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Derivative of plant phenolic compound inhibits the type III secretion system of *Dickeya dadantii* via HrpX/HrpY two-component signal transduction and Rsm systems

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SUMMARY

The type III secretion system (T3SS) is a major virulence factor in many Gram-negative bacterial pathogens and represents a particularly appealing target for antimicrobial agents. Previous studies have shown that the plant phenolic compound *p*-coumaric acid (PCA) plays a role in the inhibition of T3SS expression of the phytopathogen *Dickeya dadantii* 3937. This study screened a series of derivatives of plant phenolic compounds and identified that *trans*-4-hydroxycinnamohydroxamic acid (TS103) has an eight-fold higher inhibitory potency than PCA on the T3SS of *D. dadantii*. The effect of TS103 on regulatory components of the T3SS was further elucidated. Our results suggest that TS103 inhibits HrpY phosphorylation and leads to reduced levels of *hrpS* and *hrpL* transcripts. In addition, through a reduction in the RNA levels of the regulatory small RNA RsmB, TS103 also inhibits *hrpL* at the post-transcriptional level via the *rsmB*-RsmA regulatory pathway. Finally, TS103 inhibits *hrpL* transcription and mRNA stability, which leads to reduced expression of HrpL regulon genes, such as *hrpA* and *hrpN*. To our knowledge, this is the first inhibitor to affect the T3SS through both the transcriptional and post-transcriptional pathways in the soft-rot phytopathogen *D. dadantii* 3937.

Keywords: plant phenolic compound, Rsm system, T3SS inhibitor, type III secretion system, two-component signal transduction system.

INTRODUCTION

Antibiotic treatment is the most commonly used strategy to control pathogenic infections. However, most antibiotics kill bacteria by inhibiting cellular processes essential for survival, which leads to strong selective pressure to develop resistance against antibiotics (Cegelski *et al*., 2008; Escaich, 2008; Rasko and Sperandio, 2010). In the face of increasing antibiotic resistance, the targeting of bacterial virulence factors rather than bacterial survivability provides a novel alternative approach for the development of new antimicrobials, as virulence-specific therapeutics would offer a reduced selection pressure for antibiotic-resistant mutations (Escaich, 2008; Rasko and Sperandio, 2010). The type III secretion system (T3SS) represents a particularly appealing target for antimicrobial agents because it is a major virulence factor in many Gram-negative plant and animal pathogens (Cornelis, 2006; Tang *et al*., 2006; Waterman and Holden, 2003; Yang *et al*., 2002). The T3SS in phytobacteria, also known as the hypersensitive response and pathogenicity (Hrp) system, is a syringe needle-like structure which is responsible for the secretion and translocation of effector proteins into the host cells, where the effector proteins subvert or inhibit the host cell's defences or facilitate pathogenicity (Alfano and Collmer, 1997; Galán and Collmer, 1999; Ghosh, 2004; Grant *et al*., 2006; Hueck, 1998; Yang *et al*., 2005).

Dickeya dadantii 3937 (formerly named *Erwinia chrysanthemi*), a member of the Enterobacteriaceae family, is a Gram-negative pathogen which causes soft rot, wilt and blight diseases on a wide range of plant species (Bauer *et al*., 1994). D. dadantii possesses a T3SS, which is encoded by the *hrp* gene cluster and thought to be coordinately regulated by various host and environmental factors (Nasser *et al*., 2005; Yang *et al*., 2002). Similar to many phytopathogens, the expression of the T3SS of *D. dadantii* 3937 is repressed in nutrient-rich media, but induced in the plant apoplast or in nutrient-deficient inducing medium, which is considered to mimic plant apoplastic conditions (Galán and Collmer, 1999; Tang *et al*., 2006). The well-studied T3SS of *D. dadantii* is regulated by the master regulator HrpL, which is a member of the extracytoplasmic function (ECF) family of alternative sigma factors that up-regulate many *hrp* genes downstream of the T3SS regulatory cascade, such as *hrpA* (encoding a structural protein of the T3SS pilus), *dspE* (encoding a T3SS effector) and *hrpN* (encoding a

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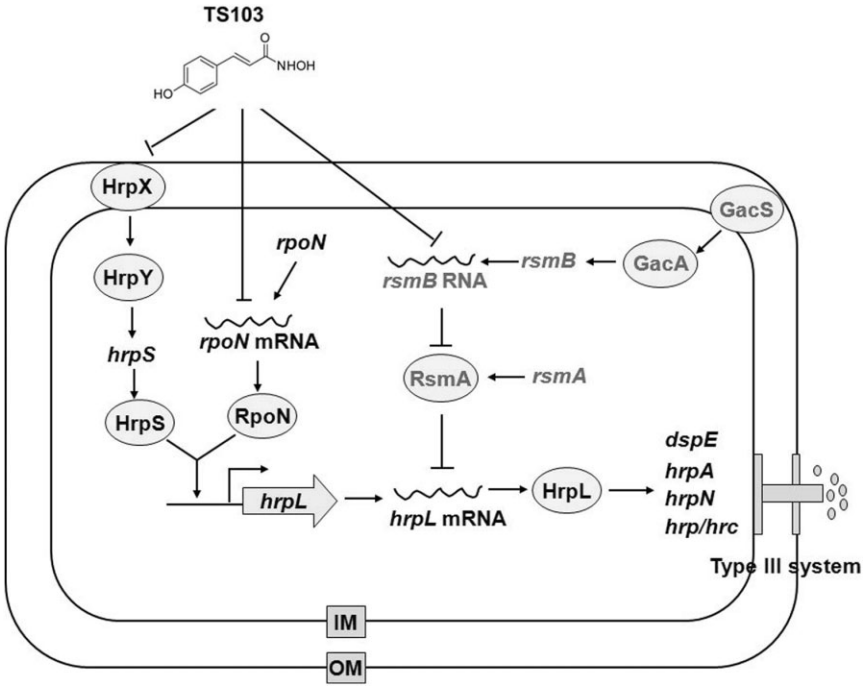

Fig. 1 Regulatory network controlling the *Dickeya dadantii* type III secretion system (T3SS). The *D. dadantii* T3SS is regulated by the HrpX/HrpY-HrpS-HrpL and GacS/GacA-*rsmB*-RsmA-HrpL regulatory pathways. The two-component signal transduction system HrpX/HrpY activates *hrpS*, which encodes a σ^{54} enhancer. HrpS is required for the expression of the alternative sigma factor, *hrpL*. HrpL activates the expression of genes encoding the T3SS apparatus and its secreted substrates. RsmA is a small RNA-binding protein that acts by decreasing the half-life of *hrpL* mRNA. GacS/GacA up-regulates the expression of *rsmB*, which increases the mRNA level of *hrpL* by sequestering RsmA. From this study, we observed that TS103 altered the *hrpA* promoter activity through both the HrpX/HrpY-HrpS-HrpL and *rsmB*-RsmA-HrpL pathways. TS103 inhibits *hrpL* at the post-transcriptional level through a decrease in expression of *rsmB*. In addition, TS103 inhibits *hrpS* transcription through suppression of phosphorylation of HrpY and also post-transcriptionally inhibits *rpoN*. ⊥, negative control; →, positive control. IM: Inner Membrane; OM: Outer Membrane.

harpin protein) (Fig. 1) (Chatterjee *et al*., 2002; Wei *et al*., 1992; Yang *et al*., 2010; Yap *et al*., 2005). The expression of *hrpL* is regulated at both the transcriptional and post-transcriptional levels. HrpX/HrpY-HrpS regulates *hrpL* at the transcriptional level. A two-component signal transduction system (TCSTS) HrpX/HrpY, encoded by genes in the centre of the *hrp* gene cluster, positively regulates *hrpS*, which encodes a σ^{54} enhancer-binding protein (Fig. 1) (Tang *et al*., 2006). HrpS interacts with the σ^{54} (RpoN)-containing RNA polymerase holoenzyme and initiates the transcription of *hrpL* (Fig. 1) (Chatterjee *et al*., 2002; Yap *et al*., 2005). The regulator of secondary metabolites (Rsm) RsmA–RsmB pair regulates *hrpL* at the post-transcriptional level. RsmA, a small RNA-binding protein, promotes *hrpL* mRNA degradation (Fig. 1) (Chatterjee *et al*., 2002; Cui *et al*., 1995). RsmB, an untranslated regulatory small RNA, binds to the RsmA protein and neutralizes the activity of RsmA on *hrpL* mRNA degradation by forming an inactive ribonucleoprotein complex with RsmA (Chatterjee *et al*., 2002; Liu *et al*., 1998) (Fig. 1). The global regulatory TCSTS, GacS/GacA, up-regulates the transcription of the regulatory small RNA RsmB (Tang *et al*., 2006). In addition to GacS/GacA, other regulators have been identified that control the expression of *rsmB* in the soft-rot pathogens *Pectobacterium* and *Dickeya*. KdgR, an IcII-like protein, has been reported to negatively control the transcription of *rsmB* by binding within the transcribed region of the *rsmB* gene in *P. carotovorum* (Miller *et al*., 2000). Polynucleotide phosphorylase (PNPase) has been reported to decrease the amount of functional *rsmB* transcripts in *D. dadantii* (Zeng *et al*., 2010).

In response to microbial attack, plants activate defence responses which lead to the induction of a broad spectrum of antimicrobial defences (Montesano *et al*., 2005; Van Loon, 2000). These induced defences are regulated by a network of interconnecting signal transduction pathways and eventually lead to the production of defence molecules, such as phenylpropanoids (Dixon and Paiva, 1995; Feys and Parker, 2000; Hahlbrock and Scheel, 1989). Phenylpropanoids are a group of secondary metabolites produced by plants from L-phenylalanine. Our previous reports have shown that the plant phenolic compound *p*-coumaric acid (PCA), an intermediate in phenylpropanoid biosynthesis, plays a role in the inhibition of T3SS expression of *D. dadantii* 3937 (Li *et al*., 2009). With the aid of structure–activity relationship (SAR) studies, the *para* positioning of the hydroxyl group in the phenyl ring and the double bond in PCA have been predicted previously to be essential for its inhibitory activity

(Li *et al*., 2009). As the regulatory mechanism of the T3SS of *D. dadantii* 3937 is well understood, to develop T3SS inhibitors which are more potent than PCA, a series of derivatives of plant phenolic compounds were screened using *D. dadantii* 3937 as a model organism. One derivative, TS103, which showed an eight-fold higher potency in the inhibition of the T3SS vs. PCA, was selected and the regulators responsible for the inhibition of T3SS gene expression by TS103 were further elucidated. Our results showed that TS103 inhibits the T3SS through both the HrpX/HrpY TCSTS and Rsm systems.

RESULTS

Screening for highly potent T3SS inhibitors of *D. dadantii*

In our previous work, the phenolic acid PCA was found to inhibit T3SS gene expression of *D. dadantii* 3937 at a concentration of 100 μM (Li *et al*., 2009). To identify T3SS inhibitors which have higher potency in the inhibition of T3SS expression, 50 derivatives of plant phenolic compounds (Fig. 2) at a concentration of 100 μM were first screened by monitoring the promoter activity of *hrpA*. The *hrpA* gene encodes the T3SS pilus, which is required for the translocation of effector proteins into plant cells and is located downstream in the T3SS regulatory pathway (Fig. 1). A reporter plasmid, pPhrpA, which carries a *hrpA-gfp* transcriptional fusion, was used to measure the effects of the derivatives of plant phenolic compounds on *hrpA* expression (Table 1). The wild-type cells containing pPhrpA were grown in T3SS-inducing medium (MM) supplemented with each of the compounds at a concentration of 100 μM. Green fluorescent protein (GFP) intensity, which is a measurement of *hrpA* promoter activity, was assayed by flow cytometry. Among the derivatives of the plant phenolic compounds screened, 13 compounds at a concentration of 100 μM showed strong inhibition of T3SS gene expression of *D. dadantii*, in which the level of *hrpA* promoter activity was reduced by more than 50% of the level in MM at both 12 and 24 h of growth after repeated measurements (Table 2).

To identify inhibitors that exhibit higher efficacy of T3SS inhibition, the *hrpA* expression of *D. dadantii* cells grown in MM supplemented with inhibitors at a concentration of 10 μM instead of 100 μM was further examined. PCA was used as a reference, in which *hrpA* expression was inhibited at a concentration of 100 μM, but not at 10 μM (Table 2 and Fig. S1, see Supporting Information)

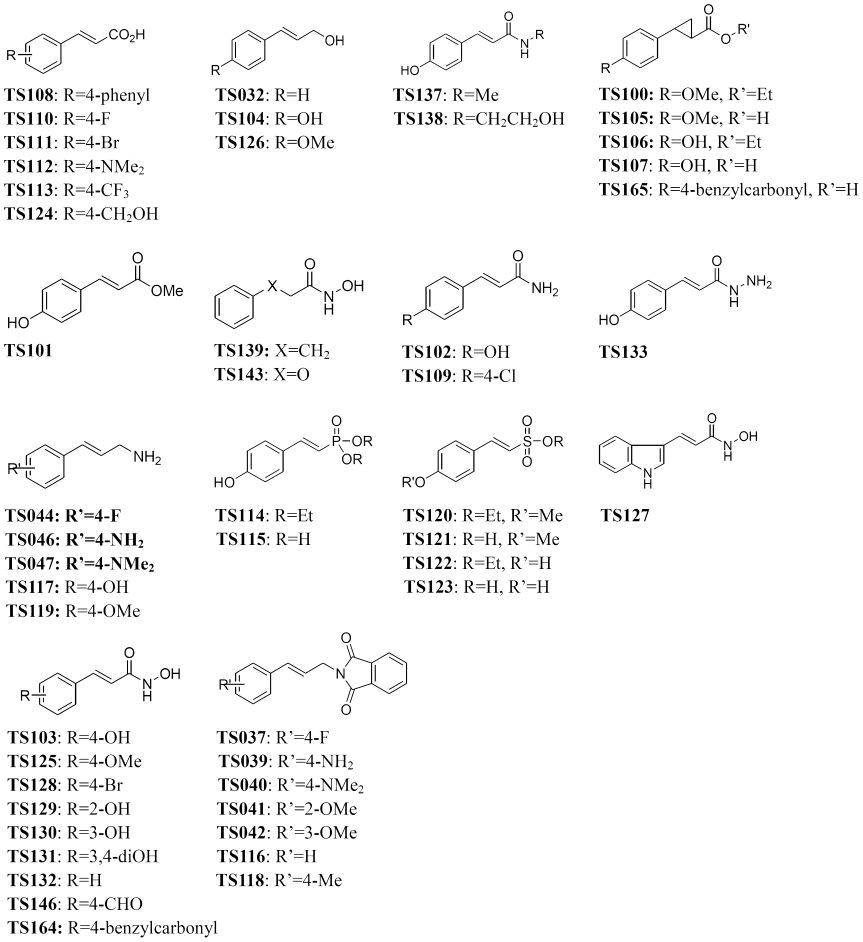

Fig. 2 Chemical structures of derivatives of plant phenolic compounds.

Table 1 Strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics	Reference or source
Strains		
Escherichia coli		
E. coli S17-1 λ-*pir*	λ-*pir* lysogen of S17-1; Sp^R	Sanchez-Romero *et al*. (1998)
Dickeya dadantii		
3937	Wild-type, *Saintpaulia* (African violet) isolate	Hugouvieux-Cotte-Pattat, N. (Microbiologie Adaptation et Pathogenie CNRS, INSA de Lyon, Universite de Lyon, Lyon F-69622, France)
Δ*hrpY*	*hrpY* deletion mutant; Km^R	This study
Δ*hrpY*D57A	3937 derivative in which the conserved aspartate residue at position 57 in HrpY was changed by nonconservative substitution to alanine; Km^R	This study
Δ*gacA*	*gacA* deletion mutant; Km^R	This study
Δ*gacA*::*gacA*	Δ*gacA* with chromosomal insertion of *lacY-gacA-cm-prt*; Km^R, Cm^R	This study
Δ*pnp*	*pnp* deletion mutant; Km^R	Zeng *et al*. (2010)
Δ*kdgR*	*kdgR* deletion mutant; Km^R	This study
3937::OpgG-His_6	3937 with a 6 × His epitope sequence tagged to the C-terminus of OpgG	Laboratory stock
Plasmids		
pAT	pProbe-AT, promoter-probe vector; Ap^R	Miller *et al*. (2000)
pPhrpA	pAT derivative with PCR fragment containing *hrpA* promoter region; Ap^R	Yang *et al*. (2008a)
pPhrpN	pAT derivative with PCR fragment containing *hrpN* promoter region; Ap^R	Yang *et al*. (2007)
pPhrpL	pAT derivative with PCR fragment containing *hrpL* promoter region; Ap^R	Yang *et al*. (2007)
pPhrpS	pAT derivative with PCR fragment containing *hrpS* promoter region; Ap^R	Li *et al*. (2009)
pPrpoN	pAT derivative with PCR fragment containing *rpoN* promoter region; Ap^R	Yi *et al*. (2010)
pPrsmB	pAT derivative with PCR fragment containing *rsmB* promoter region; Ap^R	This study
pWM91	Sucrose-based counter-selectable plasmid; Ap^R	Metcalf *et al*. (1996)
pKD4	Template plasmid for kanamycin cassette; Km^R	Datsenko and Wanner (2000)
pGEM-T Easy	Cloning vector; Ap^R	Promega (Madison, WI, USA)
phrpY	pGEM-T Easy derivative with PCR fragment containing the *hrpY* ORF and its flanking regions; Ap^R	This study
phrpYD57A	phrpY derivative in which the conserved aspartate residue at position 57 in HrpY was changed by nonconservative substitution to alanine; Ap^R	This study
pTCLSCm	6.4-kb lacY-cm-prt region cloned in pGEM-T Easy; Cm^R	Yap *et al*. (2008)
pML123	Broad-host-range cloning vector; Gm^R	Labes *et al*. (1990)
pMLkdgR	Derivative of pML123 carrying *kdgR*; Gm^R	This study
pCL1920	Expression vector; Sp^R	Lerner and Inouye (1990)
pCLhrpXY	Derivative of pCL1920 carrying *hrpXY* operon; Sp^R	This study

Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Km^R, kanamycin resistance; Gm^R, gentamicin resistance; ORF, open reading frame; Sp^R, spectinomycin resistance.

(Li *et al*., 2009). Our results showed that the level of *hrpA* expression of *D. dadantii* 3937 cells grown in MM supplemented with three of the inhibitors (TS103, TS126 and TS131) at a concentration of 10 μM was less than 50% of the level in MM at 12 h of growth (Fig. S1). Among these three compounds, the addition of TS103 at a concentration of 10 μM resulted in the greatest reduction in *hrpA* expression at 12 h of bacterial growth, in which the level of *hrpA* expression of *D. dadantii* cells grown in MM supplemented with TS103 was less than 25% of the level in MM (Fig. S1). The addition of all the selected inhibitors at a concentration of 1 μM did not result in a significant reduction in *hrpA* promoter activity at 12 h of bacterial growth (data not shown). In addition, growth inhibition was not observed in TS103 at the tested concentrations (Figs S2 and S3, see Supporting Information). These results suggest that TS103 has the highest potency of T3SS inhibition among all the derivatives of plant phenolic compounds screened in this study. To further compare the inhibitory efficacy on the T3SS between TS103 and PCA, we tested the half-maximal inhibitory concentration (IC_{50}) of these two compounds on T3SS expression. Here, IC_{50} is defined as the concentration of compound that is required for the inhibition of 50% of the *hrpA* promoter activity compared with MM. The results showed that the IC_{50} of TS103 was 2.2 μM, which is one-eighth of that of PCA (Fig. 3).

TS103 inhibits the transcription and production of T3SS structural- and harpin-encoding genes

To confirm the inhibitory effect of TS103 on the T3SS of *D. dadantii*, the promoter activities and mRNA levels of two representative *hrp* genes, *hrpA* and *hrpN*, were examined in the presence and absence of TS103. Similar to the results obtained from the screening above (Table 2), a lower *hrpA* promoter activity was observed in MM supplemented with TS103 compared with that in MM alone (Table 3). Considerably lower promoter activity of *hrpN* was observed in the cells grown in MM supplemented with TS103 in comparison with that in MM (Table 3). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis revealed a significant decrease in *hrpA* (relative expression ratio

Table 2 The *hrpA* expression of *Dickeya dadantii* 3937 in type III secretion system-inducing medium (MM) and MM supplemented with different derivatives of plant phenolic compounds.

	12 h		24 h	
Phenolic compound†	Ave MFI ± SD‡	%MM§	Ave MFI ± SD	%MM
MM	35.4 ± 0.8		90.6 ± 11.7	
TS100, ethyl *trans*-2-(4-methoxyphenyl)-1-cyclopropanecarboxylate	45.3 ± 3.4*	128.2	119.1 ± 22.1	131.5
TS101, methyl *para*-coumarate	17.5 ± 0.7*	49.6	19.9 ± 0.5*	22.0
TS102, *trans*-4-hydroxycinnamide	21.1 ± 1.2*	59.7	60.8 ± 10.6	67.1
TS103, *trans*-4-hydroxycinnamohydroxamic acid	8.7 ± 0.3*	24.7	11.9 ± 0.8*	13.2
TS104, *para*-coumaryl alcohol	3.0 ± 0.1*	8.6	12.9 ± 1.8*	14.2
TS105, *trans*-2-(4-methoxyphenyl)-1-cyclopropanecarboxylic acid	22.9 ± 2.6*	64.8	82.4 ± 5.8	90.9
TS106, ethyl *trans*-2-(4-hydroxyphenyl)-1-cyclopropanecarboxylate	10.5 ± 0.8*	29.8	49.4 ± 4.1*	54.5
TS107, *trans*-2-(4-hydroxyphenyl)-1-cyclopropanecarboxylic acid	45.9 ± 2.7*	129.9	122.1 ± 17.9	134.7
TS108, *trans*-4-phenylcinnamic acid	31.6 ± 2.5	89.3	109.0 ± 19.8	120.3
TS109, *trans*-4-chlorocinnamide	35.7 ± 6.3	100.8	81.5 ± 3.8	89.9
TS110, *trans*-4-fluorocinnamic acid	63.4 ± 8.0*	179.4	183.8 ± 19.2*	202.8
TS111, *trans*-4-bromocinnamic acid	23.9 ± 5.2	67.7	54.7 ± 7.2	60.3
TS112, *trans*-4-dimethylaminocinnamic acid	23.8 ± 4.3	67.4	60.7 ± 6.4	67.0
TS113, *trans*-4-trifluoromethylcinnamic acid	24.4 ± 0.9*	69.1	68.5 ± 5.7	75.6
MM	51.4 ± 6.7		77.1 ± 9.1	
TS114, diethyl *trans*-2-(4-hydroxyphenyl)-vinylphosphonate	55.4 ± 1.0	107.8	76.5 ± 5.6	99.2
TS115, *trans*-2-(4-hydroxyphenyl)-vinylphosphonic acid	57.9 ± 1.6	112.7	79.6 ± 3.3	103.2
TS116, *N*-(*para*-coumaryl)phthalimide	24.3 ± 1.5*	47.2	39.3 ± 3.5*	51.0
TS117, *para*-coumarylamine	49.8 ± 1.6	96.9	73.1 ± 2.0	94.9
TS118, *N*-(4-methoxycinnamyl)phthalimide	59.0 ± 0.4	114.7	83.8 ± 1.7	108.8
TS119, *trans*-4-methoxycinnamylamine	47.8 ± 3.2	93.0	68.2 ± 10.4	88.5
TS120, ethyl *trans*-2-(4-methoxyphenyl)-ethenylsulphonate	49.9 ± 2.6	97.0	68.6 ± 6.0	89.1
TS121, *trans*-2-(4-methoxyphenyl)ethenylsulphonic acid tetra(*n*-butyl)ammonium salt	57.1 ± 10.0	111.1	72.2 ± 10.6	93.7
TS122, ethyl *trans*-2-(4-hydroxyphenyl)-ethenylsulphonate	38.6 ± 1.8	75.1	60.9 ± 0.8	79.0
TS123, *trans*-2-(4-hydroxyphenyl)ethenylsulphonic acid tetra(*n*-butyl)ammonium salt	52.3 ± 2.0	101.6	77.7 ± 6.8	100.8
TS124, *trans*-4-hydroxymethylcinnamic acid	44.9 ± 3.5	87.2	78.1 ± 3.4	101.3
TS125, *trans*-4-methoxycinnamohydroxamic acid	17.4 ± 0.9*	33.8	25.6 ± 2.0*	33.2
TS126, *trans*-4-methoxycinnamyl alcohol	7.8 ± 0.2*	15.2	13.4 ± 0.3*	17.4
TS127, *trans*-3-indoleacrylohydroxamic acid	21.0 ± 3.0*	40.8	35.1 ± 3.0*	45.6
MM	30.1 ± 6.9		74.0 ± 7.9	
TS128, *trans*-4-bromocinnamohydroxamic acid	7.5 ± 0.7*	25.0	10.9 ± 4.4*	14.7
TS129, *trans*-2-hydroxycinnamohydroxamic acid	12.0 ± 0.4	39.8	20.2 ± 1.3*	27.3
TS130, *trans*-3-hydroxycinnamohydroxamic acid	28.7 ± 2.5	95.6	41.0 ± 1.1*	55.4
TS131, *trans*-3,4-dihydroxycinnamohydroxamic acid	6.1 ± 0.8*	20.2	16.4 ± 0.6*	22.2
MM	37.7 ± 2.6		56.9 ± 1.4	
TS132, *trans*-cinnamohydroxamic acid	12.3 ± 0.2*	32.7	44.2 ± 2.9*	77.7
TS133, *trans*-3-(4-hydroxyphenyl)acrylohydrazide	7.2 ± 0.9*	19.2	17.1 ± 2.5*	30.1
MM	78.7 ± 6.3		92.1 ± 17.1	
TS032, cinnamyl alcohol	27.8 ± 5.0*	35.3	53.8 ± 2.9*	58.4
MM	57.9 ± 1.7		165.4 ± 4.5	
TS037, *N*-(4-fluorocinnamyl)phthalimide	51.6 ± 3.2	89.1	130.3 ± 1.3*	78.8
TS039, *N*-(4-aminocinnamyl)phthalimide	70.8 ± 2.1*	122.4	138.9 ± 1.0*	84.0
TS040, *N*-(4-dimethylaminocinnamyl)phthalimide	49.6 ± 3.2	85.7	136.1 ± 5.4*	82.3
TS041, *N*-(2-methoxycinnamyl)phthalimide	47.6 ± 1.4*	82.2	120.6 ± 4.8*	72.9
TS042, *N*-(3-methoxycinnamyl)phthalimide	64.4 ± 5.8	111.3	146.8 ± 6.1	88.7
TS138, *N*-(2-hydroxyethyl)-4-hydroxycinnamamide	36.4 ± 3.7*	63.0	148.1 ± 10.0	89.6
TS139, 3-phenylpropionohydroxamic acid	47.9 ± 1.3*	82.8	64.7 ± 2.1*	39.1
TS146, *trans*-4-formylcinnamohydroxamic acid	50.0 ± 2.4*	86.4	93.7 ± 5.7*	56.6
MM	51.5 ± 10.9		149.2 ± 22.2	
TS044, *trans*-4-fluorocinnamylamine	43.5 ± 0.9	84.6	118.3 ± 19.1	79.3
TS046, *trans*-4-aminocinnamylamine	45.9 ± 3.9	89.3	109.6 ± 19.8	73.4
TS047, *trans*-4-dimethylaminocinnamylamine	20.9 ± 1.8*	40.5	36.2 ± 6.5*	24.2
TS137, *N*-methyl-4-hydroxycinnamamide	47.4 ± 2.5	92.1	140.6 ± 6.9	94.2
TS143, 2-phenoxyacetohydroxamic acid	23.4 ± 1.0	45.4	74.0 ± 5.6*	49.6
TS164, *trans*-4-(benzylcarbonyl)cinnamic acid	101.9 ± 7.9*	198.1	198.9 ± 1.8	133.3
TS165, *trans*-2-(4'-benzylcarbonyl)phenylcyclopropane-1-carboxylic acid	19.4 ± 0.2*	37.6	45.6 ± 1.6*	30.6
p-Coumaric acid	15.9 ± 1.2*	30.9	22.9 ± 1.8*	15.4

*Statistically significant differences in green fluorescent protein (GFP) mean fluorescence intensity (MFI) between bacterial cells grown in MM and MM supplemented with the different compounds ($P < 0.01$, Student's *t*-test).

†MM was supplemented with 100 μM of the indicated compounds. The compounds were assayed at two different times, with MM supplemented with dimethylsulphoxide (DMSO) as the control treatment (indicated by 'MM') for each set of experiments. The compound numbers are as used in Fig. 2.

‡*Dickeya dadantii* 3937 cells carrying the GFP reporter pPhrpA were used in this study. The promoter activities at 12 and 24 h of bacterial growth were determined. GFP MFI was determined for gated populations of bacterial cells by flow cytometry. Values are representative of two independent experiments, and three replicates were used for each experiment.

§The relative promoter activity of *hrpA* in *D. dadantii* 3937 cells grown in MM supplemented with 100 μM of the indicated compounds compared with that in MM (indicated by '%MM') was calculated by the formula: %MM = 100 × MFI(compound)/MFI(MM).

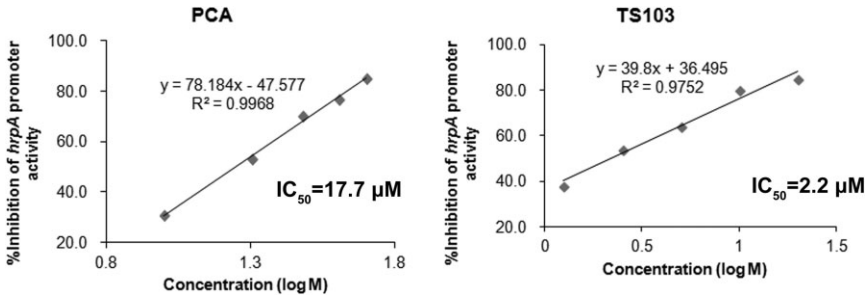

Fig. 3 Effectiveness of TS103 and *p*-coumaric acid (PCA) to inhibit *hrpA* promoter activity. *hrpA* promoter activity of *Dickeya dadantii* was determined in the presence of TS103 or PCA at the respective concentrations. The IC_{50} of these compounds represents the inhibition of 50% of the promoter activity of *hrpA* compared with the dimethylsulphoxide (DMSO) control. The data are representative of two independent experiments. Three replicates were used in each experiment.

Table 3 The expression of type III secretion system (T3SS) genes *hrpA*, *hrpN*, *hrpS*, *hrpL* and *rpoN* of *Dickeya dadantii* 3937 (3937) in T3SS-inducing medium (MM) and MM supplemented with TS103 (MM103).

	Average MFI ± SD for growth in the indicated medium†					
	12 h			24 h		
Strain	MM	MM103	%MM§	MM	MM103	%MM§
3937 (pPhrpA)	58.7 ± 6.1	8.9 ± 0.7*	15.2	66.5 ± 5.4	8.9 ± 0.2*	13.4
3937 (pPhrpN)	46.8 ± 2.9	5.8 ± 0.7*	12.4	49.9 ± 2.1	5.4 ± 0.3*	10.8
3937 (pPhrpS)	73.2 ± 0.6	27.7 ± 1.5*	37.8	90.9 ± 1.2	26.4 ± 0.6*	29.0
3937 (pPhrpL)	20.2 ± 1.8	7.6 ± 0.2*	37.6	21.5 ± 0.5	7.7 ± 0.2*	35.8
3937 (pPrpoN)	215.3 ± 26.1	175.8 ± 15.5		—‡	—	
3937 (pAT)	4.2 ± 0.5	3.0 ± 0.6		5.7 ± 1.8	4.3 ± 0.6	

*Statistically significant differences in green fluorescent protein (GFP) intensity between bacterial cells grown in MM (MM) and MM supplemented with 100 µM TS103 (MM103) ($P < 0.01$, Student's *t*-test).

†The promoter activities were compared in MM and MM supplemented with 100 µM TS103 at 12 and 24 h of bacterial growth. GFP mean fluorescence intensity (MFI) was determined for gated populations of bacterial cells by flow cytometry. Values are representative of two independent experiments, and three replicates were used for each experiment.

‡—, not determined.

§The relative promoter activity of *hrp* genes in *D. dadantii* 3937 cells grown in MM supplemented with 100 µM TS103 compared with that in MM (indicated by '%MM') was calculated by the formula: %MM = 100 × MFI(MM103)/MFI(MM).

0.221, $P = 0.049$) and *hrpN* (relative expression ratio 0.276, $P = 0.049$) mRNA levels in the cells grown in MM supplemented with TS103 compared with that in MM (Fig. 4). HrpN protein production was further analysed by Western blot in the presence of TS103. Compared with that in MM, less HrpN was detected in protein extracts from *D. dadantii* 3937 grown in MM supplemented with TS103 (Fig. 5). These results demonstrate that TS103 inhibits *hrpA* and *hrpN* expression and HrpN protein production.

TS103 inhibits *hrpL* transcription through both HrpS and RpoN

In *D. dadantii*, HrpL is a master regulator that controls the expression of genes encoding T3SS -associated filamentous structure and harpin proteins (Chatterjee *et al*., 2002; Wei *et al*., 1992; Yang *et al*., 2010; Yap *et al*., 2005). We hypothesize that TS103 lowers the level of *hrpL* transcription, which further leads to a decrease in the expression of *hrpA* and *hrpN*. To test this, the promoter activity of *hrpL* was investigated in cells in the presence and absence of TS103. About a three-fold decrease in *hrpL* promoter activity was observed in the cells grown in MM supplemented with TS103 compared with that in MM (Table 3), suggesting that TS103 inhibits *hrpL* at the transcriptional level. Previous reports have shown that HrpS, a σ^{54} enhancer-binding protein, interacts with the σ^{54} (RpoN)-containing RNA polymerase holoenzyme and initiates the transcription of *hrpL* (Chatterjee *et al*., 2002; Yap *et al*., 2005). As TS103 inhibits *hrpL* promoter activity, expression levels of *hrpS* and *rpoN* were examined in cells grown in MM supplemented with TS103 and in MM. Interestingly, a reduction in *hrpS* promoter activity was observed in the cells grown in MM supplemented with TS103 compared with that in MM, whereas *rpoN* transcriptional levels were similar (Table 3). qRT-PCR analysis revealed a significant decrease in *hrpS* (relative expression ratio 0.178, $P = 0.049$) and *rpoN* (relative expression ratio 0.265, $P = 0.046$) mRNA levels in *D. dadantii* 3937 grown in MM amended with TS103 in comparison with that in MM (Fig. 4). These results suggest that TS103 inhibits *hrpL* transcription through both *hrpS* and *rpoN*.

TS103 inhibits *hrpL* at the post-transcriptional level through *rsmB*

In addition to the regulation at the transcriptional level through HrpS and RpoN, *hrpL* is also regulated post-transcriptionally by the RsmA–RsmB pair (Chatterjee *et al*., 2002; Liu *et al*., 1998). To

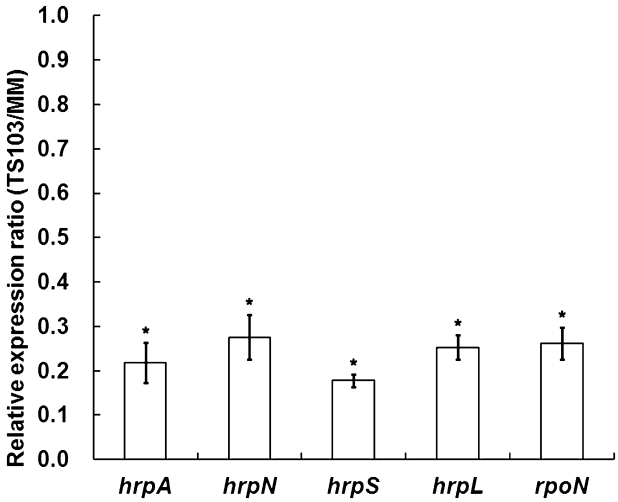

Fig. 4 Relative mRNA levels of *hrpA*, *hrpN*, *hrpS*, *hrpL* and *rpoN* as determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Relative mRNA levels of *hrpA*, *hrpN*, *hrpS*, *hrpL* and *rpoN* genes of *Dickeya dadantii* 3937 in type III secretion system-inducing medium (MM) supplemented with 100 μM TS103 (TS103) compared with that in MM (MM). Asterisks indicate statistically significant differences in mRNA level of cells grown in MM supplemented with 100 μM TS103 compared with that in MM. *rplU* was used as an endogenous control for data analysis (Pfaffl *et al*., 2002). The data are representative of two independent experiments. Three replicates were used in each experiment.

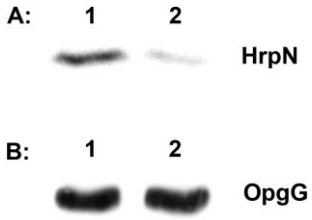

Fig. 5 HrpN (A) and OpgG (B) protein expression of *Dickeya dadantii* 3937 in type III secretion system-inducing medium (MM) and MM supplemented with TS103. Lane 1, *D. dadantii* 3937 grown in MM; lane 2, *D. dadantii* 3937 grown in MM supplemented with 100 μM TS103.

determine whether TS103 inhibits the expression of *rsmA* and *rsmB*, a Northern blot was performed to examine the RNA levels of *rsmA* and *rsmB* in *D. dadantii* 3937 grown in MM with and without TS103. Similar levels of *rsmA* mRNA were observed in cells grown in MM with and without TS103 (Fig. 6A). However, significantly lower *rsmB* RNA levels were observed in cells grown in MM supplemented with TS103 in comparison with that in MM (Fig. 6B). These results indicate that TS103 inhibits *hrpL* at the post-transcriptional level through *rsmB*.

Given that *hrpS*, *rpoN* and *rsmB* RNA levels were reduced in cells grown in MM supplemented with TS103, the *hrpL* mRNA level was further examined in cells grown in MM supplemented with TS103. qRT-PCR analysis was performed and our data revealed that a significant reduction in the *hrpL* mRNA level (relative expression ratio 0.254, $P < 0.05$) was observed in cells grown in MM supplemented with TS103 compared with that in MM (Fig. 4). Together, these results suggest that TS103 inhibits *hrpL* through both the HrpS-RpoN and *rsmB*-RsmA pathways, and consequently lowers the expression of HrpL regulon genes, such as *hrpA* and *hrpN*.

TS103 inhibits *hrpS* transcription through phosphorylation of HrpY

The phosphorylation of HrpY is mandatory for the transcriptional activation of *hrpS* in *D. dadantii* and other phytopathogens (Nizan-Koren *et al*., 2003; Yap *et al*., 2008). The aspartate residue at position 57 (D57) in HrpY has been proven to be the phosphorylation site needed for its activity in *Pantoea stewartii* ssp. *stewartii* and *Erwinia herbicola* pv. *gypsophilae* (Merighi *et al*., 2006; Nizan-Koren *et al*., 2003). We hypothesize that the inhibition of HrpY phosphorylation by TS103 leads to low levels of *hrpS* transcript of *D. dadantii* 3937. To test this, Δ*hrpY*D57A was constructed by site-directed mutagenesis, in which the conserved D57 in HrpY was changed by nonconservative substitution to alanine (D57A), and *hrpS* promoter activity was assayed in both the wild-type and Δ*hrpY*D57A strains. A lower level of *hrpS* promoter activity was observed in Δ*hrpY*D57A in comparison with that in the wild-type strain (Fig. 7). Moreover, similar levels of *hrpS* promoter activity were observed in Δ*hrpY*D57A and Δ*hrpY* (Fig. 7). As HrpX/HrpY is the TCSTS that activates the expression of *hrpS* (Tang *et al*., 2006), *hrpS* promoter activity was also determined in Δ*hrpX*. As expected, in Δ*hrpX*, the transcription of *hrpS* was reduced in comparison with that in the wild-type strain (Fig. 7). Furthermore, the promoter activity of *hrpS* was restored to the wild-type level when Δ*hrpY*D57A, Δ*hrpY* and Δ*hrpX* were complemented with pCLhrpXY *in trans* (Fig. 7). These results indicate that D57 of HrpY is the phosphorylation site required for the activity of HrpY in *D. dadantii* 3937. To further investigate whether TS103 inhibits *hrpS* transcription through its influence on the phosphorylation of HrpY, the promoter activity of *hrpS* was measured in Δ*hrpY*D57A grown in MM and MM supplemented with TS103. Similar levels of *hrpS* promoter activity were observed in Δ*hrpY*D57A grown in MM and MM supplemented with 100 μM TS103 (Table 4). Furthermore, similar levels of *hrpS* promoter activity were observed among Δ*hrpY*D57A, Δ*hrpX* and the wild-type strain when they were grown in MM supplemented with 100 μM TS103 (Table 4). These results indicate that TS103 inhibits *hrpS* expression through its influence on the phosphorylation of HrpY.

GacS/GacA is another TCSTS that regulates T3SS through the Rsm system (Tang *et al*., 2006), but has no regulatory effect on HrpS as no significant difference in *hrpS* promoter activity was observed between Δ*gacA* and its parental strain when they were grown in MM (Table 4). To demonstrate that TS103-inhibited *hrpS* expression is HrpY specific, we compared the *hrpS* promoter activities in Δ*gacA*

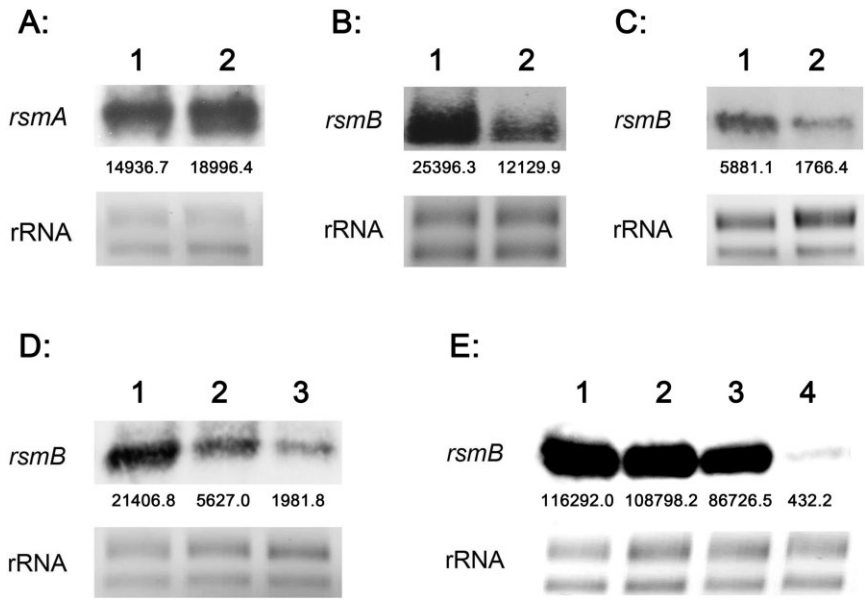

Fig. 6 The relative RNA levels of *rsmA* and *rsmB* as determined by Northern blot. Cells were cultured in type III secretion system-inducing medium (MM) or MM supplemented with 100 μM TS103 for 12 h before RNA isolation. rRNA was used as an internal control. (A) Lane 1, 3937 grown in MM; lane 2, 3937 grown in MM supplemented with TS103. (B) Lane 1, 3937 grown in MM; lane 2, 3937 grown in MM supplemented with TS103. (C) Lane 1, Δ*pnp* grown in MM; lane 2, Δ*pnp* grown in MM supplemented with TS103. (D) Lane 1, Δ*csrD* grown in MM; lane 2, Δ*csrD* grown in MM supplemented with TS103; lane 3, 3937 grown in MM. (E) Lane 1, Δ*kdgR* (pMLkdgR) grown in MM; lane 2, 3937 (pML123) grown in MM; lane 3, Δ*kdgR* (pML123) grown in MM; lane 4, Δ*kdgR* (pML123) grown in MM supplemented with TS103. Numbers below the Northern blots indicate the relative intensity of *rsmA*/*rsmB* RNA provided by ImageJ.

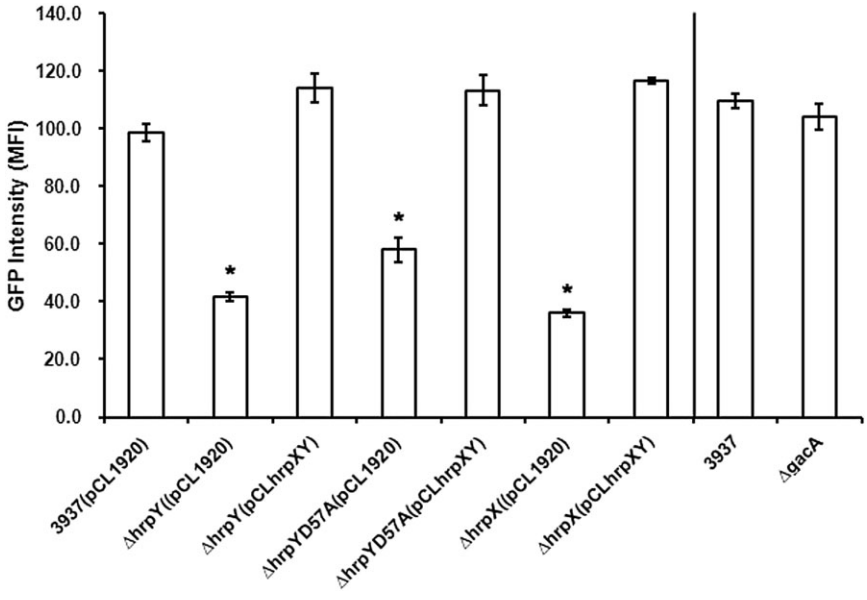

Fig. 7 The *hrpS* promoter activity of *Dickeya dadantii* derivatives in type III secretion system-inducing medium (MM) at 12 h of growth. Strains carrying the green fluorescent protein (GFP) reporter pPhrpS were used in this study. The promoter activities at 12 h of bacterial growth were determined. GFP mean fluorescence intensity (MFI) was determined for gated populations of bacterial cells by flow cytometry. Values are representative of two experiments, and three replicates were used for each experiment. Asterisks indicate statistically significant differences in GFP MFI between the wild-type strain and mutants or complementary strains ($P < 0.01$, Student's *t*-test).

and the wild-type *D. dadantii* 3937 when they were grown in MM supplemented with TS103. The addition of TS103 led to a similar reduction in *hrpS* promoter activity in the Δ*gacA* and wild-type strains (Table 4), suggesting that the inhibition of *hrpS* by TS103 is HrpY specific and is not via GacS/GacA. Together, these results suggest that specific inhibition of HrpY phosphorylation by TS103 leads to low levels of *hrpS* transcript of *D. dadantii* 3937.

TS103 inhibits *rsmB* through unknown regulators

As a reduced level of *rsmB* RNA was observed when *D. dadantii* 3937 was grown in MM supplemented with TS103 compared with that in MM (Fig. 6B), the regulators of *rsmB* by TS103 were further investigated. The TCSTS GacS/GacA has been reported to be the transcriptional regulator of *rsmB* in several bacteria (Liu *et al*.,

Table 4 The *hrpS* promoter activity of *Dickeya dadantii* 3937 (3937) and its derivatives in type III secretion system-inducing medium (MM) and MM supplemented with TS103 (MM103).

	Average MFI ± SD for growth in the indicated medium†			
	12 h		24 h	
Strain	MM	MM103	MM	MM103
3937	92.0 ± 6.6	37.5 ± 0.9*	63.2 ± 6.4	28.5 ± 1.1*
Δ*hrpY*D57A	40.7 ± 1.1	35.2 ± 0.7	32.6 ± 1.8	26.4 ± 0.5
Δ*hrpX*	33.2 ± 2.0	34.2 ± 0.2	25.5 ± 0.6	25.4 ± 0.6
Δ*gacA*	94.2 ± 2.5	35.9 ± 1.6*	57.7 ± 3.0	24.5 ± 0.4*

*Statistically significant differences in green fluorescent protein (GFP) intensity between bacterial cells grown in MM (MM) and MM supplemented with 100 μM TS103 (MM103) ($P < 0.01$, Student's *t*-test).

†The promoter activities were compared in MM and MM supplemented with 100 μM TS103 at 12 and 24 h of bacterial growth. GFP mean fluorescence intensity (MFI) was determined for gated populations of bacterial cells by flow cytometry. Values are representative of two independent experiments, and three replicates were used for each experiment.

1999; Mukherjee *et al*., 2000; Tang *et al*., 2006). To confirm that *rsmB* is a GacA regulon gene in *D. dadantii* 3937, the promoter activity of *rsmB* in Δ*gacA* was examined. A dramatic reduction in *rsmB* promoter activity was observed in Δ*gacA* compared with the wild-type strain (Table 5). Complementation of Δ*gacA* with a *gacA* gene coupled with its native promoter into the chromosome of the mutant strain at an intergenic region (Δ*gacA*::*gacA*) restored the transcription of *rsmB* to near wild-type levels (Table 5). These results demonstrate that GacA is a transcriptional regulator of *rsmB* in *D. dadantii* 3937. To investigate whether TS103 inhibits the activity of GacS/GacA, and leads to a lower level of *rsmB* RNA, the promoter activity of *rsmB* was examined in the wild-type and Δ*gacA* strains grown in MM and MM supplemented with TS103. Similar levels of *rsmB* promoter activity were observed in the cells grown in MM and MM supplemented with TS103 (Table 5). These results suggest that GacS/GacA positively regulates *rsmB* transcription in *D. dadantii* 3937 and the inhibition of TS103 on *rsmB* does not occur through the transcriptional regulator GacS/GacA.

PNPase plays an important role in reducing *rpoN* mRNA stability and *rsmB* RNA turnover, which, in turn, down-regulates *hrpL* and HrpL regulon genes, such as *hrpA* and *hrpN* in *D. dadantii* 3937 (Zeng *et al*., 2010). As a significant reduction in *rpoN* mRNA and *rsmB* RNA levels was observed in cells grown in MM supplemented with TS103 compared with that in MM (Figs 4 and 6B), we speculate that TS103 may inhibit T3SS through PNPase. If this is the case, a similar *rsmB* RNA level should be observed in the *pnp* mutant (Δ*pnp*) grown in MM and MM supplemented with TS103. To test this, Northern blot analysis was performed to compare the *rsmB* RNA level in Δ*pnp* grown in MM with and without TS103. Unexpectedly, significantly smaller amounts of *rsmB* RNA were observed in the Δ*pnp* cells grown in TS103 compared with that in MM (Fig. 6C), suggesting that the inhibition of TS103 on *rsmB* is not through PNPase.

CsrD, a regulator of RNA turnover, was found to be essential for the decay of the small RNAs CsrB (homologue of RsmB) and CsrC in *Escherichia coli* (Suzuki *et al*., 2006). Inactivation of *csrD* resulted in an increase in the *csrB* RNA level (Suzuki *et al*., 2006). In *D. dadantii*, an increased *rsmB* RNA level was also observed in Δ*csrD* compared with the wild-type strain (Fig. 6D), suggesting that CsrD also negatively regulates the *rsmB* RNA level in *D. dadantii*. To determine whether TS103 inhibits *rsmB* RNA through CsrD, Northern blot analysis was performed to compare the *rsmB* RNA levels in Δ*csrD* grown in MM with and without TS103. Smaller amounts of *rsmB* RNA were observed in the Δ*csrD* cells grown in MM supplemented with TS103 compared with that in MM (Fig. 6D). These results suggest that *csrD* negatively regulates *rsmB* RNA in *D. dadantii* 3937 and the inhibition of TS103 on *rsmB* is not through CsrD.

Our recent work has demonstrated that OpgG, a component of Osmo-regulated periplasmic glucans, positively regulates *rsmB* at the post-transcriptional level in *D. dadantii* (X. Wu *et al*., in press). To test whether the inhibition of TS103 on *rsmB* is through OpgG, the original promoter and the whole open reading frame (ORF) of *opgG* were fused in frame with the His × 6 tag, and the OpgG protein level of the wild-type cells grown in MM and MM supplemented with TS103 was examined by Western blot. Similar levels of the OpgG protein were observed in the cells grown in MM and MM supplemented with TS103 (Fig. 5B), suggesting that the inhibition of TS103 on *rsmB* is not through OpgG.

The IclR-like regulator KdgR has been reported to be a regulator of *rsmB* in *Pectobacterium* (Liu *et al*., 1999). To study whether KdgR is a regulator of *rsmB* in *D. dadantii* 3937, and whether TS103 inhibits *rsmB* through KdgR, the promoter activity and RNA level of *rsmB* in Δ*kdgR* and the wild-type strains were examined. Similar levels of *rsmB* promoter and RNA were observed in Δ*kdgR* compared with that in the wild-type strain grown in MM (Table 5 and Fig. 6E). Furthermore, significantly smaller amounts of *rsmB* RNA were observed in Δ*kdgR* cells grown in MM supplemented with TS103 compared with that in MM (Fig. 6E). These results suggest that KdgR is not a regulator of *rsmB* in *D. dadantii* 3937 and the inhibition of TS103 on *rsmB* is not through KdgR.

DISCUSSION

The T3SS is an essential virulence factor of many Gram-negative bacterial pathogens. This secretion system has emerged as an attractive target for small-molecule anti-virulence therapeutics (Cegelski *et al*., 2008; Duncan *et al*., 2012; Escaich, 2008). Recently, a number of T3SS inhibitors have been identified in multiple bacterial species, including *Yersinia* spp., *Chlamydia* spp., *Salmonella* spp., *Pseudomonas aeruginosa*, *Erwinia amylovora*

Table 5 The *rsmB* promoter activity of *Dickeya dadantii* 3937 (3937) and its derivatives in type III secretion system-inducing medium (MM) and MM supplemented with 100 μM TS103 (MM103).

	Average MFI ± SD for growth in the indicated medium*			
	6 h		12 h	
Strain	MM	MM103	MM	MM103
3937	1525.3 ± 20.2	1603.4 ± 28.0	2531.4 ± 150.9	2628.6 ± 86.9
Δ*gacA*	74.6 ± 2.0	53.5 ± 1.4	86.0 ± 6.7	63.1 ± 4.0
Δ*gacA*::*gacA*	1956.2 ± 140.8	2052.3 ± 60.8	2779.6 ± 48.1	2905.1 ± 104.4
3937 (pML123)	—†	—	1724.8 ± 9.1	—
Δ*kdgR* (pML123)	—	—	1700.4 ± 12.0	—
Δ*kdgR* (pMLkdgR)	—	—	1582.2 ± 42.0	—

*The promoter activities were compared in MM and MM supplemented with 100 μM TS103 at 12 and 24 h of bacterial growth. Green fluorescent protein (GFP) mean fluorescence intensity (MFI) was determined for gated populations of bacterial cells by flow cytometry. Values are representative of two independent experiments, and three replicates were used for each experiment.
†—, not determined.

and *D. dadantii*, using screening-based approaches under interdisciplinary efforts between chemists and microbiologists (Hudson *et al*., 2007; Khokhani *et al*., 2013; Li *et al*., 2009; Muschiol *et al*., 2006; Pan *et al*., 2007; Yamazaki *et al*., 2012). The T3SS regulatory pathways and regulatory components of *D. dadantii* are well understood (Lebeau *et al*., 2008; Li *et al*., 2010; Yang *et al*., 2008b, 2010; Yi *et al*., 2010; Zeng *et al*., 2010). In this study, *D. dadantii* 3937 was used to screen high-potency T3SS inhibitors and to identify inhibitor targets in the T3SS pathways. The compound TS103 was found to be a highly potent inhibitor of the T3SS master regulator HrpL via HrpX-HrpY and the *rsmB*-RsmA regulatory pathway. Although no growth inhibition was observed in TS103 at low concentrations (1 or 10 μM), a further study showed that a slight promotion of bacterial growth was observed in TS103 at higher concentrations (100 μM) (Figs S2 and S3). This indicates that *D. dadantii* 3937 may possibly use TS103 as a carbon or energy source for growth. Nevertheless, TS103 dramatically inhibits the T3SS at all three concentrations used in this study. It is worth mentioning that TS103 also plays a role in inhibiting the T3SS of other *hrp* Group I phytobacteria, such as *E. amylovora* (Khokhani *et al*., 2013). However, the effect of TS103 on the T3SS of *hrp* Group II phytobacteria remains to be determined.

Bacteria use a wide variety of mechanisms to sense and respond to environmental changes, with TCSTSs being the dominant methods (Calva and Oropeza, 2006). The signal transduction lies in the recognition and interpretation of environmental signals that are related to host infection, and conversion of these signals into specific protein–protein interactions and transcriptional activation (Calva and Oropeza, 2006; Hoch, 2000). HrpX/Y is a TCSTS encoded within the *hrp* gene cluster of *D. dadantii*. HrpX, a transmembrane sensor histidine kinase, senses environmental stimuli and activates its cognate response regulator HrpY by phosphorylation. The phosphorylated HrpY then binds the *hrpS* promoter and promotes the transcription of *hrpS* (Yap *et al*., 2008). In *P. stewartii* ssp. *stewartii* and *E. herbicola* pv. *gypsophilae*, the aspartate residue at position 57 has been proven to be the phosphorylation site of HrpY (Merighi *et al*., 2006; Nizan-Koren *et al*., 2003). Although there is no direct evidence on the phosphorylation site of HrpY in *D. dadantii*, the HrpY protein without acetyl phosphate treatment was unable to bind the *hrpS* promoter, suggesting that *in vitro* phosphorylation is required for HrpY activity in *D. dadantii* (Yap *et al*., 2008). In this study, conservative and structurally neutral amino acid substitutions of aspartate to alanine at position 57 reduced the expression of *hrpS* (Table 4 and Fig. 7). In agreement with the observations in *P. stewartii* ssp. *stewartii* and *E. herbicola* pv. *gypsophilae*, our data suggest that D57 is needed for the activity of HrpY in *D. dadantii* 3937 and that D57 is the phosphorylation site of HrpY. Moreover, a similar level of *hrpS* promoter activity was observed in Δ*hrpY*D57A grown in MM and MM supplemented with 100 μM TS103 (Table 4), indicating that TS103 inhibits *hrpS* transcription through the phosphorylation of HrpY. This is the first report of a phenolic compound targeting the specific phosphorylated response regulator HrpY to inhibit the T3SS.

Rsm, also known as Csr in *E. coli*, is one of the most studied post-transcriptional regulators in bacteria (Romeo *et al*., 2012; Timmermans and Van Melderen, 2010). The Rsm/Csr system is present in many plant- and animal-associated pathogenic bacteria (Babitzke and Romeo, 2007; Bejerano-Sagie and Xavier, 2007; Liu *et al*., 1998; Suzuki *et al*., 2002; Toledo-Arana *et al*., 2007). It is composed of two regulatory components: the RNA-binding protein (RsmA in soft rot phytopathogens, RsmA and RsmE in *Pseudomonas*, CsrA in *E. coli*) and non-coding regulatory small RNAs (RsmB in soft rot phytopathogens, RsmY and RsmZ in *Pseudomonas*, CsrB in *E. coli*) (Gudapaty *et al*., 2001; Romeo, 1998). The central component of the Rsm/Csr systems is a homodimeric RNA-binding protein (CsrA or RsmA), which either represses or activates the expression of target mRNAs post-transcriptionally (Babitzke and Romeo, 2007; Mercante *et al*., 2009). A common feature of Rsm/Csr systems is that a TCSTS is responsible for the activation of the transcription of each small RNA in response to an unknown signal(s) (Babitzke and Romeo,

2007). In *D. dadantii*, the conserved TCSTS GacA/GacS regulates the small RNA RsmB at the transcriptional level (Table 5). In this TCS, GacS, a tripartite sensor histidine kinase, senses environmental stimuli and activates its cognate response regulator, GacA, by phosphorylation, which, in turn, induces the expression of the regulatory small RNA RsmB (Blumer *et al*., 1999; Cui *et al*., 2001; Heeb and Haas, 2001). *rsmB* transcripts then bind to and sequester RsmA, which eventually affects the expression of downstream genes (Chatterjee *et al*., 2002; Liu *et al*., 1998). In *P. aeruginosa*, the authors deduced that TS103 affects the transcripts of the small RNAs *rsmY* and *rsmZ* via the activation of GacA through GacS and/or other two-component sensor proteins that cross-talk to GacS/GacA (Yamazaki *et al*., 2012). Although we speculate that the strong negative effect of TS103 on *rsmB* of *D. dadantii* might involve the interference of GacS/GacA, our observations do not support such a hypothesis, because TS103 has no inhibitory effect on *rsmB* promoter activity in the wild-type strain, whereas *rsmB* promoter activity was dramatically reduced in Δ*gacA* compared with that in the wild-type strain (Table 5).

RNA turnover is an important process in the regulation of gene expression and is tightly regulated (Mata *et al*., 2005). In *E. coli*, RNA decay is often initiated by an endoribonuclease, RNase E, which preferentially binds to the 5' monophosphorylated terminus of transcripts, with cleavage occurring in A/U-rich regions adjacent to stem-loop structures. The resulting cleavage products are then rapidly degraded by the processive 3' to 5' exoribonucleases PNPase and RNase II (Carpousis, 2007; Romeo *et al*., 2012). Recently, the turnover of the small RNAs CsrB and CsrC in *E. coli* was reported to require a novel regulator, CsrD, in addition to RNase E and PNPase (Romeo *et al*., 2012; Suzuki *et al*., 2006). In *D. dadantii*, PNPase also plays an important role in *rsmB* RNA turnover (Zeng *et al*., 2010). Consistent with our previous work (X. Wu *et al*., in press), an increased *rsmB* RNA level was observed in Δ*csrD* compared with that in the wild-type strain (Fig. 6E), suggesting that CsrD negatively regulates the *rsmB* RNA level in *D. dadantii*. However, smaller amounts of *rsmB* RNA were observed in both Δ*pnp* and Δ*csrD* cells grown in MM supplemented with TS103 compared with that in MM (Fig. 6C,D), suggesting that the inhibition of TS103 on *rsmB* RNA is not through CsrD and PNPase. In addition, our recent work found that CsrD regulates the *rsmB* RNA level through OpgG in *D. dadantii* (X. Wu *et al*., in press). In this study, similar levels of OpgG protein were observed in the cells grown in MM and MM supplemented with TS103 (Fig. 5B), suggesting that the inhibition of TS103 on *rsmB* is not through CsrD. Overall, the knowledge of the post-transcriptional regulators of the small RNA RsmB is limited. Moreover, the regulation of RsmB by TS103 remains to be determined.

In summary, this study screened a series of derivatives of plant phenolic compounds and identified that TS103 has the highest inhibitory potency on T3SS of *D. dadantii*. The effect of TS103 on the regulatory components of the T3SS was further elucidated and revealed that the inhibition goes through both the HrpX/Y-HrpS-HrpL and *rsmB*-RsmA-HrpL regulatory pathways. To our knowledge, this is the first inhibitor which affects the T3SS through both transcriptional and post-transcriptional pathways in the soft-rot pathogen *D. dadantii* 3937.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, primers and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *D. dadantii* were grown in Luria–Bertani (LB), MM or mannitol–glutamic acid (MG) medium at 37 °C and 28 °C, respectively (Yang *et al*., 2007, 2008b; Zeng *et al*., 2010; Zou *et al*., 2012). Medium was supplemented with chloramphenicol (20 μg/mL), ampicillin (100 μg/mL), kanamycin (50 μg/mL), spectinomycin (50 μg/mL) and gentamicin (15 μg/mL) when required. The primers used for PCR in this work are listed in Table S1 (see Supporting Information).

Sources of the screened compounds

Compounds TS32 and TS108–TS113 were purchased from commercial sources (Sigma-Aldrich, Louis, MO, USA; Alfa Aesar, Ward Hill, MA, USA or TCI, Cambridge, MA, USA). TS37, TS39–TS42, TS44, TS46 and TS47 were synthesized by the methods described in Methods S1. The remaining compounds were synthesized via the routes described in our recent publications (Khokhani *et al*., 2013; Yamazaki *et al*., 2012). DMSO stock solutions were prepared and stored at −20 °C. The compounds were added at a final concentration of 100 μM, except when indicated otherwise. DMSO was used as a control (indicated by 'MM').

Flow cytometry analysis

Promoter activities of *hrpA*, *hrpN*, *hrpS*, *hrpL*, *rsmB* and *rpoN* were measured by flow cytometry as described previously (Peng *et al*., 2006). Briefly, the bacterial cells carrying the promoter–GFP transcriptional fusion plasmid were cultured in LB broth at 28 °C overnight and subcultured 1:100 in MM and MM supplemented with compounds in 20-mL glass culture tubes. Samples were diluted to the appropriate concentration with 1 × phosphate-buffered saline (PBS) at 12 and 24 h after inoculation. The promoter activities were analysed by measuring the GFP intensity using flow cytometry (BD Biosciences, San Jose, CA, USA). Meanwhile, the optical density of the samples was also measured at 600 nm (OD_{600}) when required.

Construction of plasmids and mutants

The Δ*gacA*, Δ*hrpY* and Δ*kdgR* deletion mutants were constructed by marker exchange mutagenesis (Yang *et al*., 2002). Briefly, two fragments flanking each target gene were obtained. A kanamycin cassette, amplified from pKD4 (Table 1), was ligated with these two fragments and cloned into the *Bam*HI and *Xho*I sites in pWM91. This construct was transferred

into *D. dadantii* 3937 by conjugation using *E. coli* S17-1λ*pir*. To select strains with chromosomal deletions, transconjugants with kanamycin and ampicillin resistance were plated onto MG containing 5% sucrose and kanamycin. Colonies having sucrose and kanamycin resistance and ampicillin sensitivity were isolated and confirmed by PCR using outside primers.

Δ*hrpY*D57A, in which the conserved aspartate residue at position 57 in HrpY was changed by nonconservative substitution to alanine, was constructed in a similar manner as above using plasmid phrpYD57A as the PCR template to obtain the flanking fragments, and a kanamycin cassette was placed at the 3' end of the *hrpY* ORF for selection. The plasmid phrpYD57A was constructed as follows. A 1830-bp fragment containing the *hrpY* ORF and its flanking region was amplified using primers hrpYH1 and hrpYH2 and 3937 genome DNA as the template. The PCR fragment was ligated into pGEM-T Easy vector to generate plasmid phrpY. phrpYD57A, bearing a D57A change in *hrpY* primary amino acid sequence, was generated by a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA, USA) according to the manual, using the primers hrpYD57A-Frd and hrpYD57A-RC and phrpY as the template.

To construct plasmids for complementation, the ORF and promoter region of target genes were amplified and cloned into low-copy-number plasmids pML123 or pCL1920 (Table 1). *gacA* was inserted into the chromosome using allelic exchange mutagenesis for complementation. To construct plasmids to complement the *gacA* mutation, the promoter and coding regions of *gacA* were PCR amplified and cloned into the chromosomal integration vector pTCLSCm (Jahn *et al*., 2008; Yap *et al*., 2008). The resulting plasmid was electrotransformed into Δ*gacA* and allelic exchange was used to insert *gacA* into the chromosome to generate the complementation strain Δ*gacA*::*gacA* (Jahn *et al*., 2008). All of the constructs and mutants described above were verified by PCR and DNA sequencing.

Northern blot analysis

About 10 μg of RNA of *D. dadantii* in MM and MM supplemented with TS103 were determined using Northern blot as described previously (Li *et al*., 2009). In brief, *D. dadantii* was grown in MM and MM supplemented with TS103 for 12 h, total RNA was isolated using TRI reagent and residual DNA was removed with the TURBO DNA-free kit (Ambion, Austin, TX, USA). RNA samples were analysed using biotin-labelled probe and a biotin detection system (BrightStar Psoralen–Biotin and Bright Star BioDetect, Ambion). rRNA was used as an internal control.

qRT-PCR analysis

The mRNA levels were measured by qRT-PCR. *Dickeya dadantii* 3937 was cultured in MM or MM supplemented with 100 μM TS103 for 12 h. Cells were harvested and total RNA was isolated as described previously (Li *et al*., 2009; Yang *et al*., 2008a). The cDNA level of target genes in different samples was quantified by real-time PCR using a Real Master Mix (Eppendorf, Westbury, NY, USA), as described previously (Peng *et al*., 2006). Data were analysed using a Relative Expression Software Tool (Pfaffl *et al*., 2002). The expression level of *rplU* was used as an endogenous control for data analysis (Mah *et al*., 2003).

Western blot analysis

Proteins were separated by 12.5% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilion poly(vinylidene difluoride) (PVDF) transfer membrane (Millipore, Bedford, MA, USA) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA). The blot (immunoblot) was then probed with an anti-HrpN peptide antibody (1:5000) or anti-His polyclonal antibody (1:2000; Southern Biotech, Birmingham, AL, USA). The blot was incubated for 2 h with the primary antibody and then washed in PBS containing 0.1% Tween 20. Antigen–antibody complexes were visualized by incubation of the blots in a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (Abcam, Cambridge, MA, USA) using an ECL detection system (GE Healthcare UK Ltd, England, UK).

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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 The relative promoter activity of *hrpA* in *Dickeya dadantii* 3937 cells grown in type III secretion system-inducing medium (MM) supplemented with 10 μM of the indicated compounds compared with that in MM (indicated by '%MM'). Green fluorescent protein (GFP) mean fluorescence intensity (MFI) was determined for gated populations of bacterial cells by flow cytometry. %MM was calculated by the formula: %MM = 100 × MFI(compound)/MFI(MM). Asterisks indicate statistically significant difference in *hrpA* promoter activity of *D. dadantii* 3937 cells grown in MM supplemented with 10 μM of the indicated compounds compared with that in MM. Two independent experiments were performed and three replicates were used for each experiment.

Fig. S2 The growth of *Dickeya dadantii* 3937 in type III secretion system-inducing medium (MM) and MM supplemented with TS103 at different concentrations at 12 and 24 h. To study the effect of TS103 on bacterial growth, 50 μL of bacterial suspension [optical density at 600 nm (OD_{600}) = 1.0] was used as the initial inoculum and added to 5 mL of MM and MM supplemented with TS103. The growth of *D. dadantii* 3937 was recorded. Results from one representative experiment are shown. Three replicates were used in this experiment, and the experiment was repeated twice.

Fig. S3 The growth curve of *Dickeya dadantii* 3937 in type III secretion system-inducing medium (MM) supplemented with TS103 at different concentrations. To detect the growth curve of *D. dadantii* 3937 in MM supplemented with TS103 at concentrations of 0, 1, 10 and 100 μM, overnight cultured bacteria were added to MM and MM supplemented with TS103 [initial inoculum optical density at 600 nm (OD_{600}) = 0.01]. The OD_{600} of *D. dadantii* 3937 was recorded at 2-h intervals. Results from one representative experiment are shown. Three replicates were used in this experiment, and the experiment was repeated twice.

Table S1 Primers used in this study.

Methods S1 General procedure for the preparation of cinnamyl amine derivatives TS37, TS39–TS42, TS44, TS46 and TS47.