Virulence of *Pseudomonas syringae* pv. *tomato* DC3000 Is Influenced by the Catabolite Repression Control Protein Crc

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Pseudomonas syringae infects diverse plant species and is widely used as a model system in the study of effector function and the molecular basis of plant diseases. Although the relationship between bacterial metabolism, nutrient acquisition, and virulence has attracted increasing attention in bacterial pathology, it is largely unexplored in *P. syringae*. The Crc (catabolite repression control) protein is a putative RNA-binding protein that regulates carbon metabolism as well as a number of other factors in the pseudomonads. Here, we show that deletion of *crc* increased bacterial swarming motility and biofilm formation. The *crc* mutant showed reduced growth and symptoms in *Arabidopsis* and tomato when compared with the wild-type strain. We have evidence that the *crc* mutant shows delayed hypersensitive response (HR) when infiltrated into *Nicotiana benthamiana* and tobacco. Interestingly, the *crc* mutant was more susceptible to hydrogen peroxide, suggesting that, in planta, the mutant may be sensitive to reactive oxygen species generated during pathogen-associated molecular pattern-triggered immunity (PTI). Indeed, HR was further delayed when PTI-induced tissues were challenged with the *crc* mutant. The *crc* mutant did not elicit an altered PTI response in plants compared with the wild-type strain. We conclude that Crc plays an important role in growth and survival during infection.

The plant pathogen strain *Pseudomonas syringae* pv. *tomato* DC3000 is the causal agent of bacterial speck on tomato (Cuppels 1986; Preston 2000) and has been studied extensively

as a model organism, with many efforts aimed at teasing apart the molecular mechanisms involved in pathogenesis and in overcoming plant defense responses (Xin and He 2013). The pathogen *P. syringae* is equipped with a number of virulence factors (Ichinose et al. 2013). Some factors shown to be important in plant-pathogen interactions include flagella, phyto-toxins such as coronatine, and extracellular polysaccharides. Flagella are important for plant colonization, invasion of the leaf apoplast, and bacterial entry through stomata (Nogales et al. 2015), as well as for the formation of cell aggregates called biofilms. *P. syringae* also produces several exopolysaccharides (EPSs), including alginate, levan, cellulose, and Psl (Arrebola et al. 2015; Laue et al. 2006; Records and Gross 2010; Yu et al. 1999). Many of these compounds have been shown to play a role in the attachment of bacterial cells to a surface (Laue et al. 2006) and formation or maintenance of biofilms (Laue et al. 2006) as well as pathogenesis of *P. syringae* (Arrebola et al. 2015; Yu et al. 1999).

Much attention has been given to the type III secretion system (T3SS) that deploys effector molecules into plant cells. This system significantly contributes to the ability of the pathogen to multiply and suppress the plant's basal defense responses or pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Alfano and Collmer 2004) and, most often, results in disease development in a compatible host. However, in an incompatible interaction with a nonhost, such as the interaction of *P. syringae* pv. *tomato* DC3000 with *Nicotiana benthamiana*, recognition of one or several T3SS effectors by appropriate resistance proteins results in the elicitation of a hypersensitive response (HR) that tends to restrict bacterial multiplication (Lamb and Dixon 1997). A considerable amount of effort has been given to the study of these interactions and the molecular events underlying disease resistance mechanisms.

P. syringae pv. *tomato* DC3000 is considered a hemibiotrophic pathogen capable of causing disease in tomato as well as *Arabidopsis*. When conditions are optimal, usually when it is wet and cool, the bacteria invade the plant via wounds or natural openings like stomata. Once inside the plant host, the bacterium resides in the apoplastic space, where it must obtain nutrients from the host in order to multiply and survive. An in-depth study of nutrient utilization by *P. syringae* (Rico and Preston 2008) showed that the sugars fructose, glucose, malate, and malonate, the organic acids citrate and succinate, and the amino acids γ -aminobutyric acid (GABA), aspartate, glutamate, glutamine, and serine are abundant in apoplastic extracts.

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DC3000 expresses pathways involved in the utilization of these molecules and, interestingly, appears to lack those pathways for assimilation of amino acids that are present in lower concentrations in the plant apoplast (Rico and Preston 2008). This lead to the hypothesis that *P. syringae* is specially adapted for growth in this particular environment. However, it is still not clear what nutrients are actually utilized by *P. syringae* during growth in plant tissue (Rico and Preston 2008).

The Crc protein is a catabolite repression control protein involved in regulating carbon metabolism in the *Pseudomonas* species (Morales et al. 2004; Rojo 2010). This system regulates the selection of preferred carbon sources by inhibiting the expression of the assimilation pathways for nonpreferred substrates when more desirable substrates are available. This optimization of bacterial metabolism allows for improved growth and ability of bacteria to thrive in the various settings they encounter (Görke and Stülke 2008; Poncet et al. 2009; Rojo 2010). Growth in poor carbon sources leads to activation of the CbrA/CbrB two-component system, although the signal that activates CbrA/CbrB is not known. The activation of this two-component system, along with the sigma factor RpoN, induces transcription of the small RNAs *crcZ* and *crcX* in *P. syringae* DC3000 (Filiatrault et al. 2013), *crcZ* and *crcY* in *P. putida* (Moreno et al. 2012), and *crcZ* in *P. aeruginosa* (Sonnleitner et al. 2009). These small RNAs are able to bind to Crc and relieve the Crc-mediated repression of mRNA targets, including genes encoding proteins involved in carbon utilization. Several models have been proposed to describe the molecular mechanism by which Crc inhibits translation of target mRNAs. Crc was initially thought to bind mRNAs directly at an AAnAAnAA motif (CA motif) near the translation start site and inhibit translation, but this idea was recently challenged (Milojevic et al. 2013). One model proposes that *crcZ* RNA binds to and sequesters the global RNA chaperone Hfq, which, in turn, relieves Hfq-mediated translational repression of mRNAs (Sonnleitner and Bläsi 2014). Another model proposes that Crc and Hfq cooperate in catabolite repression (Moreno et al. 2015). Further studies will help clarify the molecular mechanisms involved in catabolite repression in the pseudomonads.

Besides modulating catabolite repression and carbon metabolism, Crc has been shown to be involved in the regulation of several other factors, some of which are important for virulence. In *P. aeruginosa*, inactivation of the *crc* gene impairs swimming, swarming, and twitching motilities and increases EPS production and biofilm formation (Linares et al. 2010; O'Toole et al. 2000). Crc mutants also exhibit reduced virulence to *Dictyostelium discoideum* and reduced expression of quorum sensing-regulated virulence determinants, such as cyanide, pyoverdine, and elastase, but increased pyocyanin production (Linares et al. 2010; Yeung et al. 2011). We hypothesized that Crc may be involved in the regulation of virulence and other cellular processes in *P. syringae* pv. *tomato* DC3000 (hereafter referred to as DC3000). In this study, we constructed a *crc* deletion mutant of DC3000 and investigated the role of this protein in multiple virulence-related phenotypes.

RESULTS

Disruption of DC3000 *crc* alters motility.

To investigate the role of Crc in DC3000, we constructed a deletion mutant in DC3000 and investigated whether disruption of Crc affected virulence-related phenotypes. The first phenotypes we investigated were swimming and swarming motilities. The DC3000 Δ *crc* strain showed increased swarming compared with the wild-type (WT) strain when tested on nutrient broth (NB) medium with glucose (NBG) and minimal media plates

containing either fructose, succinate, or mannitol as the sole carbon source (Fig. 1). In minimal media with glucose as the sole carbon source, swarming of Δ *crc* was reduced compared with that in WT. We also observed that Δ *crc* displayed very little to no growth in liquid minimal medium supplemented with glucose (data not shown). The *crc* gene was cloned and was used to complement in *trans* the Δ *crc* strain. Swarming was lower than WT levels when Δ *crc* is complemented (Fig. 1), presumably because overexpression of Crc leads to a tighter control of target genes than in WT. No difference in swimming was observed between the WT, Δ *crc*, and complemented strain (data not shown).

Deletion of *crc* results in increased biofilm formation.

Since *P. aeruginosa* mutants that display altered swarming also are influenced in their ability to form biofilms (Wang et al. 2014), we tested the ability of the DC3000 Δ *crc* mutant to form a biofilm, using the microtiter biofilm cell attachment assay (O'Toole 2011). The Δ *crc* strain showed an enhancement in biofilm formation in comparison with the WT strain when performed under static conditions, using mannitol as the carbon source (Fig. 2A). However, under shaking conditions, we did not observe a difference in biofilm formation (Fig. 2A). In addition, we performed the biofilm experiments using various carbon sources (Supplementary Fig. S1). Biofilm formation was similar between the WT and *crc* mutant except when glucose was present and cultures were shaken, when we observed an increase in biofilm formation for the *crc* mutant compared with the WT. Contrary to the literature, we did not observe an inverse correlation between swarming and biofilm formation in the different media, with the exception of glucose. Biofilm formation was not restored to WT levels in the complemented strain in all cases (Fig. 2A). We hypothesize that, because a multicopy plasmid was used for the complementation, overexpression of Crc is occurring and this results in a different phenotype, depending on the media. No growth differences were observed when the optical density at 600 nm (OD₆₀₀) of planktonic cells was measured as a function of time during the period of growth in the microtiter wells (data not shown).

The *crc* mutant is not altered in EPS production.

The enhanced biofilm formation of the Δ *crc* mutant led us to investigate whether this mutant was altered in EPS production. Cellulose, Psl, and Pel have been shown to play a role in biofilm formation and swarming (Schurr 2013; Ude et al. 2006). While other strains of *Pseudomonas* contain the locus for Pel, *P. syringae* DC3000 does not contain this locus (Buell et al. 2003). Psl and cellulose polysaccharides can be observed by the addition of the dye Congo red to media. The strains were grown on Luria medium (LM) or NB agar containing 40 μ g of Congo red per milliliter. After three days of growth, there was no difference in Congo red-binding between the WT, Δ *crc*, or *crc*-complemented strain (Δ *crc*[pCrc]) when grown on LM or NB agar plates (Fig. 2B), suggesting that the EPS composition of the Δ *crc* strain was not altered. The Δ *cbrA* strain (Filiatrault et al. 2013) was used as a positive control, since this mutant has been shown to display enhanced binding of Congo red (Yeung et al. 2011).

Crc plays a role in virulence of *P. syringae* DC3000.

In order to test the involvement of Crc in virulence, both *Arabidopsis* and tomato plants were dip-inoculated in suspensions of WT and the Δ *crc* mutant. The Δ *crc* mutant caused fewer disease symptoms as compared with WT on both species (Fig. 3A and B). We also evaluated symptom formation in syringe-inoculated tomato leaves and observed that Δ *crc*

caused reduced symptoms, as seen by reduced chlorosis, compared with the WT strain (Fig. 3C). We performed growth assays in dip-inoculated *Arabidopsis* plants and observed that the Δcrc mutant grew less than WT (Supplementary Fig. S2).

We then performed bacterial growth assays in dip- or syringe-inoculated tomato and found that the Δcrc mutant displayed reduced growth compared with the WT strain at earlier time points (Fig. 4). Lower growth was significantly different 4 or 2 days postinoculation in dipped or syringe-inoculated plants, respectively. The growth of Δcrc was still lower than WT at later time points, but the difference in growth between the strains was not statistically significant (Fig. 4). Therefore, disease symptoms or growth of Δcrc , or both, were reduced, irrespective of the mode of bacterial entry into plant tissues in both tomato and *Arabidopsis* (dipping versus syringe infiltration). Based on these results, we conclude that *crc* is necessary for initial growth and fitness in planta.

Deletion of *Crc* reduces the ability to elicit HR.

DC3000 elicits a HR in tobacco and *N. benthamiana* (Alfano et al. 1997; Wei et al. 2007). The HR is a localized defense response associated with immunity and involves rapid, programmed plant cell death (Coll et al. 2011; Greenberg and Yao 2004). We found that the Δcrc strain showed reduced or delayed HR in *N. benthamiana* and tobacco compared with the WT strain (Fig. 5A). Delayed HR was also observed in tobacco, but the number of times the *crc* mutant caused delayed HR was not

found to be significant. This phenotype was restored when the *crc* mutant was complemented (Fig. 5A). Since the HR response due to Δcrc was reduced but not abolished, we used a cell-death scoring system in order to obtain a better comparison between Δcrc and WT in *N. benthamiana* (Fig. 5B). This scoring system has been used previously to compare samples undergoing cell death (Oh et al. 2010; Rosli et al. 2013) and categorizes the degree of cell death observed into different classes, allowing for better visualization of the data. This analysis clearly shows that overall Δcrc is reduced in the ability to elicit HR. A comparison was made between the total number of times full HR was observed in inoculated leaf areas of WT (28 of 33 times) versus Δcrc -inoculated leaf areas (eight of 33 times), in six independent experiments. Fisher's exact test showed that Δcrc was significantly different from WT ($P < 0.05$).

We compared growth of WT versus Δcrc in *N. benthamiana* leaves undergoing HR and found that they grew to similar levels 18 h after inoculation (Supplementary Fig. S3).

The *Crc* mutant is not affected in its ability to translocate effectors.

Since Δcrc displayed a delayed or reduced HR phenotype and decreased virulence, we speculated about the ability if the *crc* mutant to translocate effectors efficiently into plant cells. *Crc* has been shown to play a role in the regulation of T3SS genes in *P. aeruginosa* (Dong et al. 2013). In order to test

Fig. 1. Inactivation of *crc* increases swarming motility of *Pseudomonas syringae*. Average diameter of swarming colonies with standard deviations of *P. syringae* strains measured after 24 h growth at 28°C. Swarming was evaluated in minimal media with glucose, fructose, mannitol, or succinate as the sole carbon source, and nutrient broth medium with glucose (NBG). The samples significantly different from wild type (WT), as measured using student's *t* test ($P < 0.05$), are shown with an asterisk. The experiment was repeated three times with similar results. The vertical bars represent the standard deviation of the mean.

T3SS-mediated effector delivery in Δcrc , we performed an effector translocation assay using the adenylate cyclase (Cya) reporter protein fused to the AvrPto effector (Schechter et al. 2004).

Accumulation of cAMP, a product of Cya activity, was measured in *N. benthamiana* leaves inoculated with WT DC3000 and Δcrc expressing AvrPto-Cya (Fig. 5C). Both strains were found to translocate at similar levels, while little cAMP accumulation occurred when leaves were inoculated with the DC3000 T3SS-deficient mutant expressing AvrPto-Cya. Therefore, since Δcrc translocates AvrPto-Cya as well as WT DC3000, this suggests that the Δcrc mutant strain has a functional T3SS and the reduced HR and virulence phenotypes observed are due to other factors.

Crc influences susceptibility to reactive oxygen species (ROS) in *P. syringae*.

HR is characterized by the induction of defense responses, including production of active oxygen species and synthesis of antimicrobial compounds like phytoalexins (Coll et al. 2011; Greenberg and Yao 2004). Therefore, we investigated whether the presence of hydrogen peroxide altered the growth of the Δcrc strain. Disruption of *crc* rendered the bacteria more sensitive to H_2O_2 at a concentration of 0.0225% in liquid King's B (KB) medium after 8 h of growth (Fig. 6A). Growth was restored in the complemented strain. This suggests that Crc may play a role in the response to H_2O_2 stress.

Fig. 2. Inactivation of *crc* causes an increase in biofilm formation under static conditions but does not affect exopolysaccharide (EPS) production. **A**, Biofilm formation in mannitol-glutamate medium was measured by the crystal violet-staining method and was expressed as optical density at 570 nm (OD_{570}). **B**, The indicated strains were spotted onto Luria medium (LM) or nutrient broth (NB) agar media containing 40 μ g of Congo red per milliliter. The addition of Congo red to the media is to visualize EPS production. The $\Delta cbrA$ strain was used as a positive control. Plates were photographed 2 days after spotting and the experiment was repeated two times with similar results.

The Δcrc strain is more sensitive to PTI.

Preinoculation of *N. benthamiana* leaves with a nonpathogen such as *P. fluorescens* to induce PTI followed by challenge-inoculation of an avirulent bacterium abolishes or delays HR because of the induction of PTI (Oh et al. 2010). We hypothesized that if the Δcrc mutant was more sensitive to PTI, we would observe total abolishment or even a more significant delay in HR when PTI is induced. To test this hypothesis, we used the overlapping challenge assay in *N. benthamiana* (Chakravarthy et al. 2009) to compare the ability to elicit HR in PTI-induced versus noninduced tissues. *N. benthamiana* leaves were inoculated with WT *P. fluorescens*, which induces PTI and a ROS response or with *P. fluorescens* $\Delta fliC$, which is unable to elicit PTI and ROS (Oh et al. 2010; Wei et al. 2013), and, 7 h later, overlapping challenge inoculations were performed with Δcrc or WT bacteria.

Cell death due to WT was seen by 24 h in the nonoverlapping area (Fig. 6B), but Δcrc , as expected, did not cause cell death in overlapping or non-overlapping areas at this same time point. After 48 h, the WT began to show partial HR in the overlapping areas, in contrast to Δcrc where little to no HR was observed in the overlapping areas in which PTI was induced (Fig. 6B and C). Δcrc caused HR in overlapping areas in control samples in which PTI was not induced (*P. fluorescens* $\Delta fliC$ or buffer). We quantitated the HR in overlapping areas of infiltration at 48 h, using a cell-death scoring system (Fig. 6C) (Oh et al. 2010; Rosli et al. 2013). We compared the number of times partial to full cell death was observed in overlapping areas challenged with WT (10 of 19) versus Δcrc (three of 19) in three independent experiments. Fisher's exact test showed that Δcrc

Fig. 3. *Pseudomonas syringae* pv. tomato DC3000 Δcrc causes reduced disease symptoms. **A**, *Arabidopsis* plants were dip-inoculated with wild type (WT) or Δcrc at 1×10^7 CFU/ml. **B**, Tomato plants were dip-inoculated with WT or Δcrc at 3×10^7 CFU/ml. **C**, Tomato leaves were syringe-inoculated with WT or Δcrc at 1×10^4 CFU/ml. Representative photographs were taken 4 days after *Arabidopsis* dip-inoculation and tomato syringe-infiltration and 6 days after tomato dip-inoculation in all treatments. The experiments were repeated at least five times with similar results.

was significantly different from WT ($P < 0.05$). Our results, therefore, show that the ability of Δcrc to elicit HR is further reduced in PTI- induced tissues. Taking into account our observation that Δcrc is sensitive to H_2O_2 stress in cultures (Fig. 6A), we hypothesize that Δcrc may be affected by ROS during the PTI response.

The Δcrc mutant elicits similar levels of PTI marker gene expression and ROS production compared with WT in *N. benthamiana* leaves.

We speculated that if the Δcrc mutant elicited a different PTI response in plants compared with WT, this may render the mutant more sensitive to ROS produced during PTI. We measured the expression of known PTI marker genes, described previously (Nguyen et al. 2010), in *N. benthamiana* leaves inoculated with WT or Δcrc . Since WT would elicit HR, we chose to examine gene expression 3 h postinoculation, prior to the translocation of type III effectors (Anderson et al. 2014; Pombo et al. 2014). This earlier time point should measure the gene expression in response to microbe-associated molecular patterns (MAMPs) (or PAMPs). Buffer-inoculated tissue was used as a control. The expression of *GRAS2*, *ACRE31*, and *Pti5* was induced in tissues inoculated with WT DC3000 as compared with buffer-inoculated control (Fig. 7A). In tissues inoculated with Δcrc , we found similar levels of induction of all three genes, compared with the buffer control, as seen with WT. Therefore, Δcrc and WT induced similar levels of PTI marker gene expression (Fig. 7A).

We also measured the production of ROS in *N. benthamiana* leaf discs incubated with WT, Δcrc , or the complemented mutant (Fig. 7B). ROS are produced as an early response to MAMPs

during PTI (Nicaise et al. 2009; Wu et al. 2014). The levels of ROS produced during coinocubation of leaf discs and the bacterial strains was similar.

Since no difference was detected in the ability of the Δcrc mutant to produce ROS compared with WT or induced gene expression of known PTI marker genes, we investigated whether Crc influences expression of known genes involved in virulence in DC3000. It was also possible that, although the Δcrc mutant was able to translocate the effector AvrPto (Fig. 5C), Crc may display altered expression of other components of the type III system or other genes reported to be involved in virulence. Therefore, we isolated RNA from WT and Δcrc strains grown in various media and performed quantitative reverse transcription-polymerase chain reaction (qRT-PCR). All the genes we tested showed no significant difference in expression between the WT and the Δcrc mutant (Supplementary Fig. S4).

Fig. 4. *Pseudomonas syringae* pv. *tomato* DC3000 Δcrc shows reduced growth in plants. **A**, Tomato plants were dip-inoculated with wild type (WT) or Δcrc at 3×10^7 CFU/ml, and bacterial growth was measured 4 and 6 days postinoculation. **B**, Tomato leaves were syringe-infiltrated with WT or Δcrc at 1×10^4 CFU/ml and bacterial growth was measured 2 and 4 days postinoculation. Growth is depicted as means with standard deviation of average Log(CFU/cm²) from three plants per sample. Inoculum (Log[CFU/ml]) is shown. Students *t* test was performed to analyze statistical significance of growth difference at $P < 0.05$, and populations significantly different from WT are indicated with an asterisk. The experiment was repeated five times with similar results.

Fig. 5. The *crc* mutant causes delayed hypersensitive response (HR) in *Nicotiana benthamiana* and tobacco and translocates AvrPto-Cya to similar levels as wild-type (WT) DC3000. **A**, *N. benthamiana* and tobacco leaves were infiltrated with the indicated strains at 2×10^7 CFU/ml (optical density at 600 nm = 0.02) and were photographed 1 day after inoculation. **B**, HR cell death in *N. benthamiana* caused by WT and Δcrc was scored as full, partial, or none. The number of spots showing cell death in each category was scored, and a 100% stacked column representing the scoring results is shown. The HR inoculation and scoring experiments were repeated at least three times with similar results. **C**, DC3000 and Δcrc mutant were compared for their ability to translocate the AvrPto-Cya translocation reporter expressed from plasmid pCPP5388 (Lam et al. 2014). The type three secretion system-deficient strain containing pCPP5388 was used as a control. Bacteria were infiltrated into *N. benthamiana* leaves at 5×10^7 CFU/ml and, 6 h later, leaf discs were harvested and the amount of cAMP measured. The average cAMP (in picomoles) with standard deviation from three plants is shown. The experiment was repeated three times with similar results.

DISCUSSION

The posttranscriptional regulator Crc has been reported to modulate motility, antibiotic susceptibility, and virulence of *P. aeruginosa* (Linares et al. 2010; O'Toole et al. 2000). Interestingly, strain-specific phenotypes for the role of Crc in bacteria have been observed. For example, a *crc* mutant of *P. aeruginosa* PA14 is defective in twitching motility and formation of biofilms (O'Toole et al. 2000), whereas in *P. aeruginosa* PAO1, lack of Crc leads to reduced swarming and increased cell aggregation (Linares et al. 2010). In the present study, we show that inactivation of Crc leads to altered motility, cell aggregation, and virulence of *P. syringae* pv. *tomato* DC3000. The reduced virulence appears to be independent of the T3SS, but due, rather, to an increased susceptibility to PTI.

Fig. 6. Susceptibility to hydrogen peroxide and pathogen-associated molecular pattern-triggered immunity (PTI) for the wild type (WT) and Δ *crc* mutant. **A**, The optical density of the indicated strains was compared by the addition of hydrogen peroxide at 0.0225% for the WT, Δ *crc* mutant, and complemented strains. The experiment was repeated three times and the values were averaged. Vertical bars represent standard deviation of the mean. **B**, Susceptibility to PTI using the overlapping challenge assay. *Nicotiana benthamiana* leaves were syringe-infiltrated with *Pseudomonas fluorescens* or *P. fluorescens* lacking *fliC* (*P.f.* Δ *fliC*) at 5×10^8 CFU/ml. Buffer was used as a control. The spots were challenged, 7 h later, with WT DC3000 or Δ *crc* at 2×10^7 CFU/ml. The areas inoculated with *P. fluorescens* or *P. fluorescens* Δ *fliC* are circled with a black marker. Photographs were taken 24 or 48 h post-challenge inoculation to observe cell death outside or inside the area of overlapping infiltration. **C**, Hypersensitive response (HR) cell death in the overlapping area of infiltration was scored as full, partial, or none. HR due to WT or Δ *crc* in PTI-induced or non-induced tissues is shown as a 100% stacked column. Five to ten plants were inoculated in each experiment and the experiment was repeated three times with similar results.

The pseudomonads can perform various forms of motility. Swimming motility is defined as movement of a single bacterium in a liquid or low-viscous medium and requires functional flagella. Swarming motility, which is coordinated multicellular movement (or group behavior) across a semisolid surface, also involves flagellum but, in addition, requires the presence of other factors, such as biosurfactants. It was also recently demonstrated that DC3000 performs a type of motility, a sliding movement, that is flagella-independent but syringofactin-dependent (Nogales et al. 2015). Motility plays an important role in the ability of bacteria to colonize different environments and attach to surfaces and in the subsequent formation of cell aggregates or cell-associated communities called biofilms. Reports indicate that there is an inverse relationship between swarming ability and cell aggregation, or biofilm formation (Caiazza et al. 2007), in *P. aeruginosa*. In most cases, impaired motility results in enhanced biofilm formation and vice versa. Therefore, it was surprising to see that the *crc* mutant in DC3000 demonstrated both an increase in swarming and biofilm formation. However, differences in biofilm formation were dependent upon media and whether or not cultures were static or shaking. Since swimming motility was not affected, this

Fig. 7. Induction of pathogen-associated molecular pattern-triggered immunity (PTI) reporter genes *Acre31*, *Gras2*, and *Pti5* and reactive oxygen species (ROS) elicitation by wild type (WT) and Δ *crc* in *Nicotiana benthamiana*. **A**, *N. benthamiana* leaves were syringe-infiltrated with 2×10^8 CFU/ml of WT or Δ *crc* and 10 mM MgCl₂ was infiltrated as a control. Leaf samples were harvested after 3 h and RNA was isolated. Quantitative reverse transcription-polymerase chain reaction was performed using cDNA prepared from the different samples. The fold induction of reporter genes in WT- or Δ *crc*-inoculated samples over that in control samples was calculated using the cycle threshold ($2^{-\Delta\Delta C_t}$) method. The average fold induction over control of three plants per strain with standard deviation is shown. The experiment was repeated three times with similar results. Students *t* test revealed no significant differences in the level of induction of genes between WT and Δ *crc* ($P < 0.05$). **B**, Leaf discs were excised and incubated with 12×10^7 CFU/ml of DC3000 WT (blue line), Δ *crc* (red line), Δ *crc* (pCrc) (green line), or 10mM MgCl₂ (purple line) as control. Production of ROS over time was measured using a L-012-based chemiluminescence assay. The average relative light units (R.L.U.) for at least 40 leaf discs per strain with standard deviation is shown. Measurements were taken every 4 min over 2 h. Students *t* test revealed no significant differences in ROS production between WT and Δ *crc* or Δ *crc* (pCrc) ($P < 0.05$).

suggests that the *crc* mutant is not attenuated in the functionality of flagella. We also found that expression of *fliC* was similar between the strains in the different media. However, our observations that disease symptoms were reduced during dip-inoculation and syringe-infiltration (Fig. 3) may imply that flagellar function is somehow affected in the *crc* mutant, since dip-inoculation would require flagella for entry into plant tissue.

We chose to investigate the production of the EPSs, Psl, and cellulose, using a Congo red binding assay, since cellulose has also been identified as a matrix component in other *Pseudomonas* spp. biofilms, including *P. syringae* (Ude et al. 2006). There were no noticeable differences in production of these molecules by the DC3000 *crc* mutant. This is in contrast to *P. aeruginosa*, in which the *crc* mutant displays Congo red binding. The observation that the *crc* mutant in DC3000 does not demonstrate differences in Congo red binding like that in *P. aeruginosa* suggests different pathways of regulation in the plant pathogen *P. syringae*.

Swarming motility, a coordinated movement of bacterial cells across a semisolid surface (Dézziel et al. 2003), generally requires production of a surface wetting material or surfactant, like rhamnolipids or rhamnolipid precursors 3-(3-hydroxyalkanoyloxy) alkanolic acid (HAA). In *P. aeruginosa*, mono- and di-rhamnolipids as well as HAAs are secreted during swarming. The production of HAA has been shown by *P. syringae* pv. *syringae* B728a (Burch et al. 2012). *P. syringae* pv. *syringae* B728a also produces lipopeptides called syringofactins, shown to be involved in swarming motility (Berti et al. 2007; Burch et al. 2010). It should be noted that *P. syringae* pv. *syringae* B728a and DC3000 differ in their lifestyles, with B728a displaying a more pronounced epiphytic growth stage in contrast to DC3000, which is considered a weak epiphyte (Boureau et al. 2002; Xin and He 2013). Although we did not investigate the ability of the *crc* mutant to produce surfactants, it is possible that the *crc* mutant has enhanced production of these molecules. Since motility, in particular HAA-mediated swarming, is favored in an epiphytic environment rather than in planta (Yu et al. 2013), one might speculate that enhanced swarming or biofilm formation may be detrimental to DC3000 once inside the plant cell. Recently, it was shown that *Pseudomonas cichorii* mutants unable to produce a lipopeptide called cicofactins displayed enhanced biofilm formation and were less pathogenic than the WT strains, due to the inability to spread within the plant (Pauwelyn et al. 2013).

Inactivation of the *crc* gene leads to reduced virulence. One explanation may be the effect Crc has on metabolism of the bacterium in the apoplast. In planta, the sugars fructose, sucrose, and citrate are highly abundant. To allow expression of these metabolic pathways that are involved in utilization of these sugars, Crc would be needed to be sequestered. Sequestration would likely involve the small RNA *crcZ* and *crcX*. Indeed, we have shown in vitro that deletion of the small RNA *crcZX* results in reduced ability to grow in media in which fructose and other sugars are provided as the main carbon source (Filiatrault et al. 2013). Therefore, the deletion of both *crcZ* and *crcX* might result in reduced growth and symptoms in planta. However, a *crcZX* deletion strain is still able to cause disease and HR (data not shown). Alternatively, *P. syringae* could preferentially use the most abundant amino acids present in apoplastic fluid (GABA, aspartic acid, glutaric acid, alanine, serine, and glutamine) (Park et al. 2010; Rico and Preston 2008) and alleviate the need to relieve Crc-mediated repression of the pathways involved in, e.g., fructose and citric acid. However, studies evaluating the transcriptional response of *P. syringae* B728a have shown that genes involved in the metabolism of sucrose and inositol as well as genes for GABA metabolism

were up-regulated in bacteria growing in the apoplast compared with bacteria on the leaf surface (Yu et al. 2013). Unfortunately, this study could not determine the regulation of genes involved in fructose, mannitol, and succinate in planta, because these components were present in the basal medium. The fact that the *crc* mutant displays reduced virulence and reduced growth in planta suggests that, in addition to regulating expression of nutritional factors, Crc regulates other factors important for virulence. Interestingly, the intracellular signaling molecule cyclic di-GMP (c-di-GMP) is a secondary messenger that controls the switch between single-celled motile and sessile lifestyles in *Pseudomonas* spp. (Valentini and Filloux 2016). C-di-GMP is responsible for the regulation of biofilm formation, dispersion, motility, EPS production, the cell cycle, and virulence (Römling et al. 2013). For *P. syringae* DC3000, increasing c-di-GMP concentrations reduced motility and increased biofilm formation (Pérez-Mendoza et al. 2014). In addition, elevated c-di-GMP levels in DC3000 inhibit flagellin synthesis, allowing bacteria to evade PTI (Engl et al. 2014; Pfeilmeier et al. 2016). However, virulence is reduced when c-di-GMP levels are increased (Engl et al. 2014; Pfeilmeier et al. 2016). Since disruption of Crc also influences motility, biofilm formation, and virulence in DC3000, one might speculate crosstalk between pathways regulated by Crc, such as catabolite repression and c-di-GMP. Although catabolite repression occurs via another mechanism in *Xanthomonas* spp., it has been shown that expression of the GGDEF domain protein XCC2731 is regulated by carbon source (Hsiao et al. 2011). Therefore, it may be worth investigating whether Crc impacts expression of c-di-GMP proteins in *P. syringae* DC3000.

The virulence of *P. syringae* DC3000 relies on the activity of effectors delivered by the T3SS and on the phytotoxin coronatine, which plays an important role in symptom development (Xin and He 2013). The *crc* mutant caused reduction of either virulence symptoms, growth, or both in tomato and *Arabidopsis*. The infected plants still showed some symptoms of bacterial speck disease in tomato and chlorosis in *Arabidopsis*, suggesting that coronatine function was not affected. The *crc* mutant also did not display growth defects in vitro (data not shown). Therefore, we ruled out the possibility that reduced virulence resulted from the lack or inability to metabolize carbohydrates in plant tissues.

The first line of active plant defense is PTI, which involves detection of conserved microbial factors, such as bacterial flagella, peptidoglycan, and lipopolysaccharides, via host-pattern recognition receptors (Monaghan and Zipfel 2012). Successful pathogens are able to overcome PTI, due to the virulence activity of secreted effectors. The recognition of effectors by plant resistance genes leads to the second line of defense, effector-triggered immunity (ETI), which is stronger and more effective at restricting the spread of pathogens. The HR has been proposed to be a manifestation of ETI. It is a localized defense response and involves rapid, programmed plant cell death (Coll et al. 2011; Greenberg and Yao 2004). The *crc* mutant caused delayed or reduced HR in tobacco and *N. benthamiana*, but because effector translocation was not altered, this indicates that the T3SS is functional in the *crc* mutant. Furthermore, since the mutant is still able to eventually elicit HR in *N. benthamiana*, this also suggests that HopQ1-1-mediated HR has not been disrupted in the *crc* mutant, since HopQ1-1 has been identified as an avirulence determinant in *N. benthamiana* (Wei et al. 2007). Further, our qRT-PCR data also examined the expression of *hopQ1-1*, and we found that it was expressed to similar levels in WT and the *crc* mutant.

The production of ROS is a hallmark of HR and PTI. We wondered if the *crc* mutant was more sensitive to ROS, and indeed, we found that it was more sensitive to oxidative stress

compared with the WT strain in vitro. Furthermore, our experiment with the challenge assay also showed that PTI causes a delay in *crc*-mediated HR, likely due to PTI-mediated ROS production. Although our results suggest that reduced virulence and altered ability to elicit HR is due to an increase in sensitivity to ROS, we cannot rule out that Δ *crc* is affected in the number or presentation of PAMPs.

We chose to study the expression of T3SS-related regulatory or structural genes *hrpL*, *hrcC*, *hrpR*, T3E *hopQ1-1* (Lam et al. 2014), flagellar component *fliC* (Haefele and Lindow 1987), harpin *hrpZ* (Preston et al. 1995), phytotoxin coronatine synthesis regulator *corR* (Sreedharan et al. 2006), and carbohydrate metabolism genes *pgm* and *mgo* (Mellgren et al. 2009; Morris et al. 1995). These genes have prior demonstrated roles in the virulence of *P. syringae*. We examined gene expression in rich medium (NB), in which Crc would be predicted to bind to its targets, and in minimal media supplemented with Fe, which has been shown to turn on the expression of T3SS genes (Bronstein et al. 2008). We observed that there was no significant up- or downregulation of any of the genes in *crc* compared with WT under all conditions tested.

Analysis of predicted Crc-binding sites in *Pseudomonas* genomes (Browne et al. 2010) suggests that some targets are genus-wide and are related to central metabolism, whereas other targets are species-specific. There are 143 genes predicted to be directly regulated by Crc in *P. syringae* DC3000. Crc-binding sites are mostly for genes predicted to be involved in carbohydrate metabolism. There are predicted binding sites in transcripts for *algP* (alginate metabolism), PSPTO_3494 (myo-inositol 2-dehydrogenase), proteins involved in chemotaxis, and several transcriptional regulators. None of the predicted targets appear to be directly involved in susceptibility to oxidative stress. Future work to investigate the effect Crc has on one or both the global transcriptome or proteome of *P. syringae* DC3000 would help elucidate more specifically the direct and indirect roles Crc plays in nutrient acquisition and virulence-related traits of *P. syringae* DC3000.

The determination of Crc-binding sites in DC3000 would help explain the effects seen on virulence, motility, and sensitivity to ROS. It is clear that Crc plays a broad role in DC3000 virulence, in addition to its role in carbohydrate metabolism. One possibility is that the increased swarming and biofilm formation seen may be detrimental to the *crc* mutant for plant cell contact and deployment of the T3SS, thereby causing the observed delay in HR and reduced growth in planta. As such, components of the T3SS seem unaffected in the mutant, as seen by the virulence gene expression and effector translocation studies. The initial entry of the *crc* mutant into apoplastic space may be similar to WT, as suggested by normal flagellar function in swimming experiments and WT levels of *fliC* gene expression in qPCR. Experiments with PTI marker genes and ROS production indicate that the mutant does not elicit an altered PTI response, which implies that major PAMPs are not different in the *crc* mutant. Therefore, we might speculate that the *crc* mutant can enter the plant leaf equally as well as WT but is unable to efficiently colonize because it produces more biofilm and is, also, affected by the ROS. It is likely that DC3000 cells need to individually make contact with plant cells, infect and overcome defenses, and then, establish colonies. Another possibility is that, although we did not observe a difference in swimming in the conditions we tested, flagella are somehow affected, and this may delay entry and, subsequently, delay all stages of the life cycle.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The strains and plasmids used for this study are available upon request. *Pseudomonas syringae* pv. *tomato* DC3000 was

routinely cultured on KB agar (King et al. 1954), NB agar, modified LM (10 g of Bacto tryptone, 6 g of yeast extract, 0.6 g of NaCl, 0.4 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.5 g K_2HPO_4 per liter) (Hanahan 1983) or mannitol-glutamate (MG) medium (per liter, 10 g of mannitol, 2 g of L-glutamic acid, 0.5 g of KH_2PO_4 , 0.2 g of NaCl, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, final pH of 7.0) (Keane et al. 1970) at 28°C or at room temperature. *Escherichia coli* DH5 α or TOP10 (Invitrogen) were used as the host for subcloning and other plasmid manipulations. *E. coli* was grown at 37°C in Luria-Bertani (LB) medium or LB medium solidified with 1.5% (wt/vol) agar.

Construction of a *crc* deletion mutant.

A Crc (PSPTO_0079) deletion mutant (Δ *crc*) was created using a pK18mobsacB plasmid (Schäfer et al. 1994). pK18mobsacB/ Δ *crc* was created by PCR amplification of DNA fragments of approximately 1.0 kb that flank *crc*. Gel-purified PCR fragments were joined by a second PCR amplification with primers containing *Xba*I restriction sites. The product was gel-purified, was digested with *Xba*I, and was cloned into pK18mobsacB digested with the same restriction enzymes. The pK18mobsacB deletion construct was confirmed by sequencing (Cornell University Life Sciences Core Laboratories Center) before introduction into DC3000 via electroporation. Integration events were selected on KB medium containing 50 μg of kanamycin per milliliter and were then transferred to 10% sucrose medium, to select for crossover events that resulted in the loss of the *sacB* gene. Sucrose-resistant colonies were screened by PCR and positive clones (those containing the deletion) were confirmed by sequencing.

Complementation of Δ *crc* mutant.

The genomic region containing the *crc* gene was PCR amplified from DC3000 chromosomal DNA using the primers 5'-GCGCTCTAGACAGTGCAGGACCGGTATTG-3' and 5'-ATATTCTAGATCAGATAGTCAGGTCAG-3', which contained restriction sites for *Xba*I. The amplified product was gel-purified, was digested with *Xba*I, and was cloned into pUCP22 (Schweizer 1991) that had been previously digested with *Xba*I. The ligation was transformed into *E. coli* DH5 α . Transformants were selected on gentamycin. Those colonies containing the correct size inserts, confirmed by sequencing to ensure no mutations, were introduced during PCR. pUCP22 containing the *crc* gene (pUCP22:*crc*) was introduced into the Δ *crc* strain by electroporation. This strain was designated Δ *crc* (pCrc). As a control, pUCP22 was introduced into the WT strain.

Evaluating susceptibility to hydrogen peroxide.

DC3000 and Δ *crc* were grown overnight at 28°C in KB liquid medium. The overnight cultures were pelleted and resuspended in fresh liquid KB to an OD₆₀₀ of 1.0. Cultures were further diluted to an OD₆₀₀ = 0.1 in 200 μl of KB only or KB containing hydrogen peroxide (0.015, 0.0225, or 0.03%) in a 96-well plate. The plate was incubated for 24 h, with shaking, in a BioTek plate reader. The OD₆₀₀ was recorded after 8 h. Each experiment was performed in triplicate for each concentration of hydrogen peroxide, and a total of three biological replicates were performed. The OD₆₀₀ values for each technical replicate were averaged and standard deviations were calculated.

Motility assays.

Swimming medium consisted of tryptone (10 g/liter), sodium chloride (7 g/liter), and 0.3% agar. Swim plates were inoculated, with a toothpick, with bacteria grown overnight on KB agar plates. Plates were incubated at room temperature for 48 h. The diameter of the swimming zone was measured at both 24 and 48 h postinoculation. Swimming assays were performed

in triplicate for each strain tested and three independent biological replicates were completed. The swimming diameter was averaged for each replicate and standard deviation was calculated.

Swarming medium consisted of nutrient broth (8 g/liter), glucose (0.5% [wt/vol]), and 0.5% (wt/vol) agar (NBG). Different carbon sources, including fructose, glucose, and succinate, were substituted for mannitol in the MG medium to a final concentration of 1% (wt/vol). Swarm plates were inoculated with bacteria that had been grown overnight in KB liquid medium at 28°C and adjusted to an OD₆₀₀ = 0.3 in KB liquid medium. Bacterial suspension (5 µl) was spotted onto the center of the swarming plate and plates were incubated at room temperature. The diameter of the swarming zone was measured 24 h postinoculation. Swarming assays were performed in triplicate for each strain tested and three independent biological replicates were completed. The swarming diameter was averaged for each replicate and standard deviation was calculated.

Biofilm formation.

Biofilm formation was assessed using the microtiter dish biofilm method described by (O'Toole 2011), with some modifications. *P. syringae* strains were grown overnight in KB liquid medium at 28°C. Overnight cultures were washed three times with desired medium. The tested medium included MG liquid medium and MG liquid medium in which carbon sources were substituted with fructose, glucose, and succinate at final concentrations of 1% (wt/vol). For measurable biofilm formation, we modified the established biofilm formation method by allowing the presence of 2 mM CaCl₂ in addition to the 100-µl OD₆₀₀ = 0.01 bacterial suspension in 96-well plates. Bacterial suspensions were added to 96-well plates prefilled with media to final OD₆₀₀ = 0.01 and were allowed to incubate at 28°C or room temperature for 24 h, with and without shaking. After 24 h, planktonic cells were removed from wells. Wells were washed gently three times with double-distilled (dd)H₂O. Approximately 150 µl of 0.1% crystal violet stain was added to each well and was allowed to incubate for 10 min. The stain was removed and wells were washed three times with ddH₂O. Stained biofilms were resuspended in 30% acetic acid and were allowed to incubate for 10 min. The OD₅₇₀ was recorded for each well and the average and standard deviation for technical replicates were computed.

Congo red assays.

P. syringae strains were grown overnight in KB liquid medium, were washed twice with NB or LM media, and were then resuspended in NB or LM at an OD₆₀₀ of 0.3. Culture (5 µl each) was spotted onto NB agar plates containing 40 µg of Congo red per milliliter (Sigma Aldrich). The plates were incubated at 28°C and were monitored for a total of 3 days.

Tomato and *Arabidopsis* dip-inoculation.

Arabidopsis was grown and dip-inoculated as described earlier (Katagiri et al. 2002). Briefly, the plants were grown at 23°C with a 12-h day and 75% relative humidity. Three- to four-week-old plants were dipped in bacterial suspensions adjusted to 1 × 10⁷ CFU ml⁻¹ in 10 mM MgCl₂ containing 0.04% Silwet L-77. Tomato inoculations were performed as described by Park et al. (2013), except that Silwet L-77 was used at a concentration of 0.04% for the dip assay. *Solanum lycopersicum* (tomato cultivar Moneymaker) was grown at 23°C in a greenhouse, with approximately 16 h of light per day. Four- to five-week-old plants were first acclimatized on a bench top for 24 h and were then bagged to create a high-humidity environment for 24 h prior to dipping. Bacterial suspensions were spread onto KB agar plates and were incubated at 28°C for 24 h.

The resulting bacterial lawns were harvested and were adjusted to 3 × 10⁷ CFU ml⁻¹ in 10 mM MgCl₂. Tomato plants were dipped in this suspension for 30 s. The inoculum was serially diluted and was plated on KB containing 50 µg of rifampicin per milliliter to verify the bacterial concentration. Following bacterial inoculation, tomato plants were allowed to air dry on the bench for 1 h, then, were incubated in a growth chamber at 25°C with 80% relative humidity, and 16 h of light. Three leaf discs per sample were harvested, using a cork borer. Bacteria were extracted from leaf samples by mechanical disruption with steel beads in 10 mM MgCl₂. Serial dilutions were spotted on KB supplemented with 50 µg of rifampicin per milliliter. Spots containing >5 and <30 colonies were used to quantify the number of CFUs per centimeter of leaf tissue.

Tomato syringe-infiltration assay.

Five- to six-week-old plants were used for syringe-infiltration assays and were acclimatized on the lab bench 1 day prior to infiltration. Bacteria were grown as described above, and were adjusted to a final concentration of 1 × 10⁴ to 5 × 10⁴ CFU/ml in 10 mM MgCl₂. Broad, well-developed leaves were syringe-infiltrated with bacterial suspensions and were maintained under the conditions described above. To enumerate bacterial growth, three leaf discs per spot were harvested, ground, diluted, and spotted as described above.

HR assay.

Four- to five-week-old *Nicotiana tabacum* cv. *Xanthi* or *Nicotiana benthamiana* were used for the HR assay. *P. syringae* strains were grown on KB agar with appropriate antibiotics at 28°C for 2 days. The bacteria were resuspended in 100 to 200 µl of liquid KB, were spread onto a fresh plate, and were grown for an additional 24 h. Cells were harvested from the plate and were resuspended in 10 mM MgCl₂. The OD of the cultures was adjusted to an OD₆₀₀ of 0.2 (approximately 2 × 10⁸ CFU/ml). Serial 10-fold dilutions were made in 10 mM MgCl₂. Cells corresponding to an OD₆₀₀ of 0.2, 0.02, or 0.002 were used to syringe-infiltrate *N. benthamiana* and tobacco leaves. Plants were left on the laboratory bench to allow for the development of HR.

Translocation assay.

The plasmid pCPP5388, containing the T3SS translocation reporter adenylate cyclase (Cya) gene fused to the effector AvrPto (Lam et al. 2014; Oh et al. 2007), was transformed into the Δ crc strain. The T3SS strain CUCPB5113 was used as a negative control. Translocation assays were performed as described by Lam et al. (2014).

Overlapping cell-death assay for PTI.

The assay was performed as described (Chakravarthy et al. 2009). Briefly, WT *P. fluorescens* or *P. fluorescens* Δ fliC adjusted to an OD₆₀₀ = 0.5 (approximately 5 × 10⁸ CFU/ml) was used to induce PTI and was challenged 7 h later with WT DC3000 or Δ crc adjusted to an OD₆₀₀ = 0.02 (approximately 2 × 10⁷ CFU/ml). Plants were kept on the lab bench and were scored for the breakdown of PTI in the overlapping area of infiltration.

RNA isolation and qPCR.

RNA for bacterial virulence genes. Overnight cultures of WT DC3000 or Δ crc were grown from fresh KB plates in NB or MG medium or MG in which the mannitol was substituted with succinate or fructose at a final concentration of 1% (wt/vol). All MG-based media used to grow cells for bacterial qPCR contained 50 µM FeSO₄, to induce expression of T3SS (Bronstein et al. 2008). The OD₆₀₀ of the overnight cultures was measured

and was adjusted to a final of $OD_{600} = 0.1$, in the same medium as the corresponding primary overnight culture. The secondary cultures were in a final volume of 10 ml in 125-ml flasks. They were grown for 7 h (NB) or 24 h (mannitol-, succinate-, or fructose-containing media). Aliquots (1 ml) were used to harvest cells, and cell pellets were frozen at -80°C . Total RNA was isolated from the frozen cells, using the Quick-RNA miniprep kit (Zymo Research), following manufacturer's instructions.

RNA for plant PTI marker genes. Five-week-old *N. benthamiana* plants were syringe-infiltrated on well-expanded leaves with bacterial suspensions of DC3000 or the Δcrc mutant ($OD_{600} = 0.2$) or 10 mM MgCl_2 . Leaf tissue (six to eight 1-cm leaf discs) was harvested and was flash-frozen 3 h after syringe-infiltration. Total RNA was isolated, using the RNeasy plant mini kit (Qiagen) (Nguyen et al. 2010).

qPCR. Isolated RNA was treated with DNase (Ambion) to remove residual DNA. RNA was then cleaned using the RNA Clean-up and concentrate kit (Zymo Research). Absence of DNA was confirmed by performing control reactions with primers to the bacterial gene *gyrA* (PSPTO_1745) or plant gene ATPase (Nguyen et al. 2010). Extracted bacterial and plant RNA was synthesized into cDNA, using the qScript cDNA Supermix (Quanta Biosciences), and qPCR was performed using Bio-Rad SSoAdvanced universal SYBR green supermix (Bio-Rad). For bacterial gene expression, the qPCR was performed in 384-well plates on a ViiA 7 real-time PCR system (Cornell Core Facility). For evaluating plant gene expression, qPCR was performed using 96-well plates on an iQ5 multicolor real-time detection system (BioRad). All primer pairs were found to yield unique products, using the dissociation protocol (data not shown). Gene expression fold change was calculated using the cycle threshold ($\Delta\Delta C_t$) method. For bacterial qPCR, the reference gene used was *gyrA* (PSPTO_1745). The ATPase gene served as the reference gene for plant qPCR (Nguyen et al. 2010). Primers used for bacterial qPCR are listed in Supplementary Table S1. Primers used for plant qPCR were as previously published (Nguyen et al. 2010).

Extracted bacterial and plant RNA was synthesized into cDNA, using the qScript cDNA supermix (Quanta Biosciences), and qPCR was performed using Bio-Rad SSoAdvanced universal SYBR green supermix (Bio-Rad). For bacterial gene expression, the PCR was performed in 384-well plates on a ViiA 7 real-time PCR system (Cornell Core Facility). For evaluating gene expression in infiltrated *N. benthamiana* plants, qPCR was performed using 96-well plates on a iQ5 multicolor real-time detection system (BioRad). All primer pairs have been previously shown to yield unique products, using the dissociation protocol (data not shown). Gene-expression fold change was calculated using the $\Delta\Delta C_t$ method. C_t values of each gene tested were normalized to the C_t values of the housekeeping gene *gap1* (PSPTO_1287).

Assay for ROS.

The method for ROS measurement was adapted from previous studies (Smith and Heese 2014; Wei et al. 2013). Leaf discs of four-week-old *N. benthamiana* were excised and placed individually into wells of a 96-well plate, containing 100 μl of distilled H_2O , and were allowed to incubate for 20 h for wound recovery. Bacterial suspensions made from fresh plates were spread onto KB agar plates and were incubated at 28°C for 24 h. Bacteria were harvested and OD_{600} was measured. Distilled H_2O was removed from the multiwell plates and the following mixture was added: 100 μl of 0.5 mM L-012 (Wako) in 10 mM morpholinepropanesulfonic acid-KOH buffer (pH 7.4), containing the bacterial suspension at a final OD_{600} of 0.1 (approximately 1×10^8 CFU ml^{-1}). Controls included wells with no bacteria. The intensity of ROS production

was determined by monitoring the chemiluminescence between 0 and 120 min, using a Biotek Synergy 2 microplate reader. Each experiment was done three independent times with similar results.

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