Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2016) 17(4), 487–500 DOI: 10.1111/mpp.12295

Plant phenolic acids affect the virulence of *Pectobacterium aroidearum* and *P. carotovorum* ssp. *brasiliense* via quorum sensing regulation

JANAK RAJ JOSHI[1,2], SAUL BURDMAN[1], ALEXANDER LIPSKY[2], SHAKED YARIV[2] AND IRIS YEDIDIA[2,*]

[1]*Department of Plant Pathology and Microbiology and the Otto Warburg Minerva Center for Agricultural Biotechnology, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, 76100, Rehovot, Israel*

[2]*Department of Plant Sciences, Agricultural Research Organization, The Volcani Center, 50250, Bet Dagan, Israel*

SUMMARY

Several studies have reported effects of the plant phenolic acids cinnamic acid (CA) and salicylic acid (SA) on the virulence of soft rot enterobacteria. However, the mechanisms involved in these processes are not yet fully understood. Here, we investigated whether CA and SA interfere with the quorum sensing (QS) system of two *Pectobacterium* species, *P. aroidearum* and *P. carotovorum* ssp. *brasiliense*, which are known to produce *N*-acyl-homoserine lactone (AHL) QS signals. Our results clearly indicate that both phenolic compounds affect the QS machinery of the two species, consequently altering the expression of bacterial virulence factors. Although, in control treatments, the expression of QS-related genes increased over time, the exposure of bacteria to non-lethal concentrations of CA or SA inhibited the expression of QS genes, including *expI*, *expR*, *PC1_1442* (*luxR* transcriptional regulator) and *luxS* (a component of the AI-2 system). Other virulence genes known to be regulated by the QS system, such as *pecS*, *pel*, *peh* and *yheO*, were also down-regulated relative to the control. In agreement with the low levels of expression of *expI* and *expR*, CA and SA also reduced the level of the AHL signal. The effects of CA and SA on AHL signalling were confirmed in compensation assays, in which exogenous application of *N*-(β-ketocaproyl)-L-homoserine lactone (eAHL) led to the recovery of the reduction in virulence caused by the two phenolic acids. Collectively, the results of gene expression studies, bioluminescence assays, virulence assays and compensation assays with eAHL clearly support a mechanism by which CA and SA interfere with *Pectobacterium* virulence via the QS machinery.

Keywords: bacterial virulence, cinnamic acid, *Pectobacterium*, quorum sensing, salicylic acid.

**Correspondence*: Email: irisy@volcani.agri.gov.il

INTRODUCTION

Pectobacterium spp., which were formerly considered to be members of the genus *Erwinia*, are plant pathogens of the *Enterobacteriaceae* family that cause soft rot on many types of fruit, ornamentals and vegetables (Ma *et al*., 2007; Park *et al*., 2012; Toth *et al*., 2003, 2011). Like other soft rot enterobacteria, *Pectobacterium* induces characteristic soft rot decay through the disruption of host cell integrity, which is promoted by a variety of plant cell wall-degrading enzymes (PCWDEs) secreted by the bacterium (Davidsson *et al*., 2013; Toth and Birch, 2005). The synthesis of PCWDEs in *Pectobacterium* and other soft rot enterobacteria is mediated by quorum sensing (QS) (Barnard and Salmond, 2007; de Kievit and Iglewski, 2000; Pirhonen *et al*., 1993).

QS is a cell-to-cell communication system that allows bacteria to monitor the environment and to modulate gene expression according to population density (Fuqua *et al*., 1994). It is largely governed by the production, secretion, detection and response to freely diffusible chemical signal molecules, called auto-inducers. Of these molecules, the most studied are the *N*-acyl-homoserine lactones (AHLs) (Fuqua *et al*., 2001; Waters and Bassler, 2005). QS plays an essential role in the virulence of pathogenic bacteria (Antunes *et al*., 2010; Deep *et al*., 2011; Rutherford and Bassler, 2012). Among soft rot enterobacteria species, the QS systems of *Pectobacterium atrosepticum*, *Pectobacterium carotovorum* and *Dickeya* spp. have been the subject of several studies (Crépin *et al*., 2012; Faure and Dessaux, 2007; Liu *et al*., 2008; Nasser *et al*., 1998).

Pectobacteria utilize 3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-AHL) and 3-oxooctanoyl-L-homoserine lactone (3-oxo-C8-AHL) as QS signalling molecules (Jafra *et al*., 2006; Pollumaa *et al*., 2012). These molecules are synthesized by ExpI and detected by the receptor protein ExpR (Barnard and Salmond, 2007; Engebrecht and Silverman, 1984; Liu *et al*., 2008). ExpR is a transcriptional regulator that undergoes a conformational change, which subsequently decreases the expression of the *rsmA* gene. The products of this gene are negative regulators of the genes involved in the synthesis of PCWDEs (Barnard and Salmond, 2007; Barnard *et al*., 2007; Pollumaa *et al*., 2012; Toth *et al*., 2003).

Therefore, the repression of *rsmA* ensures high levels of expression of PCWDEs, as well as type III secretion and other virulence factors (Burr *et al*., 2006; Cui *et al*., 2005; Smadja *et al*., 2004). Collectively, AHL-mediated effects switch bacteria into a brute-force mode to promote the rotting of the host tissue (Liu *et al*., 2008).

The recognition of the crucial role of QS in bacterial virulence has made QS an attractive target for new control strategies against bacterial infections. QS inhibitors have been shown to successfully reduce or attenuate the virulence of *Pseudomonas aeruginosa* (Hentzer *et al*., 2003; O'Loughlin *et al*., 2013), *Vibrio harveyi* (Manefield *et al*., 2000), *Staphylococcus aureus* (Murray *et al*., 2014) and *P. carotovorum* (Manefield *et al*., 2001). Moreover, the expression of heterologous AHL lactonase and co-inoculation with bacteria producing this or other AHL-degrading enzymes have been shown to be effective, novel biocontrol strategies for the management of *P. carotovorum* and *Erwinia amylovora* (Dong *et al*., 2001; Molina *et al*., 2003, 2005).

The development of QS interference strategies is becoming an increasingly important approach for the control of bacterial infections (Defoirdt *et al*., 2013; Galloway *et al*., 2012; Helman and Chernin, 2015; Kalia, 2013). Some plant phenolic compounds, such as coumaric acid and salicylic acid (SA), have been shown to interfere with the QS system of the plant pathogens *Agrobacterium tumefaciens* and *P. carotovorum* (Bodini *et al*., 2009; Lagonenko *et al*., 2013; Yuan *et al*., 2007). The elucidation of the mechanisms underlying the action of these compounds may pave the way to the development of effective measures for the control of soft rot pectobacteria.

Lagonenko *et al*. (2013) showed that SA inhibits the biofilm formation and motility of *P. carotovorum* and *Pseudomonas syringae* pv. *syringae*. Moreover, SA has been found to reduce infection in tissue-cultured potato infected by *Dickeya solani* (Czajkowski *et al*., 2015). In agreement with these findings, we have shown recently that non-lethal concentrations of several plant phenolic compounds, including SA and cinnamic acid (CA), affect several virulence-associated traits and reduce the virulence of several *Pectobacterium* strains in different hosts (Joshi *et al*., 2015). To unravel the mechanism by which plant secondary metabolites, such as CA and SA, affect soft rot bacterial virulence, we investigated the effect of these compounds on the expression patterns of selected virulence-related genes in *Pectobacterium aroidearum* and *P. carotovorum* ssp. *brasiliense*. The virulence genes were categorized as follows: (i) QS system (AI-1 and AI-2) genes; (ii) QS-regulated genes; and (iii) other genes (related to membrane transporters, type III secretion, flagella and motility). Next, we attempted to elucidate the effect of CA and SA on AHL production using reporter bacteria that detect QS signals. Finally, bacterial infection assays were performed in the presence and absence of CA and SA to evaluate the effects of these compounds on virulence. Virulence assays were also performed to assess the ability of exogenous application of QS signal molecules to compensate for the impaired virulence caused by the phenolic compounds.

RESULTS

Expression of QS genes in the presence of phenolic compounds

We have shown previously that several plant phenolic compounds, including CA and SA, influence virulence-associated traits and reduce the virulence of several *Pectobacterium* species on different hosts when applied at non-lethal concentrations (Joshi *et al*., 2015). To further investigate the mechanism by which plant phenolic compounds influence bacterial virulence factors, we assessed the effects of CA and SA on the expression of selected genes of two bacterial strains representing two *Pectobacterium* species: *P. aroidearum* PC1 and *P. carotovorum* ssp. *brasiliense* Pcb1692. Exponentially growing bacterial cells of these strains were used to inoculate fresh Lysogeny-Broth (LB) with or without non-lethal concentrations of CA or SA (0.25 and 0.21 mg/mL, respectively) at an initial bacterial concentration of 10^7 colony-forming units (CFU)/mL. Gene expression levels in the presence of the compounds were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) at three time points that corresponded to different bacterial growth phases: acclimatization [1 h after inoculation (hai)], exponential phase (8 hai) and stationary phase (24 hai), as described in Experimental Procedures. At each of the mentioned time points, bacterial cell growth was evaluated by serial dilution plating to verify that bacterial concentrations of SA-/CA-treated and control cultures were at similar levels (Fig. S1, see Supporting Information).

Under these conditions, in the two strains, both CA and SA affected the expression of *expI*/*expR* QS system genes (Fig. 1) and the expression patterns of several genes whose expression is mediated by this QS system (Fig. 2). In control treatments, the relative expression of *expI* and *expR* increased with time and, after 24 h of growth, the expression of these genes was increased significantly ($P = 0.05$). At this time point, we observed an increase of about 2.5- and four-fold for *expI* in PC1 and Pcb1692, respectively, and an increase of about 10- and four-fold for *expR* in strains PC1 and Pcb1692, respectively, relative to the expression of these genes measured after 1 h of growth (Fig. 1). In contrast, during the same time period, bacterial cultures of both strains grown in the presence of CA and SA did not display such a time-dependent increase in the expression of these genes, which was significantly lower than that of the untreated bacteria.

The expression levels of *PC1_1442* (*luxR* transcription regulator belonging to the AI-1 system) also increased significantly ($P = 0.05$) with time under control conditions in both strains, whereas exposure to the phenolic compounds did not allow for an increase in *PC_1442* expression over time (Fig. 1). Interestingly,

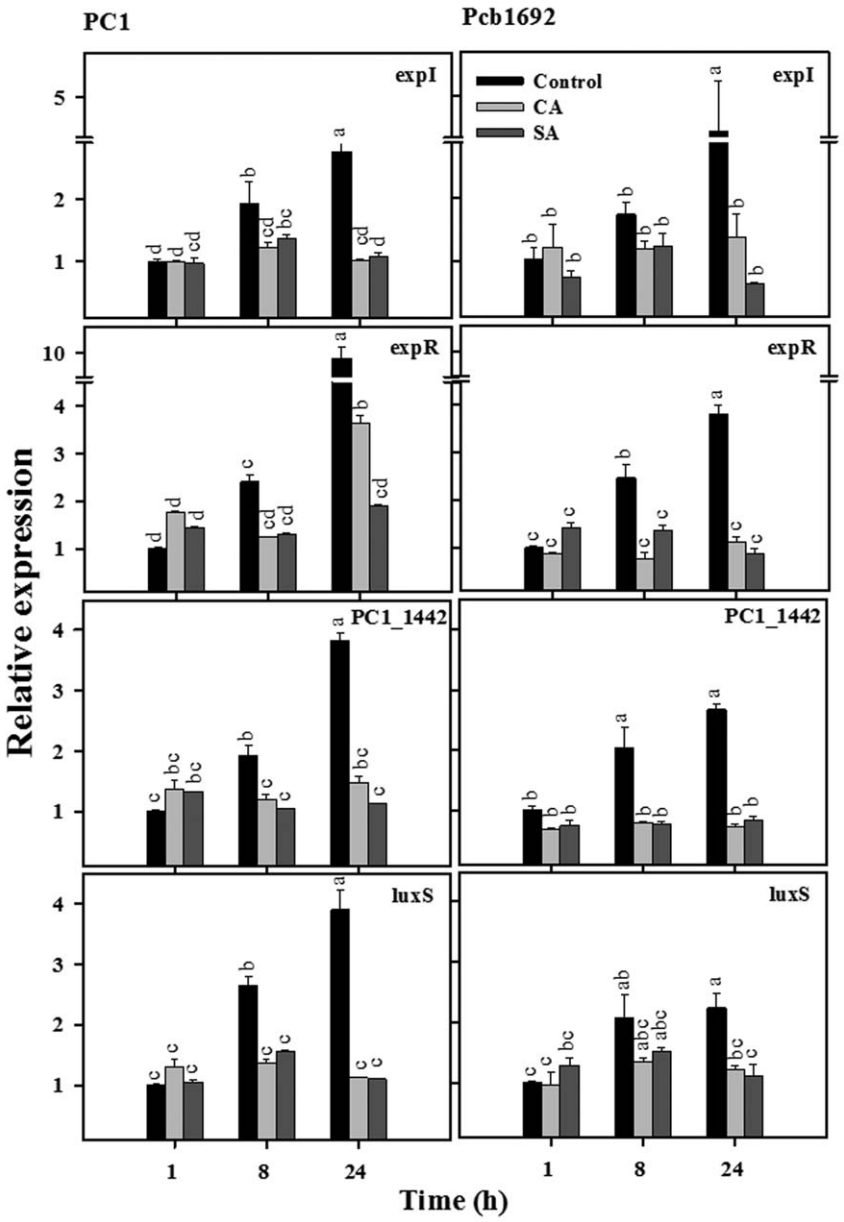

Fig. 1 Effects of cinnamic acid (CA; 0.25 mg/mL) and salicylic acid (SA; 0.21 mg/mL) on transcript levels of quorum sensing (QS) system genes in *Pectobacterium aroidearum* PC1 and *Pectobacterium carotovorum* ssp. *brasiliense* Pcb1692. The transcript levels of QS (AI-1 system) genes [*expI*, *expR*, *PC1_1442* (*luxR* transcription regulator)] and a QS (AI-2 system) gene (*luxS*) were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) of cDNA samples prepared from RNA extracts of cultures grown in Lysogeny-Broth (LB) (28 °C, continuous shaking at 150 rpm) with or without the phenolic acids. The data represent the results from one representative experiment, out of three with similar results. Means ± standard errors (SE) of the relative expression of each gene (three replicates per treatment) are shown. Different letters indicate significant differences ($P = 0.05$) among treatments for each time point in each gene/strain combination.

similar results were also observed for the expression of the *luxS* gene, which is part of the AI-2 system (Fig. 1).

Overall, under control conditions, a significant increase ($P = 0.05$) in the relative expression of all of the tested QS system genes was observed following 8 h of growth, whereas no such increase in the relative expression of these genes was observed following exposure to CA and SA (Fig. 1).

Expression of QS-regulated genes in the presence of phenolic compounds

To further characterize the response of the *Pectobacterium* strains to CA and SA, we also analysed the expression of genes contributing to production of PCWDEs: *pecS*, *pel*, *peh* and *yheO*, and their negative regulator *rsmA* (Fig. 2). These genes are located downstream of the QS cascade and are considered to be QS dependent (Barnard and Salmond, 2007; Barnard *et al*., 2007; Kõiv *et al*., 2013; Liu *et al*., 2008; Smith *et al*., 2006). The relative expression of *yheO* increased significantly ($P = 0.05$), in a gradual manner, during the experiments, reaching a maximum at 24 h in the control cultures. A pattern of increased expression over time was also observed for *pecS*, *pel* and *peh*. In contrast, the expression of these genes in the CA- and SA-treated cultures did not change substantially with time under the experimental conditions, with significantly lower expression levels ($P = 0.05$) observed in these cultures relative to controls after 24 h (Fig. 2).

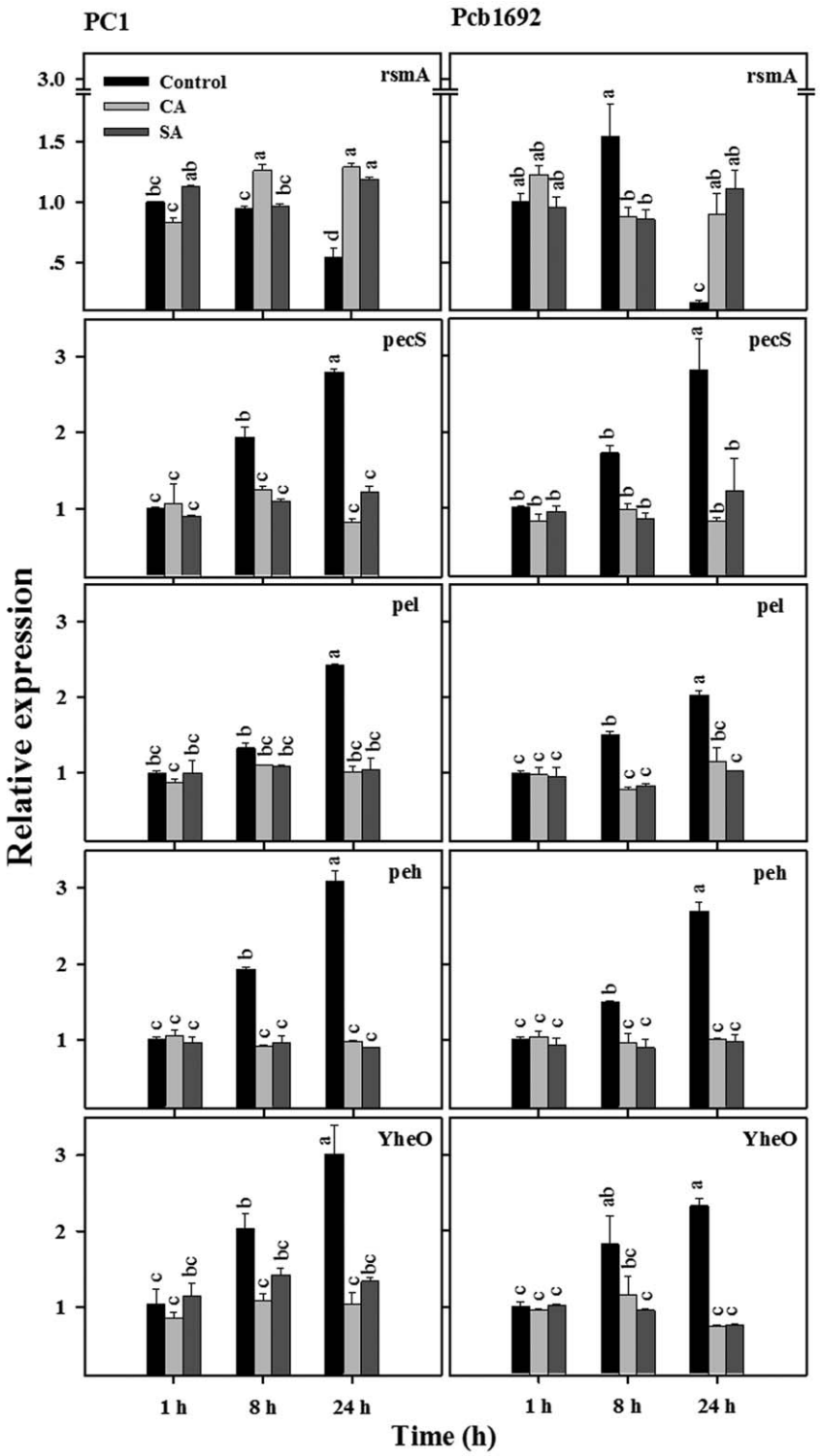

Fig. 2 Effects of cinnamic acid (CA; 0.25 mg/mL) and salicylic acid (SA; 0.21 mg/mL) on transcript levels of quorum sensing-controlled genes in *Pectobacterium aroidearum* PC1 and *Pectobacterium carotovorum* ssp. *brasiliense* Pcb1692. The transcript levels of *rsmA*, *pecS*, *pel*, *peh* and *yheO* in DNA-free RNA prepared from the bacterial strains grown in Lysogeny-Broth (LB) medium (28 °C, continuous shaking at 150 rpm) with or without CA and SA were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Means ± standard errors (SE) of relative expression of each gene (three replicates per treatment) are shown. Different letters indicate significant differences ($P = 0.05$) among treatments for each time point in each gene/strain combination.

In contrast with *pecS*, *pel*, *peh* and *yheO*, expression levels of the post-transcriptional master regulator of PCWDEs, *rsmA*, decreased significantly ($P = 0.05$) at the stationary phase (24 h) in the controls of both strains (Fig. 2). Exposure to the phenolic compounds did not alter significantly *rsmA* expression over time in either strain. In contrast with the untreated cells, for which a significant ($P = 0.05$) reduction in the expression of *rsmA* was observed at 24 h relative to 8 h, CA- and SA-treated cells did not

exhibit this reduction in the relative expression of this gene. Moreover, relative *rsmA* expression values at 24 h were significantly ($P = 0.05$) higher in the CA- and SA-treated cells relative to the controls, with the exception of the SA treatment in PC1, in which a higher level of expression was observed in the control.

Expression of other virulence genes in the presence of phenolic compounds

In this study, we also assessed the effects of CA and SA on the relative expression of genes that have been associated with virulence, but whose expression is not necessarily associated with QS (Table 1). This group of genes included efflux pump genes (*acrD* and *PC1_3330*), a membrane transporter gene (*nssA*), hypersensitive response genes (*hrpN* and *hrpL*) and a stress-related gene (*mtlD*). Overall, in contrast with the effects observed among QS and QS-regulated genes, the expression of most of the above genes was not affected by exposure to CA or SA over time (Fig. 3). Moreover, QS-independent transcription activators (hor and hrpS), which were also tested, were not affected by phenolics treatment (Fig. S2, see Supporting Information).

Effects of CA and SA on the production of QS signalling molecules

AHLs are known signalling molecules in QS systems that control the synthesis of PCWDEs in *Pectobacterium* spp. and other soft rot bacteria (de Kievit and Iglewski, 2000), among other processes. To gain further insight into the effects of CA and SA on QS and on the virulence of *Pectobacterium*, we assessed how these compounds influence the production of AHLs, using bacterial reporter strains. The first assay was based on the reporter bacterium *Chromobacterium violaceum* CV026. In the presence of external AHLs, this strain produces the purple pigment violacein (McClean *et al*., 1997). Figure 4A clearly shows that PC1 and Pcb1692 cells pretreated with CA or SA induced lower purple coloration in *C. violaceum* CV026. The effect was also studied quantitatively using an *Escherichia coli* strain carrying the bioluminescence QS reporter plasmid pSB401. Supernatants of PC1 and Pcb1692 cultures grown with or without (control) exposure to CA or SA were compared for their ability to induce bioluminescence in the reporter strain. Supernatants of cell cultures that were exposed to both SA and CA induced lower bioluminescence levels, as

Table 1 Genes assessed in this study.

	Genes/gene ID	Description of function	References
QS (AI-1 system)	*expI* (8135146)	QS signal generator; acyl-homoserine lactone synthase	Nasser *et al*. (1998)
	expR (8135145)	QS transcriptional regulator	Nasser *et al*. (1998)
	PC1_1442 (8132381)	Transcriptional regulator of LuxR-like proteins; alignment score showing 71% similarity with *expR*	NCBI
QS (AI-2)	*luxS* (8134173)	*S*-Ribosylhomocysteinase; QS autoinducer-2 production	Schauder *et al*. (2001); Taga *et al*. (2001)
Genes regulated by QS	*rsmA* (8134182)	Controls the production of PCWDEs, carbon storage and cell motility	Broberg *et al*. (2014); Kõiv *et al*. (2013)
	pecS (8133198)	Transcriptional regulator that controls the production of various virulence factors including pectinase and cellulase.	Hommais *et al*. (2008); Praillet *et al*. (1996); Reverchon *et al*. (1994)
	pel (8133114)	Secretion of pectate lyase	NCBI
	peh (8131918)	Secretion of polygalacturonase	NCBI
	PC1_2249 (*yheO*) (8133193)	*yheO* domain-containing protein; DNA-binding protein controlling the production of pectolytic enzymes	Lee *et al*. (2013)
Other genes	*acrD* (8134910)	Multidrug efflux pump	NCBI
	nssA (8130937)	Putative sodium/sulfate symporter or membrane transporter	NCBI
	mtlD (2885531)	Mannitol dehydrogenase; protects against multiple types of stress (high salt and oxidative stress)	Hema *et al*. (2014)
	hrpN (8133133)	Hypersensitive response gene	Jang *et al*. (2006)
	hrpL (8133148)	Controls expression of *hrp* genes, including *hrpN*; elicits hypersensitive response	Wei and Beer (1995)
	PC1_3330 (8134303)	Efflux pump membrane protein; multidrug resistance protein	NCBI

Gene IDs mentioned in this table were taken from GenBank (National Center for Biotechnology Information, NCBI).
PCWDEs, plant cell wall-degrading enzymes; QS, quorum sensing.

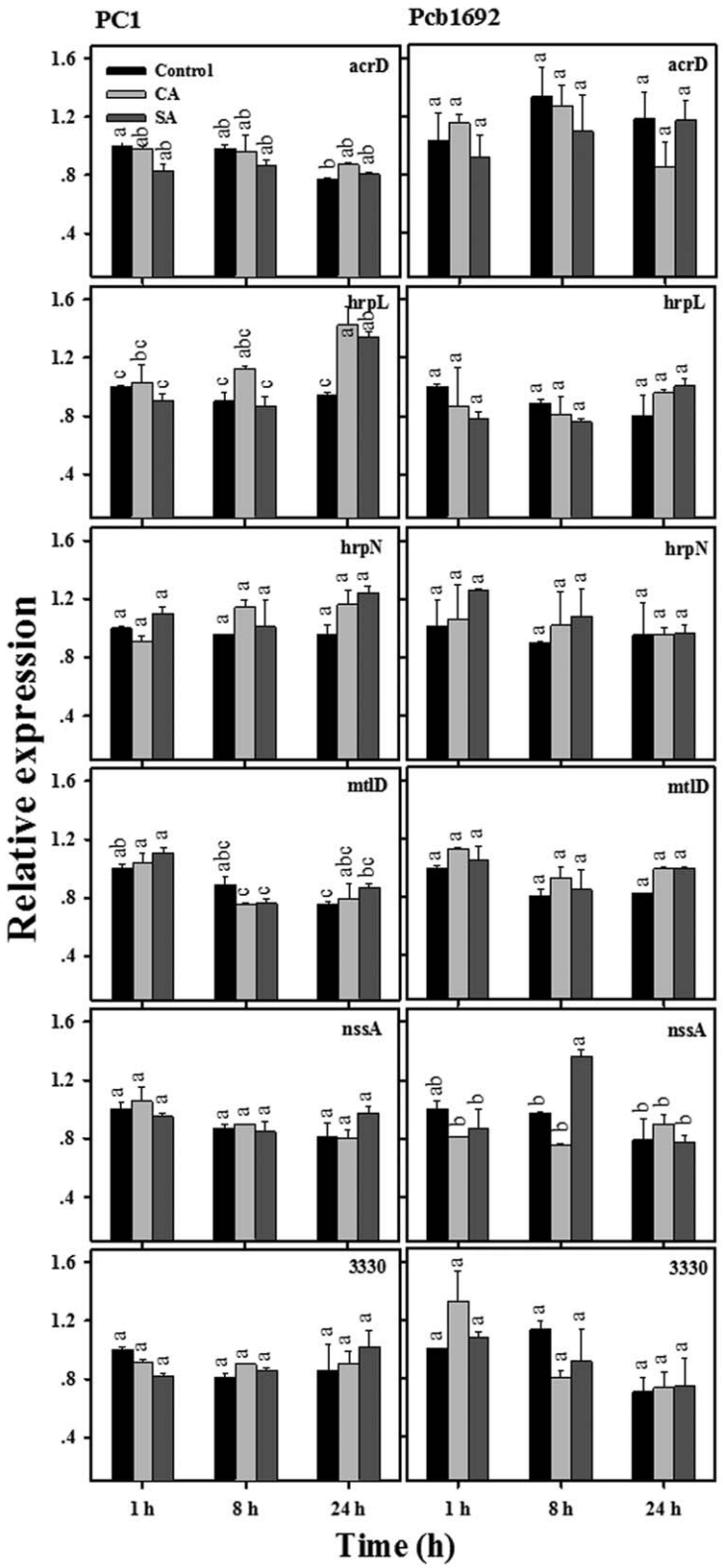

Fig. 3 Effects of cinnamic acid (CA; 0.25 mg/mL) and salicylic acid (SA; 0.21 mg/mL) on transcript levels of other genes (associated with transporters, efflux pumps and multidrug resistance) in *Pectobacterium aroidearum* PC1 and *Pectobacterium carotovorum* ssp. *brasiliense* Pcb1692. The transcript levels of *acrD*, *hrpL*, *hrpN*, *mtlD*, *nssA* and *PC1_3330* in DNA-free RNA prepared from the strains grown in Lysogeny-Broth (LB) medium (28 °C, continuous shaking at 150 rpm) with or without CA and SA were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Means ± standard errors (SE) of relative expression of each gene (three replicates per treatment) are shown. Different letters indicate significant differences ($P = 0.05$) among treatments for each time point in each gene/strain combination.

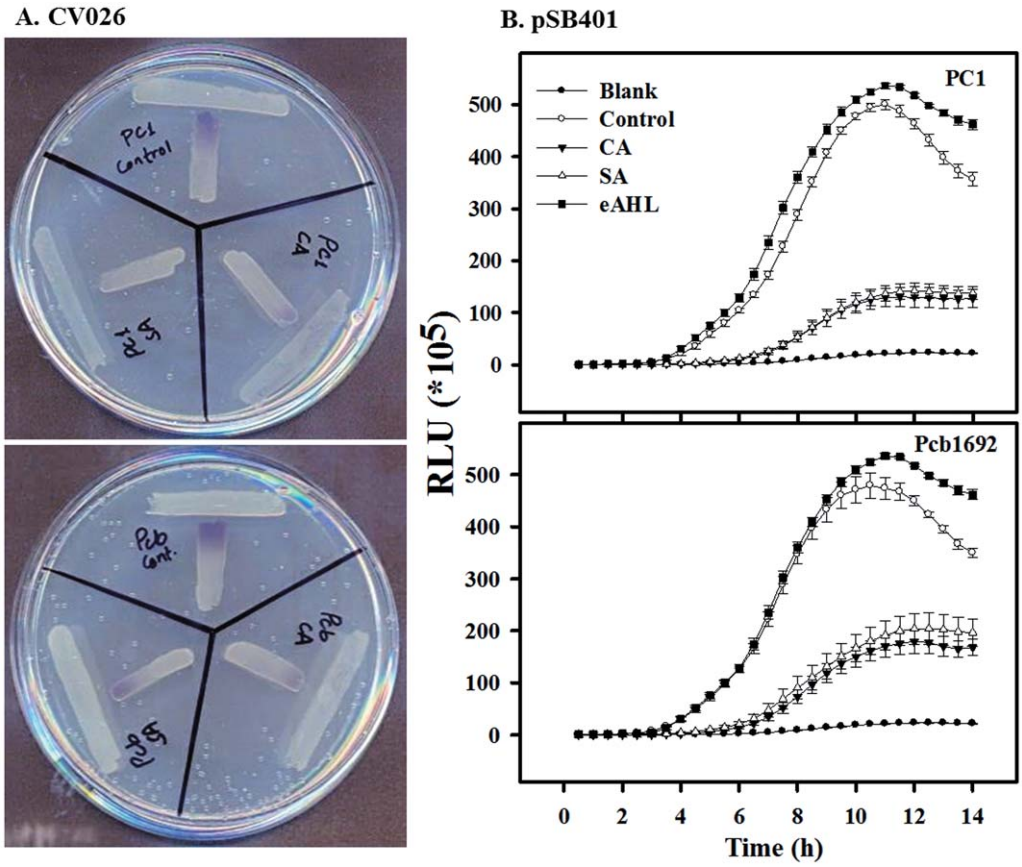

Fig. 4 Effects of cinnamic acid (CA; 0.25 mg/mL) and salicylic acid (SA; 0.21 mg/mL) on the production of quorum sensing (QS) signalling molecules by *Pectobacterium aroidearum* PC1 and *Pectobacterium carotovorum* ssp. *brasiliense* Pcb1692. (A) Purple colour exhibited by CV026, as response to *N*-acyl-homoserine lactones (AHLs) produced by PC1 (top) or Pcb1692 (bottom) that were grown with (control) and without non-lethal concentrations of CA or SA. (B) Intensity of luminescence produced by *Escherichia coli* pSB401 induced by supernatants of strains PC1 (top) and Pcb1692 (bottom) grown with or without non-lethal concentrations of CA or SA. Luminescence (250 ms) and absorbance (600 nm) were measured every 30 min for 14 h, and the relative luminescence (RLU = LU/$OD_{600\ nm}$) was calculated. Blank was *E. coli* pSB401 grown in the absence of supernatants of *Pectobacterium* strains, and also *E. coli* pSB401 was supplemented with exogenous *N*-(β-ketocaproyl)-L-homoserine lactone (eAHL) at 100 nM. Each data point represents the mean ± standard error (SE) of eight replicates per treatment of one experiment, representative of two independent experiments with similar results. OD, optical density; LU, light units; RLU, relative light units.

compared with the bioluminescence induced by supernatants of control cells (Fig. 4B).

Restoration of virulence of CA- or SA-treated cells by the addition of exogenous AHL

We have reported recently that CA, SA and other plant phenolics significantly reduce the virulence of *Pectobacterium* strains on *Zantedeschia aethiopica* (calla lily) and *Solanum tuberosum* (potato) (Joshi *et al*., 2015). To determine whether the impairment of the virulence in *Pectobacterium* by CA and SA is associated with the direct interference of these compounds with the QS system, we assessed whether exogenous application of AHL can restore wild-type levels of virulence in PC1 and Pcb1692 cells pretreated with CA or SA. Bacterial cultures of strains PC1 and Pcb1692 were grown in the absence (controls) or presence of non-lethal concentrations of CA or SA. Next, each culture was divided into two groups: in one group, *N*-(β-ketocaproyl)-L-homoserine lactone (eAHL) was added at a final concentration of 100 nM and the other group served as a no-eAHL control. Bacterial cultures were used in virulence assays on calla lily leaf discs and potato tubers as described in Experimental Procedures.

Untreated bacterial cultures (controls) produced characteristic tissue necrosis in both hosts (Fig. 5), typical of the development of soft rot and indicative of extensive exoenzyme-mediated tissue damage. The addition of eAHL to the control cultures did not increase disease severity in either host, relative to the control (data not shown). As expected from previous studies (Joshi *et al*., 2015), CA- and SA-treated bacterial cultures failed to induce disease symptoms (Fig. 5). However, exogenous application of AHL

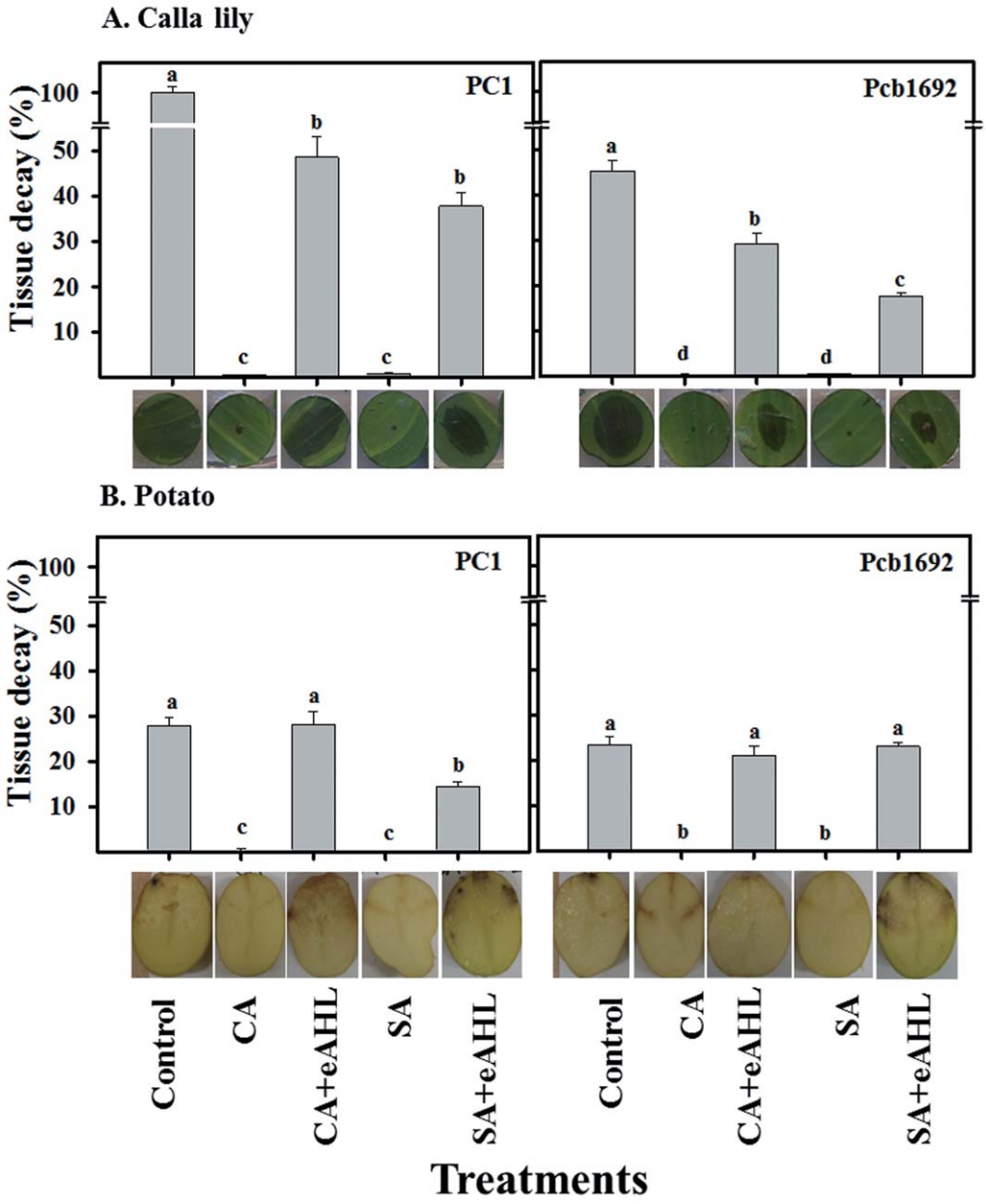

Fig. 5 Effects of cinnamic acid (CA; 0.74 mg/mL), salicylic acid (SA; 0.42 mg/mL) and *N*-(β-ketocaproyl)-L-homoserine lactone (eAHL) on the virulence of *Pectobacterium aroidearum* PC1 and *Pectobacterium carotovorum* ssp. *brasiliense* Pcb1692 on calla lily (A) and potato (B). Bacterial strains were exposed to non-lethal concentrations of CA and SA before they were used to inoculate the plant tissue. Parts of the CA- and SA-treated cultures were supplemented with 100 nM of eAHL before inoculation. Calla lily leaf discs and potato tubers were injected with 10 μL of bacterial suspension [10^6 colony-forming units (CFU)] and incubated at 28 °C. Virulence was determined as the percentage of decayed tissue 15 and 48 h after the inoculation of the calla lily leaf discs and potato tubers, respectively, relative to the decay induced by untreated bacterial cultures (control). Data represent the means ± standard errors (SE) of two independent experiments with 10 replicates for calla lily and four replicates for potato in each experiment. Treatments that are not labelled with the same letter in each panel are significantly ($P = 0.05$) different. Representative photographs of infected discs of calla lily and potato tubers are shown for each treatment.

to these cultures successfully restored their virulence on both hosts (Fig. 5). In some of the treatments, exogenous application of AHL to CA- or SA-treated cells fully complemented their virulence (i.e. CA-treated cultures of PC1 and both CA- and SA-treated cultures of Pcb1692 in potato). In other cases, a partial restoration of virulence relative to untreated controls was observed. This was the case for both strains on calla lily. Importantly, however, in all cases, disease severity induced by CA/eAHL- and SA/eAHL-treated cultures was significantly higher ($P = 0.05$) than that induced by CA and SA treatments (Fig. 5). Overall, these results support the

hypothesis that SA and CA affect bacterial virulence, at least in part, by impairing the QS machinery, as also evidenced by gene expression and AHL reporter assays.

DISCUSSION

Plant colonization by *Pectobacterium* spp. is a very complex process in which a variety of factors, including the secretion and activity of PCWDEs, are involved. Several studies have suggested that, in soft rot enterobacteria, PCWDE activity is strictly under the control of the QS system (Barnard and Salmond, 2007; Barnard *et al*., 2007; Jones *et al*., 1993; Liu *et al*., 2008). The best-studied QS systems are dependent on AHL signalling molecules which, at high cell densities, bind to receptor proteins belonging to the LuxR family of transcriptional regulators, resulting in altered gene expression (Jones *et al*., 1993). In soft rot enterobacteria, these systems have been reported to regulate not only PCWDEs, but other virulence factors, including the virulence regulators *expR*, *rsmA* and *virR* (Liu *et al*., 2008).

There is increasing evidence indicating that pectobacteria attack the cell wall and simultaneously target host defence mechanisms (Liu *et al*., 2008). However, plants respond to pathogen attack by the activation of defence responses. In this regard, small plant molecules, such as phenolic acids, are known to play a role in the elicitation of plant defence responses and also contribute to plant defence by exerting direct antimicrobial activity or by targeting virulence factors of the pathogen (Daglia, 2012; Matern and Kneusel, 1988). Indeed, some plant phenolic compounds have been reported to directly affect the production of virulence factors by *P. carotovorum* and *Ps. syringae* pv. *syringae* (Lagonenko *et al*., 2013; Sova, 2012). For example, SA has been shown to reduce the production of AHL in *Ps. aeruginosa* and *P. carotovorum*, and also to interfere with the QS system of *A. tumefaciens* (Chang *et al*., 2014; Lagonenko *et al*., 2013; Yuan *et al*., 2007). Furthermore, *o*-coumaric acid and *t*-cinnamic acid enhance the expression of type III secretion system (T3SS) genes in *Dickeya dadantii* 3937 (Yang *et al*., 2008). In contrast, *p*-coumaric acid has been shown to repress the T3SS genes of the same pathogen, indicating that different phenolics, including different isomers of certain phenolic acids, can induce or repress the expression of the same set of genes in a given pathogen (Li *et al*., 2009; 2015). Phenolic acids are also known to regulate the infection efficiency during the exchange of signals between legumes and rhizobia (Mandal *et al*., 2010). However, despite the known effects of phenolic compounds on bacterial virulence, the mechanisms of action behind them are, as yet, unresolved (Chow *et al*., 2011; Mirzoeva *et al*., 1997; Prithiviraj *et al*., 2005).

Recently, we have shown that non-lethal concentrations of various phenolic compounds affect PCWDE activity, biofilm formation and the motility of several *Pectobacterium* species, as well as their virulence in interactions with various host plants (Joshi *et al*., 2015). In the present study, we investigated the effects of two phenolic acids, CA and SA, on the expression of several virulence-associated genes in pectobacteria. As a result of the demonstrated role of QS in PCWDE activity in soft rot enterobacteria, our efforts were focused on QS genes and on genes that are known to be regulated by the QS machinery in these bacteria. Two strains that are considered to be type strains of two *Pectobacterium* species, PC1 (*P. aroidearum*) and Pcb1692 (*P. carotovorum* ssp. *brasiliense*), were used in this study (Nabhan *et al*., 2013; Onkendi and Moleleki, 2014). Pathogens belonging to these species are able to infect monocot and dicot hosts of agricultural importance, especially under warm conditions (Nabhan *et al*., 2012, 2013; Onkendi and Moleleki, 2014).

In the present study, we explored the direct effects of CA and SA on the expression of virulence genes in *Pectobacterium*, distinct from their recognized role as bactericidal compounds (Daglia, 2012), or as signal molecules involved in plant defence, a well-known role of SA (Zhang *et al*., 2010). The direct mechanism of action of such compounds on pectobacteria has hardly been studied, and the accumulation of information with regard to this subject from other soft rot enterobacterial models points to the importance of the examination of this mechanism. On the basis of previous studies, several virulence genes reported to be involved in *Pectobacterium* virulence were selected and their expression over time was analysed quantitatively using qRT-PCR with and without exposure to the phenolic acids. As QS is a cell density-dependent phenomenon, we followed the bacterial densities in treated and untreated cultures under the experimental conditions, with the results showing no differences between treatments in this parameter. Moreover, bacterial levels in all treatments considerably exceeded the threshold level required for the production of QS signalling molecules by *Pectobacterium* (Liu *et al*., 2008). We also studied the transcriptional patterns of QS system (AI-1 and AI-2) genes, QS-regulated genes and other genes, mainly coding for membrane transporters and efflux pumps.

The most significant effects of the two phenolic acids were observed for the QS system genes *expI*, *expR* and *PC1_1442*, which are part of the AI-1 QS system, and *luxS*, which is involved in the AI-2 QS system. *expI* and *expR* define the AI-1 QS system and are involved in a two-component signal transduction system in *Pectobacterium* (Marchler-Bauer *et al*., 2013; Pollumaa *et al*., 2012). The increased expression of QS system genes from the exponential growth phase and onwards was observed in both *P. aroidearum* PC1 and *P. carotovorum* ssp. *brasiliense* Pcb1692. Similar observations have been reported in several studies, where the efficient performance of the QS system was observed under high cell densities, represented by higher levels of AHL in *Photobacterium fischeri* and *Ps. aeruginosa* (Nealson *et al*., 1970; Wagner *et al*., 2003). However, neither of the *Pectobacterium* spp. displayed such an increase in QS system genes in the presence of CA or SA, although bacterial densities were similar to those of

untreated controls, indicating that CA and CA directly repress the QS system. Our findings are also in line with those of Bandara *et al*. (2006) and Yang *et al*. (2009), who claimed that SA is a potential QS inhibitor in *Ps. aeruginosa*.

The second group of genes included a negative regulator of PCWDEs (*rsmA*) and four genes (*pecS*, *pel*, *peh* and *yheO*) that are involved in synthesis of PCWDEs identified in previous studies (Table 1). The regulator displayed a different expression pattern, with the lowest level of expression observed at a later stage of growth, when expression levels of QS system genes and genes contributing to the secretion of PCWDEs were at their peak. In addition, rsmB (small regulatory RNA of Rsm system) displayed a high level of expression, whereas rsmC (post-transcriptional regulator of Rsm system), similar to rsmA, showed a low level of expression (data not shown). This observation fits the existing QS model already defined in *P. atrosepticum*, according to which *rsmA* negatively regulates the secretion of PCWDE (Chatterjee *et al*., 1995; Mole *et al*., 2007; Mukherjee *et al*., 1996; Pollumaa *et al*., 2012). These findings are in line with those of Cui *et al*. (2005), indicating that the availability of AHL (synthesized by ExpI) engages ExpR, thus making it unavailable for its target (*rsmA* promoter) and resulting in decreased *rsmA* production. The smaller amount of *rsmA* followed by higher levels of *pecS*, *pel*, *peh* and *yheO* expression in control treatment at the last stage of growth is typical of negative regulation of *rsmA*, which accounts for synthesis of virulence factors, such as PCWDEs. (Table 1; Charkowski *et al*., 2012). Exposure of the bacteria to CA or SA did not allow the normal expression pattern of the regulator *rsmA* or of the PCWDE genes *pecS*, *pel*, *peh* and *YheO*. Furthermore, the inhibition of any increase in the expression of QS genes might have contributed to the down-regulation of virulence genes (such as *pecS*, *pel*, *peh* and *yheO*), downstream of the QS apparatus, and consequently the impairment of bacterial virulence (Chatterjee *et al*., 2005; Cui *et al*., 2005). No response of genes related to membrane transport was observed following the exposure to CA or SA, suggesting that efflux pumps and type III secretion were not part of the inhibitory effect of the compounds.

The inhibitory effects of CA and SA on the expression of QS-related genes made it reasonable to assume that these compounds might affect directly the accumulation of QS signalling molecules (AHL in *Pectobacterium*). This hypothesis was confirmed employing two commonly used reporter assays for the presence of AHL molecules (Krishnan *et al*., 2012; McClean *et al*., 1997). Our results clearly demonstrate that treatment of PC1 and Pcb1692 with CA and SA reduces the production/accumulation of AHL.

To further clarify the results, virulence assays were conducted on two hosts representing two taxonomic plant groups, namely the monocot host calla lily and the dicot host potato. According to our hypothesis, if exposure to SA or CA actually affects virulence via QS signalling, the exogenous application of QS molecules (eAHL) might compensate for the deficiency of AHL molecules, leading to the recovery of virulence. As expected from our previous findings (Joshi *et al*., 2015), exposure of both *Pectobacterium* strains to CA and SA impaired their virulence. However, the supply of eAHL to the CA- or SA-treated bacteria prior to infection restored bacterial virulence, fully in potato tubers and partially in calla lily leaves. This differential recovery might be a result of the nature of the tissue, where potato is a storage organ, rich in starch, whereas the calla lily leaf is a less favourable target. The time of exposure to the bacteria was also different, 48 h for potato and 15 h for calla lily, as no remarkable disease symptoms were developed in potato after 15 h. Nevertheless, this observation indicates that CA and SA interfere directly with the QS machinery of pectobacteria. Also, it indicates that, during plant infection, the accumulation of the AHL signal is sufficient for efficient PCWDE production, even in the absence of the auto-inducer AI-2, which, according to gene expression analyses, is also suppressed by CA or SA. In agreement with this notion, Laasik *et al*. (2006) observed that an AI-2 mutant of *P. carotovorum* does not substantially differ from the wild-type in PCWDE activity.

To conclude, the results of this work strongly support the hypothesized existence of a mechanism by which the plant phenolic acids CA and SA act through the QS machinery. CA and SA reduce the virulence of *Pectobacterium* spp., apparently by the inhibition of AHL production/accumulation and, ultimately, the production and secretion of PCWDEs. Further work is required to determine what is/are the exact site(s) of action of these compounds. The understanding of such aspects could hold potential for the future development of control measures against soft rot bacteria, as well as other plant pathogenic bacterial species that rely on AHL-mediated QS systems for their fitness and virulence.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 2. PC1, Pcb1692 and CV026 were cultivated at 28 °C, whereas *E. coli* pSB401 was cultivated at 37 °C. All strains were grown in LB medium (Difco Laboratories, Detroit, MI, USA) under continuous shaking (150 rpm) in a TU-400 incubator shaker (MRC, Holon, Israel). Murashige and Skoog minimal medium (MS; Duchefa, Haarlem, the Netherlands) was used for plant inoculation assays.

RNA extraction and cDNA preparation

Pectobacterium strains were grown overnight at 28 °C in LB medium under continuous shaking. Then, 20–40 μL from these cultures were transferred to 10 mL of fresh LB containing non-lethal concentrations of cinnamic acid (CA, 0.25 mg/mL) or SA (0.21 mg/mL), and brought to a concentration of 10^7 CFU/mL. Controls were treated in the same manner, but with the absence of CA or SA. The fresh cultures were then grown at 28 °C under continuous shaking. Two-millilitre samples were collected at

Table 2 Strains used in this study.

Strain	Description	Reference/source
Pectobacterium aroidearum PC1	Monocot strain isolated from *Ornithogalum dubium*, NCBI Accession no. PRJNA31289	Yishay *et al*. (2008)
Pectobacterium carotovorum ssp. *brasiliense* Pcb1692	Dicot strain isolated from *Solanum tuberosum*, NCBI Accession no. PRJNA31121	Yishay *et al*. (2008)
Chromobacterim violaceum CV026	Mini-Tn5 mutant derived from *C. violaceum* ATCC 31532 Hg^R, *cvil*::Tn5 *xyl*E, Kan^R, plus spontaneous Str^R. AHL (C_4–C_8) biosensor, produces violacein (pigment) only in the presence of eAHL	McClean *et al*. (1997)
Escherichia coli pSB401	*luxRluxI*' (*Photobacterium fischeri* [ATCC 7744])::*lux*CDABE (*Photorhabdus luminescens* [ATCC 29999]) fusion; pACYC184-derived, Tet^R, AHL bioluminescent biosensor	Winson *et al*. (1998)

AHL, *N*-acyl-homoserine lactone; eAHL, *N*-(β-ketocaproyl)-L-homoserine lactone; Hg^R, hygromycin-resistant; Kan^R, kanamycin-resistant; NCBI, National Center for Biotechnology Information; Str^R, streptomycin-resistant; Tet^R, tetracycline-resistant.

1, 8 and 24 h (corresponding to lag, log and stationary phases, respectively; Fig. S3a, see Supporting Information) for RNA extraction. RNA was extracted using the EZ-RNA II kit (Biological Industries, Kibbutz Beit Haemek, Israel) following the manufacturer's instructions. Extracted RNA was employed to prepare cDNA using a cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). A minimum of 1 μg of RNA was used with a total reaction volume of 20 μL. The cDNA reverse transcription reaction was performed using a programmable thermal controller (MJ Research, St. Bruno, QC, Canada) programmed to one cycle at 42 °C for 30 min, followed by inactivation at 95 °C for 2 min, after which the cDNA was stored at −20 °C for future use.

Quantification of mRNA by qRT-PCR

Virulence genes from *P. aroidearum* and *P. carotovorum* ssp. *brasiliense* were divided into three different categories, as described in Table 1: QS genes, QS-regulated genes and others. These two bacterial strains were found to contain a similar gene repertoire for the QS and Rsm systems (with high sequence similarity, >90%) as established in *P. atrosepticum*. Primers employed for mRNA reverse transcription were designed using the National Center for Biotechnology Information (NCBI) primer BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and are detailed in Table S1 (see Supporting Information). The primers were designed to generate 100–120-bp-sized amplicons and primer melting temperatures were designed for 60 °C, with a melting temperature difference of less than 5 °C for each primer pair. Primer sequences were subjected to BLAST analysis (using NCBI BLAST software) against the database for the genus *Pectobacterium* to eliminate the likelihood of non-specific binding. qRT-PCR mixtures contained 3.4 μL (17 ng) of cDNA, 5 μL of fast Syber green master mix (Applied Biosystems) and 0.8 μL (5 μM) of each forward and reverse primer. Reactions were performed using a Step One Plus Real-Time PCR system (Applied Biosystems) with the following cycling parameters: holding stage, 95 °C for 20 s; cycling stage for 40 cycles of 95 °C for 3 s and 60 °C for 30 s; and melting curve stage, 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The data were analysed by the comparative C_T ($\Delta\Delta C_T$) method, with expression normalized to the expression of the reference gene *recA*, as described by Takle *et al*. (2007).

Qualitative assays for the detection of AHL molecules

Strain CV026 is a mini-Tn5 mutant of *C. violaceum* in which the purple pigment violacein is induced in the presence of AHL compounds with *N*-acyl C_4–C_8 side chains (McClean *et al*., 1997). This assay was performed as a qualitative tool to assess the effect of CA and SA on the production of AHLs in *Pectobacterium* strains. PC1 and Pcb1692 were grown in LB overnight as described above. Cultures were then centrifuged (7000 ***g***, 5 min, at 28 °C) and bacterial pellets were resuspended in fresh LB supplemented or not with non-lethal concentrations of CA or SA, and incubated for 8 h at 28 °C. The reporter strain CV026 was grown in fresh LB medium supplemented with kanamycin (10 μg/mL). Then, CV026 and *Pectobacterium* strains were spread perpendicularly (in a T-shape) on LB plates, with the reporter strain being spread a few millimetres away from the tested bacteria. The plates were incubated overnight at 28 °C and the intensity of the purple colour exhibited by the reporter strain was then assessed.

Quantitative assay for AHL molecules using a bioluminescence-based assay

Escherichia coli pSB401 is a bioluminescence-based QS biosensor, which was generated on the background of *E. coli* strain JM109. This strain carries the plasmid pSB401, which possesses the *luxRI':luxCDABE* bioluminescent reporter gene fusion (Winson *et al*., 1998). This system can detect AHLs with acyl chains ranging from six to eight carbons in length (C_6–C_8 AHLs; Middleton *et al*., 2002). This strain was used to quantitatively assess the secretion of AHL molecules by *Pectobacterium* strains PC1 and Pcb1692 in the presence of CA and SA. Bacteria were grown overnight (PC1 and Pcb1692 at 28 °C and *E. coli* pSB401 at 37 °C) in liquid LB medium under continuous shaking (150 rpm), centrifuged (7000 ***g***, 5 min, at 28 °C) and the supernatants were removed. Bacterial pellets of *Pectobacterium* strains were suspended in fresh LB supplemented (or not) with non-lethal concentrations of CA (0.25 mg/mL) or SA (0.21 mg/mL), and incubated for 8 h at 28 °C, whereas psB401 was suspended in fresh LB with tetracycline (10 μg/mL) and incubated at 37 °C. Control and treated suspensions of strains PC1 and Pcb1692 were then centrifuged (7000 ***g***, 5 min, at 28 °C) and 10 μL of supernatant were mixed with 190 μL of 5×10^6 CFU/mL *E. coli* pSB401 in fresh LB medium in 96-well microtitre plates. The supernatant used (10 μL) contained CA and SA, diluted 20-fold, to a final volume of 200 μL of fresh LB. As the concentration used for the *Pectobacterium* strains did not affect the growth of psB401 (Fig. S3b, see Supporting Information), the effect of the diluted phenolics concentration on the reporter strain could be neglected. Two hundred microlitres of the reporter strain were used as a blank for the experiment and 100 nM of

synthetic eAHL (Sigma, St. Louis, MO, USA) was added to the reporter strain as a positive control. The plates were incubated at 37 °C for 15 h in an Enspire 2300 multilabel reader (Perkin-Elmer, Santa Clara, CA, USA). Bioluminescence and optical density (OD) were automatically and simultaneously determined every 30 min at 250 ms and 600 nm, respectively. Bioluminescence was calculated as the number of relative light units (RLU) per unit of optical density at 600 nm, which accounted for the influence of the different treatments on total bioluminescence, as described by Winzer *et al*. (2000).

AHL virulence compensation assays

Virulence was measured by the assessment of symptom severity in two plants, *Zantedeschia aethiopica* (calla lily) and *Solanum tuberosum* (potato) 'Lady Rosetta', as described by Yishay *et al*. (2008). To explore the effect of eAHL, higher concentrations of CA and SA (0.74 mg/mL and 0.42 mg/mL, respectively) were used in these experiments. These concentrations did not lead to the development of visible symptoms within 12 h of infection and were found to reduce the growth of the strains by 50% (Joshi *et al*., 2015). The experiment was performed on fully expanded young leaves of calla lily and small (about 25–50 g) potato tubers that were surface sterilized by soaking in 0.4% sodium hypochlorite for 20 min, and then washed twice with sterilized distilled water. In the case of calla lily, leaf discs (20 mm in diameter) were excised from disinfected leaves and transferred to Petri dishes containing MS medium. Whole disinfected potato tubers were used for infection assays. Bacterial strains were grown overnight in LB liquid medium at 28 °C with continuous shaking and diluted to 10^8 CFU/mL (OD_{600} = 0.1) in sterile double-distilled water containing CA or SA alone or in combination with 100 nM eAHL. The bacterial suspensions were shaken (150 rpm) in a TU-400 incubator shaker for 2 h at 28 °C before inoculation. Leaf discs and potato tubers were then pierced at the centre with a sterile tip and inoculated with 10 μL of bacterial suspension (10^6 CFU). The inoculated plant material was incubated at 28 °C. For calla lily, the disease severity was expressed as the percentage of decayed tissue relative to the total area of the disc, and was recorded 15 h after inoculation. For potato tubers, the disease severity was expressed as the percentage of rotten tissue, which was determined by weighing decayed tissues after 48 h of inoculation. Two independent experiments were carried out, with 10 and four replicates for each treatment, for the leaf disc and tuber assays, respectively.

Data analysis

Data were analysed by one-way analysis of variance (ANOVA) using JMP software (Version 5, Medmenham, Buckinghamshire, UK). When ANOVA indicated a significant difference ($P < 0.05$), a Tukey–Kramer multiple comparison test was performed. Graphs were generated with Sigma Plot Version 10.0 (Systat Software, San Jose, CA, USA).

ACKNOWLEDGEMENTS

We thank Dr Leonid Chernin and Dr Yael Helman for providing quorum sensing reporter strains (*Chromobacterium violaceum* CV026 and *Escherichia coli* pSB401), and Dr Leonid Chernin for critical reading of the manuscript.

REFERENCES

Antunes, L.C., Ferreira, R.B., Buckner, M.M. and Finlay, B.B. (2010) Quorum sensing in bacterial virulence. *Microbiology*, **156**, 2271–2282.

Bandara, M.B., Zhu, H., Sankaridurg, P.R. and Willcox, M.D. (2006) Salicylic acid reduces the production of several potential virulence factors of *Pseudomonas aeruginosa* associated with microbial keratitis. *Invest. Ophthalmol. Vis. Sci.* **47**, 4453–4460.

Barnard, A.L. and Salmond, G.C. (2007) Quorum sensing in *Erwinia* species. *Annal. Bioanal. Chem.* **387**, 415–423.

Barnard, A.M., Bowden, S.D., Burr, T., Coulthurst, S.J., Monson, R.E. and Salmond, G.P. (2007) Quorum sensing, virulence and secondary metabolite production in plant soft-rotting bacteria. *Philos. Trans. R. Soc. London, B: Biol. Sci.* **362**, 1165–1183.

Bodini, S.F., Manfredini, S., Epp, M., Valentini, S. and Santori, F. (2009) Quorum sensing inhibition activity of garlic extract and p-coumaric acid. *Lett. Appl. Microbiol.* **49**, 551–555.

Broberg, M., Lee, G.W., Nykyri, J., Lee, Y.H., Pirhonen, M. and Palva, E.T. (2014) The global response regulator ExpA controls virulence gene expression through RsmA-mediated and RsmA-independent pathways in *Pectobacterium wasabiae* SCC3193. *Appl. Environ. Microbiol.* **80**, 1972–1984.

Burr, T., Barnard, A.M.L., Corbett, M.J., Pemberton, C.L., Simpson, N.J.L. and Salmond, G.P.C. (2006) Identification of the central quorum sensing regulator of virulence in the enteric phytopathogen, *Erwinia carotovora*: the VirR repressor. *Mol. Microbiol.* **59**, 113–125.

Chang, C.-Y., Krishnan, T., Wang, H., Chen, Y., Yin, W.-F., Chong, Y.-M., Tan, L.Y., Chong, T.M. and Chan, K.-G. (2014) Non-antibiotic quorum sensing inhibitors acting against N-acyl homoserine lactone synthase as druggable target. *Sci. Rep.* **4**, 1–8.

Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., Hugouvieux-Cotte-Pattat, N., Solanilla, E.L., Low, D., Moleleki, L., Pirhonrn, M., Pitman, A., Perna, N., Reverchon, S., Palenzuela, P.R., Francisco, M.S., Toth, I., Tsuyumu, S., Waals, J.D., Wolf, J.V.D., Gijsegem, F.V., Yang, C.-H. and Yedidia, I. (2012) The role of secretion systems and small molecules in soft-rot Enterobacteriaceae pathogenicity. *Annu. Rev. Phytopathol.* **50**, 425–449.

Chatterjee, A., Cui, Y., Liu, Y., Dumenyo, C.K. and Chatterjee, A.K. (1995) Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulases, and proteases in *Erwinia carotovora* subsp. *carotovora* in the absence of the starvation/cell density-sensing signal, N-(3-oxohexanoyl)-L-homoserine lactone. *Appl. Environ. Microbiol.* **61**, 1959–1967.

Chatterjee, A., Cui, Y., Hasegawa, H., Leigh, N., Dixit, V. and Chatterjee, A.K. (2005) Comparative analysis of two classes of quorum-sensing signaling systems that control production of extracellular proteins and secondary metabolites in *Erwinia carotovora* subspecies. *J. Bacteriol.* **187**, 8026–8038.

Chow, S., Gu, K., Jiang, L. and Nassour, A. (2011) Salicylic acid affects swimming, twitching and swarming motility in *Pseudomonas aeruginosa*, resulting in decreased biofilm formation. *J. Exp. Microbiol. Immunol.* **15**, 22–29.

Crépin, A., Barbey, C., Beury-Cirou, A., Hélias, V., Taupin, L., Reverchon, S., Nasser, W., Faure, D., Dufour, A., Orange, N., Feuilloley, M., Heurlier, K., Burini, J.-F. and Latour, X. (2012) Quorum sensing signaling molecules produced by reference and emerging soft-rot bacteria (*Dickeya* and *Pectobacterium* spp. *PLoS ONE*, **7**, e35176.

Cui, Y., Chatterjee, A., Hasegawa, H., Dixit, V., Leigh, N. and Chatterjee, A.K. (2005) ExpR, a LuxR homolog of *Erwinia carotovora* subsp. *carotovora*, activates transcription of *rsmA*, which specifies a global regulatory RNA-binding protein. *J. Bacteriol.* **187**, 4792–4803.

Czajkowski, R., van der Wolf, J., Krolicka, A., Ozymko, Z., Narajczyk, M., Kaczynska, N. and Lojkowska, E. (2015) Salicylic acid can reduce infection symptoms caused by *Dickeya solani* in tissue culture grown potato (*Solanum tuberosum* L.) plants. *Eur. J. Plant Pathol.* **141**, 545–558.

Daglia, M. (2012) Polyphenols as antimicrobial agents. *Curr. Opin. Biotechnol.* **23**, 174–181.

Davidsson, P.R.R., Kariola, T., Niemi, O. and Palva, T. (2013) Pathogenicity of and plant immunity to soft rot pectobacteria. *Front. Plant Sci.* **4**, 191.

Deep, A., Chaudhary, U. and Gupta, V. (2011) Quorum sensing and bacterial pathogenicity: from molecules to disease. *J. Lab. Physicians*, **3**, 4–11.

Defoirdt, T., Brackman, G. and Coenye, T. (2013) Quorum sensing inhibitors: how strong is the evidence? *Trends Microbiol.* **21**, 619–624.

Dong, Y.-H., Wang, L.-H., Xu, J.-L., Zhang, H.-B., Zhang, X.-F. and Zhang, L.-H. (2001) Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature*, **411**, 813–817.

Engebrecht, J. and Silverman, M. (1984) Identification of genes and gene products necessary for bacterial bioluminescence. *Proc. Natl. Acad. Sci. USA*, **81**, 4154–4158.
Faure, D. and Dessaux, Y. (2007) Quorum sensing as a target for developing control strategies for the plant pathogen *Pectobacterium*. *Eur. J. Plant Pathol.* **119**, 353–365.
Fuqua, C., Parsek, M.R. and Greenberg, E.P. (2001) Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* **35**, 439–468.
Fuqua, W.C., Winans, S.C. and Greenberg, E.P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **176**, 269–275.
Galloway, W.R., Hodgkinson, J.T., Bowden, S., Welch, M. and Spring, D.R. (2012) Applications of small molecule activators and inhibitors of quorum sensing in Gram-negative bacteria. *Trends Microbiol.* **20**, 449–458.
Helman, Y. and Chernin, L. (2015) Silencing the mob: disrupting quorum sensing as a means to fight plant disease. *Mol. Plant Pathol.* **16**, 316–329.
Hema, R., Vemanna, R.S., Sreeramulu, S., Reddy, C.P., Senthil-Kumar, M. and Udayakumar, M. (2014) Stable expression of *mtlD* gene imparts multiple stress tolerance in finger millet. *PLoS ONE*, **9**, e99110.
Hentzer, M., Wu, H., Andersen, J.B., Riedel, K., Rasmussen, T.B., Bagge, N., Kumar, N., Schembri, M.A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J.W., Molin, S., Eberl, L., Steinberg, P., Kjellebreg, S., Hoiby, N. and Givskov, M. (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J.* **22**, 3803–3815.
Hommais, F., Oger-Desfeux, C., Van Gijsegem, F., Castang, S., Ligori, S., Expert, D., Nasser, W. and Reverchon, S. (2008) PecS is a global regulator of the symptomatic phase in the phytopathogenic bacterium *Erwinia chrysanthemi* 3937. *J. Bacteriol.* **190**, 7508–7522.
Jafra, S., Jalink, H., Van Der Schoor, R. and Van Der Wolf, J.M. (2006) *Pectobacterium carotovorum* subsp. *carotovorum* strains show diversity in production of and response to N-acyl homoserine lactones. *J. Phytopathol.* **154**, 729–739.
Jang, Y.-S., Sohn, S.-I. and Wang, M.-H. (2006) The hrpN gene of *Erwinia amylovora* stimulates tobacco growth and enhances resistance to *Botrytis cinerea*. *Planta*, **223**, 449–456.
Jones, S., Yu, B., Bainton, N.J., Birdsall, M., Bycroft, B.W., Chhabra, S.R., Cox, A.J., Golby, P., Reeves, P.J. and Stephens, S. (1993) The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. *EMBO J.* **12**, 2477–2482.
Joshi, J.R., Burdman, S., Lipsky, A. and Yedidia, I. (2015) Effects of plant antimicrobial phenolic compounds on virulence of the genus *Pectobacterium*. *Res. Microbiol.* **166**, 535–545. doi: 10.1016/j.resmic.2015.04.004.
Kalia, V.C. (2013) Quorum sensing inhibitors: an overview. *Biotechnol. Adv.* **31**, 224–245.
de Kievit, T.R. and Iglewski, B.H. (2000) Bacterial quorum sensing in pathogenic relationships. *Infect. Immun.* **68**, 4839–4849.
Kõiv, V., Andresen, L., Broberg, M., Frolova, J., Somervuo, P., Auvinen, P., Pirhonen, M., Tenson, T. and Mae, A. (2013) Lack of RsmA-mediated control results in constant hypervirulence, cell elongation, and hyperflagellation in *Pectobacterium wasabiae*. *PLoS ONE*, **8**, e54248.
Krishnan, T., Yin, W.F. and Chan, K.G. (2012) Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* PAO1 by Ayurveda spice clove (*Syzygium aromaticum*) bud extract. *Sensors*, **12**, 4016–4030.
Laasik, E., Andresen, L. and Mäe, A. (2006) Type II quorum sensing regulates virulence in *Erwinia carotovora* ssp. *carotovora*. *FEMS Microbiol. Lett.* **258**, 227–234.
Lagonenko, L., Lagonenko, A. and Evtushenkov, A. (2013) Impact of salicylic acid on biofilm formation by plant pathogenic bacteria. *J. Biol. Earth Sci.* **3**, B176–B181.
Lee, D.H., Lim, J.A., Lee, J., Roh, E., Jung, K., Choi, M., Oh, C., Ryu, S., Yun, J. and Heu, S. (2013) Characterization of genes required for the pathogenicity of *Pectobacterium carotovorum* subsp. *carotovorum* Pcc21 in Chinese cabbage. *Microbiology*, **159**, 1487–1496.
Li, Y., Peng, Q., Selimi, D., Wang, Q., Charkowski, A.O., Chen, X. and Yang, C.-H. (2009) The plant phenolic compound p-coumaric acid represses gene expression in the *Dickeya dadantii* type III secretion system. *Appl. Environ. Microbiol.* **75**, 1223–1228.
Li, Y., Hutchins, W., Wu, X., Liang, C., Zhang, C., Yuan, X., Khokhani, D., Chen, X., Che, Y., Wang, Q. and Yang, C.-H. (2015) Derivative of plant phenolic compound inhibits the type III secretion system of *Dickeya dadantii* via HrpX/HrpY two-component signal transduction and Rsm systems. *Mol. Plant Pathol.* **16**, 150–163.
Liu, H., Coulthurst, S.J., Pritchard, L., Hedley, P.E., Ravensdale, M., Humphris, S., Burr, T., Takle, G., Brurberg, M.-B., Brich, P.R.J., Salmond, G.P.C. and Toth, I.K. (2008) Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. *PLoS Path.* **4**, e1000093.
Ma, B., Hibbing, M.E., Kim, H.-S., Reedy, R.M., Yedidia, I., Breuer, J., Breuer, J., Glasner, J.D., Perna, N.T., Kelman, A. and Charkowski, A.O. (2007) Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*. *Phytopathology*, **97**, 1150–1163.
Mandal, S.M., Chakraborty, D. and Dey, S. (2010) Phenolic acids act as signaling molecules in plant–microbe symbioses. *Plant Signal. Behav.* **5**, 359–368.
Manefield, M., Harris, L., Rice, S.A., de Nys, R. and Kjelleberg, S. (2000) Inhibition of luminescence and virulence in the black tiger prawn (*Penaeus monodon*) pathogen *Vibrio harveyi* by intercellular signal antagonists. *Appl. Environ. Microbiol.* **66**, 2079–2084.
Manefield, M., Welch, M., Givskov, M., Salmond, G.P. and Kjelleberg, S. (2001) Halogenated furanones from the red alga, *Delisea pulchra*, inhibit carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen *Erwinia carotovora*. *FEMS Microbiol. Lett.* **205**, 131–138.
Marchler-Bauer, A., Zheng, C., Chitsaz, F., Derbyshire, M.K., Geer, L.Y., Geer, R.C., Gonzales, N.R., Gwadz, M., Hurwitz, D.I., Lanczcki, C.J., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Yamashita, R.A., Zhang, D. and Bryant, S.H. (2013) CDD: conserved domains and protein three-dimensional structure. *Nucleic Acid Res.* **41**, 348–352.
Matern, U. and Kneusel, R. (1988) Phenolic compounds in plant disease resistance. *Phytoparasitica*, **16**, 153–170.
McClean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift, S., Bycroft, B.W., Stewart, G.S. and Williams, P. (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology*, **143**, 3703–3711.
Middleton, B., Rodgers, H.C., Cámara, M., Knox, A.J., Williams, P. and Hardman, A. (2002) Direct detection of *N*-acylhomoserine lactones in cystic fibrosis sputum. *FEMS Microbiol. Lett.* **207**, 1–7.
Mirzoeva, O.K., Grishanin, R.N. and Calder, P.C. (1997) Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria. *Microbiol. Res.* **152**, 239–246.
Mole, B.M., Baltrus, D.A., Dangl, J.L. and Grant, S.R. (2007) Global virulence regulation networks in phytopathogenic bacteria. *Trends Microbiol.* **15**, 363–371.
Molina, L., Constantinescu, F., Michel, L., Reimmann, C., Duffy, B. and Défago, G. (2003) Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. *FEMS Microbiol. Ecol.* **45**, 71–81.
Molina, L., Rezzonico, F., Defago, G. and Duffy, B. (2005) Autoinduction in *Erwinia amylovora*: evidence of an acyl-homoserine lactone signal in the fire blight pathogen. *J. Bacteriol.* **187**, 3206–3213.
Mukherjee, A., Cui, Y., Liu, Y., Dumenyo, C.K. and Chatterjee, A.K. (1996) Global regulation in *Erwinia* species by *Erwinia carotovora rsmA*, a homologue of *Escherichia coli csrA*: repression of secondary metabolites, pathogenicity and hypersensitive reaction. *Microbiology*, **142**, 427–434.
Murray, E.J., Crowley, R.C., Truman, A., Clarke, S.R., Cottam, J.A., Jadhav, G.P., Steele, V.R., O'Shea, P., Lindholm, C., Cockayne, A., Chhabra, S.R., Chan, W.C. and Williams, P. (2014) Targeting *Staphylococcus aureus* quorum sensing with nonpeptidic small molecule inhibitors. *J. Med. Chem.* **57**, 2813–2819.
Nabhan, S., De Boer, S.H., Maiss, E. and Wydra, K. (2012) Taxonomic relatedness between *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium carotovorum* subsp. *odoriferum* and *Pectobacterium carotovorum* subsp. *brasiliense* subsp. nov. *J. Appl. Microbiol.* **113**, 904–913.
Nabhan, S., De Boer, S.H., Maiss, E. and Wydra, K. (2013) *Pectobacterium aroidearum* sp. nov., a soft rot pathogen with preference for monocotyledonous plants. *Int. J. Syst. Evol. Microbiol.* **63**, 2520–2525.
Nasser, W., Bouillant, M.L., Salmond, G. and Reverchon, S. (1998) Characterization of the *Erwinia chrysanthemi expI-expR* locus directing the synthesis of two N-acyl-homoserine lactone signal molecules. *Mol. Microbiol.* **29**, 1391–1405.
Nealson, K.H., Platt, T. and Hastings, J.W. (1970) Cellular control of the synthesis and activity of the bacterial luminescent system. *J. Bacteriol.* **104**, 313–322.
O'Loughlin, C.T., Miller, L.C., Siryaporn, A., Drescher, K., Semmelhack, M.F. and Bassler, B.L. (2013) A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. *Proc. Natl. Acad. Sci. USA*, **110**, 17 981–17 986.
Onkendi, E. and Moleleki, L. (2014) Characterization of *Pectobacterium carotovorum* subsp. *carotovorum* and *brasiliense* from diseased potatoes in Kenya. *Eur. J. Plant Pathol.* **139**, 557–566.

Park, T.-H., Choi, B.-S., Choi, A.-Y., Choi, I.-Y., Heu, S. and Park, B.-S. (2012) Genome sequence of *Pectobacterium carotovorum* subsp. *carotovorum* strain PCC21, a pathogen causing soft rot in Chinese cabbage. *J. Bacteriol.* **194**, 6345–6346.

Pirhonen, M., Flego, D., Heikinheimo, R. and Palva, E.T. (1993) A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *EMBO J.* **12**, 2467–2476.

Pollumaa, L., Alamae, T. and Mae, A. (2012) Quorum sensing and expression of virulence in pectobacteria. *Sensors*, **12**, 3327–3349.

Praillet, T., Nasser, W., Robert-Baudouy, J. and Reverchon, S. (1996) Purification and functional characterization of PecS, a regulator of virulence-factor synthesis in *Erwinia chrysanthemi*. *Mol. Microbiol.* **20**, 391–402.

Prithiviraj, B., Bais, H.P., Weir, T., Suresh, B., Najarro, E.H., Dayakar, B.V., Schweizer, H.P. and Vivanco, J.M. (2005) Down regulation of virulence factors of *Pseudomonas aeruginosa* by salicylic acid attenuates its virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*. *Infect. Immun.* **73**, 5319–5328.

Reverchon, S., Nasser, W. and Robert-Baudouy, J. (1994) *pecS*: a locus controlling pectinase, cellulase and blue pigment production in *Erwinia chrysanthemi*. *Mol. Microbiol.* **11**, 1127–1139.

Rutherford, S.T. and Bassler, B.L. (2012) Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb. Perspect. Med.* **2**, 1–25.

Schauder, S., Shokat, K., Surette, M.G. and Bassler, B.L. (2001) The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol. Microbiol.* **41**, 463–476.

Smadja, B., Latour, X., Faure, D., Chevalier, S., Dessaux, Y. and Orange, N. (2004) Involvement of N-acylhomoserine lactones throughout plant infection by *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium atrosepticum*). *Mol. Plant–Microbe Interact.* **17**, 1269–1278.

Smith, D., Wang, J.H., Swatton, J.E., Davenport, P., Price, B., Mikkelsen, H., Stickland, H., Nishikawa, K., Gardiol, V., Spring, D.R. and Welch, M. (2006) Variations on a theme: diverse N-acyl homoserine lactone-mediated quorum sensing mechanisms in gram-negative bacteria. *Prog. Surf. Sci.* **89**, 167–211.

Sova, M. (2012) Antioxidant and antimicrobial activities of cinnamic acid derivatives. *Mini Rev. Med. Chem.* **12**, 749–767.

Taga, M.E., Semmelhack, J.L. and Bassler, B.L. (2001) The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium*. *Mol. Microbiol.* **42**, 777–793.

Takle, G.W., Toth, I.K. and Brurberg, M.B. (2007) Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen *Pectobacterium atrosepticum*. *BMC Plant Biol.* **7**, 50.

Toth, I.K. and Birch, P.R.J. (2005) Rotting softly and stealthily. *Curr. Opin. Plant Biol.* **8**, 424–429.

Toth, I.K., Bell, K.S., Holeva, M.C. and Birch, P.R.J. (2003) Soft rot Erwiniae: from genes to genomes. *Mol. Plant Pathol.* **4**, 17–30.

Toth, I.K., van der Wolf, J.M., Saddler, G., Lojkowska, E., Hélias, V., Pirhonen, M., Tsror (Lahkim), L. and Elphinstone, J.G. (2011) *Dickeya* species: an emerging problem for potato production in Europe. *Plant Pathol.* **60**, 385–399.

Wagner, V.E., Bushnell, D., Passador, L., Brooks, A.I. and Iglewski, B.H. (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J. Bacteriol.* **185**, 2080–2095.

Waters, C.M. and Bassler, B.L. (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.* **21**, 319–346.

Wei, Z.M. and Beer, S.V. (1995) *hrpL* activates *Erwinia amylovora hrp* gene transcription and is a member of the ECF subfamily of sigma factors. *J. Bacteriol.* **177**, 6201–6210.

Winson, M.K., Swift, S., Fish, L., Throup, J.P., Jørgensen, F., Chhabra, S.R., Bycroft, B.W., Williams, P. and Stewart, G.S.A.B. (1998) Construction and analysis of *luxCDABE*-based plasmid sensors for investigating N-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol. Lett.* **163**, 185–192.

Winzer, K., Falconer, C., Garber, N.C., Diggle, S.P., Camara, M. and Williams, P. (2000) The *Pseudomonas aeruginosa* lectins PA-IL and PA-IIL are controlled by quorum sensing and by RpoS. *J. Bacteriol.* **182**, 6401–6411.

Yang, L., Rybtke, M.T., Jakobsen, T.H., Hentzer, M., Bjarnsholt, T., Givskov, M. and Tolker-Nielsen, T. (2009) Computer-aided identification of recognized drugs as *Pseudomonas aeruginosa* quorum-sensing inhibitors. *Antimicrob. Agents Chemother.* **53**, 2432–2443.

Yang, S., Peng, Q., San Francisco, M., Wang, Y., Zeng, Q. and Yang, C.H. (2008) Type III secretion system genes of *Dickeya dadantii* 3937 are induced by plant phenolic acids. *PLoS ONE*, **3**, e2973.

Yishay, M., Burdman, S., Valverde, A., Luzzatto, T., Ophir, R. and Yedidia, I. (2008) Differential pathogenicity and genetic diversity among *Pectobacterium carotovorum* ssp. *carotovorum* isolates from monocot and dicot hosts support early genomic divergence within this taxon. *Environ. Microbiol.* **10**, 2746–2759.

Yuan, Z.C., Edlind, M.P., Liu, P., Saenkham, P., Banta, L.M., Wise, A.A., Ronzone, E., Binns, A.N., Kerr, K. and Nester, E.U. (2007) The plant signal salicylic acid shuts down expression of the vir regulon and activates quormone-quenching genes in *Agrobacterium*. *Proc. Natl. Acad. Sci. USA*, **104**, 11790–11795.

Zhang, Y., Xu, S., Ding, P., Wang, D., Cheng, Y.T., He, J., Gao, M., Xu, F., Li, Y., Zhu, Z., Li, X. and Zhang, Y. (2010) Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proc. Natl. Acad. Sci. USA*, **107**, 18220–18225.

SUPPORTING INFORMATION

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

Fig. S1 Effects of cinnamic acid (CA) and salicylic acid (SA) on the growth of *Pectobacterium aroidearum* PC1 and *Pectobacterium carotovorum* ssp. *brasiliense* Pcb1692. The strains were grown in liquid Lysogeny-Broth (LB) and LB with non-lethal concentrations of CA or SA (0.25 mg/mL and 0.21 mg/mL, respectively). After 1, 8 and 24 h of growth, the bacterial cultures were serially diluted and plated on LB agar plates. The numbers of colonies were counted and CFU (colony forming units)/mL are expressed on a logarithmic scale.

Fig. S2 Effects of cinnamic acid (CA; 0.25 mg/mL) and salicylic acid (SA; 0.21 mg/mL) on the transcript levels of hor and hrpS. These genes are transcriptional regulators, which were not affected by the supplied phenolic compounds.

Fig. S3 (a) Growth curve of *Pectobacterium* strains (*P. aroidearum* PC1 and *P. carotovorum* ssp. *brasiliense* Pcb1692) in the control and in the presence of non-lethal concentrations of cinnamic acid (CA) and salicylic acid (SA). Bacteria were grown at 28 °C under continuous shaking for 24 h under control and treatment conditions, and growth was assessed by measuring the absorbance (optical density, O.D.) at 600 nm every hour. The data represent means ± standard errors with at least five replicates for each experiment. (b) Growth curves of psB401 in control and in the presence of non-lethal concentrations of CA and SA. Bacteria were grown at 37 °C under continuous shaking with measurements taken every 30 min for 24 h.

Table S1 Primers used for real-time polymerase chain reaction (PCR).