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Type III secretion inhibitors for the management of bacterial plant diseases

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SUMMARY

The identification of chemical compounds that prevent and combat bacterial diseases is fundamental for crop production. Bacterial virulence inhibitors are a promising alternative to classical control treatments, because they have a low environmental impact and are less likely to generate bacterial resistance. The major virulence determinant of most animal and plant bacterial pathogens is the type III secretion system (T3SS). In this work, we screened nine plant extracts and 12 isolated compounds—including molecules effective against human pathogens—for their capacity to inhibit the T3SS of plant pathogens and for their applicability as virulence inhibitors for crop protection. The screen was performed using a luminescent reporter system developed in the model pathogenic bacterium *Ralstonia solanacearum*. Five synthetic molecules, one natural product and two plant extracts were found to down-regulate T3SS transcription, most through the inhibition of the regulator *hrpB*. In addition, for three of the molecules, corresponding to salicylidene acylhydrazide derivatives, the inhibitory effect caused a dramatic decrease in the secretion capacity, which was translated into impaired plant responses. These candidate virulence inhibitors were then tested for their ability to protect plants. We demonstrated that salicylidene acylhydrazides can limit *R. solanacearum* multiplication *in planta* and protect tomato plants from bacterial speck caused by *Pseudomonas syringae* pv. *tomato*. Our work validates the efficiency of transcription reporters to discover compounds or natural product extracts that can be potentially applied to prevent bacterial plant diseases.

Keywords: bacterial speck, bacterial wilt, disease control, *Pseudomonas syringae*, *Ralstonia solanacearum*, type III secretion system, virulence inhibitors

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INTRODUCTION

Few effective management options are available against bacterial plant diseases, such as bacterial wilt caused by *Ralstonia solanacearum* or bacterial speck caused by *Pseudomonas syringae* pv. *tomato*. Antibiotics and copper-based compounds have traditionally been used (Zaumeyer, 1958); however, their application is now restricted in many countries (Duffy *et al.*, 2005; Mackie *et al.*, 2012) because of their environmental impact. An important emerging strategy to combat pathogens seeks to block the ability of bacteria to harm the host by inhibiting bacterial virulence factors (Rasko and Sperandio, 2010). Unlike antibiotics, virulence inhibitors do not kill the pathogen and should thus preserve the host endogenous microbiome and exert little selective pressure, avoiding the rapid appearance of resistance (Clatworthy *et al.*, 2007).

The type III secretion system (T3SS) is an attractive target for antimicrobial compounds as it is essential for virulence in many pathogenic Gram-negative bacteria (Puri and Bogyo, 2009). This system injects bacterial effector proteins into host cells to subvert their defences (Buttner, 2016). In bacterial plant pathogens, the T3SS is encoded by the *hrp* genes, so called because they play a key role in both hypersensitive response (HR) elicitation and pathogenicity (Boucher *et al.*, 1987). The HR is a programmed cell death reaction that takes place locally in plants on pathogen recognition at the site of infection (Huysmans *et al.*, 2017). In the model phytopathogenic bacterium *R. solanacearum*, the regulator HrpB directly activates the transcription of the genes encoding the structural units of the T3SS and its associated effectors (Genin *et al.*, 1992; Occhialini *et al.*, 2005; Valls *et al.*, 2006). Amongst the genes controlled by HrpB is *hrpY*, which codes for the major constituent of the T3SS pilus (Van Gijsegem *et al.*, 2000).

As a strategy to block bacterial virulence, interdisciplinary efforts have identified some small molecules that can specifically inhibit the synthesis or functionality of the T3SS in human pathogens of the genera *Yersinia*, *Salmonella*, *Chlamydia*

and *Pseudomonas* (Hudson *et al.*, 2007; Kauppi *et al.*, 2003; Muschiol *et al.*, 2006; Yamazaki *et al.*, 2012). Compounds with such activity include salicylidene acylhydrazides, *N*-hydroxybenzimidazoles, cytosporone B, *p*-coumaric acid (PCA) and (–)-hopeaphenol (Davis *et al.*, 2014; Kauppi *et al.*, 2003; Kim *et al.*, 2009; Li *et al.*, 2009, 2013; Zetterstrom *et al.*, 2013). Most of these anti-virulence agents lack bacteriocidal activity and have been proven in *in vitro* or *in vivo* studies to inhibit symptoms or infections, showing no toxic effects on the host (Duncan *et al.*, 2012). The treatment of infected animals has shown promising results for *Citrobacter rodentium* (Kimura *et al.*, 2011), *Yersinia pseudotuberculosis* (Garrity-Ryan *et al.*, 2010), *Chlamydia trachomatis* (Slepenkin *et al.*, 2011) and *Salmonella enterica* (Hudson *et al.*, 2007; Nesterenko *et al.*, 2016) infections. More recently, the plant phenolic compound PCA has been identified as an inhibitor of T3SS transcription in the phytopathogen *Dickeya dadantii* (Li *et al.*, 2009). Recent reports have shown that some PCA derivatives can suppress T3SS functionality in *Xanthomonas oryzae* (Fan *et al.*, 2017) and *Erwinia amylovora* (Yang *et al.*, 2014) in rice and apple flower infection, respectively. Other PCA derivatives have been shown to be efficient in reducing blossom blight caused by *E. amylovora* on apple trees in the field (Sundin *et al.*, 2016).

In this work, we have determined the effect of several plant extracts and some molecules already described as T3SS inhibitors of bacterial animal pathogens against plant pathogens. We have taken advantage of a luminescent reporter system developed for *R. solanacearum* (Monteiro *et al.*, 2012a) to select those compounds/extracts that inhibit the transcription of *R. solanacearum hrpB* and *hrpY* genes without affecting the transcription of a constitutive control promoter. Positive candidates were tested for their ability to suppress T3SS functionality *in vitro* and *in vivo*. Finally, their efficiency in the control of bacterial wilt or bacterial speck in tomato plants was examined.

Table 1 List of compounds and plant extracts evaluated in this work.

ID	Compound or the most abundant compound in extract	Source of the material (Reference)
PP1	*p*-Coumaric acid	Synthetic plant phenylpropanoid (Li *et al.*, 2009)
PP2	2,4-Dihydroxycinnamic acid (umbellic acid)	Synthetic plant phenylpropanoid (Li *et al.*, 2009)
PP3	4-Chlorocinnamic acid	Synthetic plant phenylpropanoid (Li *et al.*, 2009)
PP4	3,4-Dihydroxycinnamic acid (caffeic acid)	Synthetic plant phenylpropanoid (Li *et al.*, 2009)
PP5	4-Methoxycinnamic acid	Synthetic plant phenylpropanoid (Li *et al.*, 2009)
PP6	4-Methylcinnamic acid	Synthetic plant phenylpropanoid (Li *et al.*, 2009)
CB	Cytosporone B	Synthetic fungal compound (Li *et al.*, 2013)
SA1	ME0054 [benzoic acid N′-(2,3,4-trihydroxy-benzylidene)-hydrazide]	Synthetic salicylidene acylhydrazide (Nordfelth *et al.*, 2005)
SA2	ME0055 [4-nitrobenzoic acid N′-(2,4-dihydroxy-benzylidene)-hydrazide]	Synthetic salicylidene acylhydrazide (Dahlgren *et al.*, 2010; Nordfelth *et al.*, 2005)
SA3	ME0177 [2-nitro-benzoic acid N′-(3,5-dichloro-2-hydroxy-benzylidene)-hydrazide]	Synthetic salicylidene acylhydrazide (Dahlgren *et al.*, 2010)
SA4	ME0192 [3,5-dichloro-benzoic acid N′-(4-diethylamino-2-hydroxy-benzylidene)-hydrazide]	Synthetic salicylidene acylhydrazide (Dahlgren *et al.*, 2010)
HP	(-)-Hopeaphenol	Plant natural compound (Davis *et al.*, 2014; Zetterstrom *et al.*, 2013)
E1	4,11-Dimethoxy-5-methyl-[1,3]dioxolo[4,5-b]acridin-10(5*H*)-one	*Melicope elleryana* leaf extract (Crow and Price, 1949)
E2	4-Methoxy-6-[(*E*)-2-(4-methoxyphenyl)ethenyl]pyran-2-one	*Piper methysticum* root extract (Bu'lock and Smith, 1960)
E3	3,7,8-Trihydroxyserrulat-14-en-19-oic acid	*Eremophila microtheca* leaf extract (Barnes *et al.*, 2013)
E4	7-[3-(5,5-Dimethyl-4-oxofuran-2-yl)but-2-enoxy]chromen-2-one	*Geijera parviflora* leaf extract (Dreyer and Lee, 1972)
E5	4,4′-((1*R*,2*R*,3*S*,4*S*)-3,4-Dimethylcyclobutane-1,2-diyl)bis(2-methoxyphenol)	*Endiandra anthropophagorum* root extract (Davis *et al.*, 2007, 2009)
E6	1a-Acetoxy-4b,8a-dihydroxy-6b,9a-dibenzoyl-b-agarofuran	*Denhamia celastroides* leaf extract (Levrier *et al.*, 2015)
E7	(*E*)-1,3-Diphenylprop-2-en-1-one	*Syzygium tierneyanum* leaf extract (Kumar *et al.*, 2016)
E8	5,6-Dimethoxy-10-methyl-2*H*-pyrano[2,3-f]quinolin-2-one	*Goniothalamus australis* bark extract (Levrier *et al.*, 2013)
E9	5-(4-Methoxybenzyl)-6-methyl-[1,3]dioxolo[4,5-g]isoquinolin-6-ium	*Doryphora sassafras* leaf extract (Carroll *et al.*, 2001)

RESULTS

In vitro screen for compounds that reduce *hrpY* transcription

We used *R. solanacearum* as a model bacterial plant pathogen to evaluate the potential T3SS inhibitory effect of a number of pure compounds and plant extracts. We tested molecules already described as T3SS inhibitors in human and animal pathogens, including PCA and analogues (plant phenylpropanoids; PP1–6), cytosporone B (CB), salicylidene acylhydrazides (SA1–4), (–)-hopeaphenol (HP) and the plant-derived extracts (E1–9). All tested molecules and their sources are summarized in Table 1, and their chemical structures are presented in Fig. S1 (see Supporting Information). To detect and quantify their inhibitory effects, we took advantage of a strain that bears a transcriptional fusion of the *hrpY* promoter (*PhrpY*), controlling the expression of the T3SS pilus component, with the *luxCDABE* operon (Monteiro *et al.*, 2012a). This strain emits luminescence and does not require antibiotic selection as the promoter::reporter fusion is stably integrated in a monocopy in the bacterial chromosome. Bacteria were grown in minimal medium—a condition ensuring maximal induction of *hrpY* expression—and luminescence was directly measured 8 h after incubation with each of the compounds and normalized by the cell density [optical density at 600 nm (OD_{600})]. Figure 1 shows *hrpY* expression levels after incubation with each extract/molecule normalized by the expression levels in control conditions [dimethylsulfoxide (DMSO) addition]. As shown in Fig. 1, CB, SA1–4, HP, E8 and E9 exhibited a statistically significant ($P < 0.05$) repression of *hrpY* expression. The inhibitory effect was mild after the addition of compounds CB, SA4, HP, E8 and E9, whereas SA1, SA2 and SA3 almost completely abolished *hrpY* expression. We thus selected these molecules, as well as a molecule and an extract with intermediate effects (SA4 and E8), for further characterization.

Salicylidene acylhydrazides inhibit T3SS expression at the *hrpB* level

We performed a time-course analysis monitoring *hrpY* expression on addition of varying amounts of the identified inhibitors to determine their minimal effective concentration (Fig. 2). This experiment revealed that a minimal concentration of 10 μM for SA2, SA3 and HP, and 50 μM for SA1, was sufficient to cause full inhibition, whereas, for SA4 and E8, 100 μM was needed for maximal effect. Next, to determine whether the analysed substances caused a general inhibition of T3SS, and not only of *hrpY*, we measured the transcription of *hrpB*—the master regulator controlling the expression of the T3SS genes—over time (Fig. S2, see Supporting Information). As can be observed in Fig. 3, six of the eight substances inhibiting *PhrpY* caused a comparable reduction in *hrpB* transcription, implying a shutdown of all T3SS-encoding genes and the associated effectors controlled by this regulator (Occhialini *et al.*, 2005).

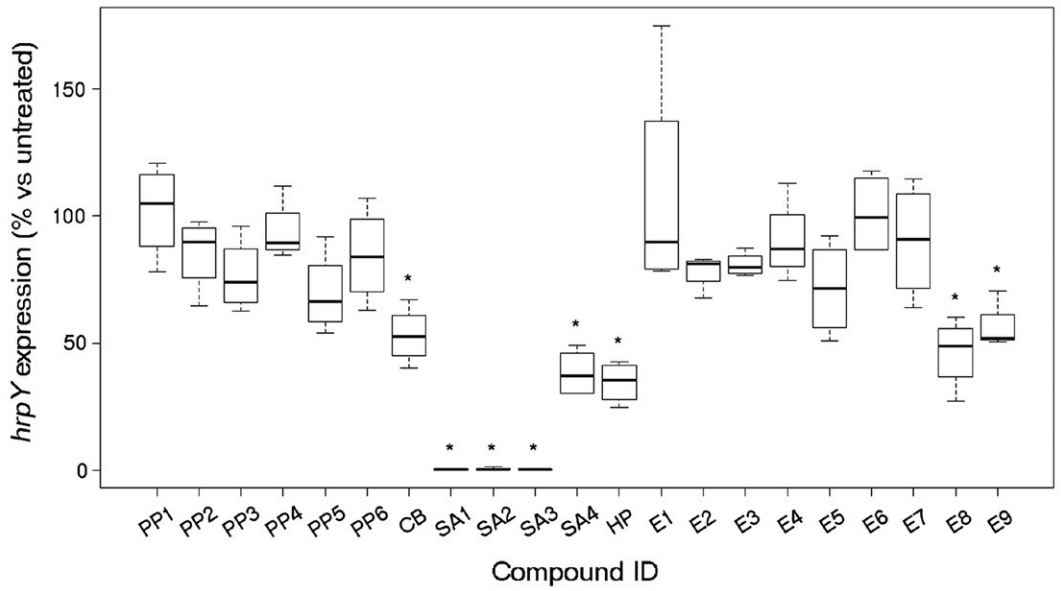

Fig. 1 Expression of the type III secretion system (T3SS) pilus gene (*hrpY*) in the presence of different compounds. *Ralstonia solanacearum* carrying the *PhrpY::luxCDABE* fusion was grown in minimal medium supplemented with each compound/extract (detailed in Table 1) at a final concentration of 100 μM, or with dimethylsulfoxide (DMSO) (control). *hrpY* expression was quantified at 8 h post-inoculation (hpi) by luminescence, normalized by cell density and represented with respect to the value obtained with DMSO (control). Compounds/extracts marked with an asterisk showed statistically significant reduction ($P < 0.05$) in *hrpY* expression compared with control conditions. Each measurement corresponds to the average of four replicates. The experiment was repeated three times with similar results.

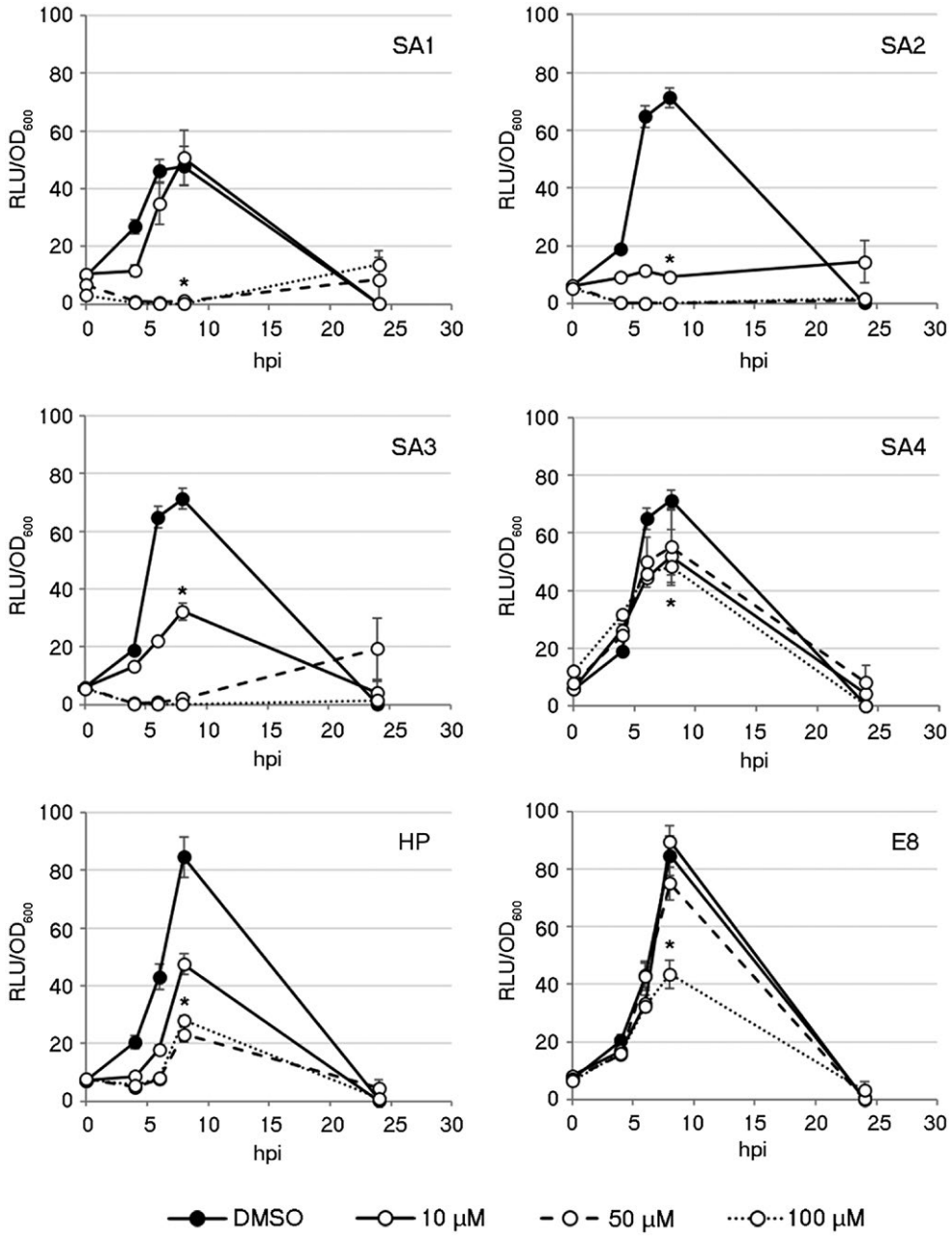

Fig. 2 Time-course expression of *hrpY* after the addition of selected type III secretion system (T3SS) inhibitors. *hrpY* expression was quantified at 4, 6, 8 and 24 h post-inoculation (hpi) by direct luminescence quantification from bacteria growing in minimal medium supplemented with SA1–4, HP or E8 at different concentrations, or with dimethylsulfoxide (DMSO) as a control. Expression is represented as relative luminescent units (RLU) normalized by the bacterial density [optical density at 600 nm (OD_{600})] at each time point. Asterisks indicate the minimal effective concentration ($P < 0.05$) of each compound or extract at the most informative time point. Each measurement represents the average of four replicates. The experiment was repeated three times with similar results.

To rule out the possibility that the observed effects were caused by a general, non-specific inhibition of gene expression, we made use of an *R. solanacearum* strain containing the luminescence reporter under the control of the heterologous promoter *PpsbA*. *PpsbA* is a chloroplastic promoter that shows strong, constitutive expression in a range of environmental conditions when introduced into Gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas fluorescens*, *Agrobacterium tumefaciens* and different *P. syringae* strains (Brixey *et al.*, 1997; Tombolini *et al.*, 1997; Wang *et al.*, 2007). As shown in Fig. 4A, at 8 h post-inoculation (hpi), a slight but significant ($P < 0.05$) induction of *PpsbA* expression was detected after bacterial

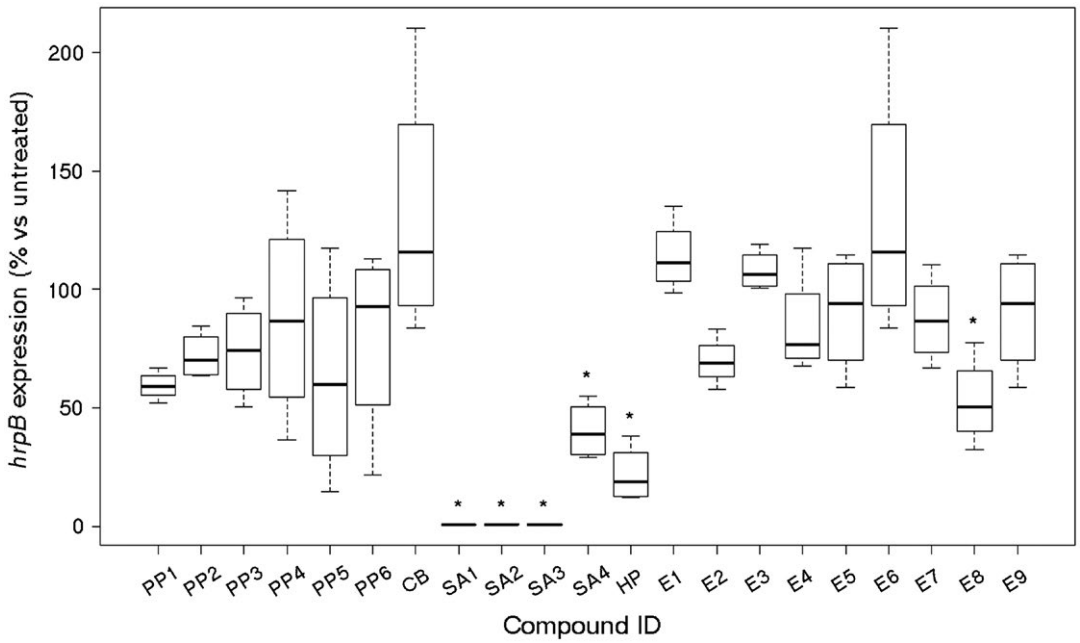

Fig. 3 Expression of the main type III secretion system (T3SS) regulator (*hrpB*) in the presence of different compounds. *Ralstonia solanacearum* bearing the *PhrpB::luxCDABE* fusion was grown in minimal medium supplemented with each compound/extract (detailed in Table 1) at a final concentration of 100 μM, or with dimethylsulfoxide (DMSO) (control). *hrpB* expression was quantified at 8 h post-inoculation (hpi) by luminescence, normalized by cell density, and represented with respect to that in DMSO. Compounds/extracts marked with an asterisk showed statistically significant reduction ($P < 0.05$) in *hrpB* expression compared with control conditions. Each measurement corresponds to the average of four replicates. The experiment was repeated three times with similar results.

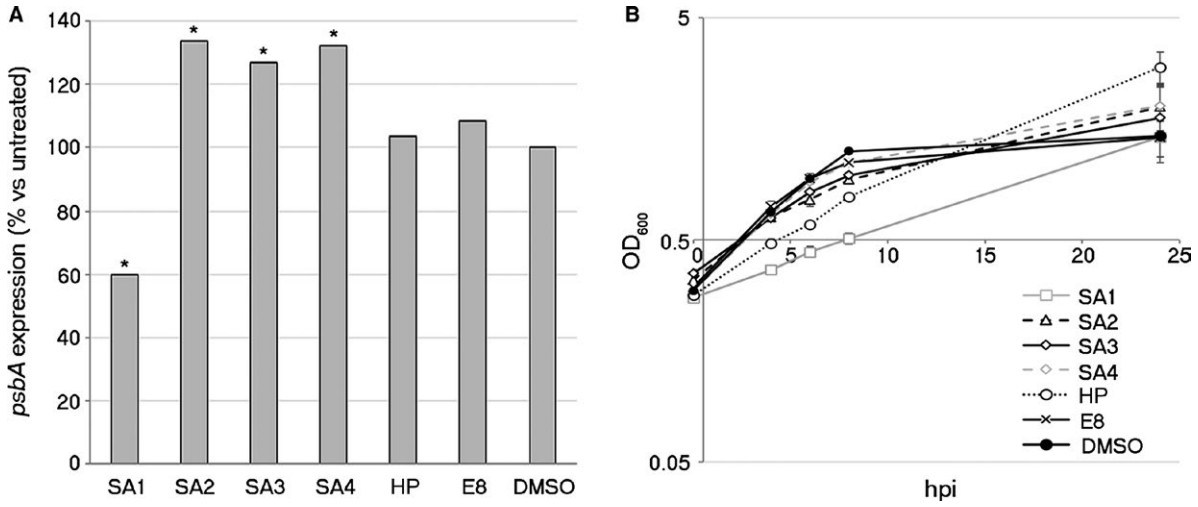

Fig. 4 *PpsbA* transcription and *Ralstonia solanacearum* growth on treatment with identified type III secretion system (T3SS) inhibitors. (a) *Ralstonia solanacearum* bearing the *PpsbA::luxCDABE* fusion was grown for 8 h in liquid minimal medium supplemented with each compound/extract at its *in vitro* minimal effective concentration (50 μM for SA1, 10 μM for SA2 and SA3, 50 μM for SA4 and HP, and 100 μM for E8). Transcription was quantified by measuring the luminescence divided by bacterial growth. The percentage of *psbA* expression in each treatment was normalized by basal expression after dimethylsulfoxide (DMSO) addition (control). Each measurement corresponds to an average of four replicates, and experiments were repeated three times with similar results. Standard errors never exceeded 25%. Compounds marked with an asterisk showed statistically significant ($P < 0.05$) reduction or increase in *psbA* expression compared with the addition of DMSO (control). (b) Bacterial growth was measured at 4, 6, 8 and 24 h in the same conditions using *R. solanacearum* containing the *PhrpY::luxCDABE* construct. Cell densities were measured as the absorbance at 600 nm and are represented in a logarithmic scale.

incubation with SA2, SA3 and SA4 (Fig. 4A), showing that inhibition of the transcription of T3SS was selective. However, one compound (SA1) caused repression of *PpsbA* expression, an effect probably caused by the slower bacterial growth produced by incubation with this compound (see below and Fig. 4B). Bacterial growth defects were not observed during our gene expression analyses using reporter strains. However, to accurately determine whether the compounds used affected bacterial viability, we measured the growth of *R. solanacearum* PhrpY-Lux in liquid culture after the addition of the transcriptional inhibitors. Figure 4B shows that, at their minimal inhibitory concentrations, SA1 and HP are slightly bacteriostatic, as their effects can only be observed at short time points and are not apparent after 24 h.

Salicylidene acylhydrazides inhibit T3SS effector translocation

To determine whether transcriptional inhibitors of T3SS impair its functionality, we tested their effect on the T3SS-dependent secretion of effector proteins *in vitro*. To this end, we used an *R. solanacearum* strain producing an HA-tagged version of the T3SS effector AvrA. To ensure AvrA-HA production in the presence of T3SS inhibitors, this tagged version was placed under the control of the constitutive *psbA* promoter, which is highly expressed under our experimental conditions (Cruz *et al.*, 2014). As shown in Fig. 5, incubation of bacteria with the strongest T3SS inhibitors—the salicylidene acylhydrazide derivatives SA1, SA2 and SA3—inhibited AvrA secretion, as this effector was

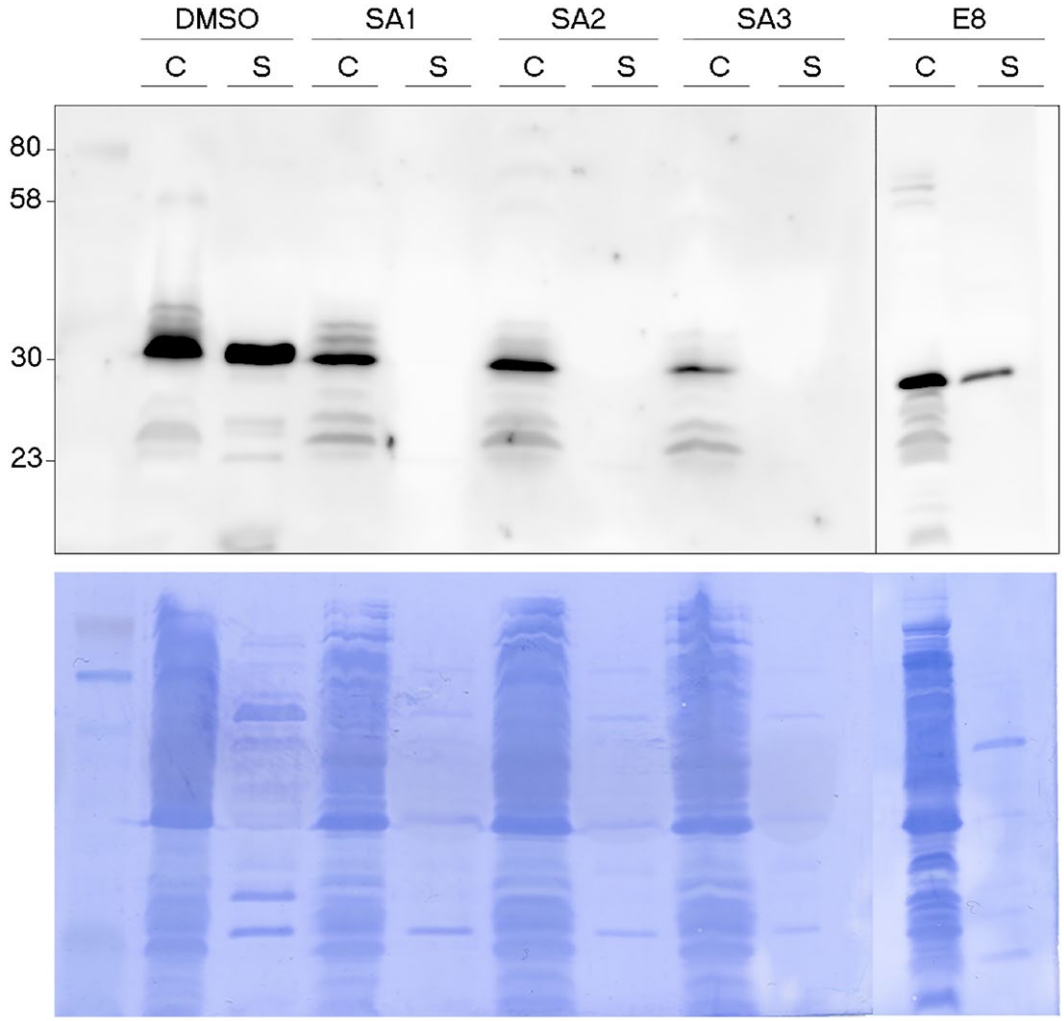

Fig. 5 Effector secretion is inhibited by bacterial pre-incubation with salycidene acylhydrazides. *Ralstonia solanacearum* bearing the *Pps-AvrA-HA* construct was grown for 8 h in minimal medium supplemented with Congo red to promote protein secretion and with each of the type III secretion system (T3SS) inhibitors (SA1–3 and E8) at 100 μM. Incubation with dimethylsulfoxide (DMSO) was used as a control to verify that protein secretion was not altered. The cytosolic (C) and secreted (S) protein fractions were separated by centrifugation followed by protein precipitation, and AvrA was detected with an anti-HA antibody. Coomassie-stained sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) membranes used in western blotting are also shown.

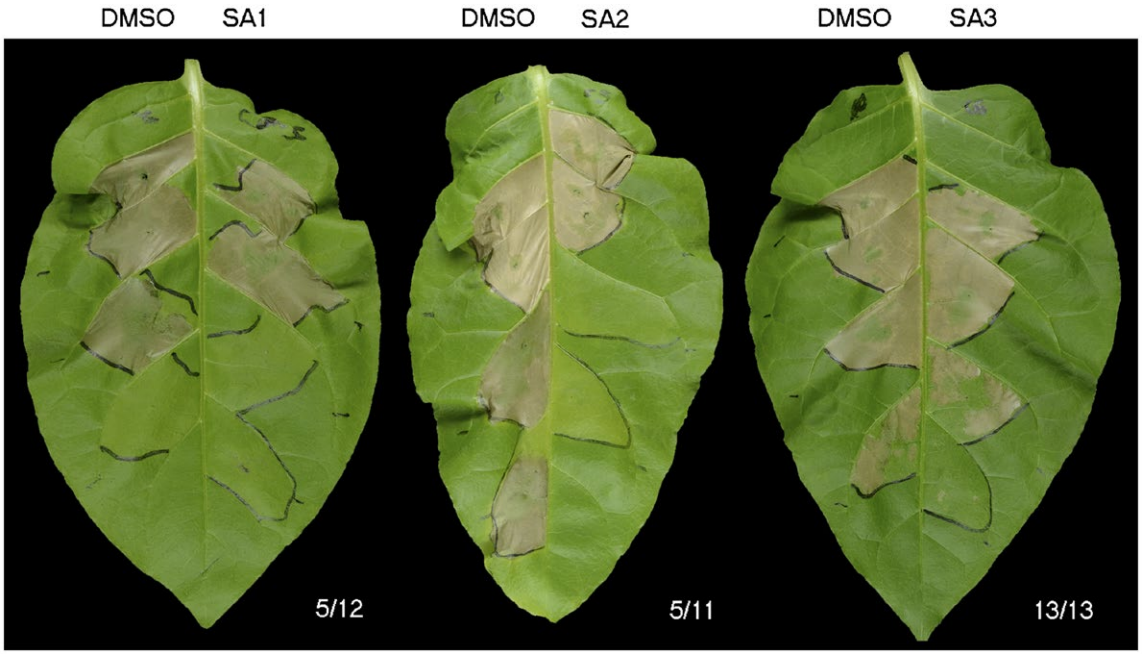

Fig. 6 Hypersensitive response (HR) inhibition by salicylidene acylhydrazides. Bacteria grown for 8 h post-inoculation (hpi) in liquid minimal medium after the addition of SA1–3 at 100 μM or dimethylsulfoxide (DMSO) alone were serially diluted five-fold in water [10^7, 5×10^6, 10^6 and 5×10^5 colony-forming units (CFU)/mL, top to bottom] and leaf infiltrated into *Nicotiana tabacum*. HRs were photographed at 2 days post-inoculation (dpi). Numbers indicate the proportion of positive leaves [showing HR inhibition as a result of type III secretion system (T3SS) suppressors] in relation to the total tested leaves.

detected only in the cytosolic bacterial fraction (C) and not in the secreted fraction (S). These results support the lack of a functional T3SS in bacteria incubated with these compounds, as AvrA could not be secreted to the medium through this apparatus. This effect was accentuated for the strongest T3SS inhibitors, as bacterial incubation with the mild inhibitor E8 allowed the detection of secreted AvrA in the culture medium, although at lower levels than in the control condition (DMSO).

The AvrA effector secreted by the *R. solanacearum* strain used in this work has been shown to trigger an HR on tobacco plants (Poueymiro *et al.*, 2009). To validate our *in vitro* results and to determine whether the inhibition of T3SS secretion is physiologically relevant *in planta*, we tested the influence of pre-incubation of bacteria with salicylidene acylhydrazides on the plant HR. *Nicotiana tabacum* and *N. benthamiana* plants were leaf infiltrated with five-fold *R. solanacearum* dilutions obtained after 8 h of incubation with SA1-3 or DMSO (control). As shown in Fig. 6, HR was inhibited when *N. tabacum* leaves were infiltrated with bacteria grown in the presence of some salicylidene acylhydrazides (SA1–3), showing that inhibition of effector secretion resulted in the evasion of recognition by the plant immune system. Similar results were obtained when using *N. benthamiana* as host (Fig. S3, see Supporting Information).

Salicylidene acylhydrazides limit *R. solanacearum* growth *in planta* and protect tomato plants from bacterial speck

The *R. solanacearum* reporter strains proved to be very useful to identify small molecules that inhibit T3SS. As a first step to validate the ability of these compounds to limit *R. solanacearum* infection, we measured the multiplication of bacteria infiltrated into tomato leaves alone or in the presence of the inhibitors. As shown in Fig. 7, a significant decrease ($P < 0.05$) in bacterial growth was observed when the most effective T3SS inhibitors (SA1–3) were present. This result demonstrates that salicylidene acylhydrazides are effective in limiting *R. solanacearum* growth *in planta*, although no differences in wilting symptoms could be observed after watering tomato plants with an *R. solanacearum* inoculum containing SA2 at 100 μM (Fig. S4, see Supporting Information). However, as *R. solanacearum* infects plants through the roots, large amounts of inhibitors would be needed to treat the soils and protect crops from bacterial wilt. Thus, we used the foliar pathogen *Pseudomonas syringae* pv. *tomato* DC3000, which requires effector translocation via T3SS to cause bacterial speck disease in tomato (Munkvold *et al.*, 2009), to test the preventative effect of the potent T3SS inhibitors (SA1–3). Tomato plants were sprayed with a solution containing these

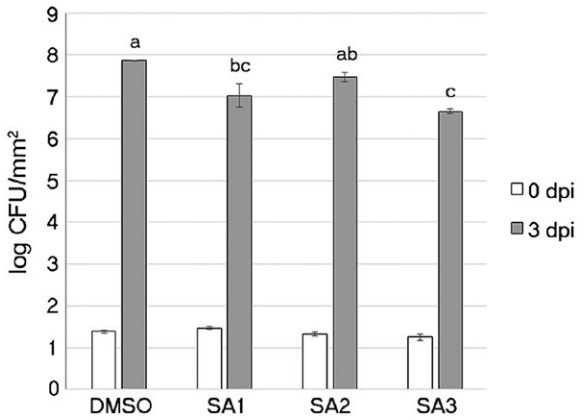

Fig. 7 *Ralstonia solanacearum* growth in tomato is impaired by the addition of compounds SA1–3. *Ralstonia solanacearum* was leaf inoculated at 10^5 colony-forming units (CFU)/mL with SA1–3 at 100 μM. Leaf discs were taken at 0 and 3 days post-inoculation (dpi) to monitor bacterial multiplication. Bacterial growth is represented as CFU/mm^2 on a logarithmic scale at day 3 and day 0 (immediately after inoculation). Each point represents the mean of three biological replicates consisting of two different leaf discs. Experiments were repeated three times with similar results. Statistically significant groups were obtained following Tukey's honestly significant difference (HSD) test using $P < 0.05$.

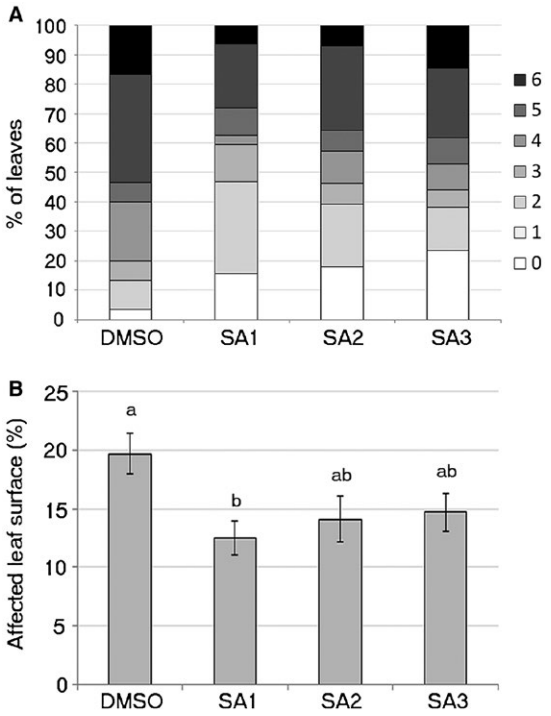

Fig. 8 Symptom development in tomato plants pre-treated with type III secretion system (T3SS) inhibitors and inoculated with *Pseudomonas syringae* pv. *tomato*. Effect of T3SS inhibitors on disease symptoms. Plants were pre-treated with SA1–3 at 100 μM or with dimethylsulfoxide (DMSO) 1 h before bacterial inoculation. Symptoms were recorded at 3 days post-inoculation (dpi) and are represented as: (A) the percentage of leaves categorized on a disease scale from 0 (no visible symptoms) to 6 (extensive necrosis on >35% of the leaf); or (B) average percentage of affected leaf surface with the corresponding standard error. Statistically significant groups were obtained with Tukey's honestly significant difference (HSD) test using $P < 0.05$. The experiment was performed three times with similar results.

compounds, or with DMSO alone (control), and subsequently inoculated by spray with a bacterial suspension. Symptoms were quantified using a necrosis index, and a clear symptom reduction was observed at 3 days post-inoculation (dpi) in plants that had been pre-treated with SA1–3 compared with control plants (Fig. 8). This was in accord with their inhibitory effect on the transcription and functionality of the main bacterial virulence determinant: the T3SS. Taken together, our results indicate that some salicylidene acylhydrazides show a protective effect against bacterial speck, suggesting that they could be utilized as virulence inhibitors to control bacterial plant diseases in the field.

DISCUSSION

An effective screening methodology to identify T3SS inhibitors

Bacterial plant diseases represent a major limitation to crop production and contribute to significant economic losses annually. Copper compounds and antibiotics have been successfully employed as management strategies in fields since the early 1900s (Elguindi *et al.*, 2011; Zaumeyer, 1958). However, the use of chemical bactericides as crop protectants represents a threat to the environment and may result in a risk for public health because of the rapid emergence of resistance that could eventually be acquired by clinical pathogens (Sundin *et al.*, 2016). In this work, we screened 21 compounds and plant extracts in search for antimicrobial alternatives that down-regulate the gene expression of the T3SS, the main virulence determinant of most pathogenic bacteria. We used a luminescent reporter strain of the model phytopathogen *R. solanacearum* to directly monitor the expression of *hrpY*, which has the highest transcriptional output amongst the *hrp* genes (Puigvert et al.). We found eight compounds and extracts (CB, SA1–4, HP, E8 and E9) capable of specifically repressing *hrpY* transcription to various degrees (Fig. 1). Six of these inhibitors also repressed *hrpB* expression (Figs 3 and 2), and thus seem to act upstream of the *hrp* regulatory cascade. The exceptions are CB and E9, which might interfere specifically with *hrpY* transcription. The effects on gene expression perfectly correlated with T3SS functional analyses, as the strongest inhibitors SA1–3 were also able to inhibit *in vitro* and *in vivo* effector production and secretion through the T3SS, whereas milder inhibitors, such as E8, had a minor effect on secretion (Fig. 5). Our screening methodology proved to be very effective, probably because of the high sensitivity of the luminescent reporter used. This system could also be scaled to 96-well plates or even be used qualitatively by the presence/absence of light emission (Kauppi *et al.*, 2003).

Ralstonia solanacearum T3SS inhibitors are effective against several plant-pathogenic bacteria

The *R. solanacearum* T3SS regulators targeted by the molecules identified here have orthologues in various *Xanthomonas* ssp. and *Burkholderia* ssp. strains (Li *et al.*, 2011; Lipscomb and Schell, 2011), showing the potential of our screening method to isolate virulence inhibitors that can be effective against other pathogens. Interestingly, we found that the salicylidene acylhydrazide SA1 inhibited *R. solanacearum* T3SS expression and could also protect plants from *P. syringae* infection. In fact, cross-inhibition is not surprising in our case, as salicylidene acylhydrazides were selected for our screening because they had already been shown to inhibit the T3SS of *E. amylovora* (Yang *et al.*, 2014), which is closely related to that of *P. syringae* (Alfano and Collmer, 1997; Tang *et al.*, 2006). In any case, our findings suggest that salicylidene acylhydrazides act on proteins that affect T3SS expression, favouring this mode of action over the alternative hypotheses proposed: direct effects on the T3SS basal apparatus proteins or possible changes in iron availability (Wang *et al.*, 2011). Interestingly, salicylidene acylhydrazides have been proven to bind to 16 *E. coli* proteins, and it has been suggested that they repress T3SS expression by blocking the function of these proteins (Wang *et al.*, 2011). As orthologues for most of these target proteins exist in *R. solanacearum* and *P. syringae*, it would be of interest to check whether they also interact with the chemicals and, if so, through which mechanism they affect the transcription of type III secretion genes.

In contrast, despite the conservation of the *hrp* genes, some molecules seem to act in a species-specific manner. This is the case for the plant phenolic compound PCA and its derivatives, which were recently found to act as T3SS inhibitors in *D. dadantii*, *E. amylovora* and *Pseudomonas aeruginosa* (Khokhani *et al.*, 2013; Li *et al.*, 2009; Yamazaki *et al.*, 2012). We showed that neither PCA nor certain derivatives (PP1–3) were effective *R. solanacearum* T3SS inhibitors, similar to the observations described for the closely related rice pathogen *X. oryzae* (Fan *et al.*, 2017).

Effectiveness of salicylidene acylhydrazides as crop protectants against bacterial pathogens

Salicylidene acylhydrazides SA1–3 proved to be powerful inhibitors of the *R. solanacearum* T3SS; our results demonstrated that SA1–3 inhibited its functionality *in vivo* and impaired bacterial multiplication *in planta* (Fig. 7). However, no symptom reduction was visible in tomato wilting assays by soil inoculation of an *R. solanacearum* suspension containing one of these potent inhibitors (Fig. 4). *Ralstonia solanacearum* is a soil-borne pathogen, and direct soil treatments are usually challenging and cost-ineffective (Yadeta and Bp, 2013). On the other hand, aerial plant treatments are widely used to control diseases caused by pathogens that infect the aerial parts of plants. Here, we demonstrated the efficiency of such treatments under laboratory conditions, as tomato plants sprayed with the potent T3SS inhibitors SA1–3 before *P. syringae* pv *tomato* inoculation displayed fewer disease symptoms compared with control plants (Fig. 8). To assess the effectiveness of inhibitors, previous studies have pre-treated bacteria prior to pathogenicity assays (Fan *et al.*, 2017; Yang *et al.*, 2014). A recent report has shown that bacterial pre-treatment with other T3SS inhibitors can impede their virulence in plants (Fan *et al.*, 2017). To simulate a more realistic application in the field, in this study, plants instead of bacteria were pre-treated with the T3SS inhibitor during the pathogenicity tests. This is the first report to demonstrate that T3SS inhibitors can be applied to plants for protection against pathogens, and opens the way to the development of analogous molecules that are cost-effective crop protectants.

T3SS inhibitor analogues thus represent a potential and cost-effective source of antimicrobials that could successfully control wilt diseases in the field. Salicylidene acylhydrazides can be efficiently prepared in one step from commercially available starting materials. Finally, the identification of such functional analogues would open the way to explore new treatment strategies for vascular wilts and other challenging bacterial plant diseases, for which no effective management strategies are currently available (Yadeta and Bp, 2013).

EXPERIMENTAL PROCEDURES

Bacterial strains and gene cloning

The *R. solanacearum* GMI1000 reporter strains for *hrpB* and *psbA* contain a fusion of either promoter to the *luxCDABE* operon integrated in the genome, and have been described elsewhere (Cruz *et al.*, 2014; Monteiro *et al.*, 2012a). Gene constructs were introduced into *R. solanacearum* GMI1000 through natural transformation of linearized plasmids and double-recombination events, as described previously (Boucher *et al.*, 1985). The hrpY reporter strain was constructed after transformation of the SfiI-digested vector pRCG-PhrpY-lux. For pRCG-PhrpY-lux construction, the *hrpY* promoter was polymerase chain reaction (PCR) amplified from the genome of strain GMI1000 with primers that added 5′AvrII and 3′KpnI flanking sites, and cloned into the pRCG-PhrpB-Lux backbone (Monteiro *et al.*, 2012b) using the introduced sites. The *R. solanacearum* GMI1000 strain expressing an HA-tagged *avrA* gene under the *psbA* promoter was generated after transformation with linearized plasmid pRCK-Pps-AvrA. pRCK-Pps-AvrA was created by Gateway LR reaction (Invitrogen, Paisley, UK) between plasmids pENTR/SD-AvrA and pRCG-Pps-GWY (Cruz *et al.*, 2014). *Ralstonia solanacearum* was routinely grown in rich B medium (10 g/L bactopeptone, 1 g/L yeast extract, 1 g/L casamino acids, 0.5% glucose) supplemented with gentamycin 10 μg/mL (solid medium) or 5 μg/mL (liquid medium) at 28 °C. For T3SS inhibition tests, the bacterial cultures

were grown in Boucher's minimal medium (Boucher *et al.*, 1985) supplemented with 20 mM glutamate and 5 μg/mL gentamycin. *Pseudomonas syringae* pv. *tomato* DC3000 was routinely grown at 28 °C on KB-agar plates supplemented with 25 μg/mL rifampicin and 50 μg/mL kanamycin or in liquid Luria–Bertani broth. The sequence of oligonucleotides used as primers is available on request.

Compound/extract supply

A list of the compounds and plant extracts used in this work can be found in Table 1 and chemical structures are indicated in Fig. 1. Synthetic plant phenylpropanoids (PP1–6) and CB were purchased from Sigma-Aldrich (Buchs, Switzerland). Salicylidene acylhydrazides (SA1–4) were provided by Dr M. Elofsson (Dahlgren *et al.*, 2010; Nordfelth *et al.*, 2005), and HP (Davis *et al.*, 2014; Zetterstrom *et al.*, 2013) and the nine plant extracts (E1–9) were provided by Dr R. Davis (Barnes *et al.*, 2013; Bu'lock and Smith, 1960; Carroll *et al.*, 2001; Crow and Price, 1949; Davis *et al.*, 2007, 2009; Dreyer and Lee, 1972; Kumar *et al.*, 2016; Levrier *et al.*, 2013, 2015).

The NatureBank biota repository (www.griffith.edu.au/gridd) was the source of the plant material from which the extracts were derived. In order to generate the plant extracts, a portion of dry plant material (300 mg) was added to a solid phase extraction (SPE) cartridge (Phenomenex polypropylene SPE, Torrance, California, USA, 10 mm × 50 mm), and dichloromethane (8 mL) followed by methanol (8 mL) were percolated through the material under gravity. Both organic extracts were combined and weighed in order to create the extract that was tested. HP (>99% purity) was obtained from the Davis Open Access Natural Product Library, which is currently housed at Compounds Australia (Griffith University; www.compoundsaustralia.com). All compounds were dissolved in DMSO at a final concentration of 100 mM and stored at –20 °C. Plant extract concentrations were calculated according to their prevalent compound (specified in Table 1) molarity, dissolved in DMSO and stored at 100 mM at –20 °C.

T3SS inhibition test

For T3SS inhibition tests, the *R. solanacearum* luminescent reporter strains described above were grown overnight in rich B medium and diluted to an OD_{600} of 0.3 in 1.5 mL of fresh Boucher's minimal medium supplemented with the test compounds. Compounds were normally evaluated at 100 μM (or 10 and 50 μM when testing the minimal effective concentration). Plant extracts were used at the equivalent molarity of their major compound (indicated in Table 1); 1.5 μL of DMSO was used as a control condition. Growth and luminescence measurements were taken at 0, 4, 6, 8 and 24 hpi. Luminescence was measured using an FB12 luminometer (Berthold Detection Systems, Pforzheim, Germany) and bacterial growth was measured as the OD_{600} in a V-1200 spectrophotometer (VWR, Radnor, Pensilvania, USA). Transcriptional output from the chosen promoters was expressed as relative luminescence units (luminometer values divided by 1000) normalized by dividing by the bacterial density of the culture. Importantly, both parameters (luminescence and cell number) have been proven to show a strong linear correlation (Cruz *et al.*, 2014; M. Planas-Marquès *et al.*, unpublished).

Effector secretion and immunodetection

To induce the production and secretion of the AvrA effector protein, 2×10^8 cells/mL were inoculated in 10 mL of minimal medium supplemented with 5 μg/mL gentamycin, 10 mM glutamate, 10 mM sucrose, 100 μg/mL Congo red and 100 μg/mL of the test compound (or 10 μL DMSO), and grown at 25 °C for 14 h. Bacterial cultures were centrifuged at 4000 *g* for 10 min and the culture medium supernatant was filter sterilized, mixed with 10 mL of cold 25% trichloroacetic acid and incubated overnight at 4 °C. Samples were then centrifuged at 6000 *g* for 30 min at 4 °C, the supernatant was discarded and the protein pellet was washed twice with cold 90% acetone. The bacterial pellet was dissolved in 50 μL of phosphate-buffered saline (PBS) once and 10 μL of Laemmli buffer five times, sonicated for 90 s (30% amplification, 10-s ON/OFF intervals) using a digital sonifier (Model 250/450, BRANSON, Danbury, Connecticut, USA) and boiled for 5 min. AvrA was detected by western blotting using a primary anti-HA rat monoclonal antibody already conjugated to horseradish peroxidase (HRP) (clone 3F10, Roche, Basel, Switzerland), diluted 1 : 4000 in 40 mL of Tris-buffered saline (TBS) supplemented with 0.1% Tween-20 and 1% skimmed milk. Immunodetected AvrA-HA was developed using Immobilon ECL (Millipore, Darmstadt, Germany) and membranes were photographed using a LAS-4000 mini system (Fujifilm, Tokyo, Japan).

Plant material and HR assays

Nicotiana benthamiana, *Nicotiana tabacum* cv. xanthi and *Solanum lycopersicum* cv. Marmande plants were grown for 3 weeks in pots containing peat soil in a glasshouse under long-day conditions (16 h light at 25 °C, 8 h dark at 22 °C).

For HR assays, *R. solanacearum* GMI1000 bearing the *PhrpY::luxCDABE* fusion was grown for 8 h in Boucher's minimal medium supplemented with glutamate and the test compound at 100 μM (or with DMSO for the non-treated condition). Bacteria were recovered by centrifugation, washed with sterile distilled water and adjusted to 10^7, 5×10^6, 10^6 and 10^5 cells/mL in sterile distilled water. Bacterial solutions were leaf infiltrated into *N. tabacum* and *N. benthamiana* plants. HR cell death was recorded at 2 days post-infiltration in *N. tabacum* plants and 5 days post-infiltration in *N. benthamiana* plants. For a better HR cell death

visualization, *N. benthamiana* leaves were ethanol bleached in 100% ethanol at 60 °C for 20 min.

Ralstonia solanacearum growth *in planta*

For *in planta* growth assays, *R. solanacearum* recovered from overnight cultures as described above was hand-infiltrated into tomato leaves at a final concentration of 10^5 colony-forming units (CFU)/mL together with compounds SA1–3 at 100 μM (or with DMSO alone in the non-treatment condition). Two 5-mm-diameter discs per biological replicate were taken from different infiltrated leaves, homogenized and 10 μL of serial ten-fold dilutions were plated in selective rich medium plates. The plates were incubated at 28 °C until the colonies could be counted. Samples were taken at day 0 and at day 3 after infiltration. Three biological replicates were used per treatment.

Virulence tests on tomato plants

Ralstonia solanacearum pathogenicity assays were performed as follows: 3-week-old tomato plants were acclimated for 3 days at 28 °C and in a 12-h/12-h photoperiod. Roots were wounded by disturbing the soil with a 1-mL pipette tip. Twenty five millilitres of a suspension containing 10^8 bacterial cells/mL supplemented with 100 μM of the test compound (or DMSO alone for the non-treated condition) were used to water each plant. Twelve plants were used in each condition and wilting symptoms were recorded per plant using an established semi-quantitative wilting scale ranging from 0 (no wilting) to 4 (death) (Vailleau *et al.*, 2007).

Pseudomonas syringae pv. *tomato* pathogenicity assays were performed as follows: 3-week-old tomato plants were sprayed with a 100 μM dilution of the test compound (or DMSO alone for the non-treated condition) and air dried for 1 h. Each plant was then sprayed with 6 mL of a *P. syringae* pv. *tomato* suspension at a final OD_{600} of 0.2. To maintain high humidity, plants were placed in trays inside transparent boxes containing a layer of water. Twenty plants were used in each test and three to four leaves were evaluated per plant. Symptoms were recorded for each leaf at 3 dpi using a necrosis scale (0, healthy leaf; 1, chlorosis; 2, necrosis in one leaflet; 3, chlorosis and necrosis in one leaflet; 4, necrosis in several leaflets; 5, chlorosis and necrosis in several leaflets; 6, general necrosis).

Statistical analyses

The effect of the compounds on gene expression and *in planta* bacterial growth was determined by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) *post hoc* test using the agricolae package (version 1.2-4) in R (version 3.3.3). Differences were considered to be statistically significant at $P < 0.05$.

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AUTHOR CONTRIBUTIONS

M.P. performed the experiments, analysed the data and wrote the manuscript. M.S. performed the experiments. B.L.-G. designed the research. N.S.C. analysed the data and wrote the manuscript. K.D.B. contributed materials and assisted in manuscript preparation. R.A.D. contributed materials and assisted in manuscript preparation. M.E. contributed materials and assisted in manuscript preparation. M.V. designed the research, analysed the data and wrote the manuscript.

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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 Chemical structures of the compounds used in this work.

Fig. S2 Analysis of time course expression of *hrpB* in the presence of candidate T3SS inhibitors. *hrpB* expression was quantified at 4, 6, 8 and 24 hpi by direct quantification of luminescence from bacteria growing in minimal medium supplemented with SA1 to 4, HP or E8 at 100 μM or with DMSO as control. Expression is represented as Relative Luminescent Units (RLU) normalized by bacterial density (OD600) at each time point. Four replicates were used in each measurement and the experiment was repeated two times with similar results.

Fig. S3 Hypersensitive response inhibition by salicylidene acylhydrazides. Bacteria grown for 8 hpi in liquid minimal medium after addition of SA1-3 at 100 μM or DMSO alone were serially diluted 5-fold in water (5×10^6, 10^6 and 5×10^5 CFUs/ml top to bottom for left and central leaves, 10^7, 5×10^6 and 10^6 CFUs/ml top to bottom for right leaf) and leaf-infiltrated in *Nicotiana benthamiana*. HR responses were photographed at 5 dpi. Leaves were ethanol-bleached for better HR visualization. Numbers indicate the proportion of positive leaves (showing HR inhibition due to T3SS suppressors) in relation to the total tested leaves, with the rest of leaves showing no effect.

Fig. S4 Wilting symptoms are unaltered in tomato plants pretreated with SA2 prior to *Ralstonia solanacearum* soil inoculation. Symptoms were recorded over time on tomato plants inoculated with *R. solanacearum* by soil drenching after watering with a DMSO solution (black triangles) or a SA2 solution (dashed line). As a control, plants watered with DMSO (white diamonds) or inoculated with *R. solanacearum* (black squares) were also included in the experiment. Disease progression was recorded per plant according to a scale ranging from 0 to 4 (0 - no wilting, 1 - 25% wilted leaves, 2 - 50%, 3 - 75%, 4 - dead plant). 12 plants were used per condition and each measurement corresponds to the mean and standard error.