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Type III secretion inhibitors for management of bacterial plant diseases

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Summary

Finding 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 they 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 9 plant extracts and 12 isolated compounds –including molecules effective against human pathogens– for their capacity to inhibit the T3SS of plant pathogens and 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 downregulate T3SS transcription, most of them through 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 in impaired plant responses. These candidate virulence inhibitors were then tested for their ability to protect plants. We demonstrate 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.

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 had traditionally been used (Zaumeyer 1958), however their application is now restricted in many countries (Duffy *et al.* 2005; Mackie *et al.* 2012), due to 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 since 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 its defences (Buttner 2016). In bacterial plant pathogens, the T3SS is encoded by the *hrp* genes, so called because they play a key role both in the hypersensitive response (HR) elicitation and in pathogenicity (Boucher *et al.* 1987). The HR is a programmed cell death reaction that takes place locally in plants upon pathogen recognition at the site of infection (Huysmans *et al.* 2017). In the model phytopathogenic bacterium *R. solanacearum,* the regulator HrpB directly activates 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*, that 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 the 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 and (–)-hopeaphenol (Davis *et al.* 2014; Kauppi *et al.* 2003; Kim *et al.* 2009; Li *et al.* 2013; Li *et al.* 2009; 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 infection showing no toxic effects on the host (Duncan *et al.* 2012). Treatment of infected animals has shown promising results for *Citrobacter rodentium* (Kimura 2011), *Yersinia pseudotuberculosis* (Garrity-Ryan 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 *p*-coumaric acid (PCA) was identified as an inhibitor of T3SS transcription in the phytopathogen *Dickeya dadantii* (Li *et al.* 2009). Recent reports show that some PCA derivatives can suppress T3SS functionality in *Xanthomonas oryzae* (Fan *et al.* 2017) and in *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.* 2012) to select those compounds/extracts that inhibit transcription of *R. solanacearum hrpB* and *hrpY* genes without affecting 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 controlling bacterial wilt or bacterial speck in tomato plants was examined.

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 *p*-coumaric acid and analogues (PP1-6), cytosporone B (CB), salicylidene acylhydrazides (SA1-4), (–)-hopeaphenol (HA) as well as the plant-derived extracts (E1-9). All tested molecules as well as their source are summarized in Table 1, and their chemical structure is represented in Supplementary Figure 1. To detect and quantify their inhibitory effects we took advantage of a strain that bears a transcriptional fusion of the *hrpY* promoter (*PhrpY*), controlling expression of the T3SS pilus component, with the *luxCDABE* operon (Monteiro *et al.* 2012). This strain emits luminescence and does not require antibiotic selection as the promoter::reporter fusion is stably integrated in 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 hours after incubation with each of the compounds and normalized by cell density (OD_{600}). Figure 1 shows *hrpY* expression levels after incubation with each extract/molecule normalized by expression levels in control conditions (DMSO addition). As shown in Figure 1 CB, SA1-4, HA, E8 and E9 showed a statistically significant (P<0.05) repression of *hrpY* expression. The inhibitory effect was mild after addition of compounds CB, SA4, HA, E8 and E9 while 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 characterisation.

Salicylidene acylhydrazides inhibit T3SS expression at the *hrpB* level

We performed a time-course analysis monitoring *hrpY* expression upon addition of varying amounts of the identified inhibitors to determine their minimal effective concentration (Figure 2). This experiment revealed that a minimal concentration of 10 μM for SA2, SA3 and HA, and 50 μM for SA1, was sufficient to cause full inhibition, while for SA4 and E8, 100 μM was needed for maximal effect. Next, to determine if the analysed substances caused a general inhibition of the T3SS, and not only on *hrpY*, we measured transcription of *hrpB* − the master regulator controlling expression of the T3SS genes − over time (Supplementary Figure 2). As can be observed in figure 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).

To rule out that the observed effects were due to a general, unspecific inhibition of gene expression we made use of a *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 in 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 Figure 4A, at 8 hpi slight but significant (P<0.05) induction of *PpsbA* expression was detected after bacterial incubation with SA2, SA3 and HA (Fig. 4A) showing that inhibition of the transcription of the T3SS was selective. However, one compound (SA1) caused repression of *PpsbA* expression, an effect probably due to the slower bacterial growth caused by incubation with this compound (see below and Figure 4B). Bacterial growth defects were not observed during our gene expression analyses using reporter strains. However, to accurately determine whether the used compounds affected

bacterial viability, we measured the growth of *R. solanacearum* PhrpY-Lux in liquid culture after addition of the transcriptional inhibitors. Figure 4B shows that, at their minimal inhibitory concentrations, SA1 and HA 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 if transcriptional inhibitors of the T3SS impaired its functionality, we tested their effect on the T3SS-dependent secretion of effector proteins *in vitro*. To this end, we used a *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 Figure 5, incubation of bacteria with the strongest T3SS inhibitors – the salicylidene acylhydrazide derivatives SA1, SA2 and SA3 – inhibited AvrA secretion, as this effector was 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 detection of secreted AvrA in the culture medium, although at lower levels than the control condition (DMSO).

The AvrA effector secreted by the *R. solanacearum* strain used in this work has been shown to trigger a Hypersensitive Response (HR) on tobacco plants (Poueymiro *et al.* 2009). To validate our *in vitro* results and determine whether inhibition of T3SS secretion was physiologically relevant *in planta*, we tested the influence of pre-incubating bacteria with salicylidene acylhydrazides on the plant HR. *N. tabacum* and *N. benthamiana* plants were

leaf-infiltrated with 5-fold *R. solanacearum* dilutions obtained after 8-hour incubation with SA1-3 or DMSO (control). As shown in Figure 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 evasion of recognition by the plant immune system. Similar results were obtained when using *N. benthamiana* as host (Supplementary Figure 3).

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 in limiting *R. solanacearum* infection, we measured multiplication of bacteria that were infiltrated on tomato leaves alone or in the presence of the inhibitors. As shown in Figure 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 a *R. solanacearum* inoculum containing SA2 at 100 μM (Supplementary Figure 4). However, as *R. solanacearum* infects plants through the roots, high 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 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 dpi in plants that had been pre-treated with SA1-3 compared to control plants (Figure 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 in crop production and contribute to significant economic losses yearly. 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 due to the rapid emergence of resistances 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 downregulate 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 expression of *hrpY*, which has the highest transcriptional output amongst the *hrp* genes (our own unpublished data). We found eight compounds and extracts (CB, SA1-4, HA, E8 and E9) capable of specifically repressing *hrpY* transcription to various degrees (Figure 1). Six of these inhibitors also repressed *hrpB* expression (Figure 3 and Supplementary Figure 2) and thus seem to act upstream of the *hrp* regulatory cascade. The exceptions are cytosporone B 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 (Figure 5). Our screening methodology has proved to be very effective, probably due to the high sensitivity of the luminescent reporter used. This system could also be scaled to 96-well plates or even be used qualitatively by presence/absence of light emission (Kauppi *et al.* 2003).

R. 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), whose T3SS is closely related to *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, privileging 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 were proven to bind to 16 *E. coli* proteins and it was suggested that they repressed T3SS expression by blocking the function of these proteins (Wang *et al.* 2011). Since orthologues for most of these target proteins exist in *R. solanacearum* and *P. syringae*, it would be of interest to check if they also interact with the

chemicals and, if so, through which mechanism that affect 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 of the plant phenolic compound *p*-coumaric acid (PCA) and its derivatives, which were recently found to act as T3SS inhibitors in *D. dadantii*, *E. amylovora* and *P. aeruginosa* (Khokhani *et al.* 2013; Li *et al.* 2009; Yamazaki *et al.* 2012). We showed that neither PCA nor some derivatives (PP1-3) were effective *R. solanacearum* T3SS inhibitors, similarly to what was 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* (Figure 7). However, no symptom reduction was visible in tomato wilting assays by soil-inoculating a *R. solanacearum* suspension containing one of these potent inhibitors (Supplementary Figure 4). *R. 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 part 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 less disease symptoms compared to control plants (Figure 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 showed that bacterial pre-treatment with other T3SS inhibitors could 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 proving 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 fields. Salicylidene acylhydrazides can be efficiently prepared in one step from commercially available starting materials. Finally, 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 strategy is 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.* 2012). Gene constructs were introduced in *R. solanacearum* GMI1000 through natural transformation of linearized plasmids and double-recombination events as described (Cruz *et al.* 2014; Monteiro *et al.* 2012). The hrpY reporter strain was constructed after transformation of SfiI-digested vector pRCG-PhrpY-lux. For pRCG-PhrpY-lux construction, the *hrpY* promoter was 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.* 2012) 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, USA) between plasmids pENTR/SD-AvrA and pRCG-Pps-GWY (Cruz *et al.* 2014). *R. solanacearum* was routinely grown in rich B medium (10g/l bactopeptone, 1 g/l yeast extract, 1 g/l casaminoacids, 0.5% glucose) supplemented with gentamycin 10 μg/ml (solid media) or 5 μg/ml (liquid media) at 28ºC. 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 28ºC 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 upon 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 Supplementary Figure 1. Synthetic plant phenylpropanoids (PP) 1–6 and cytosporone B (CB) were purchased from Sigma-Aldrich. Salicylidene acylhydrazides (SA) 1–4 were provided by Dr. M. Elofsson (Dahlgren *et al.* 2010; Nordfelth *et al.* 2005), and (–)-hopeaphenol (HA) (Davis *et al.* 2014; Zetterstrom *et al.* 2013) and the 9 plant extracts (E) 1–9 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.* 2009; Davis *et al.* 2007; Dreyer and Lee 1972; Kumar *et al.* 2016; Levrier *et al.* 2013; Levrier *et al.* 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, 10 mm x 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. (–)-Hopeaphenol (>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 a FB12 luminometer (Berthold detection systems) and bacterial growth was measured as the OD_{600} in a V-1200 spectrophotometer (VWR). Transcriptional output from the chosen promoters is expressed as Relative Luminescence Units (luminometer values divided by 1000) normalised 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), Planas-Marquès, M *et al*. unpublished).

Effector secretion and immunodetection

To induce production and secretion of AvrA effector protein, $2x10^8$ 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 ºC for 14 h. Bacterial cultures were centrifuged at 4000 *x g* for 10 minutes and the culture medium supernatant was filter sterilized, mixed with 10 ml of cold 25% trichloroacetic acid and incubated overnight at 4ºC. Samples were then centrifuged at 6000 *x g* for 30 minutes at 4 ºC, the supernatant was discarded and the protein pellet was washed twice with cold 90% acetone. The bacterial pellet was dissolved in 50 μl phosphate buffered saline (PBS) 1X and 10 μl Laemmli buffer 5X, sonicated for 90 sec (30% amplification, 10 sec ON/OFF intervals) using a Digital Sonifier (Model 250/450, Branson) and boiled for 5 min. AvrA was detected by Western Blotting using a primary anti-HA rat monoclonal antibody already conjugated to HRP (clone 3F10, Roche), diluted 1:4000 in 40 ml Tris-buffered saline (TBS) buffer supplemented with 0.1% tween 20 and 1% skimmed milk. Immunodetected AvrA-HA was developed using Immobilon ECL (Millipore) and membranes were photographed using a LAS-4000 mini system (Fujifilm).

Plant material and hypersensitive response 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 greenhouse under long-day conditions (16 h light at 25 ºC, 8 h dark at 22 ºC).

For hypersensitive response assays, *R. solanacearum* GMI1000 bearing the *PhrpY::luxCDABE* fusion was grown for 8 hours 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 in *Nicotiana tabacum* and *Nicotiana benthamiana* plants. Hypersensitive response 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 minutes.

R. solanacearum growth *in planta*

For *in planta* growth assays, *R. solanacearum* recovered from overnight cultures as described above were hand-infiltrated in tomato leaves at a final concentration of 10^5 CFU/ml together with compounds SA 1-3 at 100 µM (or with DMSO alone in the non-treatment condition). Two 5 mm-diameter disks per biological replicate were taken from different infiltrated leaves, homogenized and 10 µl of serial ten-fold dilutions plated in selective rich medium plates. Plates were incubated at 28 °C until 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

R. solanacearum pathogenicity assays were performed as follows: 3-week old tomato plants were acclimated for three days at 28 °C and 12/12 hour-photoperiod conditions. Roots were wounded by disturbing the soil with a 1 ml pipette tip. Twenty five ml of a suspension containing 10^8 bacterial cells/ml supplemented with 100 µM of the test compound (or DMSO alone for non-treated) was 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).

P. 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 non-treated) and air dried for 1 hour. Each plant was then sprayed with 6 ml of a *P. syringae* pv. tomato suspension at a final $OD_{600}= 0.2$. To maintain high humidity, plants were placed in trays inside transparent boxes containing a layer of water. A total of 20 plants were used in each test and 3-4 leaves were evaluated per plant. Symptoms were recorded for each leaf 3 days post inoculation 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 the Analysis of Variance (One-Way ANOVA) followed by the Tukey's HSD posthoc test using the agricolae package (version 1.2-4) in R (version 3.3.3). Differences were considered to be statistically significant at $P<0.05$.

Author contributions

M.P. performed experiments, analysed data and wrote the manuscript

M.S. performed experiments

B. L-G designed the research

N.S.C. analysed 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 data and wrote the manuscript

Conflict of interest: The authors declare that they have no conflict of interest.

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Figure legends

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

Figure 1. Expression of the 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 100 μM final concentration or with DMSO (control). *hrpY* expression was quantified at 8 hpi by luminescence, normalised by cell density and represented with respect to the value obtained with DMSO (control). Compounds/extracts marked with an asterisk showed statistical reduction (P<0.05) in *hrpY* expression compared to control conditions. Each measurement corresponds to the average of four replicates. The experiment was repeated three times with similar results.

Figure 2. Time course expression of *hrpY* after addition of selected T3SS inhibitors. *hrpY* expression was quantified at 4, 6, 8 and 24 hpi by direct luminescence quantification from bacteria growing in minimal medium supplemented with SA1 to 4, HA or E8 at different concentrations or with DMSO as control. Expression is represented as Relative Luminescent Units (RLU) normalized by bacterial density (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.

Figure 3. Expression of the main 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 100 μM final concentration or with DMSO (control). *hrpB* expression was quantified at 8 hpi by luminescence, normalised by cell density and represented with respect to that in DMSO. Compounds/extracts marked with an asterisk showed statistical reduction (P<0.05) in *hrpB* expression compared to control conditions. Each measurement corresponds to the average of four replicates. The experiment was repeated three times with similar results.

Figure 4. *PpsbA* transcription and *R. solanacearum* growth upon treatment with identified T3SS inhibitors. (a) *R. solanacearum* bearing the *PpsbA::luxCDABE* fusion was grown for 8 h in liquid minimal medium supplemented with each compound/extract at their *in vitro* minimal effective concentration (50 μM for SA1, 10 μM for SA2 and SA3, 50 μM for SA4 and HA, and 100 μM for E8). Transcription was quantified by measuring luminescence divided by bacterial growth. Percentage of *psbA* expression in each treatment was normalized by basal expression after 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 statistical ($P<0.05$) reduction or increase in *psbA* expression compared to 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 absorbance at 600 nm and are represented in a logarithmic scale.

Figure 5. Effector secretion is inhibited by bacterial pre-incubation with salycidene acylhydrazides. *R. solanacearum* bearing the *Pps-AvrA-HA* construct was grown for 8 hours in minimal medium supplemented with congo red to promote protein secretion and with each of the T3SS inhibitors (SA1-3 and E8) at 100 μM. Incubation with 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 SDS-PAGE membranes used in the Western Blotting are also shown.

Figure 6. 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 (10^7, $5 \cdot 10^6$, 10^6 and $5 \cdot 10^5$ CFUs/ml top to bottom) and leaf-infiltrated in *Nicotiana tabacum*. HR responses were photographed at 2 dpi. Numbers indicate the proportion of positive leaves (showing HR inhibition due to T3SS suppressors) in relation to the total tested leaves.

Figure 7. *Ralstonia solanacearum* growth in tomato is impaired by the addition of compounds SA1-3. *R. solanacearum* was leaf-inoculated at 10^5 CFU/ml with SA1-3 at 100 μM. Leaf disks were taken at 0 and 3 days after inoculation to monitor bacterial multiplication. Bacterial growth is represented as colony forming units (CFU) per mm^2 in

logarithmic scale at day 3 and day 0 (immediately after inoculation). Each point represents the mean of three biological replicates consisting on two different leaf disks. Experiments were repeated three times with similar results. Statistical groups were obtained following Tukey's HSD test using P<0.05.

Figure 8. Symptom development in tomato plants pre-treated with 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 DMSO 1 hour before bacterial inoculation. Symptoms were recorded three days post inoculation and are represented as A) percentage of leaves categorized in a disease scale from 0 (no visible symptoms) to 6 (extensive necrosis on >35% of the leaf), or as B) average on percentage of affected leaf surface with the corresponding standard error. Statistical groups were obtained with Tukey's HSD test using P<0.05. The experiment was performed three times with similar results.

Supplementary Figure 1. Chemical structures of the compounds used in this work.

Supplementary Figure 2. 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, HA or E8 at 100 μM or with DMSO as control. Expression is represented as Relative Luminescent Units (RLU) normalized by bacterial density (OD_{600}) at each time point. Four replicates were used in each measurement and the experiment was repeated two times with similar results.

Supplementary Figure 3. 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 ($5 \cdot 10^{6}$, 10^{6} and $5 \cdot 10^{5}$ CFUs/ml top to bottom for left and central leaves, 10^{7}, $5 \cdot 10^{6}$ 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.

Supplementary Figure 4. Wilting symptoms are unaltered in tomato plants pre-treated 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.

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

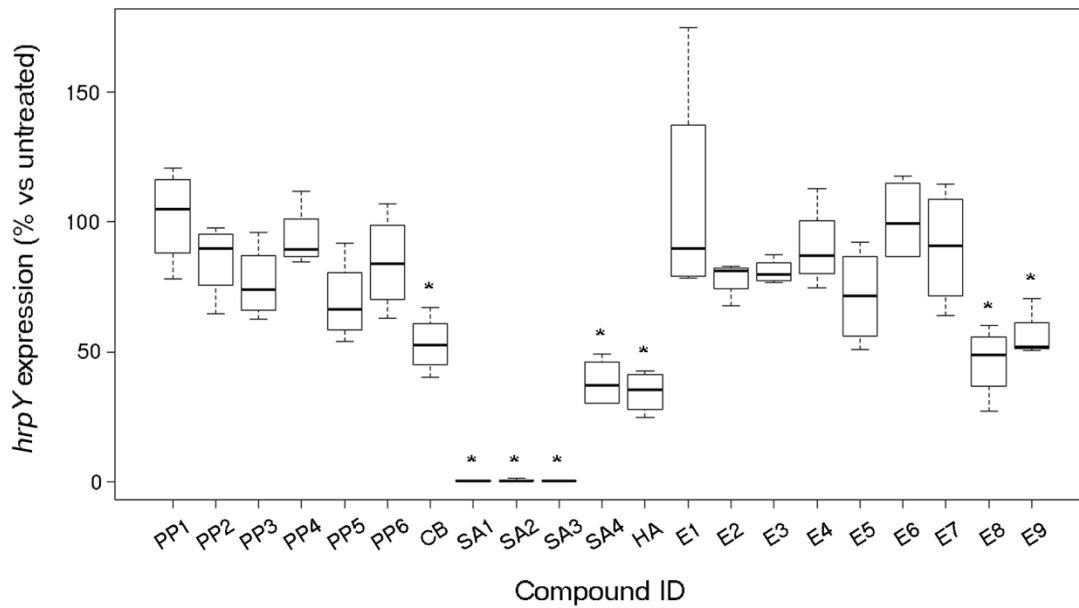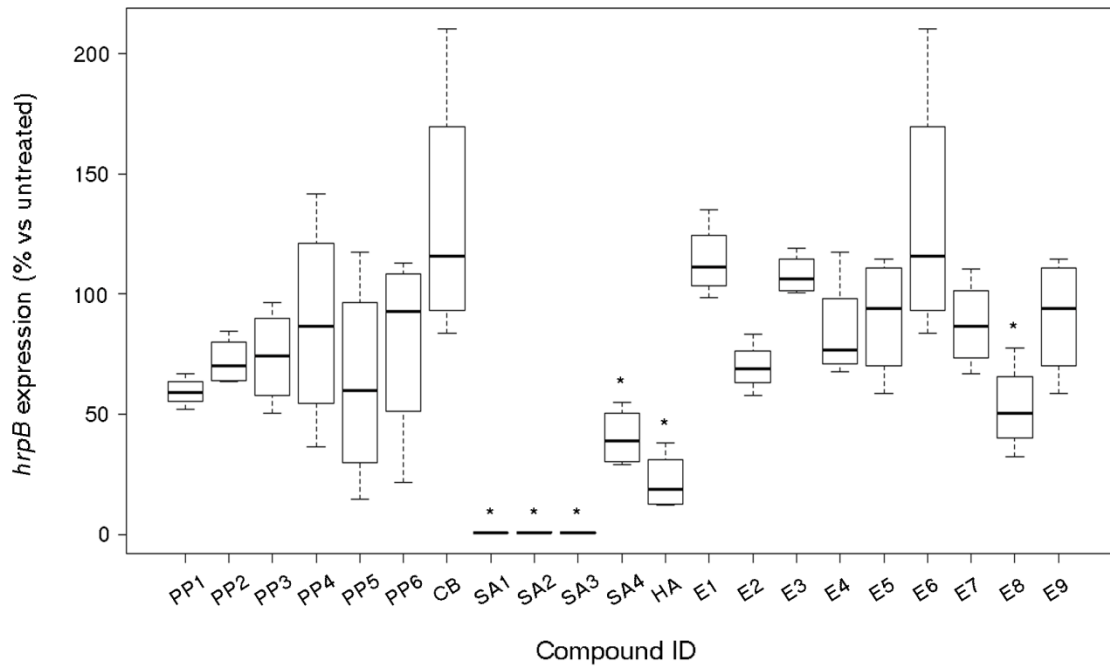| ID	Compound or the most abundant compound in extract	Source of the material (Reference)
PP1	*p*-coumaric acid	Synthetic plant phenylpropanoid (Li *et al.* 2009)
PP2	2,4-dihydroxycinnamic acid (umbellic acic)	Synthetic plant phenylpropanoid (Li *et al.* 2009)
PP3	4-chlorocinnamic acid	Synthetic plant 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-trihydoxy-benzylidene)-hydrazide)	Synthetic salicylidene acylhydrazide (Nordfelth *et al.* 2005)
SA2	ME0055 (4-nitrobenzoic acid N´-(2,4-dihydoxy-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)
HA	(-)-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.* 2009; Davis *et al.* 2007)
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)

Figure 1. Expression of the 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 100 µM final concentration or with DMSO (control). *hrpY* expression was quantified at 8 hpi by luminescence, normalised by cell density and represented with respect to the value obtained with DMSO (control). Compounds/extracts marked with an asterisk showed statistical reduction (P<0.05) in *hrpY* expression compared to control conditions. Each measurement corresponds to the average of four replicates. The experiment was repeated three times with similar results.

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hrpY expression was quantified at 4, 6, 8 and 24 hpi by direct luminescence quantification from bacteria growing in minimal medium supplemented with SA1 to 4, HA or E8 at different concentrations or with DMSO as control. Expression is represented as Relative Luminescent Units (RLU) normalized by bacterial density (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.

Figure 3. Expression of the main 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 100 μM final concentration or with DMSO (control). *hrpB* expression was quantified at 8 hpi by luminescence, normalised by cell density and represented with respect to that in DMSO. Compounds/extracts marked with an asterisk showed statistical reduction (P<0.05) in *hrpB* expression compared to control conditions. Each measurement corresponds to the average of four replicates. The experiment was repeated three times with similar results.
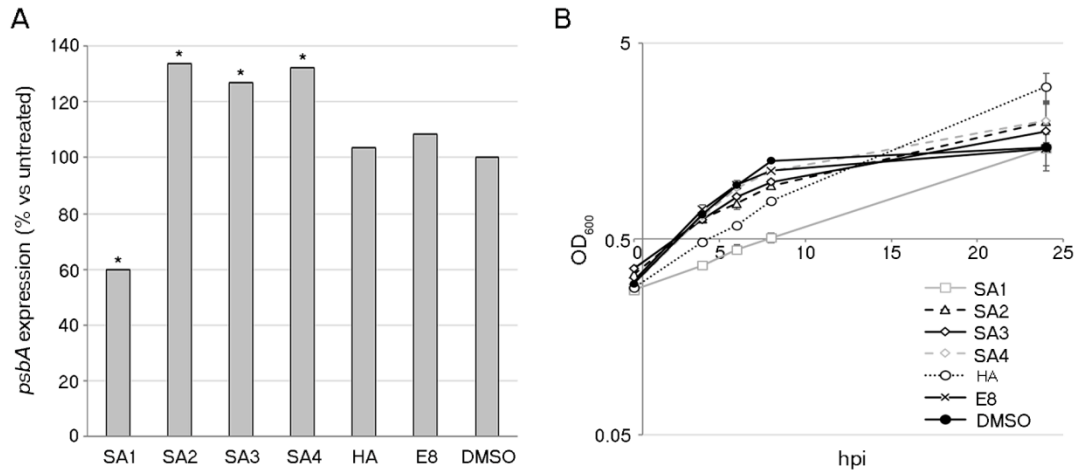
Figure 4. *PpsbA* transcription and *R. solanacearum* growth upon treatment with identified T3SS inhibitors.

(a) *R. solanacearum* bearing the *PpsbA::luxCDABE* fusion was grown for 8 h in liquid minimal medium supplemented with each compound/extract at their *in vitro* minimal effective concentration (50 µM for SA1, 10 µM for SA2 and SA3, 50 µM for SA4 and HA, and 100 µM for E8). Transcription was quantified by measuring luminescence divided by bacterial growth. Percentage of *psbA* expression in each treatment was normalized by basal expression after 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 statistical ($P<0.05$) reduction or increase in *psbA* expression compared to 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 absorbance at 600 nm and are represented in a logarithmic scale.
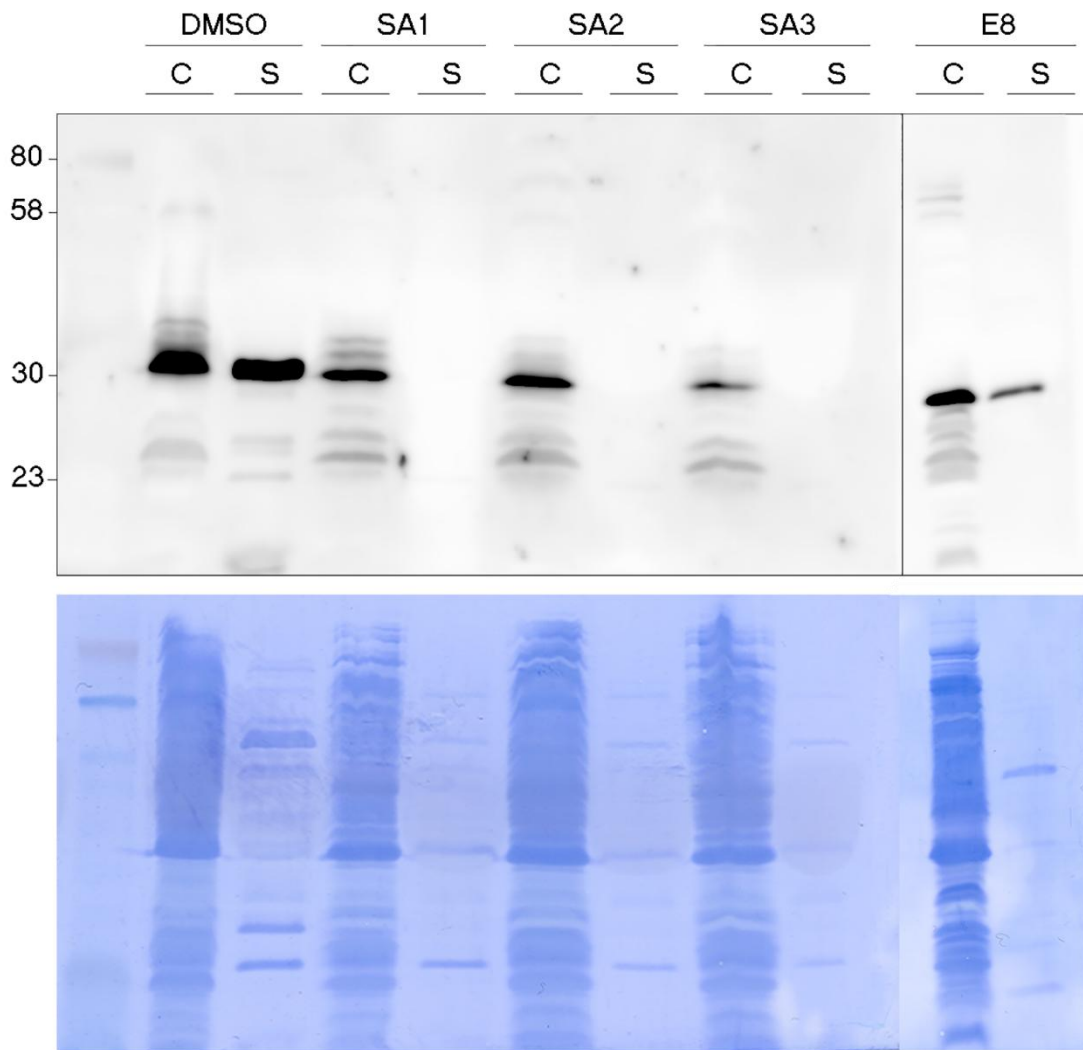
Figure 5. Effector secretion is inhibited by bacterial pre-incubation with salycidene acylhydrazides.

R. solanacearum bearing the *Pps-AvrA-HA* construct was grown for 8 hours in minimal medium supplemented with congo red to promote protein secretion and with each of the T3SS inhibitors (SA1-3 and E8) at 100 µM. Incubation with 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 SDS-PAGE membranes used in the Western Blotting are also shown.
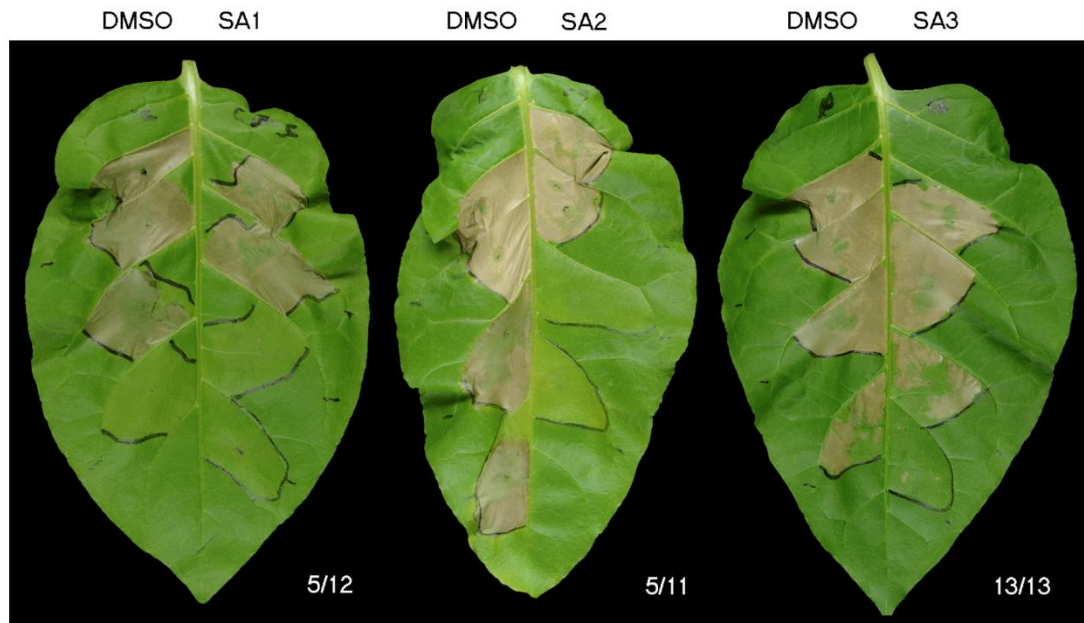
Figure 6. Hypersensitive response inhibition by salicylidene acylhydrazides.

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 (10^7, $5 \cdot 10^6$, 10^6 and $5 \cdot 10^5$ CFUs/ml top to bottom) and leaf-infiltrated in *Nicotiana tabacum*. HR responses were photographed at 2 dpi. Numbers indicate the proportion of positive leaves (showing HR inhibition due to T3SS suppresors) in relation to the total tested leaves.
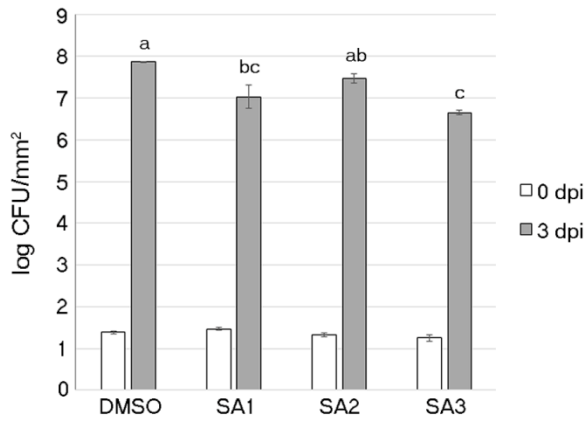
Figure 7. *Ralstonia solanacearum* growth in tomato is impared by the addition of compounds SA1-3.

R. solanacearum was leaf-inoculated at 10^5 CFU/ml with SA1-3 at 100 μM. Leaf disks were taken at 0 and 3 days after inoculation to monitor bacterial multiplication. Bacterial growth is represented as colony forming units (CFU) per mm^2 in logarithmic scale at day 3 and day 0 (immediately after inoculation). Each point represents the mean of three biological replicates consisting on two different leaf disks. Experiments were repeated three times with similar results. Statistical groups were obtained following Tukey's HSD test using $P < 0.05$.
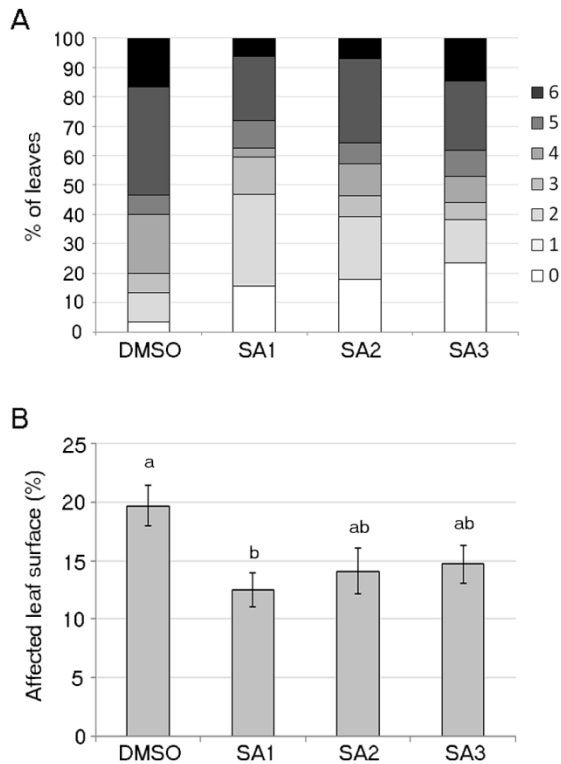
Figure 8. Symptom development in tomato plants pre-treated with T3SS inhibitors and inoculated with *Pseudomonas syringae* pv. tomato.

Effect of T3SS inhibitors on disease symptoms. Plants were pre-treated with SA1 to 3 at 100 µM or with DMSO 1 hour before bacterial inoculation. Symptoms were recorded three days post inoculation and are represented as A) percentage of leaves categorized in a disease scale from 0 (no visible symptoms) to 6 (extensive necrosis on >35% of the leaf), or as B) average on percentage of affected leaf surface with the corresponding standard error. Statistical groups were obtained with Tukey's HSD test using P<0.05. The experiment was performed three times with similar results.