^r , Amp ^r ; contains a 1.3-kb <i>EcoRI</i> fragment with the Km ^r gene used for mutagenesis of <i>rpfc</i> and <i>rpff</i> in <i>Xhp</i>	Pharmacia, Piscataway, NJ, USA
pBBR1MCS	Gm ^r , high copy number cloning vector for complementation and overexpression	Antoine and Loch (1992)
pKT-Kn	Km ^r , <i>gfp</i> expression under Km promoter in pVSP61	Hallmann <i>et al.</i> (2001)
pJP-Cm	Cm ^r , suicide plasmid for mutagenesis	Tamir-Ariel <i>et al.</i> (2007)
pOKU9-Cm	Amp ^r , Cm ^r , containing a 1.5-kb <i>Bam</i> HI fragment with a Cm ^r cassette	Gartemann and Eichenlaub (2001)
pKT-Cm	Cm ^r , GFP expression vector, containing a 1.5-kb fragment with Cm ^r cassette cloned into <i>Hind</i> III site	This study
pGEM-3kb	Amp ^r , pGEM-T Easy carrying a 3-kb PCR product containing <i>rpff</i> , <i>rpfc</i> and partial <i>rpfb</i> ORF of <i>Xhp305</i>	This study
pJET-4kb	Amp ^r , pJET carrying a 4-kb PCR product containing part of the genes of the <i>rpff</i> gene cluster of <i>Xhp305</i>	This study
pJET- <i>rpff</i>	Amp ^r , pJET carrying a 1.5-kb PCR product containing <i>rpff</i> ORF of <i>Xhp305</i>	This study
pJET- <i>rpff</i> /Km	Amp ^r , Km ^r , pJET carrying a 1.5-kb PCR product containing <i>rpff</i> ORF with an insertion of 1.3-kb blunt ended Km cassette into <i>Eco</i> 721 site of <i>rpff</i>	This study
pJET- <i>rpfc</i>	Amp ^r , pJET carrying a 2.1-kb PCR product containing <i>rpfc</i> ORF of <i>Xhp305</i>	This study
pJET- <i>rpfc</i> /Km	Amp ^r , Km ^r , pJET carrying a 2.1-kb PCR product containing <i>rpfc</i> ORF with an insertion of 1.3-kb blunt ended Km cassette into <i>Afl</i> II site of <i>rpfc</i>	This study
pJP-Cm- <i>rpff</i> /Km	Cm ^r , Km ^r , pJP-Cm carrying a 2.8-kb <i>NotI/Xba</i> I fragment of <i>rpff</i> disrupted by a Km resistance cassette	This study
pJP-Cm- <i>rpfc</i> /Km	Cm ^r , Km ^r , pJP-Cm carrying a 3.4-kb <i>Bgl</i> II fragment of <i>rpfc</i> disrupted by a Km resistance cassette	This study
pJET- <i>rpff</i> -prom	Amp ^r , pJET carrying a 0.99-kb PCR product containing the estimated promoter region of <i>rpff</i>	This study
pBBR1- <i>rpff</i>	Gm ^r , pBBR1 carrying a 0.99-kb <i>Bgl</i> II fragment of <i>rpff</i> cloned into pBBR1, under <i>lac</i> promoter	This study
pBBR1- <i>rpfc</i>	Gm ^r , pBBR1 carrying a 2.1-kb <i>Apal/SacI</i> fragment of <i>rpfc</i> cloned into pBBR1, under <i>lac</i> promoter	This study

the *rpff* mutant with the corresponding gene *in trans* using pBBR1-*rpff* restored DSF production to 1.2-fold of the wild-type level [~3000 fluorescence units (FUs)]. In the *rpfc* mutant, DSF production was five-fold higher than that of the wild-type.

Pathogenicity assays were carried out by puncture inoculation of the stem of 3-week-old pelargonium plants with the wild-type and QS mutants at a concentration of 10⁶ cells/mL. Disease symptoms monitored at 21 days post-inoculation (dpi) were substantially less severe in plants inoculated with both *rpff* and *rpfc* mutants relative to those inoculated with the wild-type (Fig. 1A). Although all plants inoculated with the wild-type developed disease symptoms, the disease incidence rates were 45% and 24% for *rpff* and *rpfc* mutants, respectively (Table 2). In addition,

although the wild-type induced disease at low, moderate and high severity levels, the mutants displayed only low severity symptoms (Table 2, Fig. 1). Complementation of the *rpff* and *rpfc* mutants with the intact coding region of the corresponding genes (pBBR1-*rpff* and pBBR1-*rpfc*) restored the disease incidence to 57% and 95%, respectively, compared with the wild-type (Table 2).

Mutation in *rpff* and *rpfc* affects colonization and movement of *Xhp* in planta

It was of interest to examine whether the reduction in symptom development by the QS mutants relative to the wild-type was associated with lower colonization and reduced movement ability

Fig. 1 Effect of *rpfF* and *rpfC* mutations on the virulence of *Xanthomonas hortorum* pv. *pelargonii* (*Xhp*) in pelargonium plants. (A) Plants inoculated with the wild-type (*Xhp305*) and mutants in *rpfF* (*rpfF*⁻) and *rpfC* (*rpfC*⁻). (B) Wild-type containing pBBR1 (*Xhp305/pBBR1*) and wild-type overproducing diffusible signal factor (DSF) (*Xhp305/pBBR1-rpfF*). Stems of 3-week-old plants were puncture inoculated with bacterial suspension (10^6 cells/mL). Experiments were repeated three times with 20 plants for each strain and photographs were taken from representative plants at 21 days post-inoculation (dpi).

Table 2 Effect of mutation in *rpfF* and *rpfC* on symptom development in pelargonium plants.

Disease incidence*	<i>Xhp305</i>	<i>rpfF</i> ⁻	<i>rpfC</i> ⁻	<i>rpfF</i> ⁻ /pBBR1- <i>rpfF</i>	<i>rpfC</i> ⁻ /pBBR1- <i>rpfC</i>
No symptoms	0	55*	76*	43*	5
Low severity	17	45*	24	25	21
Moderate severity	70	0*	0*	32*	63
High severity	13	0*	0*	0*	11

*Disease incidence: the percentage of symptomatic plants in each treatment determined at 21 days post-inoculation. For each treatment, 20 plants were used and the experiment was repeated three times. Asterisks denote significant differences ($P < 0.05$) in the mutants and the complemented strains compared with the wild-type at each disease severity level according to Student's *t*-test.

in the plant. Stems of pelargonium plants were puncture inoculated with suspensions of *Xhp305*, *rpfF* and *rpfC* mutants, and bacterial populations were monitored at different stem heights at 21 dpi. The wild-type and the two mutants showed a similar bacterial titre of approximately 10^8 colony-forming units (CFU)/g at the inoculation site (IS) (Fig. 2). In contrast, 1.5 cm above the IS, the wild-type population was about 10^8 CFU/g, whereas the mutants grew to approximately 10^6 and 10^3 CFU/g for the *rpfF* and *rpfC* mutants, respectively. Moreover, at the apex (~20 cm above the IS), only the wild-type could be detected with a titre of 10^5 CFU/g (Fig. 2).

To further examine the differences in colonization and movement of the wild-type and the *rpfF* and *rpfC* mutants, confocal laser scanning microscopy (CLSM) was employed to observe GFP-labelled strains during host infection. The presence of the GFP-expressing plasmid pKT-Cm in the wild-type and mutants did not affect bacterial growth *in vitro* and virulence *in planta* (data not shown). A longitudinal section of pelargonium stem inoculated by the wild-type and taken at 21 dpi showed that the pathogen homogeneously colonized the lumen of xylem vessels (Fig. 3A).

Transverse sections of young stems showed that many vessel lumens in the vascular bundle were filled with bacterial cells (Fig. 3B). Extensive colonization was also observed in parenchymal intercellular spaces of transverse mesophyll sections taken from necrotic leaf adjacent to the apical region (Fig. 3C). In contrast with the uniform distribution of the wild-type in the xylem vessels (Fig. 3A), the colonization patterns of both GFP-labelled mutants exhibited a noticeably discontinuous growth, forming large clumps of bacterial aggregates (Fig. 3D, E). These results are in agreement with those presented in Fig. 2, supporting the findings that the wild-type can heavily colonize the whole plant and can be detected at the apex, whereas *rpfF* and *rpfC* mutants are confined to the first few centimetres of the vessels above the IS.

Effect of mutation in *rpfF* and *rpfC* on aggregate formation by *Xhp*

The differences in aggregate formation by the wild-type *Xhp305* and the *rpfF* and *rpfC* mutants were further examined *in vitro* and viewed by CLSM. GFP-labelled strains were grown in fresh pelar-

Fig. 2 Effect of *rpfF* and *rpfC* mutations on the colonization of pelargonium plants by *Xanthomonas hortorum* pv. *pelargonii* (*Xhp*). Plants were puncture inoculated with the wild-type *Xhp305*, the quorum sensing (QS) mutants (*rpfF*⁻ and *rpfC*⁻) or *Xhp305/pBBR1-rpfF*, as described for Fig. 1. Stem segments were collected at 21 days post-inoculation (dpi) from the infection site (IS), 1.5 cm above IS and from the apex. Results represent the means and standard deviations of three independent experiments with three replicates per treatment in each experiment. Asterisks denote significant differences ($P < 0.05$) in the mutants compared with the wild-type according to Student's *t*-test. CFU, colony-forming units.

gonium extract on the surface of microscopic glass slides. After 7 days, *Xhp305* formed microcolonies, whereas the mutants formed extended aggregates (Fig. 4). Aggregates produced by the *rpfF* mutant were more dense and larger than those of the *rpfC* mutant, leading to the formation of biofilm-like structures of ~40 µm in thickness (Fig. 4C). To further support these qualitative observations, quantification of aggregate formation in pelargonium extract, Luria–Bertani (LB) medium and minimal M9 medium was measured by crystal violet staining assay. Aggregate formation by *rpfF* and *rpfC* mutants was 3.0- and 3.2-fold higher, respectively, than in the wild-type when cells were grown in the presence of plant extract, and these differences were statistically significant (Fig. 5). In minimal M9 medium, aggregation of *rpfF* and *rpfC* mutants was significantly lower, by approximately three-fold, when compared with cells of the same mutants grown in plant extract. Nevertheless, the levels of aggregation observed for the mutant strains were significantly higher than that observed for the wild-type in this medium (Fig. 5). Complementation of *rpfF* and *rpfC* mutants with the intact corresponding genes resulted in similar levels of aggregation as observed for the wild-type in the presence of plant extract and in M9 medium. Overall, the levels of aggregation in rich (LB) medium were low, with no significant differences being observed among the strains (Fig. 5).

Xhp* virulence is inhibited by the overexpression of *rpfF

It has been reported previously that high concentrations of the QS signal AHL may substantially reduce the virulence of plant-

pathogenic bacteria (Chalupowicz *et al.*, 2008; Quinones *et al.*, 2005). This possibility was examined in *Xhp* by overexpression of the DSF synthase in the wild-type strain through transformation of the wild-type with plasmid pBBR1-*rpfF* containing the entire coding region of *rpfF* under the *lac* promoter (Table 1). The transconjugant, designated as *Xhp/pBBR1-rpfF*, showed DSF levels that were 5.3-fold higher (FUs ~ 16 000) than those of the wild-type, and this strain was not able to induce symptoms on pelargonium when compared with the wild-type carrying the 'empty' vector pBBR1 (Fig. 1B). The inability of *Xhp/pBBR1-rpfF* to induce symptoms correlated with its low colonization ability *in planta* (Fig. 2). At 21 dpi, *Xhp/pBBR1-rpfF* could be detected at the IS and at 1.5 cm above the IS, but not in the apex. Nevertheless, aggregation assay using crystal violet staining in the presence of pelargonium extracts or in minimal medium revealed that the *rpfF*-overexpressing mutant, as well as the wild-type strain, hardly produced aggregates, in contrast with the *rpfF* and *rpfC* mutants (Fig. 5).

Effect of QS on the expression of putative virulence genes at early stages of infection

The effect of the QS system on the development of disease symptoms on pelargonium was further investigated by measuring the expression of various putative virulence factors of *Xhp*. The genes were selected as they were shown to be under RpfF/DSF control and presumably contribute to the virulence of various xanthomonads (Guo *et al.*, 2012; He *et al.*, 2006b; Tao *et al.*, 2010). Ten genes were selected including *pelE1* (encoding a pectate lyase), *egl*, *xc_0027* and *xc_0639* (encoding endoglucanases/cellulases), *gumM* [involved in exopolysaccharide (EPS) synthesis], *xagB* and *manA* (encoding biofilm formation/dispersion factors), *prtA* (encoding a serine protease), and *virK* and *virB* (encoding putative virulence factors) (Guo *et al.*, 2012; He *et al.*, 2006b). Expression of these genes in the wild-type was examined following inoculation of pelargonium plants. Transcript levels were measured at different hours post-inoculation (hpi) using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with the primers listed in Table 3, and expression was calculated relative to zero time (inoculation time). All the tested genes were expressed upon inoculation with the wild-type strain. The relative expression of *pelE1* (encoding a pectate lyase) was increased by 30-, 35- and 25-fold at 16, 24 and 48 hpi, respectively (Fig. 6). Expression of *gumM* was induced by 3.8- and three-fold at 24 and 48 hpi, respectively. A substantial increase of five- and 3.8-fold in the transcription of *virK* and *virB*, respectively, was observed at the initial 16 hpi. In contrast with the above genes, *virB* expression was reduced at 24 and 48 hpi by two- and 1.9-fold, respectively (Fig. 6). The endoglucanase encoding gene *xc_0027* showed significant expression levels at 16–48 hpi, whereas transcript levels of the endoglucanase *xc_0639* were only

Fig. 3 Confocal laser scanning microscopy (CLSM) of pelargonium plants inoculated with green fluorescent protein (GFP)-labelled *Xanthomonas hortorum* pv. *pelargonii* (*Xhp*) strains. Plants were puncture inoculated as described in Fig. 1 and samples were taken from stems 1.5 cm above the inoculation site and from symptomatic leaves at 21 days post-inoculation (dpi). Longitudinal (A, D, E) and transverse (B, C) sections of representative samples are shown. Images of plants inoculated by *Xhp*305-GFP: stems (A, B) and symptomatic young leaf (C). Images of stem samples taken from plants inoculated with *rpff*-GFP (D) and *rpfc*-GFP (E).

Fig. 4 Aggregate formation by green fluorescent protein (GFP)-labelled *Xanthomonas hortorum* pv. *pelargonii* (*Xhp*) (A) and its mutants in *rpff* (B) and *rpfc* (C). Bacteria (10^9 cells/mL) were grown in pelargonium extract on a microscope slide surface. Seven days after incubation, images were taken by confocal laser scanning microscopy (CLSM). Three independent experiments were performed for each strain, and similar results were obtained. Scale bars in the images represent 50 μ m. Images at the bottom are sagittal (xz) planes.

Fig. 5 Spectrophotometric determination of aggregate formation by *Xanthomonas hortorum* pv. *pelargonii* (*Xhp*) strains using the crystal violet staining assay. Bacterial strains were grown in microtitre plate wells in the presence of fresh pelargonium extract (PE), minimal M9 medium (M9) and Luria–Bertani medium (LB) for 5 days following staining with crystal violet and optical density measurements at 595 nm. The strains tested were: wild-type (*Xhp305*), quorum sensing (QS) mutants (*rpff*⁻ and *rpfC*⁻), complemented mutants (*rpff*⁻/*pBBR1-rpff* and *rpfC*⁻/*pBBR1-rpfC*) and diffusible signal factor (DSF)-overexpressing strain (*Xhp305/pBBR1-rpff*). Data represent the average and standard deviation of two experiments. Asterisks denote significant differences ($P < 0.05$) in optical density of *rpff* and *rpfC* mutants compared with *Xhp305* in plant extract and minimal M9 medium.

detected at 48 hpi (data not shown). Low transcriptional activities were measured for *egl*, *prtA*, *xagB* and *manA* during the first 48 hpi (data not shown).

The expression levels of these 10 genes were also measured on infection of pelargonium plants with the *rpff* and *rpfC* mutants, as well as the *rpff*-overexpressing strain *Xhp/pBBR1-rpff*. Expression of *pelE1* and *gumM* showed a substantial reduction of 6–12-fold at 16–48 hpi when compared with the wild-type (Fig. 6). Expression of *virK* and *virB* was reduced significantly in the *rpff* and *rpfC* mutants relative to the wild-type, whereas the *rpff*-overexpressing strain showed similar levels to the wild-type (Fig. 6). The expression levels of the other six examined genes did not differ from that of the wild-type in any of the mutants (data not shown).

As a result of the importance of bacterial cell attachment and movement in vascular infection and colonization, it was hypothesized that genes associated with these traits might be differentially expressed during the early stages of infection. Therefore, the expression levels of five additional genes associated with these traits, namely *fim* (encoding a fimbrial protein), *pilC* (a fimbrial assembly protein), *yapH* (a filamentous haemagglutinin-related protein), *pilT* (a twitching motility protein) and *papD* (a pili assembly chaperone), were measured in pelargonium plants at 24 hpi with wild-type *Xhp305*, *rpff* and *rpfC* mutants and the *rpff*-overexpressing strain *Xhp/pBBR1-rpff*. The results showed that the expression levels of *fim*, *pilC* and *papD* were high in the wild-type (increasing by 34-, 14- and 44-fold relative to zero time, respectively), as well as in the overexpressing mutant (29-, 16- and 40-fold, respectively). In contrast, the expression of these genes could not be detected in *rpff* and *rpfC* mutants (Fig. S4, see

Supporting Information). The expression of *pilT* and *yapH* was detected only in the wild-type and was higher by 42- and 20-fold, respectively, compared with zero time.

DISCUSSION

Cell-to-cell communication mediated by the DSF family is a common form of gene regulation and plays a crucial role in virulence across *Xanthomonas* spp., *Xf* and some human- and animal-pathogenic bacteria (Ryan and Dow, 2011). Although the genes of the *rpff* cluster that are involved in production and signalling by the DSF are generally conserved, the patterns of gene expression that they mediate may differ among *Xanthomonas* species and, perhaps, even pathovars. For example, although DSF-deficient mutants in both *Xcc*, a pathogen of crucifers, and *Xoo*, a pathogen of rice, exhibit reduced virulence, the regulation of virulence traits in each of these species is varied (Barber *et al.*, 1997; Chatterjee and Sonti, 2002). In addition, in contrast with *Xcc*, in which DSF deficiency reduces virulence, a DSF-deficient mutant of *Xf* was shown to be hypervirulent on grape (Newman *et al.*, 2004). Thus, diverse virulence mechanisms might be expressed among different bacteria that use DSF as their QS signal. The present research was undertaken to characterize DSF signalling in relation to the virulence of *Xhp*, the causal agent of bacterial blight in pelargonium, the most threatening disease of this ornamental plant. Despite the economic importance of the disease, little is known about basic aspects of the disease, and the DSF-mediated QS system in *Xhp* has not been studied. Our long-term goal is to explore whether DSF signalling could be manipulated for pelargonium bacterial blight control.

Table 3 Sequences of primers used in this study.

Primer	Sequence (5' → 3')	Used for
5' int	CTGGCATCGGACAGCGACGTGTTCAATCTGGG	Cloning of partial open reading frame (ORF) of <i>rpfF</i>
3' int	CGCCGCACCAGGCGGTCCATGGTACGCAGTGA	
rpf-inv-f	CAGCGGATCATGCTCAAT	<i>rpfF</i> internal primers for inverse polymerase chain reaction (PCR)
rpf-inv-r	ATGAAAGAGTAGGCACCCATG	
rpfB-inv-f	GCACGCTCTCGTTCCTCTCACT	<i>rpfB</i> internal primers for inverse PCR
rpfB-inv-r	CGACCGCTGATTCTATGCGTGC	
rpfG-f	ATGCAGGATGTTTTAGGGAAT	Sequencing of <i>rpfG</i> and <i>rpfH</i>
rpfG-r	CATGGCAATGGAGACTTCAT	
rpfF-f	GACGAAAAGTCCGGCGAGAT	Cloning of <i>rpfF</i> into pJET
rpfF-r	GATGGAACCCGTTTCGGATT	
rpfC-f	ATGAAGTCTCCATTGCCATGG	Cloning of <i>rpfC</i> into pJET
rpfC-r	CTATTCGCTATTACGGGGGGA	
rpfF-prom-f	GCGCCGAAGGGGTAATCCTAC	Cloning of <i>rpfF</i> with its promoter region
rpfF-prom-r	CCCGTAATAGCGAATAGGGCGG	
rpfF-probe-f	GATCATGCTCAATGGCAAT	Creating a probe of <i>rpfF</i> for Southern hybridization analysis
rpfF-probe-r	TCATTTGCGGTACCTGCT	
rpfC-probe-f	CACTGGCATCGAGCACAC	Creating a probe of <i>rpfC</i> for Southern hybridization analysis
rpfC-probe-r	CGATTTGCTTAAGGGTGACA	
rpfC-comp-f	CAGCAAGGAAGGATCCCAAC	Complementation of <i>rpfC</i>
rpfC-comp-r	GATGCAGTTGGGCGAAAAAT	
rpoD-f	CGCTTCGGTATCGACATGAA	Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of <i>rpoD</i>
rpoD-r	CACGCGTGACATCGAACTG	
gyrB-f	TGGACGGCTCGCACATC	qRT-PCR of <i>gyrB</i>
gyrB-r	CCTTGAGATACAGCTCGCTCTTG	
pelE1-f	CCGGGTGCGTCATTATGG	qRT-PCR of <i>pelE1</i>
pelE1-r	CTTCGCGGTTGGCATAACAG	
gumM-f	ATGCTGACGTACTGCATGCAA	qRT-PCR of <i>gumM</i>
gumM-r	CGTCGCTGAATGGCTGATC	
virK-f	TTACCGTGGCCAACGA	qRT-PCR of <i>virK</i>
virK-r	CGCAGCTGATAACGCATGA	
manA-f	AGATTGTTGCTGCGGAAGGT	qRT-PCR of <i>manA</i>
manA-r	CGTGCACATGTACGGGTGTT	
xagB-f	GCGTGGTCTTCTCCAGTAGA	qRT-PCR of <i>xagB</i>
xagB-r	GCGGCCTACAAGGGTCTGT	
virB-f	TCTCTGGAAGCGCAACAATTG	qRT-PCR of <i>virB</i>
virB-r	GGAGGGTTGTTCTTCTGCAT	
xc_0027-f	TCGTTATCATAGCCCGAAAA	qRT-PCR of <i>xc_0027</i>
xc_0027-r	TGGCCATCGCGTTCAAG	
xc_0639-f	CGGCGAACTGACCGAAAT	qRT-PCR of <i>xc_0639</i>
xc_0639-r	TCGTAATTCAACGACAGCAACTTC	
egl-f	GGCTTGGTGCCGCTGTAGT	qRT-PCR of <i>egl</i>
egl-r	AGGACCCCGCAACAATC	
prtA-f	TTGAAGCGCTCGGTGATG	qRT-PCR of <i>prtA</i>
prtA-r	ATGCGTTGGTGGACCAGATC	
fim-f	CGGCATTCTGGCTGCAAT	qRT-PCR of <i>fim</i>
fim-r	TGCCTTGGCGACTTTGGT	
yapH-f	TCAGTTGCATGGATTACTCTCAA	qRT-PCR of <i>yapH</i>
yapH-r	GGTGCTTAGGGAAGCTCTGAAC	
pilC-f	GGCATGCTGCAGACCTTCAT	qRT-PCR of <i>pilC</i>
pilC-r	CTGGCTGCTCTCCCTTCATC	
pilT-f	TGCGCACCTTCGATCGA	qRT-PCR of <i>pilT</i>
pilT-r	GCGCAGTGGCTCTTCGTA	
papD-f	CAGCAGCGGATACCACTACGT	qRT-PCR of <i>papD</i>
papD-r	AGACGCCCGGTGATG	

Aggregation and biofilm formation are regulated by cell-to-cell signalling in many pathogenic bacteria (Danhorn and Fuqua, 2007). Adherence to the host cells and biofilm formation might efficiently augment the secretion of virulence factors, including extracellular enzymes, lipopolysaccharides, EPS synthesis and

suppression of basal defences, to name a few (Tao *et al.*, 2010). However, attachment must also be down-regulated to allow the dispersal of cells, migration within the host and colonization of new host sites (Dow *et al.*, 2003). Thus, the pathogen has to switch between biofilm and planktonic growth lifestyle in different

Fig. 6 Effect of quorum sensing (QS) mutants on the expression of *Xanthomonas hortorum* pv. *pelargonii* (*Xhp*) putative virulence genes at the early stages of pelargonium infection. Relative expression of *pelE1*, *gumM*, *virK* and *virB* was measured in the wild-type *Xhp305*, its mutants *rpff*⁻ and *rpfC*⁻, and the *rpff*-overexpressing strain. Three-week-old pelargonium plants were puncture inoculated as described in Fig. 1. RNA was extracted from the inoculation site at different time points (hours post-inoculation, hpi) and gene expression was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) following cDNA synthesis. Gene expression was normalized with *rpoD* and *gyrB* as endogenous controls, and inoculated plants at time zero were used as controls. Results represent the mean and standard deviations of three different experiments with three replicates per treatment.

phases of its life cycle within the host. The reversible transition from biofilm to planktonic forms in *Xanthomonas* spp. is mediated by DSF and is essential for pathogenicity (He and Zhang, 2008).

The results of this study, demonstrating the significant role of the DSF-mediated QS system in the pathogenicity of *Xhp*, could be best analysed in the light of the prevailing concept that virulence in *Xanthomonas* spp. is tightly controlled by DSF. An optimal DSF concentration is absolutely required to maintain an appropriate balance between these two bacterial lifestyles in order to achieve successful infection (Guo *et al.*, 2012; He and Zhang, 2008; Ryan and Dow, 2011). Thus, the overexpression of *rpff*, which produced a considerably higher level of DSF (5.3-fold) when compared with the wild-type, was non-pathogenic (Fig. 1). However, only partial restoration of disease incidence (57%) was detected in the complemented *rpff* mutant, although the DSF level was 1.2-fold when compared with the wild-type. This discrepancy is not clear. It might be speculated that the DSF level obtained in the complemented mutant is not optimal under *in planta* conditions. It is also possible that the constitutive expression of *rpff* (in pBBR1) disrupts the sequential timing of DSF accumulation for optimal virulence. It is noteworthy that the *rpfC* mutant produced a high level of DSF (five-fold) when compared with the wild-type. This phenomenon has been observed previously in *Xcc*, and was attributed to a possible negative regulation of the DSF level by RpfC (Fouhy *et al.*, 2006).

A significantly higher aggregation by *Xhp* mutants was found in the presence of pelargonium extract relative to rich or minimal medium (Fig 5). These results differ from those obtained for *Xcc*, in which growth in minimal or rich medium induced aggregation

(Dow *et al.*, 2003; Torres *et al.*, 2007), suggesting that aggregation depends on environmental conditions. It might be postulated that pelargonium extract contains compounds that trigger aggregate formation.

The enhanced aggregation detected in the *rpff* and *rpfC* mutants (Figs. 3–5) could presumably involve bacterial cell-to-cell attachment, as well as attachment to surfaces; both parameters could be under Rpf/DSF control. The loss of DSF or RpfC may lead to elevated levels of cellular cyclic di-GMP through a failure to activate RpfG for cyclic di-GMP degradation (as shown in other *Xanthomonas* species). It might be hypothesized that the *rpff* and *rpfC* mutants disrupt the signalling patterns of genes expressed via the degradation of cyclic di-GMP to allow the up-regulation of fimbrial and afimbrial adhesins, which enhance adherence and preclude bacterial movement, as reported for *Xf* (Wang *et al.*, 2012). However, the results of this study demonstrated that three putative genes (*fim*, *pilC* and *papD*) related to cell attachment were down-regulated in the mutants relative to the wild-type (Fig. S4). It is possible that other genes are involved in *Xhp* attachment. However, the putative genes related to movement (*pilT* and *yapH*) were expressed only in the wild-type and not in the QS mutants, including the overexpressing strain. The latter results are in accordance with the lack of movement of these strains observed *in planta* (Fig. 2).

The down-regulation of *pelE*, *gumM*, *virK* and *virB* by the *rpff* and *rpfC* mutants (Fig. 6) may reflect the dysfunction of signalling by cyclic di-GMP. However, this hypothesis should be examined further. Although PelE might support cell dissemination, GumM is involved in xanthan biosynthesis, an important polysaccharide

component of the mature biofilm in *Xanthomonas* (Fouhy *et al.*, 2006), although not mandatory for biofilm formation (Dow *et al.*, 2003). However, both were down-regulated by the dysfunction of DSF signalling. We posit that the hyperaggregation phenotype observed for the *Xhp* *rpfF* and *rpfC* mutants on the one hand, and the absence of bacterial dispersion on the other, are caused by the loss of reversible transition between these two bacterial lifestyles of *Xhp*, resulting in arrested infection.

The effect of QS on the structural organization of the biofilm in relation to virulence is not unique to the DSF signal. For example, virulence of the maize pathogen *Pantoea stewartii* ssp. *stewartii*, is strongly dependent on stewartan, an EPS that accumulates in the xylem vessels and is controlled by the AHL class of signals (Koutsoudis *et al.*, 2006; von Bodman *et al.*, 2003).

Pelargonium plants inoculated with *rpfF* and *rpfC* mutants or with the wild-type overexpressing *rpfF* resulted in a substantial decrease in virulence (Fig. 1). These three strains were severely defective in their upward mobility within the xylem vessels and could not reach the plant apex (Fig. 2). The abortive movement of the *rpfF* and *rpfC* mutants in the xylem vessels was associated with discontinuous growth as a result of the formation of large bacterial aggregates as opposed to the dispersed appearance of the wild-type (Fig. 3). The enhanced aggregation of the *rpfF* and *rpfC* mutants was also evident *in vitro* in contrast with the dispersed appearance of the wild-type (Fig. 4). Crystal violet assay demonstrated that the overexpressed mutant, similar to the wild-type, did not form aggregates as seen for the *rpfF* and *rpfC* mutants (Fig. 5). Thus, although the suppression of mobility and virulence was common to the *rpfF* and *rpfC* mutants and the *rpfF*-overexpressing strain, the mechanisms responsible for the output of these phenotypes are probably different. Enhanced aggregation in *rpfF* and *rpfC* mutants has been reported previously in *Xcc* grown in rich medium containing glucose (Torres *et al.*, 2007). Interestingly, an *rpfC* mutant of *Xf* has also been reported to exhibit a hyperattachment phenotype as well as deficiency in virulence and migration along xylem vessels (Chatterjee *et al.*, 2008).

Disease control by excessive DSF as a result of the overproduction of *rpfF* could be assigned to the premature expression of virulence factors by the pathogen, which triggers an early recognition by the host plant and thus a more successful host defence response. A similar phenomenon has been observed with the gall-forming bacterium *Pantoea agglomerans* pv. *gypsophillae*, where the addition of exogenous AHL signal reduced the gall size by up to 90% (Chalupowicz *et al.*, 2008). Moreover, transgenic tobacco plants producing AHL exhibited enhanced resistance to the soft-rot pathogen *Erwinia carotovora* (Mae *et al.*, 2001). A similar result was obtained with transgenic grapes overexpressing *rpfF* that were shown to be more resistant to *Xf* (Lindow *et al.*, 2014). Overall, these antecedents and the data emerging from our study support the possible potential of the expression of *rpfF* in

pelargonium plants to obtain resistance against *Xhp* as an effective means to cope with bacterial blight disease.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type and mutant strains of *Xhp* 305 were grown in LB broth or agar at 28 °C, whereas *Escherichia coli* strains were cultured on the same medium at 37 °C. Antibiotics were used at the following concentrations (µg/mL): Km, 50; rifampicin (Rif), 150; ampicillin (Amp), 150; gentamycin (Gm), 30; chloramphenicol (Cm), 10. All strains were maintained as glycerol stocks at -80 °C.

Pathogenicity assay and colonization test

Pelargonium × *hortorum* 'Anthony' (Adler Nurseries, Kibbutz Nir Oz, Israel) plants were grown in individual pots placed in a glasshouse at 25–28 °C, and computational drip irrigation was supplied once a day for 10 min. Three-week-old plants were inoculated by puncturing the stems three times with a needle 2 cm above the ground. Then, 10 µL of bacterial suspension containing 10⁸ cells/mL, or 10 µL of sterilized distilled water in mock-inoculated plants, were placed at the wounds. The inoculated plants were inspected visually at weekly intervals and the appearance of typical symptoms was recorded. Disease severity was determined using a four-grade scale as follows: no symptoms, plants look as healthy as mock-inoculated plants; low severity, small necrotic areas were observed on one or two leaves close to the IS; moderate severity, more than three leaves developed necrotic areas; high severity, wilting and necrosis symptoms were observed on most leaves or the plant died. Colonization of pelargonium plants by the different strains was determined at 21 dpi. One centimetre stem pieces were excised from the IS, 1.5 cm above the IS, and from the apex. The tissue was macerated in 1 mL of distilled water and 10-fold serial dilutions were plated onto LB agar plates amended with Rif. The numbers of CFU per gram of stem fresh weight were determined after 48 h. The experiment was performed three times and the mean for each treatment was calculated from three plants.

Bioassay for the detection of DSF

Detection of DSF production was carried out using the DSF biosensor strain *Xcc* 8523 (pKLN55). This strain was constructed by insertion of plasmid pKLN55 harbouring the DSF-inducible *eng:gfp* reporter gene (Newman *et al.*, 2004). Essentially, both the reporter and the tested *Xhp* strains were grown to an optical density at 595 nm (OD₅₉₅) of 0.25 in 50 mL of LB broth amended with the appropriate antibiotics at 28 °C. The reporter culture (100 µL) was spread onto an LB agar plate and paper discs (5 mm in diameter) were laid on the surface. The tested *Xhp* strains (50 µL) were added to the individual discs and the plates were incubated for 48 h at 28 °C. In this assay, a fluorescent signal is detected in the area surrounding the discs. Fluorescence was viewed with a fluorescent binocular microscope (model Leica MZFLIII, Wetzlar, Germany) and measured as follows. Cells of the reporter culture surrounding the paper disc, containing the

tested *Xhp* strains or the water control, were harvested with a plastic loop (2 mm in diameter) and suspended in 500 µL of sterile water. Aliquots of 100 µL of each suspension were adjusted to an OD₅₉₅ of 0.5, transferred to wells of a black microtitre plate and read in a fluorescence microplate reader (Synergy 2 Multi-Mode Microplate Reader; Bio-Tek, Winooski, VT, USA) using excitation at 485 nm and emission at 528 nm. FUs of each well were normalized by subtracting the background fluorescence value of control wells containing only the reporter. For each treatment, the average readings of three wells were calculated. Experiments were repeated at least three times.

DNA manipulation

Isolation of genomic DNA from *Xhp* or plasmid DNA from *E. coli*, cloning, ligation, transformations, colony hybridizations, Southern blots and other DNA manipulations were performed according to standard procedures (Ausubel *et al.*, 1995) or as recommended by the supplier. The cloning vectors used in this study are listed in Table 1. DNA fragments were amplified by PCR with *Taq* (Super-Therm polymerase JMR-801; Roche, Mannheim, Germany), and synthetic oligonucleotides (Table 3) were purchased from Sigma-Aldrich (Rehovot, Israel). PCR was performed directly from bacterial colonies. Amplifications were carried out in a Biometra thermocycler (Biometra GmbH, Gottingen, Germany) programmed for one cycle of 5 min at 95 °C, 34 cycles of 1 min at 95 °C, 30 s at 65 °C and 1 min at 72 °C, and a final extension of 5 min at 72 °C. The *E. coli* and *Xhp* strains were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. DNA sequencing was performed by Hylabs Ltd. (Rehovot, Israel).

Isolation of *Xhp* QS genes

A partial fragment of *rpff* from *Xcp305* was obtained by PCR amplification with the primers 3' int and 5' int (Table 3) based on the sequence of homologous genes of *Xcc* strain 8004 and *Xf* Temecula. The 0.7-kb fragment obtained was cloned into pGEM-T easy vector (Table 1). To complete the gene sequence, total genomic DNA of *Xcp305* was digested with several restriction enzymes, followed by self-ligation of the DNA fragments. The resulting circular DNAs were subjected to inverse PCR with the internal primers *rpff*-inv-f and *rpff*-inv-r (Table 3). A 3-kb *EcoRI* fragment was obtained, extracted from the gel and cloned into pGEM-T easy vector for sequencing and open reading frame (ORF) analysis. This fragment contains the entire ORFs of *rpff* and *rpfc* and the partial ORF of *rpfb*. The strategy described above for the isolation of *rpff* and *rpfc* was also employed to complete the sequence of *rpfb* and other genes of the *rpf* cluster using inverse PCR amplification with the primers *rpfb*-inv-f, *rpfb*-inv-r, *rpfg*-inv-f and *rpfg*-inv-r (Table 3). Further isolation and characterization of an additional 4-kb DNA fragment revealed the entire ORFs of *rpfb*, *rpfh* and *rpfg* genes.

Generation of mutants

To construct the *rpff* knockout mutant, a 1.5-kb fragment containing the entire *rpff* gene was amplified using genomic DNA from *Xhp305* as a

template and the primers *rpff*-r and *rpff*-f (Table 3). The fragment harbouring *rpff* was cloned into pJET cloning vector to yield pJET-*rpff*. The construct was then disrupted by insertion of a Km resistance cassette into the internal *Eco721* site of *rpff*. The Km resistance cassette was obtained after digestion of plasmid pUC4K with *EcoRI* and subsequent blunt end reaction with DNA Polymerase I, Klenow fragment before insertion into pJET-*rpff*. The DNA fragment of the resulting construct, named pJET-*rpff*/Km, was sequenced to confirm the deletion before transformation into *E. coli* DH5α. To generate the marker exchange mutant of *rpff* in *Xhp305*, an *XbaI*-*NotI* 8-kb DNA fragment containing the *rpff*/Km insertion was excised from the above plasmid, blunt ended and ligated into pJP-Cm predigested with *HincII*. The resulting plasmid, carrying the origin of replication of plasmid R6K without its *pir* gene (Penfold and Pemberton, 1992; Tamir-Ariel *et al.*, 2007), was replicated in *E. coli* S17-1/λ*pir*. Mutants, designated as *rpff*⁻, were generated following electroporation of pJP-Cm-*rpff*/Km into *Xhp305* and selection on LB plates amended with Km. Mutation was verified by Southern blot analysis using a 0.25-kb PCR product of *rpff* as probe, which was generated with primers *rpff*-probe-f and *rpff*-probe-r (Table 3).

For complementation of the *rpff* mutant, a fragment containing the entire *rpff* gene was amplified using genomic DNA from *Xhp305* as template and the primers *rpff*-prom-f and *rpff*-prom-r. Following cloning of the PCR product into pJET vector, a *BglII* fragment was blunt ended and then cloned into pBBR1 predigested with *EcoRV*. The derivative construct was introduced by electroporation into the *rpff* mutant and selected on LB agar plates containing Rif and Gm.

For generation of the *rpfc* mutant, a 2.1-kb fragment containing the entire *rpfc* gene was amplified using genomic DNA from *Xhp305* as template and the primers *rpfc*-r and *rpfc*-f (Table 3). The fragment harbouring *rpfc* was cloned into pJET to yield pJET-*rpfc*. The *rpfc* gene in this plasmid was then disrupted by insertion of a Km resistance cassette (as described above) into the unique and internal *AflIII* site of *rpfc*. The resulting construct, namely pJET-*rpfc*/Km, was sequenced to confirm the deletion. To generate the marker exchange mutant in *rpfc* of *Xhp305*, a *BglII* 3.4-kb DNA fragment containing *rpfc*/Km was excised from the plasmid, blunt ended and ligated into pJP-Cm predigested with *HincII*. The mutants, designated as *rpfc*⁻, were generated following electroporation of pJP-Cm-*rpfc*/Km into *Xhp305* and selection on LB agar plates amended with Km. Mutations were verified by Southern blot analysis using a 0.25-kb PCR product of *rpfc* as probe, which was amplified with the primers *rpfc*-probe-f and *rpfc*-probe-r (Table 3). Complementation of the *rpfc* mutant was performed by introducing *in trans* the wild-type gene cloned with its promoter region into pBBR1, as described above for complementation of the *rpff* mutant.

A mutant overexpressing *rpff* was generated by cloning the coding region of *rpff* into the plasmid pBRR1 under its *lac* promoter. The resulting construct was then electroporated into strain *Xhp305* to yield the transconjugant *Xhp305*/pBRR1-*rpff*. Strain *Xhp305* harbouring the cloning ('empty') vector (*Xhp305*/pBRR1) was used as control for pathogenicity assays on pelargonium.

GFP-labelled strains were obtained by introducing *in trans* vector pKT-Cm, which carries the *gfp* gene. This plasmid was constructed following excision of a *BamHI* 1.5-kb fragment containing a Cm resistance cassette from pOKU9 and insertion of this fragment into the *HindIII* site of pKT-Km.

CLSM

For examination of *in planta* colonization and movement, stems of pelargonium plants were inoculated with GFP-labelled *Xhp305*, *rpff* and *rpfc* mutants. At 21 dpi, stem pieces located 1.5 cm above the IS and symptomatic leaves were collected. The plant samples were transversely or longitudinally sectioned and examined with a confocal laser scanning microscope (model CLSM Fluo View 500a; Olympus, Tokyo, Japan). At least 50 images taken from 25 plants were used.

For *in vitro* studies, GFP-labelled strains were grown in 3 mL of LB broth for 48 h at 28 °C. Bacterial cultures were centrifuged, and the pellet was washed twice with sterile water and resuspended in 3 mL of plant extract prepared as follows: sections of 2-week-old pelargonium stems (2 g) were macerated with 5 mL of distilled water and the liquid portion of the extract was filtered through a 0.22-µm membrane filter (Minisart; Sartorius Stedim Biotech, Aubagne, France). The resulting bacterial suspension (200 µL) was placed on the surface of a glass microscope slide and incubated at 28 °C inside Petri dishes sealed with paraffin film to avoid evaporation. After 7 days, the slides were examined by CLSM.

Crystal violet assay for aggregate formation

Cultures of *Xhp* strains were grown in 3 mL of LB broth for 48 h at 28 °C, and then diluted into fresh LB medium at 1:1000. The cultures were grown at 28 °C until reaching an OD₅₉₅ of 0.5, centrifuged and the pellet was washed once and resuspended in 3 mL of sterile water. The suspensions (50 µL) were added to individual wells in 24-well multidishes (Nalgene Nunc, NY, USA) containing 150 µL of LB, minimal M9 medium (Chalupowicz *et al.*, 2010) or fresh pelargonium extracts. The multidishes were incubated for 5 days at 28 °C. After incubation, the cultures were gently removed by pipetting the supernatants and the wells were washed twice with 200 µL of sterile water. Following fixation at 60 °C for 20 min, surface-attached bacteria were stained with 0.1% crystal violet solution for 1 h at room temperature. The wells were then washed gently three times with 200 µL of sterile water and air dried for 1 h prior to visual analysis. For quantitative determination, crystal violet in each well was solubilized by adding 1 mL of 95% ethanol, and a 1:10 dilution was measured at OD₅₉₅.

qRT-PCR analysis

Three-week-old pelargonium plants were puncture inoculated with wild-type and mutant strains of *Xhp* as described above. Total RNA was extracted from inoculated plants at 16, 24 and 48 hpi using the following procedure: two separate pools of three inoculated stems (1 cm around the ISs) were cut and used for the isolation of RNA with a MasterPure RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). RNA quantification was performed with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Epsom, Surrey, UK). To remove residual genomic DNA, the isolated RNA was treated with DNase (Turbo DNA-free; Ambion Inc., Austin, TX, USA). Reverse transcription was carried out from 1 µg of total RNA using a qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA). The cDNAs obtained were subjected to PCR amplification with Power SYBR Green PCR Master Mix (Applied

Biosystems, Warrington, UK) in a final volume of 20 µL. The specific primers for qRT-PCR were designed by the Primer Express 3.0 program (Applied Biosystems Instrument, Foster City, CA, USA) and are listed in Table 3. The primers for *pelE1*, *egl*, *xc_0027*, *xc_0639*, *gumM*, *priA*, *virK*, *virB*, *xagA* and *manB*, as well as the internal normalization control genes *rpoD* and *gyrB* of *Xhp305*, were designed based on the genome sequence of Xcc strain 8004, and tested for their ability to amplify the appropriate product from the genomic DNA of *Xhp305*. The primers for *fim*, *pilC*, *papD*, *pilT* and *yapH* were designed based on the draft genome sequence of wild-type *Xhp305*, which was recently performed by our group (T. Pupko *et al.*, unpublished data). The sequences of these genes were selected based on their similarity to putative genes associated with bacterial cell attachment and movement in other *Xanthomonas* species. Real-time detection was performed in triplicate with a 7300 Real Time PCR System (Applied Biosystems). The values at the inoculation time (zero time) were used as controls for the relative expression of the genes. Relative quantification and statistical analysis were performed with Real-time PCR Data Analysis software (Applied Biosystems Instrument). The results represent the mean of three different experiments with three replicates per time point.

Sequence analyses

DNA and protein sequence homology searches (e.g. BLASTN, BLASTX and PSI BLAST) were performed using the National Center for Biotechnology Information (NCBI) BLAST programs. Alignments of amino acid sequences were carried out with the programs CLUSTALX and GeneDoc. Restriction maps, ORF searches and additional molecular analyses were performed with Sci Ed Central for Windows 95, Scientific and Educational Software (Cary, NC, USA).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Physical map and transcriptional orientation of the quorum sensing (QS)-related genes in *Xanthomonas hortorum* pv. *pelargonii* (Xhp) strain 305. A 6.5-kb DNA fragment was sequenced using primers designed on the basis of homologous

genes in the related genomes of *Xanthomonas campestris* pv. *campestris* (Xcc) strain 8004 and *Xylella fastidiosa* (Xf) Temecula.

Fig. S2 Alignment of the deduced protein sequences of RpfF of *Xanthomonas hortorum* pv. *pelargonii* (Xhp) strain 305, *Xanthomonas campestris* pv. *campestris* (Xcc) ATCC 33913, Xcc 8004 and *X. campestris* pv. *vesicatoria* (Xcv) 85-10. RpfF_{Xhp305} is 92% similar in predicted amino acids with the corresponding proteins of Xcc and Xcv. The alignments were generated using CLUSTALX and GeneDoc programs. Numbers on the right-hand side indicate the positions of the amino acid residues.

Fig. S3 Alignment of the deduced protein sequences of RpfC of *Xanthomonas hortorum* pv. *pelargonii* (Xhp) strain 305, *Xanthomonas campestris* pv. *campestris* (Xcc) ATCC 33913, Xcc 8004 and *X. campestris* pv. *vesicatoria* (Xcv) 85-10. RpfC_{Xhp305} is

88% similar in predicted amino acids with the corresponding proteins of Xcc and Xcv. The alignments were generated using CLUSTALX and GeneDoc programs. Numbers on the right-hand side indicate the positions of the amino acid residues.

Fig. S4 Relative expression of putative genes related to cell attachment and movement 24 h after pelargonium infection by the wild-type and quorum sensing (QS) mutants. Transcript levels of *fim*, *pilC*, *papD*, *pilT* and *yapH* were examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Gene expression was normalized with *rpoD* and *gyrB* as endogenous controls, and inoculated plants at time zero were used as controls. Results represent the mean and standard deviations of three different experiments with three replicates per treatment.