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Small-molecule inhibitors suppress the expression of both type III secretion and amylovoran biosynthesis genes in *Erwinia amylovora*

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SUMMARY

The type III secretion system (T3SS) and exopolysaccharide (EPS) amylovoran are two essential pathogenicity factors in *Erwinia amylovora*, the causal agent of the serious bacterial disease fire blight. In this study, small molecules that inhibit T3SS gene expression in *E. amylovora* under *hrp* (*hypersensitive response and pathogenicity*)-inducing conditions were identified and characterized using green fluorescent protein (GFP) as a reporter. These compounds belong to salicylidene acylhydrazides and also inhibit amylovoran production. Microarray analysis of *E. amylovora* treated with compounds 3 and 9 identified a total of 588 significantly differentially expressed genes. Among them, 95 and 78 genes were activated and suppressed by both compounds, respectively, when compared with the dimethylsulphoxide (DMSO) control. The expression of the majority of T3SS genes in *E. amylovora*, including *hrpL* and the *avrRpt2* effector gene, was suppressed by both compounds. Compound 3 also suppressed the expression of amylovoran precursor and biosynthesis genes. However, both compounds induced significantly the expression of glycogen biosynthesis genes and siderophore biosynthesis, regulatory and transport genes. Furthermore, many membrane, lipoprotein and exported protein-encoding genes were also activated by both compounds. Similar expression patterns were observed for compounds 1, 2 and 4. Using crab apple flower as a model, compound 3 was capable of reducing disease development in pistils. These results suggest a common inhibition mechanism shared by salicylidene acylhydrazides and indicate that small-molecule inhibitors that disable T3SS function could be explored to control fire blight disease.

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INTRODUCTION

Erwinia amylovora is the causative agent of fire blight, a bacterial disease of apples (*Malus sylvestris*) and pears (*Pyrus communis* L.). Fire blight is a devastating necrotic disease, resulting in serious economic losses to the apple and pear industry. In the USA alone, average regional losses and cost of fire blight control reach over $100 million annually (Norelli *et al*., 2003). Since the 1970s, streptomycin applications during bloom time in spring have been the most effective in controlling blossom blight. However, the occurrence of streptomycin-resistant *E. amylovora* strains in Washington, New York, Oregon, Missouri, Michigan, Canada and elsewhere has rendered this antibiotic ineffective (Coyier and Covey, 1975; Loper *et al*., 1991; Manulis *et al*., 1998; McGhee and Sundin, 2011; Norelli *et al*., 2003; Russo *et al*., 2008; Shaffer and Goodman, 1985). Thus, alternative new strategies for the control of fire blight are needed to prevent severe losses in susceptible orchards (Kim *et al*., 2012).

The discovery of the *hypersensitive response (HR) and pathogenicity* (*hrp*) gene cluster, which encodes a type III secretion system (T3SS) common to Gram-negative bacterial pathogens, is a breakthrough discovery in modern molecular plant bacteriology (He *et al*., 2004). Like many other Gram-negative plant-pathogenic bacteria, *E. amylovora* also contains an *hrp*-T3SS that delivers effector proteins into host plants (Oh and Beer, 2005; Oh *et al*., 2005). In *E. amylovora*, the T3SS is required to elicit an HR on nonhost plants and to cause disease on susceptible host plants. The *hrp* gene cluster of *E. amylovora* is located within ~60 kb of pathogenicity island 1 (PAI-1) that includes T3SS regulatory genes (*hrpL*, *hrpS* and *hrpXY*), genes encoding structural components of T3SS and effectors (Oh and Beer, 2005; Oh *et al*., 2005; Zhao and Qi, 2011). Based on current knowledge, *E. amylovora* T3SS secretes at least 15 virulence-associated proteins, including HrpA, HrpN, HrpW, HrpJ, HrpK, HopAK1 (Eop2), DspE, HopC1, HopX1 (Eop3), AvrRpt2 (Eop4) and Eop1 (EopB) (Bogdanove *et al*., 1998; Nissinen *et al*., 2007; Zhao *et al*., 2005, 2006).

Thus far, two pathogenicity factors, including T3SS and its effectors and the acidic exopolysaccharide (EPS) amylovoran, are required for *E. amylovora* to cause disease (Zhao and Qi, 2011; Zhao *et al*., 2009a). Amylovoran biosynthetic genes are located within a 12-gene amylovoran biosynthetic (*ams*) operon, designated as *ams*A to *ams*L, with *amsG* as the first gene in the operon (Bernhard *et al*., 1993; Bugert and Geider, 1995). Two genes adjacent to the *ams* cluster, *galE* and *galF*, and located on the right are involved in amylovoran precursor formation. Amylovoran biosynthesis is strictly regulated by the Rcs phosphorelay system, which is also required for virulence (Wang *et al*., 2009, 2012; Zhao *et al*., 2009b). Additional determinants contribute to virulence and plant colonization of *E. amylovora*, including the extracellular EPS levan, protease, iron-scavenging siderophore desferrioxamine and genes involved in sorbitol and sucrose metabolism (Aldridge *et al*., 1997; Bogs and Geider, 2000; Dellagi *et al*., 1998, 1999; Du and Geider, 2002; Khan *et al*., 2012; Zhao and Qi, 2011; Zhao *et al*., 2009b).

As a member of the family Enterobacteriaceae, *E. amylovora* is closely related to many mammalian pathogens, including *Escherichia coli*, *Salmonella enterica*, *Shigella flexneri* and *Yersinia pestis*, which also utilize T3SS to deliver effector proteins into their eukaryotic hosts (Büttner and He, 2009; He *et al*., 2004). The T3SS-homologous macromolecular apparatus has been identified only in Gram-negative bacteria and is a key virulence determinant, and many studies have conducted chemical screenings by targeting known T3SS processes, including gene expression, effector secretion and translocation, and symptom development (Baron, 2010; Keyser *et al*., 2008; Kline *et al*., 2011). This is based on the speculation that T3SS is a logical target for chemotherapeutic intervention, and disabling or blocking the function of T3SS could serve as a strategy for the control of bacterial diseases. These large-scale chemical screening studies have led to the discovery of several classes of T3SS-inhibiting compounds, including salicylidene acylhydrazides (Baron, 2010; Keyser *et al*., 2008; Kline *et al*., 2011). Subsequent studies have shown that salicylidene acylhydrazide family compounds broadly interfere with the functionality of T3SS in different mammalian pathogenic bacteria, including *Yersinia pseudotuberculosis*, *S. enterica*, *Escherichia coli* O157: H7, enteropathogenic *Escherichia coli* (EPEC), *Shigella flexneri*, *Pseudomonas aeruginosa* and *Chlamydia* spp. (Bailey *et al*., 2007; Hudson *et al*., 2007; Kauppi *et al*., 2003; Layton *et al*., 2010; Muschiol *et al*., 2006, 2009; Negrea *et al*., 2007; Nordfelth *et al*., 2005; Tree *et al*., 2009; Veenendaal *et al*., 2009; Wolf *et al*., 2006). Furthermore, salicylidene acylhydrazides protect mice from vaginal infection by *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Chu *et al*., 2010; Slepenkin *et al*., 2011).

Other classes of small-molecule inhibitors of T3SS have also been reported (Felise *et al*., 2008; Kimura *et al*., 2011; Kline *et al*., 2009; Pan *et al*., 2009). Tethered thiazolidinone blocked T3SS protein secretion of *Salmonella typhimurium* and *Yersinia enterocolitica*, and also prevented HR when *Pseudomonas syringae* was co-infiltrated into tobacco (Felise *et al*., 2008; Kline *et al*., 2009). Aurodox, a linear polyketide compound isolated from *Streptomyces* sp. K01-0509, has been reported recently to inhibit the protein secretion of *Citrobacter rodentium* (Kimura *et al*., 2011). The oral administration of aurodox contributed to the survival of mice that had received a lethal dose of *C. rodentium*. However, it was unclear whether or not these small-molecule inhibitors were also effective against plant-pathogenic enterobacteria, such as *E. amylovora*.

One of the goals of this study was to determine whether or not small-molecule inhibitors, such as salicylidene acylhydrazides, were effective in inhibiting T3SS gene expression in *E. amylovora*. Moreover, the global effects of salicylidene acylhydrazide compounds on the transcriptomic profiles of *E. amylovora* were evaluated. Finally, the efficacy of these compounds in controlling fire blight by either disabling or blocking of T3SS function during blossom infection was also investigated.

RESULTS

Screening of small-molecule inhibitors that suppress T3SS gene expression *in vitro*

To define *in vitro* growth conditions that induce T3SS gene expression in *E. amylovora*, two media [*hrp*-inducing minimal medium (HMM) and M9] amended with different carbon sources, including galactose, glucose, mannitol and fructose, each at 10 and 20 mM, were used. The promoter activities of T3SS genes were determined by flow cytometry (Wang *et al*., 2009). HMM containing 20 mM galactose elicited the strongest inducing effect on T3SS gene promoter activities (data not shown; Fig. S1A, see Supporting Information) (Gaudriault *et al*., 1997). Moreover, following a time-course study of *hrp* gene promoter activities in HMM containing 20 mM galactose, the expression of *hrpL*, *hrpN*, *dspE* and *hrpA* genes increased with time and reached their highest levels at 18 h (Fig. S1B). Thus, HMM containing 20 mM galactose was selected as the medium of choice, and bacteria were grown for 18 h at 18 °C in all subsequent chemical screening experiments.

Fifteen small-molecule inhibitors were selected and tested for the suppression of the promoter activities of *hrpN*, *dspE*, *hrpA* and *hrpL* genes in *E. amylovora*. These compounds were selected as either they have been reported to inhibit T3SS gene expression in related enterobacterial pathogens (Felise *et al*., 2008; Pan *et al*., 2009; Tree *et al*., 2009) or they shared similar structures with T3SS inhibitor compounds (chemical compounds 9–12). Based on their abilities to inhibit the promoter activities of the four target T3SS genes, these compounds were divided into three groups. Chemical compounds 1, 2, 3 and 9 strongly inhibited the promoter activities of *hrpN*, *dspE*, *hrpA* and *hrpL* genes (Fig. 1). However, chemical compounds 4, 12, 14 and 16 had low to modest inhibitory effects

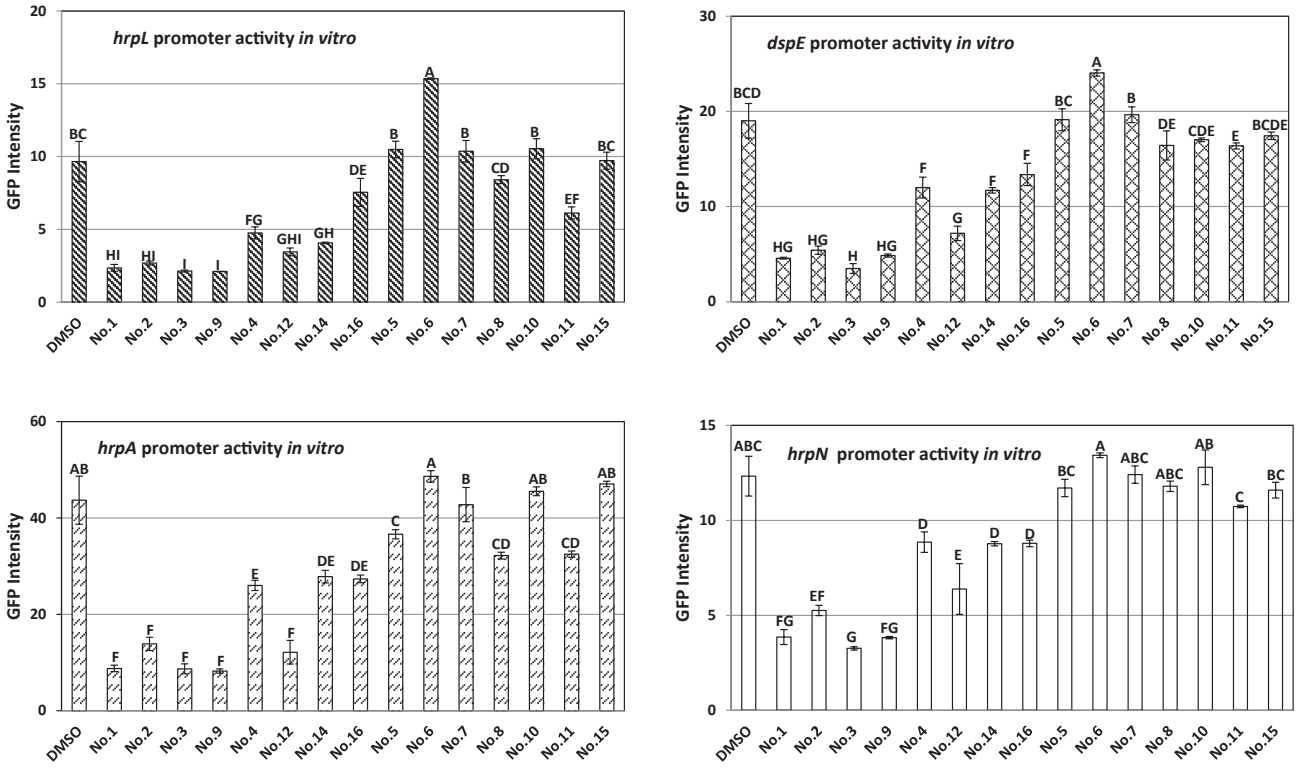

Fig. 1 Effect of small-molecule inhibitors on the promoter activities of *hrpN*, *dspE*, *hrpL* and *hrpA* genes in *Erwinia amylovora* strain Ea273. Bacterial strains were grown in *hrp* (*hypersensitive response and pathogenicity*)-inducing minimal medium (HMM) in the presence of 100 μM of the corresponding compounds at 18 °C for 18 h. The equivalent volume of dimethylsulphoxide (DMSO) was added as a control. Error bars represent the standard deviation. One-way analysis of variance (ANOVA) and Turkey's W-test ($P = 0.05$) were carried out to determine the difference in the means using the SAS program.

on the promoter activities of one to four genes, whereas chemical compounds 5–8, 10, 11 and 15 had either minimal or no inhibitory effects on the promoter activities of these T3SS genes (Fig. 1). Thus, for the remainder of this study, experiments were conducted using chemical compounds 3 and 9, as compound 3 is the most potent in suppressing T3SS gene expression and compound 9 has not been reported previously.

Both chemical compounds 3 and 9 exhibited similar inhibitory effects on the promoter activities of the four target T3SS genes at different concentrations (Fig. S2, see Supporting Information). To eliminate the likelihood that these compounds resulted in growth defects of *E. amylovora*, bacterial growth was monitored *in vitro* in the presence of these two compounds under *hrp*-inducing conditions, and no significant effects on *E. amylovora* growth were observed (Fig. S3A, see Supporting Information).

Effects of chemical compounds 3 and 9 on the transcriptome of *E. amylovora*

Microarray analysis was used to evaluate the transcriptomic profiles of *E. amylovora* following the treatment of bacterial cultures with compounds 3 and 9 relative to dimethylsulphoxide (DMSO), all grown in HMM containing 20 mM galactose (Fig. 2). A total of 588 genes were significantly differentially expressed, of which 320 and 268 genes were suppressed or induced by the compounds, respectively (Table S1, see Supporting Information). For treatment with compound 3, 251 genes were up-regulated and 279 genes were down-regulated, whereas, for compound 9 treatment, 112 genes were up-regulated and 119 genes were down-regulated when compared with DMSO treatment (Fig. 2, Table S1). Based on the combined results from both compounds, 78 genes were commonly suppressed and 95 genes were commonly induced by compounds 3 and 9 (Fig. 2).

Overall, the expression of the majority of the 38 known T3SS genes was down-regulated or suppressed by compound 3, and some were suppressed by compound 9, when compared with DMSO treatment (Table 1). These genes included regulatory genes (*hrpL* and *hrpS*), all known effectors (*avrRpt2*, *hopC1*, *hopAk1*, *hopX1*, *dspE*, *eop2* and *hrpW*), chaperones (*dspF*, *esc1* and *esc3*) and T3SS structural genes (Table 1). However, both *hrpN* and *hrpA* hybridization spots on the microarray were saturated, thus resulting in a ratio of 1.0 (Table 1). In addition, compound 3 suppressed the expression of 14 amylovoran precursor and biosynthesis genes (*galEF* and *amsABCDEFGHIJKL*) (Table 1). All *ams* genes also

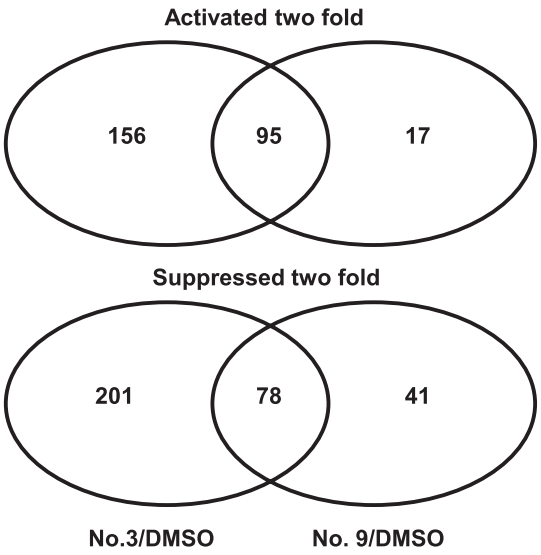

Fig. 2 Venn diagrams showing the number of differentially expressed genes in compound 3- and 9-treated samples relative to dimethylsulphoxide (DMSO) control. Differentially expressed genes were those that were activated (>twofold) or suppressed (<twofold) by compounds 3 (50 μM) and 9 (50 μM), respectively. *Erwinia amylovora* strain Ea273 was grown in *hrp* (*hypersensitive response and pathogenicity*)-inducing minimal medium (HMM) at 18 °C for 18 h.

exhibited lower levels of expression following treatment with compound 9 when compared with DMSO treatment. These results indicated that both compounds inhibited T3SS and amylovoran biosynthesis genes. Furthermore, the membrane-encoding protein operon (*EAM_2938-2935*), reported to be positively regulated by HrpL and RcsBC (McNally *et al*., 2012; Wang *et al*., 2012), was also inhibited by compounds 3 and 9 (Table 1).

Among the up-regulated genes, five glycogen metabolism genes (*glgABCPX*) and 13 exogenous iron uptake system genes, including siderophore desferrioxamine E biosynthesis (*dfoJAC*), siderophore-interacting protein (*EAM_3350*), TonB-dependent ferrioxamine receptors (*foxR*, *EAM_1726*), TonB-dependent receptor complex (*tonB*, *exbBD*) and TonB-dependent transporter genes [haemin ABC transporter (*hmuVUTS*)], were highly induced by both compounds (Table 2). In addition, yet another 35 genes were commonly up-regulated by both compounds, and these encoded membrane, lipoprotein, exported proteins and oxidative stress-related proteins and transporters (Table 2). Furthermore, some iron–sulphur cluster genes (*sufAC*) were also induced by compound 3 (Table S1), suggesting that this compound might also interfere with endogenous iron function.

Validation of microarray data by quantitative real-time polymerase chain reaction (qRT-PCR)

To validate the results of the microarray data, qRT-PCR was carried out for 10 selected genes, including *hrpA*, *dspE*, *hrpL*, *hrpN*, *avrRpt2*, *amsG*, *amsD*, *glgB*, *foxR* and *EAM_3350*. The results of qRT-PCR were mostly consistent with those of microarray analysis, except for *hrpN* and *hrpA*, which showed strong inhibition in qRT-PCR analysis (Fig. 3). The expression of *hrpA* in qRT-PCR for compounds 3 and 9 was suppressed by 0.13- and 0.66-fold, respectively, whereas the expression of *hrpN* was suppressed by fold changes of 0.07 and 0.29, respectively.

Other small-molecule inhibitors exhibit similar gene expression patterns

qRT-PCR analysis was also performed to evaluate the influence of compounds 1, 2 and 4 on the expression levels of selected target genes, including four *hrp* genes (*hrpA*, *hrpN*, *hrpL* and *dspE*), an amylovoran biosynthesis gene (*amsG*) and two iron uptake genes (*EAM_359* and *hmuS*) in *E. amylovora*. Similar to the results obtained with compounds 3 and 9, all three compounds, 1, 2 and 4, suppressed both T3SS and *ams* gene expression, but induced the expression of iron acquisition genes, when compared with DMSO treatment (Fig. 4). For example, *hrpL* expression levels exhibited fold changes of 0.51, 0.20, 0.24, 0.7 and 0.19 for compounds 1, 2, 3, 4 and 9, respectively. In contrast, expression of the haemin transport protein-encoding gene *hmuS* exhibited fold changes of 52.27, 5.09, 50.12, 9.99 and 22.91 for compounds 1, 2, 3, 4 and 9, respectively. These results suggest that the suppression of either T3SS or *ams* expression, together with the induction of iron uptake gene expression, may represent a common transcriptional effect of these compounds.

Small-molecule inhibitors suppress amylovoran production

To determine whether small-molecule inhibitors influence the production of amylovoran, a cetylpyrimidinium chloride (CPC) assay was performed as described previously (Zhao *et al*., 2009b). At a concentration of 25 μM, the optical densities at 600 nm (OD_{600}), which reflect amylovoran production, were 1.09, 1.09, 0.99, 1.19 and 1.32 for compounds 1, 2, 3, 4 and 9, respectively, when compared with DMSO (1.40) (Fig. 5). This indicates that all of these chemical compounds suppress amylovoran production slightly, although this is not statistically significant for compound 9 relative to DMSO treatment.

Compound 3 delays disease progress on crab apple flowers

To determine whether small-molecule inhibitors could prevent fire blight disease development on stigmas, a crab apple flower assay was carried out as described previously (Pusey, 1997). As chemical compound 3 is the most potent in suppressing T3SS gene expression, it was selected for this experiment. Six days post-inoculation,

Table 1 Suppression of type III secretion system (T3SS) and amylovoran biosynthesis genes after treatment with compounds 3 and 9.

Gene ID	Gene	Compound 3/DMSO*	Compound 9/DMSO*	Protein description
EAM_0423	*avrRpt2(eop4)*	0.27	0.38	Cysteine protease protein AvrRpt2
EAM_2190	*hopX1(eop3)*	0.24	0.55†	Type III effector protein HopC1
EAM_2697	*hopC1*	0.31	0.41	Putative type III effector protein
EAM_2780	*hopAK1(eop2)*	0.23	0.52	Type III effector (pectin lyase)
EAM_2871	*dspF*	0.27	0.89†	Putative avirulence protein
EAM_2872	*dspE*	0.28	0.73	Type III effector protein
EAM_2873	*hrpW*	0.37	0.70†	Putative pectate lyase
EAM_2874	*esc3(orfC)*	0.37	0.63†	Chaperone
EAM-2875	*eop1(orfB)*	0.38	0.62†	Effector protein Eop1
EAM_2876	*esc1(orfA)*	0.26	0.43	Chaperone for Eop1
EAM_2877	*hrpN*	**0.92**†	**1.04**†	Harpin
EAM_2878	*hrpV*	0.21	0.44	Type III secretion system protein
EAM_2879	*hrpT*	0.25	0.54†	Type III secretion system protein
EAM_2880	*hrcC*	0.22	0.42	Type III secretion system protein
EAM_2881	*hrpG*	0.28	0.56	Type III secretion system protein
EAM_2882	*hrpF*	0.18	0.43	Type III secretion system protein
EAM_2883	*hrpE*	0.19	0.40	Type III secretion system protein
EAM_2884	*hrpD*	0.17	0.32	Type III secretion system protein
EAM_2885	*hrcJ*	0.24	0.44	Type III secretion system protein
EAM_2886	*hrpB*	0.39	0.64	Type III secretion system protein
EAM_2887	*hrpA*	**0.77**	**1.11**†	Type III secretion system protein
EAM_2891	*hrpS*	0.41	0.97†	Sigma 54 enhancer-binding protein
EAM_2894	*hrpL*	0.16	0.28	Sigma factor HrpL
EAM_2895	*hrpJ*	0.19	0.37	Type III secretion system protein
EAM_2896	*hrpI*	0.18	0.37	Type III secretion system protein
EAM_2897	*hrpQ*	0.26	0.53	Type III secretion system protein
EAM_2898	*hrcN*	0.21	0.41	Type III secretion system protein
EAM_2899	*hrpO*	0.30	0.47	Type III secretion system protein
EAM_2900	*hrpP*	0.25	0.53†	type III secretion system protein
EAM_2901	*hrcQ*	0.18	0.48	type III secretion system protein
EAM_2902	*hrcR*	0.23	0.53†	type III secretion system protein
EAM_2903	*hrcS*	0.17	0.44	type III secretion system protein
EAM_2904	*hrcT*	0.32	0.57	type III secretion system protein
EAM_2905	*hrcU*	0.19	0.45	type III secretion system protein
EAM_2908	*hsvC*	0.06	0.35	Conserved hypothetical protein
EAM_2909	*hsvB*	0.04	0.22	Conserved hypothetical protein
EAM_2910	*hsvA*	0.06	0.30	Putative amidinotransferase
EAM_2911	*hrpK*	0.39	0.47	Type III secretion system protein
EAM_2161	*galE*	0.42	0.94†	UDP-glucose 4-epimerase
EAM_2162	*galF*	0.33	0.77†	UTP-glucose-1-phosphate uridylyltransferase
EAM_2163	*amsL*	0.38	0.85†	Amylovoran biosynthesis protein
EAM_2164	*amsK*	0.26	0.56	Glycosyltransferase
EAM_2165	*amsJ*	0.33	0.69†	Amylovoran biosynthesis protein
EAM_2166	*amsF*	0.45	0.98†	Amylovoran biosynthesis protein
EAM_2167	*amsE*	0.26	0.65	Amylovoran glycosyltransferase
EAM_2168	*amsD*	0.30	0.65	Glycosyltransferase
EAM_2169	*amsC*	0.34	0.84†	Oligosaccharide repeat unit polymerase
EAM_2170	*amsB*	0.28	0.71	Glycosyltransferase
EAM_2171	*amsA*	0.24	0.69†	Tyrosine-protein kinase
EAM_2172	*amsI*	0.24	0.68†	Protein-tyrosine-phosphatase
EAM_2173	*amsH*	0.28	0.72†	Amylovoran export protein
EAM_2174	*amsG*	0.34	0.93†	UDP-galactose-lipid carrier transferase
EAM_2935		0.29	0.53	γ-Glutamyltranspeptidase
EAM_2936		0.15	0.36	Putative exported protein
EAM_2937		0.26	0.5	Putative disulphide bond formation membrane protein
EAM_2938		0.38	0.62	Putative membrane protein

*Expression ratio ≤0.5 indicates genes were suppressed in compound treatments.

†$P > 0.05$; all others with $P < 0.05$.

Bold type indicates that corresponding spots were saturated in the array.

Table 2 Genes activated by compounds 3 and 9.

Gene ID	Gene	Compound 3/DMSO*	Compound 9/DMSO*	Protein description
EAM_3268	*glgP*	5.58	3.32	Glycogen phosphorylase
EAM_3269	*glgA*	6.91	3.24	Glycogen synthase
EAM_3270	*glgC*	7.18	4.03	Glucose-1-phosphate adenylyltransferase
EAM_3271	*glgX*	5.06	2.43	Glycogen debranching enzyme
EAM_3272	*glgB*	7.34	3.75	1,4-α-Glucan branching enzyme
EAM_0358	*foxR*	13.17	7.35	Ferrioxamine TonB-dependent receptor
EAM_0359	*dfoC*	60.30	43.63	Decarboxylase
EAM_0360	*dfoA*	236.45	133.50	Siderophore biosynthesis protein
EAM_0361	*dfoJ*	214.85	120.14	Siderophore biosynthesis
EAM_1639	*hmuV*	59.27	37.76	Haemin ABC transporter
EAM_1640	*hmuU*	64.50	46.38	Haemin ABC transporter
EAM_1641	*hmuT*	118.68	73.26	Haemin ABC transporter
EAM_1642	*hmuS*	99.19	71.24	Haemin ABC transporter
EAM_1726		32.4	4.87	TonB-dependent receptor
EAM_1888	*tonB*	2.92	4.02	TonB protein
EAM_2955	*exbD*	4.53	4.44	Biopolymer transport protein
EAM_2956	*exbB*	6.20	6.13	Biopolymer transport protein
EAM_3350		15.4	22.4	Putative siderophore-interacting protein
EAM_2594	*nrdH*	18.3	10.5	Glutaredoxin-like protein
EAM_2595	*nrdI*	15.3	9.1	Putative flavodoxin
EAM_2596	*nrdE*	9.1	5.72	Ribonucleoside-diphosphate reductase 2 α chain
EAM_2597	*nrdF*	5.7	4.72	Ribonucleoside-diphosphate reductase 2 β chain
EAM_0425	*blc*	3.46	2.03	Outer membrane lipoprotein
EAM_0561		2.15	2.03	Putative exported protein
EAM_0565		2.11	2.0	Putative membrane protein
EAM_0611	*osmY*	3.0	2.41	Osmotically inducible protein Y
EAM_0612		2.11	2.0	Putative membrane protein
EAM_0624		6.16	2.65	Putative lipoprotein
EAM_9625		5.15	2.61	Putative polypeptide transport protein
EAM_0893		6.86	2.60	Putative exported protein
EAM_0927		7.55	3.65	Putative membrane protein
EAM_0965		10.0	3.75	Putative lipoprotein
EAM_0966		8.43	3.79	Putative phospholipid-binding protein
EAM_1076	*osmC*	7.79	2.69	Peroxiredoxin (osmotically-inducible protein C)
EAM_1077		3.26	9.37	Putative membrane protein
EAM_1078		3.45	12.4	Putative membrane protein
EAM_1086		2.40	2.34	Putative lipoprotein
EAM_1190		17.7	3.77	Putative exported protein
EAM_1231		9.74	5.19	Putative membrane protein
EAM_1543		8.05	3.62	Putative lipoprotein
EAM_1544		9.17	3.82	Putative exported protein
EAM_1616	*osmE*	3.40	2.14	Osmotically inducible lipoprotein E
EAM_1617		6.47	10.6	Putative membrane protein
EAM_1958	*osmB*	4.38	1.49	Osmotically inducible lipoprotein B
EAM_1984		6.84	2.77	Putative membrane protein
EAM_2038		3.46	2.04	Putative membrane protein
EAM_2057		5.74	7.49	Putative peroxidase
EAM_2058		5.96	7.4	Putative exported protein
EAM_2059		6.7	7.98	Putative permease
EAM_2467	*prt1*	4.91	2.82	Extracellular metalloprotease
EAM_2941		20.2	19.2	Putative ABC transporter
EAM_3038		4.12	2.03	Putative exported protein
EAM_3377		5.93	3.20	Putative exported protein

*Expression ratio ≥2.0 indicates genes were activated in compound treatments; $P < 0.05$.

a significant difference in symptom development in pistils was observed for compound 3-treated flowers relative to treatment with DMSO alone (Fig. 6). Pistils of flowers inoculated with a *dspE* mutant of *E. amylovora*, which is nonpathogenic, remained healthy and showed the least amount of necrosis at the inoculation site. Similarly, compound 3-treated flowers exhibited slight necrotic symptoms. In contrast, flowers inoculated with either the wild-type (WT) strain alone or the WT strain treated with DMSO exhibited heavy necrosis along entire pistils (Fig. 6).

To investigate whether compound 3 also affected *E. amylovora* growth *in vivo*, bacterial growth was determined using dilution plating. Two and four days post-inoculation, bacterial populations

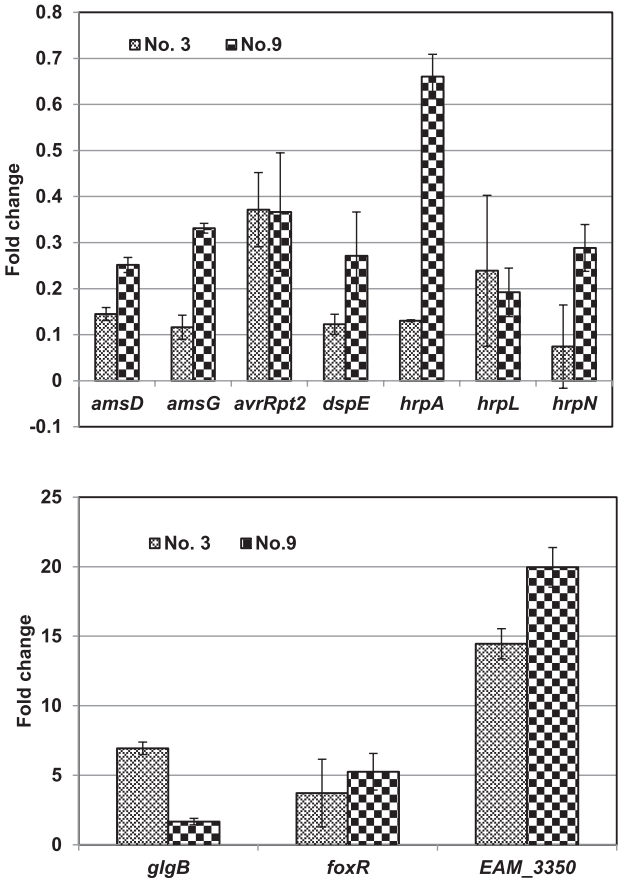

Fig. 3 Verification of microarray data by quantitative real-time polymerase chain reaction (qRT-PCR). The relative fold change of each gene was derived from the comparison of the compound-treated versus dimethylsulphoxide (DMSO)-treated *Erwinia amylovora* strain Ea273 in *hrp* (*hypersensitive response and pathogenicity*)-inducing minimal medium (HMM) for compounds 3 and 9 using qRT-PCR. The 16S rDNA (*rrsA*) gene was used as a control. The values of the relative fold change were means of three replicates. The experiments were repeated three times with similar results. Error bars indicate the standard deviation.

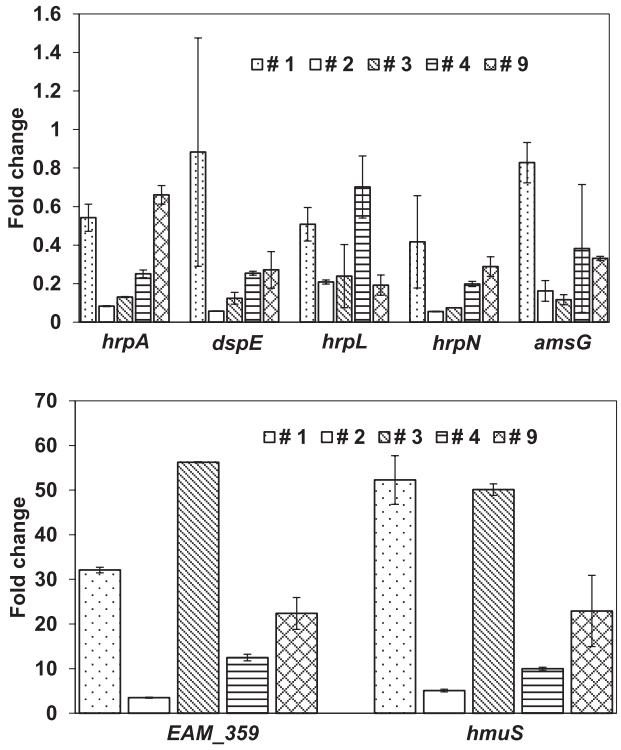

Fig. 4 Effect of salicylidene acylhydrazide inhibitors on virulence gene expression. Relative fold changes were determined by quantitative real-time polymerase chain reaction (qRT-PCR) for *hrpA*, *dspE*, *hrpL*, *hrpN*, *amsG*, *EAM_359* and *hmuS* genes. *Erwinia amylovora* Ea273 was grown in the presence of compounds 1–4 and 9 (50 μM) in *hrp* (*hypersensitive response and pathogenicity*)-inducing minimal medium (HMM) at 18 °C for 18 h. The 16S rDNA (*rrsA*) gene was used as a control. The values of the relative fold change were means of three replicates. The experiments were repeated three times with similar results. Error bars indicate the standard deviation.

were found to be comparable between DMSO- and compound 3-treated flowers. By six days post-inoculation, compound 3-treated flowers showed about a two-fold reduction in bacterial population when compared with those inoculated with WT treated with DMSO alone, although this difference was not statistically significant (Fig. S3B).

We also tested whether these compounds could inhibit the elicitation of HR by *E. amylovora* in tobacco. Surprisingly, none of the 15 compounds inhibited HR when infiltrated with *E. amylovora* strain Ea273 at levels of 10–100 μM. When *E. amylovora* strain Ea1189 was used, at 50 μM or higher, three compounds (6, 8 and 11) inhibited HR development at 24 h (Fig. S4, see Supporting Information; data not shown for compound 6); however, normal HR was observed at 48 h for all compounds at all levels.

DISCUSSION

As a landmark discovery, advances in the study of bacterial virulence factors have provided mounting evidence that T3SS is a potent virulence mechanism shared by a broad spectrum of pathogenic Gram-negative bacteria that infect both plant and mammalian hosts (He *et al*., 2004). Thus, the T3SS apparatus is essential for bacterial cells to evade host immune defenses, and T3SS could serve as a universal target for the development of novel antibacterial agents (Baron, 2010; Kline *et al*., 2011). It is likely that agents that inhibit T3SS can result in an antibacterial response without killing bacteria (Keyser *et al*., 2008). In this study, 15 chemical compounds known to either act or share similar structures to those reported as T3SS inhibitors were evaluated for their suppression of T3SS gene expression in *E. amylovora*. Among these, four compounds were found to either strongly or moderately suppress T3SS gene expression. Two of these compounds (9 and 12) have not been reported previously to act as T3SS

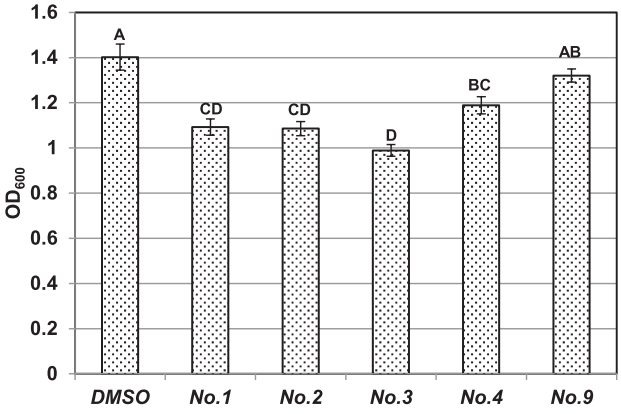

Fig. 5 Effect of small-molecule inhibitors on amylovoran production. *Erwinia amylovora* strain Ea273 was grown in MBMA medium with 1% sorbitol at 28 °C for 24 h with shaking. Chemical compounds and an equal volume of dimethylsulphoxide (DMSO) were added to the cell culture at a final concentration of 25 μM. Amylovoran concentrations were measured by the cetylpyrimidinium chloride (CPC) method and normalized to a cell density of unity. Data points represent the means of three replicates ± standard deviations. Each experiment was performed at least three times with similar results.

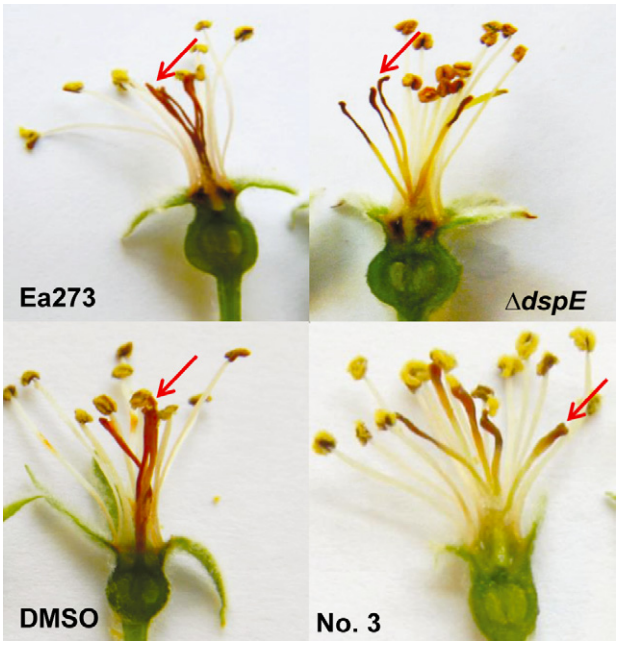

Fig. 6 Virulence assay on crab apple (*Malus mandshurica*) flowers. Symptoms of crab apple flowers at 6 days post-inoculation with *Erwinia amylovora* strain Ea273, *dspE* mutant, Ea273 treated with dimethylsulphoxide (DMSO) or compound 3 at 50 μM. Photographs were taken at 6 days post-inoculation. The experiments were repeated three times with similar results. Arrow indicates representative inoculation site (stigma) and symptoms below on style and ovary.

inhibitors. Moreover, five compounds (5–8 and 15), reported previously to inhibit T3SS in other bacteria, were not found to be effective against *E. amylovora*.

High-throughput screening is a powerful tool in the identification of small-molecule inhibitors that disable T3SS function. Recent studies have identified several classes of synthetic compounds, as well as natural products, as active T3SS inhibitors in a wide range of Gram-negative bacterial pathogens, including *Escherichia coli*, *Salmonella*, *Yersinia*, *Shigella* and *Chlamydia* (Baron, 2010; Gauthier *et al*., 2005; Keyser *et al*., 2008; Kline *et al*., 2011; Muschiol *et al*., 2006). These inhibitors include salicylanilides, salicylidene anilides, salicylidene acylhydrazides, thiazolidinone, sulphonylaminobenzanilides, caminosides and guadinomines, and are likely to target T3SS gene expression, conserved outer membrane proteins, effector secretion and needle assembly. Pan *et al*. (2009) have also described a group of T3SS-inhibiting compounds of diverse chemical structures that are distinct from all previously reported T3SS inhibitors.

In this study, all compounds that suppressed the expression of T3SS genes in *E. amylovora* under *hrp*-inducing conditions belonged to the salicylidene acylhydrazides, except for one compound: 16. This is the largest family of small-molecule inhibitors identified thus far (Keyser *et al*., 2008; Wang *et al*., 2011). Recent evidence suggests that this group of compounds interferes with various T3SS functions, including gene expression, effector secretion or translocation, and needle assembly (Kauppi *et al*., 2003; Muschiol *et al*., 2006, 2009; Veenendaal *et al*., 2009; Wolf *et al*., 2006). In this study, the suppression of T3SS gene expression by compounds 3 and 9 was found to be dose dependent in *E. amylovora*, and similar results were observed for all five compounds (1–4 and 9) tested for their effect on gene expression.

Global gene expression profiles for compounds 3 and 9 have indicated that the transcriptional profiling for *E. amylovora* is highly similar to that reported for *Escherichia coli* O157: H7 and *S. typhimurium* after salicylidene acylhydrazide treatment (Layton *et al*., 2010; Tree *et al*., 2009). Previously, it has been demonstrated that salicylidene acylhydrazides suppress the transcription of all T3SS genes of *Escherichia coli* O157: H7 and *S. typhimurium* (Layton *et al*., 2010; Tree *et al*., 2009). In this study, compounds 3 and 9 also exhibited strong inhibition of all known T3SS genes in *E. amylovora*. It is important to note that this is the first report of the T3SS inhibitory effect of compound 9. These findings indicate that salicylidene acylhydrazides broadly inhibit T3SS gene expression not only in mammalian pathogens, but also in plant-pathogenic enterobacteria. Furthermore, this suggests that the suppression of T3SS by salicylidene acylhydrazide is a conserved transcriptional response among different bacterial species. However, other compounds, such as 6, 7 and 8, also inhibit T3SS in some organisms (Felise *et al*., 2008, Pan *et al*., 2009), but are not effective in *E. amylovora*, thus suggesting that these compounds are likely to have specific targets.

Amylovoran is another essential pathogenicity factor of *E. amylovora* (Zhao *et al*., 2009a, b). Transcriptomic analysis has revealed that salicylidene acylhydrazides also inhibit amylovoran production and amylovoran biosynthesis gene expression, but promote glycogen gene expression. These findings demonstrate, for the first time, that salicylidene acylhydrazides may also target other bacterial virulence factors in addition to T3SS. It has been reported that salicylanilide class compounds inhibit two-component signal transduction systems in bacteria (Macielag *et al*., 1998). In *E. amylovora*, the RcsBCD phosphorelay two-component system plays an essential role in controlling amylovoran and glycogen production, as well as virulence (Wang *et al*., 2009, 2012). Therefore, it is likely that salicylidene acylhydrazides may act on the RcsBCD system as well.

Another unique feature of the salicylidene acylhydrazide-induced transcriptome is the activation of the iron uptake system. Layton *et al*. (2010) have observed that about one-quarter of genes in *S. typhimurium* regulated significantly following treatment with 100 μM INP0403 (compound 2 in this study) are genes involved in iron acquisition, including the TonB-dependent receptor complex (*tonB*, *exbBD*), ABC ferric transporter (*fepABCDEG*), iron transport (*feoAB*, *fhuABCDEF*) and iron uptake regulator (*fur*). Similarly, in this study, most of the activated genes of the *E. amylovora* transcriptome by compounds 3 and 9 were exogenous iron uptake genes, including genes involved in siderophore desferrioxamine E biosynthesis (*dfoJAC*), siderophore-interacting protein (*EAM_3350*), TonB-dependent ferrioxamine receptors (*foxR*, *EAM_1726*), TonB-dependent receptor complex (*tonB*, *exbBD*), TonB-dependent transporter genes [haemin ABC transporter (*hmuVUTS*)] and endogenous iron–sulphur assembly genes (*sufAC*). These findings further suggest that a common mechanism may be shared by salicylidene acylhydrazides in regulating bacterial transcription. However, both compounds inhibit the expression of the *ftnA* gene (*EAM_2940*) by more than five-fold (Table S1). Ferritin, encoded by *ftnA*, is a primary intracellular iron storage protein that stores iron and contributes to its controlled release. Reduced levels of ferritin proteins result in reduced iron storage. This indicates that iron uptake/release may be fundamental in protecting bacteria from salicylidene acylhydrazides. As siderophore desferrioxamine is a virulence factor in *E. amylovora* (Dellagi *et al*., 1998, 1999), this new finding may suggest a unique role for siderophores in protecting bacteria from stress conditions, in addition to their role in virulence.

Both compounds 9 and 12 suppressed the expression of a membrane-encoding protein operon (*EAM_2938-2935*) (Table 1), previously reported to be positively regulated by HrpL and RcsBC, and to be a novel virulence factor (McNally *et al*., 2012; Wang *et al*., 2012). Interestingly, both compounds also activated 35 genes that encode for membrane, lipoprotein, exported proteins, oxidative stress-related proteins (such as OsmC) and ABC transporters (Table 2). However, the precise role of these membrane and lipoprotein-encoding genes remains unknown. Recently, thiol peroxidase Tpx and oxidoreductase WrbA have been shown to be putative targets of salicylidene acylhydrazide compounds, and conformational changes of Tpx between reduced and oxidized states affect the binding ability to these compounds (Gabrielsen *et al*., 2012; Wang *et al*., 2011). However, in this study, there were no observed differences in expression of *tpx* and other peroxiredoxin genes, such as *ahpC* and *sodA*. However, several genes encoding redox proteins and central metabolism, such as *fpr* (*EAM_0114*), encoding ferredoxin-NADP reductase, the *nuo* operon (*nuoABCEFGHIJKLMN*), encoding the energy-conserving NADH dehydrogenase, the succinate dehydrogenase operon (*sdhABCD*) and the succinyl-CoA synthetase operon (*sucABCD*), are highly suppressed either by both compounds or by compound 3 (Table S1). Recruitment of 'free' ferrous iron has been found to generate destructive hydroxyl radicals through the Fenton reaction. The ferrous iron can also be released from Fe–S clusters when superoxide production is induced. As Tpx and WrbA are scavengers of reactive oxygen species (ROS), and alterations in central metabolism related to NADP consumption are critical for superoxide-mediated Fe–S cluster destabilization and for the stimulation of the Fenton reaction (Davies *et al*., 2009; Dwyer *et al*., 2007; Kohanski *et al*., 2010), it is likely that salicylidene acylhydrazides may increase iron uptake, which, in turn, promotes the formation of ROS. Recent studies have also suggested that ROS can regulate both the structure and function of a variety of biomolecules by altering their oxidation–reduction (redox) states (Fang, 2011). Thus, it is tempting to speculate that exposure to these small molecules could also result in ROS that act as redox signals, thus disrupting T3SS function and amylovoran biosynthesis. Future analysis is necessary to fully address the mode of action of these salicylidene acylhydrazides in inhibiting T3SS functions.

In the spring, blossom blight infection is the main source of dissemination of *E. amylovora*, and the pathogen must establish large epiphytic populations on stigmas of flowers for further successful infection of host plants. The control of fire blight is achieved mainly following the application of antibiotics to flowers to suppress pathogen infection. Using crab apple flowers as a model, we have shown that compound 3 is capable of reducing disease development in pistils. These results suggest that small-molecule inhibitors that disable T3SS function could be useful in the control of fire blight disease.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth media

The bacterial strains and plasmids used in this study are listed in Table 3. Luria–Bertani (LB) medium was routinely used for the culture of *E. amylovora*. Amylovoran production was determined by growing bacte-

Table 3 Bacterial strains, plasmids and small-molecule inhibitors used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
Erwinia amylovora		
Ea273	Wild-type, isolated from apple	Wang *et al*. (2010)
Ea1189	Wild-type, isolated from apple	Burse *et al*. (2004)
Ea273a	Rifampicin-resistant derivative of Ea273, Rif^R	This study
ΔdspE	*dspE*::Km; Km^R-insertional mutant of *dspE* of Ea1189, Km^R	Zhao *et al*., 2009b
Plasmids		
pFPV25	Ap^R, GFP-based promoter trap vector containing a promoterless gfpmut3a gene	Valdivia and Falkow (1997)
pHrpN-GFP	735-bp *Eco*RI-*Bam*HI DNA fragment containing promoter sequence of *hrpN* gene in pFPV25	This study
pHrpA-GFP	708-bp *Eco*RI-*Bam*HI DNA fragment containing promoter sequence of *hrpA* gene in pFPV25	This study
pZW2	608-bp *Kpn*I-*Xba*I DNA fragment containing promoter sequence of *hrpL* gene of Ea273 in pFPV25	Wang *et al*. (2010)
pZW3	570-bp *Sma*I DNA fragment containing promoter sequence of *dspE* gene of Ea273 in pFPV25 in forward orientation	Wang *et al*. (2010)
Small-molecule inhibitors		
Compound 1	ME0052, INP0010	Nordfelth *et al*. (2005)
Compound 2	ME0053, INP0403	Nordfelth *et al*. (2005)
Compound 3	ME0054, INP0401	Nordfelth *et al*. (2005)
Compound 4	ME0055, INP0031	Nordfelth *et al*. (2005)
Compound 5	INP0002	Negrea *et al*. (2007)
Compound 6	Compound 1, TTS29	Felise *et al*. (2008)
Compound 7	Compound 2, CAS #3530-35-6	Pan *et al*. (2009)
Compound 8	Compound 3, CAS #27885-92-3	Pan *et al*. (2009)
Compound 9	5277768	This study
Compound 10	5472847	This study
Compound 11	5551447	This study
Compound 12	5569869	This study
Compound 14	INP0007	Negrea *et al*. (2007)
Compound 15	Compound 1, CAS #329057-04-7	Pan *et al*. (2009)
Compound 16	Compound 3-didiproprionate, CAS #55750-06-6	Pan *et al*. (2009)

Ap^R, ampicillin resistance; CAS, Chemical Abstracts Service; GFP, green fluorescent protein; Km^R, kanamycin resistance; Rif^R, rifampicin resistance. Compounds 9–12 are from ChemBridge and have unique numbers.

rial cells in MBMA medium (3 g KH_2PO_4, 7 g K_2HPO_4, 1 g $[NH_4]_2SO_4$, 2 mL glycerol, 0.5 g citric acid and 0.03 g $MgSO_4$) amended with 1% sorbitol (Zhao *et al*., 2009b). Bacterial strains were also grown in HMM (1 g $[NH_4]_2SO_4$, 0.346 g $MgCl_2.6H_2O$, 0.099 g NaCl, 8.708 g K_2HPO_4 and 6.804 g KH_2PO_4) or M9 minimal medium (12.8 g $Na_2HPO_4.7H_2O$, 3.0 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.24 g $MgSO_4$ and 0.011 g $CaCl_2$) to activate T3SS gene expression by the addition of carbon sources as indicated. When necessary, the following antibiotics were added to the medium: 50 μg/mL kanamycin (Km), 100 μg/mL ampicillin (Ap) and 50 μg/mL rifampicin (Rif).

Small-molecule inhibitors and sources

Chemical compounds 1–4 (Nordfelth *et al*., 2005), previously reported to inhibit T3SS of enterobacterial pathogens, were prepared according to published procedures (Dahlgren *et al*., 2010), and kindly supplied by Dr Mikael Elofsson (Fig. S5, see Supporting Information, Table 3). Previously, other names used for these compounds included ME0052–ME0054 and INP0010, INP0403, INP0401 and INP0031, respectively (Negrea *et al*., 2007; Tree *et al*., 2009; Veenendaal *et al*., 2009). Compounds 5–16 were purchased from ChemBridge (San Diego, CA, USA), Timtec (Newark, DE, USA) and Sigma-Aldrich (St. Louis, MO, USA) (Fig. S5). Compounds 5 and 14 were also known as INP0002 and INP0007, respectively (Negrea *et al*., 2007). Compound 6 was reported by Felise *et al*. (2008), and compounds 7, 8, 15 and 16 were reported by Pan *et al*. (2009) to inhibit T3SS, but referred to as compounds #2, 3, 1 and #3-diproprionate, respectively (Table 3).

Construction of promoter–green fluorescent protein (GFP) fusions for promoter activity assays

The promoter region of *hrpN* and *hrpA* genes was amplified by PCR. Primer pairs hrpN1–hrpN2 and hrpA1–hrpA2, with restriction sites, were used to amplify 735 and 708 bp for *hrpN* and *hrpA* promoter sequences from *E. amylovora* strain, respectively (Table S2, see Supporting Information). PCR products and the promoter trapping vector pFPV25 were both digested with *Eco*RI and *Bam*HI. The resulting fragments were gel purified, ligated together and cloned upstream of the promoterless GFP gene. The final plasmids were designated as pHrpN-GFP for *hrpN* and pHrpA-GFP for *hrpA*, which were confirmed by restriction enzyme digestion and sequencing.

GFP reporter assays by flow cytometry

The BD FACSCanto flow cytometer (BD Bioscience, San Jose, CA, USA) was used to monitor the GFP intensities of bacterial strains containing the corresponding promoter–GFP constructs as described previously (Wang *et al*., 2009). For *hrp*-inducing condition assays, *E. amylovora* strain Ea273, containing GFP–promoter fusion plasmids, was grown overnight in LB medium, and washed twice with phosphate-buffered saline (PBS). The

bacterial suspension was re-inoculated into either HMM or M9 minimal medium amended with different carbon sources at different levels. Bacterial cultures were harvested by centrifugation at different time points, and resuspended in PBS for flow cytometry.

For chemical screening, bacterial strains containing GFP–promoter fusion plasmids were washed with PBS and resuspended in HMM containing 20 mM galactose. One milliliter of cell suspension was seeded into 24-well plates at a density of OD_{600} = 0.2. Compounds were added to each well to yield final concentrations of 10, 25, 50 and 100 μM, and DMSO was added as a control. GFP intensities were measured by flow cytometry after incubation at 18 °C for 18 h.

Flow cytometry was performed on a BD LSRII 10 parameter multilaser analyzer (BD Bioscience). Data were collected for a total of 100 000 events and analysed statistically by gating using flow cytometry software FCS Express V3 (De Novo Software, Los Angeles, CA, USA). A geometric mean was calculated for each sample. Each treatment was performed in triplicate and each experiment was repeated three times.

CPC assays for the determination of amylovoran concentrations

The amylovoran concentration in supernatants of bacterial cultures was determined quantitatively using a turbidity assay with CPC, as described previously (Bellemann *et al*., 1994; Wang *et al*., 2009; Zhao *et al*., 2009b). Briefly, wild-type *E. amylovora* Ea273 was grown overnight in LB broth and washed with PBS three times. After the final wash, the bacterial pellet was resuspended in 200 μL of PBS. A total of 100 μL of bacterial cells was inoculated into 10 mL of MBMA medium with 1% sorbitol. A 3-mL cell suspension was seeded in Falcon tubes. Chemicals were added to the bacterial suspension to a final concentration of 25 μM, and DMSO was added as a control. After incubation for 24 h at 28 °C with shaking, 50 μL of CPC at 50 mg/mL was added to 1 mL of supernatant following centrifugation. After 10 min of incubation at room temperature, the amylovoran concentration was determined by measuring OD_{600} turbidity. The final concentration of amylovoran production was normalized for a cell density of 1.0. Each treatment was performed in triplicate and each experiment was repeated three times.

Crab apple flower assays

Bacterial suspensions were grown overnight in LB broth, harvested by centrifugation and resuspended in PBS at a density of OD_{600} = 0.2. Bacterial suspensions were incubated with compounds at 50 μM or an equivalent volume of DMSO for 4 h at room temperature in the dark. One-day-old fully opened crab apple (*Malus mandshurica*) flowers were detached and immediately transferred to a microcentrifuge tube containing 2 mL of 10% sucrose as described previously (Pusey, 1997). Two microlitres of bacterial suspension were evenly delivered to the stigmas per flower by pipette under a microscope. The inoculated flowers were held in a sealed container that was incubated in a growth chamber at 24 °C and 90% relative humidity. Symptoms were recorded at 6 days post-inoculation. For the bacterial population assays, flowers were homogenized at 0, 2, 4 and 6 days post-inoculation. Populations were estimated by plating serial dilutions and calculating colony-forming units (CFU). Each treatment was performed in triplicate and each experiment was repeated three times.

RNA isolation

WT *E. amylovora* strain Ea273 was grown overnight in LB broth at 28 °C. Bacterial cells were washed and re-inoculated into HMM after washing twice with PBS. Two millilitres of cell suspension were seeded into 12-well tissue culture plates at a density of OD_{600} = 0.3 cells/well. Compounds were added to each well at a final concentration of 50 μM, and an equal volume of DMSO was used as a control. The plates were incubated at 18 °C for 18 h with shaking. The 1.5-mL cultures were stabilized using 3 mL of RNA protect reagent (Qiagen, Hilden, Germany). Cells were harvested by centrifugation for 10 min at 4000 ***g***, and RNA was extracted using a Qiagen Bacterial RNA Protect Mini Kit as recommended by the manufacturer (Qiagen). On-column DNA digestion was performed using Qiagen DNase. RNA was quantified using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies; Wilmington, DE, USA) and RNA quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Microarray hybridization and data analysis

Microarray hybridization and data analysis were carried out as described previously (McNally *et al*., 2012; Wang *et al*., 2012). Briefly, a 60-mer *E. amylovora* microarray (8 × 15 K) was designed at the James Hutton Institute, Aberdeen, UK and synthesized by Agilent Technologies. Each slide contains eight arrays, and each array contains ~15 000 spots, with each probe spotted in triplicate. A detailed description and design of the oligonucleotide microarray is available at the ArrayExpress website (accessions: microarray A-MEXP-2000). All microarray data are available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo, accession number GSE45602). Four biological replicates for each chemical treatment (two samples were combined) were hybridized to three arrays. Four biological replicates (two each combined) for DMSO treatment were hybridized to two arrays. All arrays were cross-compared as technical replicates.

Synthesis and labelling of cDNA were performed using a total of 10 μg of total RNA and a FairPlay III microarray labelling kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. After labelling, the concentration of cDNA was determined by a NanoDrop ND-100 spectrophotometer. The labelled cDNA (600 ng) was then hybridized to the slide for 17 h at 65 °C in an Agilent rotating oven (10 rpm) in the presence of a 2 × hybridization buffer (Agilent Technologies). After successive washing by gene expression wash buffers (Agilent Technologies), the hybridized slide was scanned using an Axon 4000B array scanner at a resolution of 5 μm (Molecular Devices, Sunnyvale, CA, USA). Microarray images were processed by GenePix Pro 6.0 image analysis software (vs. 6.0.1.26, Molecular Devices). Raw data were processed through logarithmic transformation and normalization using R software (R.2.2.1). Statistical comparisons were performed using multiple testing procedures to evaluate the statistical significance of differentially expressed genes. A modified *t*-test was computed to measure the significance associated with each differential expression value. A gene expression value was assumed to be significantly different when the *P* value was less than 0.05 (except otherwise mentioned) and the expression ratio was ≥2.0 or ≤0.5.

qRT-PCR

To validate the microarray results, gene expression levels of *hrpA*, *dspE*, *hrpL*, *hrpN*, *avrRpt2*, *amsG*, *amsD*, *glgB*, *foxR* (*EAM_0358*), *hmuS*, *dfoJ* (*EAM_359*) and *EAM_3350* were determined by qRT-PCR. For each sample, synthesis of cDNA was performed using 1 μg of total RNA and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Primers were designed using Primer3 software with high specificity and are listed in Table S2. qRT-PCR was conducted in an ABI 7300 System (Applied Biosystems, Foster City, CA, USA) using Fast SYBR Green PCR master mix (Applied Biosystems). All reactions were run on 96-well optical reaction plates. Thermal cycling conditions included a step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and a final dissociation curve analysis step from 65 °C to 95 °C. Technical replicate experiments were performed for each biological triplicate sample. The amplification specificity for each reaction was confirmed by dissociation curve analysis. The Ct values determined were then exploited for further analysis. The relative quantification method (ΔΔCt method) was used to determine the expression level of selected genes. The expression of the 16S rRNA gene was used to normalize gene expression across samples. The gene expression values obtained from DMSO-treated samples were used to calculate a relative quantification (RQ) value for each gene (Wang *et al*., 2012).

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SUPPORTING INFORMATION

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

Fig. S1 Promoter activities of type III secretion system (T3SS) genes of *Erwinia amylovora* in M9 and *hrp* (*hypersensitive response and pathogenicity*)-inducing minimal medium (HMM) with different concentrations of carbon sources. (A) Promoter activities of *hrpN*, *dspE*, *hrpL* and *hrpA* in *E. amylovora* strain Ea273 in M9 and HMM containing 10 and 20 mM of galactose at 18 °C for 18 h. (B) Time course of promoter activities of *hrpN*, *dspE*, *hrpL* and *hrpA* in *E. amylovora* strain Ea273 in HMM containing 20 mM galactose at 18 °C.

Fig. S2 Effect of different concentrations of small-molecule inhibitors 3 and 9 on promoter activities of *hrpN*, *dspE*, *hrpL* and *hrpA* genes. *Erwinia amylovora* strain Ea273 was grown in *hrp* (*hypersensitive response and pathogenicity*)-inducing minimal

medium (HMM) at 18 °C for 18 h. The highest volume of dimethylsulphoxide (DMSO) added (equivalent to 100 μM of chemicals) is shown as the control.

Fig. S3 (A) Effect of small molecules 3 and 9 on bacterial growth in *hrp* (*hypersensitive response and pathogenicity*)-inducing minimal medium (HMM) containing 20 mM galactose at 18 °C. (B) Effect of small molecule 3 on bacterial growth on flowers. Flowers were homogenized at 0, 2, 4 and 6 days post-inoculation. The population was measured by plating serial dilutions and calculating colony-forming units (CFU).

Fig. S4 Small-molecule inhibitors delayed the hypersensitive response (HR) in tobacco. Tobacco leaf was infiltrated with *Erwinia amylovora* Ea1189 (OD_{600} = 0.15) in the presence of compounds 8 (left) and 11 (right) at the different concentrations indicated. The same volume of dimethylsulphoxide (DMSO) was added as a control as 100 μM of compounds. Photographs were taken 24 h post infiltration.

Fig. S5 Structures of the small-molecule inhibitors used in this study. Not shown are compounds 6 (see Felise *et al*., 2008; compound #1, TTS29), 7, 8, 15 and 16 (see Pan *et al*., 2009; compounds #2, 1, 3 and 3-diproprionate, respectively).

Table S1 Differentially expressed genes in all comparisons.

Table S2 Promoter cloning and quantitative real-time polymerase chain reaction (qRT-PCR) primers used in this study.