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The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*

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SUMMARY

Successful pathogen infection likely involves the suppression of general antimicrobial host defences. One *Pseudomonas syringae* virulence factor proposed to act in this manner is coronatine (COR), a phytotoxin believed to function as an analogue of one or more jasmonates, a family of plant growth regulators. COR biosynthetic (COR$^-$) mutants of *P. syringae* pv. *tomato* strain DC3000 exhibit reduced virulence on *Arabidopsis thaliana* and tomato. In the present study, three genetically and biochemically defined COR$^-$ mutants of DC3000 were used to explore potential effects of COR and its precursors, coronafacic acid (CFA) and coronamic acid (CMA), on defence signalling pathways in *A. thaliana*. Inoculation with wild-type DC3000 resulted in the accumulation of several jasmonate-responsive transcripts, whereas infection with a mutant strain that accumulates CFA, which is structurally similar to methyl jasmonate (MeJA), did not. Thus, COR, but not CFA, stimulates jasmonate signalling during *P. syringae* infection of *A. thaliana*. The ability of the COR$^-$ mutants to grow to high levels *in planta* was fully restored in *A. thaliana* lines deficient for salicylic acid (SA) accumulation. Although the COR$^-$ mutants grew to high levels in SA-deficient plants, disease symptoms were reduced in these plants. Collectively, these results indicate that COR is required both for overcoming or suppressing SA-dependent defences during growth in plant tissue and for normal disease symptom development in *A. thaliana*.

INTRODUCTION

To cause disease successfully, a pathogen must be capable of entering host tissue, evading or suppressing general antimicrobial defences, obtaining nutrients and water, multiplying to high levels and inducing tissue damage. The bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000, the causative agent of bacterial speck disease of tomato and crucifers (Cuppels, 1986; Whalen *et al.*, 1991; Zhao *et al.*, 2000), is a valuable model in which to study the molecular basis of pathogenicity. Recently, substantial progress has been made towards identifying virulence factors in *P. syringae* that contribute to pathogenesis, including those associated with the type III secretion system (TTSS) and the phytotoxin coronatine (COR) (Bender and Scholz-Schroeder, 2004; Collmer *et al.*, 2002; Greenberg and Vinatzer, 2003; Jin *et al.*, 2003; Kunkel and Chen, 2005; Ponciano *et al.*, 2003).

COR is a non-host-specific phytotoxin produced by several members of the *P. syringae* group of pathovars (Bender *et al.*, 1999; Mitchell, 1982). COR is a hybrid molecule consisting of the polyketide coronafacic acid (CFA) (Parry *et al.*, 1994; Rangaswamy *et al.*, 1998) and coronamic acid (CMA), a cyclized derivative of isoleucine (Mitchell, 1985). Both moieties of the COR molecule have been predicted to function as analogues of endogenous plant signalling molecules. The CFA moiety is structurally and functionally similar to several jasmonates, a family of endogenous plant signalling molecules that includes jasmonic acid (JA), methyl jasmonate (MeJA) and 12-oxo-phytodienoic acid (OPDA), the C_{18} precursor of JA (Feys *et al.*, 1994; Uppalapati *et al.*, 2005; Weiler *et al.*, 1994). The CMA portion of the molecule resembles the plant compound aminocyclopropyl carboxylic acid (ACC), the immediate precursor to ethylene (Bleecker and Kende, 2000). However, results from recent gene expression profiling experiments investigating the effects of applying purified CMA to tomato tissue indicate that, although CMA has biological activity *in planta*, it does not function as an analogue of ACC (Uppalapati *et al.*, 2005). Thus, the precise mechanism of COR function in altering host physiology and promoting pathogen virulence is unclear.

COR plays a vital role in the virulence of DC3000 on several host species (Brooks *et al.*, 2004; Mittal and Davis, 1995; Penaloza-Vazquez *et al.*, 2000; S. Elizabeth and C. Bender, unpublished observations). COR$^-$ mutants of DC3000 do not

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grow to wild-type levels or induce typical disease symptoms on either *Arabidopsis thaliana* or tomato (Brooks *et al*., 2004; Mittal and Davis, 1995; Penaloza-Vazquez *et al*., 2000). COR has been implicated in chlorosis and lesion expansion in tomato (Bender *et al*., 1987; Penaloza-Vazquez *et al*., 2000). However, experiments to separate growth defects from lesion development, which presumably requires growth to high levels within plant tissue, have not been carried out. Recently, several COR^- DC3000 mutants characterized at the genetic and biochemical levels have been described (Brooks *et al*., 2004). These include Tn*5* insertion mutants that disrupt synthesis of CMA (*cmaA*), CFA (*cfa6*) or both, as in the case of a *cmaA cfa6* double mutant that produces neither precursor nor COR. All three mutants exhibited reduced virulence on *A. thaliana*, indicating that the intact COR molecule is an important virulence factor in the *P. syringae*/*A. thaliana* interaction (Brooks *et al*., 2004).

In response to microbial attack, plants induce a variety of host defences, including a rapid oxidative burst, accumulation of endogenous signalling molecules, such as salicylic acid (SA), jasmonates and ethylene, induction of several defence-related genes (e.g. pathogenesis related or *PR* genes), and the production of antimicrobial phytoalexins and lytic enzymes (Felix *et al*., 1999; Glazebrook *et al*., 1997; Hammond-Kosack and Jones, 1996; Schmelz *et al*., 2003). Presumably, in order to colonize host tissue successfully, plant pathogens must be able to suppress these basal antimicrobial host defence responses (Kunkel and Chen, 2005; Nomura *et al*., 2005; Ponciano *et al*., 2003), and it has been hypothesized that COR and/or its intermediates (CFA and CMA) may be involved in this process (Kunkel and Brooks, 2002; Nomura *et al*., 2005). Several recent observations support the hypothesis that COR suppresses host defences. For example, the overproduction of CFA by *P. syringae* pv. *glycinea* PG4180 was correlated with a delay in the induction of localized programmed cell death events (e.g. the hypersensitive response or 'HR') associated with plant defences induced by non-host resistance on tobacco plants (Budde and Ullrich, 2000). Likwise, inoculation of *A. thaliana* plants with a COR^- mutant of *P. syringae* pv. *maculicola* elicited a stronger systemic resistance response than did inoculation with the wild-type strain (Cui *et al*., 2005). Infection of tomato plants with a DC3000 COR^- mutant (DC3118) resulted in elevated expression of several *PR* genes (Zhao *et al*., 2003). Similarly, *A. thaliana* plants infected with a different COR^- mutant of DC3000 (DC3661) induced elevated expression of two defence-related genes, *PAL* and *GST*, which encode phenylalanine ammonia lyase and glutathione-S-transferase, respectively (Mittal and Davis, 1995). The effect of COR and its intermediates on expression of *PR* genes during *P. syringae* infection of *A. thaliana* plants has not been previously reported and is one objective of the current study.

In *A. thaliana,* the SA-dependent defence response pathway is important for defence against infection by virulent *P. syringae*. Transgenic *A. thaliana* plants expressing the *P. putida nahG* gene, which encodes salicylate hydroxylase, an enzyme that degrades SA to catechol, are unable to accumulate elevated levels of SA in response to pathogen attack and are more susceptible to infection by both virulent and avirulent *P. syringae* strains (Delaney *et al*., 1994). Similarly, *A. thaliana sid2* mutants that are blocked for SA production via the isochorismate synthase pathway do not accumulate SA during infection with *P. syringae* pv. *maculicola* strain ES4326 and exhibit enhanced disease susceptibility after infiltration with this virulent pathogen (Nawrath and Metrauz, 1999; Wildermuth *et al*., 2001). Expression of several SA-dependent *PR* genes is correlated with infection, and the induction of these genes is often monitored to assess the activity of the SA-dependent defence pathway (Glazebrook, 2001). However, the role of most *PR* gene products in pathogen defence is unclear.

The jasmonate signalling pathway is also important in pathogen defence, and is induced during wounding, herbivore attack, and infection by several bacterial and fungal pathogens (Farmer *et al*., 2003). However, this pathway does not appear to contribute to resistance against hemi-biotrophic pathogens such as *P. syringae* (Kunkel and Brooks, 2002). Rather, an intact jasmonate signalling pathway is required for full susceptibility to *P. syringae* in both *A. thaliana* and tomato and may be modulated by this pathogen in order to promote disease susceptibility (Block *et al*., 2005; Feys *et al*., 1994; Kloek *et al*., 2001; Nickstadt *et al*., 2004; Uppalapati *et al*., 2005; Zhao *et al*., 2003; N. Laurie-Berry and B. Kunkel, unpublished data). One hypothesis, based on mounting evidence that COR functions as a jasmonate mimic and that the SA- and jasmonate-dependent defence signalling pathways are mutually antagonistic (Glazebrook *et al*., 2003; Kunkel and Brooks, 2002), is that COR induces jasmonate signalling within plants and results in inhibition of SA-dependent defence responses (Kunkel and Brooks, 2002; N. Laurie-Berry and B. Kunkel, unpublished data).

This hypothesis is supported by the finding that reduced disease susceptibility to *P. syringae* in *A. thaliana* and tomato COR-insensitive mutant plants is associated with increased signalling through the SA-dependent defence pathway (Kloek *et al*., 2001; Nickstadt *et al*., 2004; Zhao *et al*., 2003; N. Laurie-Berry and B. Kunkel, unpublished data), and the observation that infection with a COR-defective strain of DC3000 results in elevated expression of several SA-dependent defence-related genes in tomato (Zhao *et al*., 2003). Furthermore, the observations that reduced disease susceptibility of *A. thaliana coi1* and *jin1* mutant plants is suppressed by introduction of the *nahG* transgene or the *sid2* mutation, both of which result in a failure to accumulate elevated levels of SA (Kloek *et al*., 2001; N. Laurie-Berry and B. Kunkel, unpublished data), are also consistent with this hypothesis. Interestingly, despite the fact that *nahG coi1* plants and *sid2 jin1* plants allow high levels of growth of DC3000, these plants do not

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develop typical disease symptoms (Kloek *et al.*, 2001; N. Laurie-Berry and B. Kunkel, unpublished data). Taken together, these findings suggest that COR may play two separate roles in pathogenesis: (i) suppression of SA-dependent plant defences and (ii) promotion of disease symptom development via an SA-independent mechanism. Recent findings by Block *et al.* (2005) are consistent with this hypothesis, and suggest that COR functions to promote *P. syringae* virulence via both SA-dependent and SA-independent mechanisms.

In this study we further explore the role of COR and its precursors, CFA and CMA, in *P. syringae* pathogenesis. To do so, we took advantage of three well-defined DC3000 COR biosynthetic mutants to investigate the hypothesis that *P. syringae* utilizes COR (or its intermediates) to modulate the jasmonate and SA signalling pathways. We show that infection of *A. thaliana* with wild-type DC3000 induced jasmonate-responsive genes, whereas infection with the COR$^-$ mutants did not, indicating that COR, but not its precursors CFA or CMA, has jasmonate activity in the context of this interaction. We also demonstrate that the ability of the COR$^-$ mutants to grow to high levels in plant tissue was fully restored in *A. thaliana sid2-2* mutants and *nahG* transgenic lines that fail to accumulate SA. These data suggest that COR is required to overcome or suppress SA-dependent defences in *A. thaliana*. Furthermore, although the COR$^-$ mutants multiplied to wild-type levels in SA-deficient plants, the disease symptoms on these plants were significantly reduced relative to those on plants infected with wild-type DC3000. Thus, COR is required both for overcoming SA-dependent defences during growth in plant tissue and for production of wild-type disease symptoms.

RESULTS

COR, but not its precursor CFA, acts as a functional mimic of jasmonates during *P. syringae* pathogenesis

The DC3000 COR$^-$ mutants used in this study included a *cmaA* (strain AK7E2; CMA$^-$ CFA$^+$ COR$^-$), a *cfa6* (strain DB4G3; CMA$^+$ CFA$^-$ COR$^-$) and a *cmaA cfa6* double mutant (strain DB29; CMA$^-$ CFA$^-$ COR$^-$) (Brooks *et al.*, 2004). These mutants exhibit reduced virulence on *A. thaliana* compared with wild-type DC3000. As shown in Fig. 1A,C, growth of the mutant strains in plant tissue was significantly lower than wild-type DC3000 by 4 days post inoculation (dpi). In some experiments, a significant reduction in growth of the COR$^-$ strains was obvious by 2 dpi. The defect in growth within host tissue of the COR$^-$ mutants was always associated with a dramatic reduction in disease symptom production (Fig. 2A). Infection of *A. thaliana* leaves with wild-type DC3000 resulted in disease symptoms consisting of small water-soaked lesions surrounded by regions of chlorosis. In contrast, infection with the COR$^-$ mutants resulted in little to no disease, consisting of limited chlorosis on fewer than 5% of infected leaves (Fig. 2A) (Brooks *et al.*, 2004).

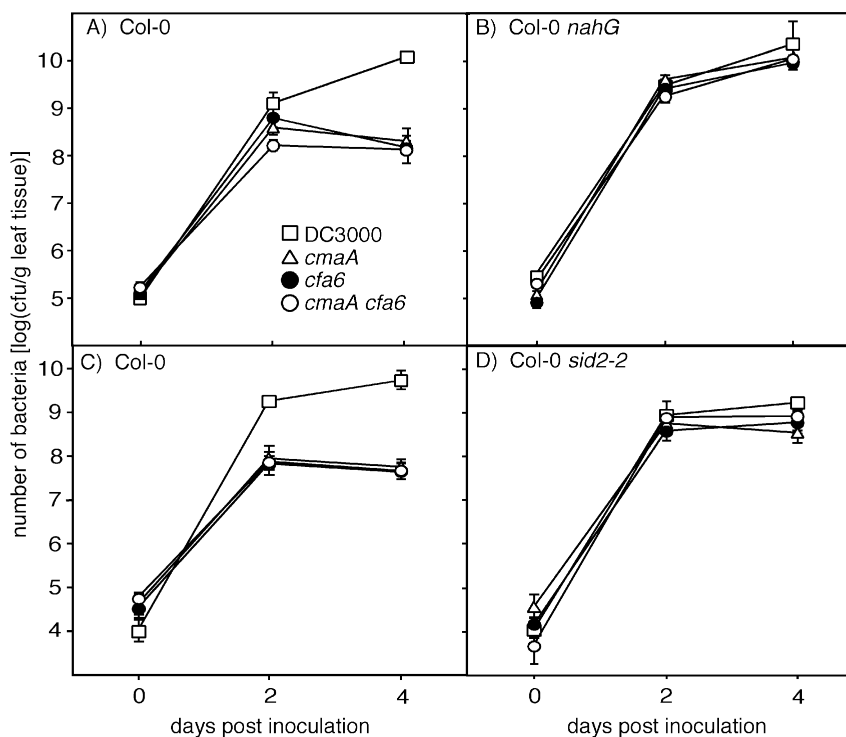

Fig. 1 Growth of *P. syringae* pv. *tomato* strain DC3000 and COR biosynthetic mutant derivatives in wild-type and SA-deficient *A. thaliana* plants. Multiplication of wild-type and COR$^-$ DC3000 strains in wild-type Col-0 (A,C) plants, Col-0 *nahG* transgenic plants (B), and Col-0 *sid2-2* plants (D). The data shown in A and B were collected in a single experiment, and data in C and D collected in a second, independent experiment. Open boxes represent growth of wild-type DC3000; open triangles represent the DC3000 *cmaA* mutant; closed circles represent the DC3000 *cfa6* mutant; and open circles represent the *cmaA cfa6* double mutant. *A. thaliana* plants were dip-inoculated in a solution containing 10 mM $MgCl_2$, 0.02% Silwet L77 and 5×10^8 cfu/mL of DC3000 or the indicated COR$^-$ mutant strain (see Materials and methods). Whole leaves were removed 0, 2 and 4 days after inoculation, weighed and homogenized in 10 mM $MgCl_2$. Tissue removed at 0 dpi was surface sterilized by immersion in 15% H_2O_2 with subsequent rinsing in sterile dH_2O prior to grinding and plating. Numbers of bacteria are expressed as the log of colony-forming units (cfu) per gram of plant tissue. Error bars represent the standard error of the mean (± SEM). These experiments were repeated at least twice with similar results.

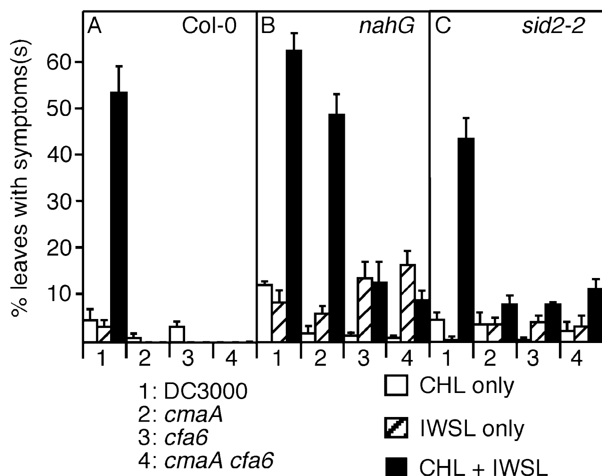

Fig. 2 Symptom production by *P. syringae* pv. *tomato* strain DC3000 and COR biosynthetic mutant derivatives on wild-type and SA-deficient *A. thaliana* plants. Disease symptom severity was quantified as the average percentage of affected leaves per plant exhibiting the indicated disease symptom from 10 to 20 plants per treatment analysed. Disease symptom severity was quantified 4 days after dip-inoculation of wild-type Col-0 plants (A), Col-0 *nahG* plants (B), or Col-0 *sid2-2* plants (C) with wild-type DC3000 or the indicated COR⁻ mutant derivatives. Open bars represent leaves displaying only chlorotic (CHL) tissue; hatched bars represent leaves displaying only individual water-soaked lesions (IWSLs); closed bars represent leaves that exhibited both chlorosis and IWSLs. Error bars represent the standard error of the mean (± SEM). This experiment was repeated a second time with similar results.

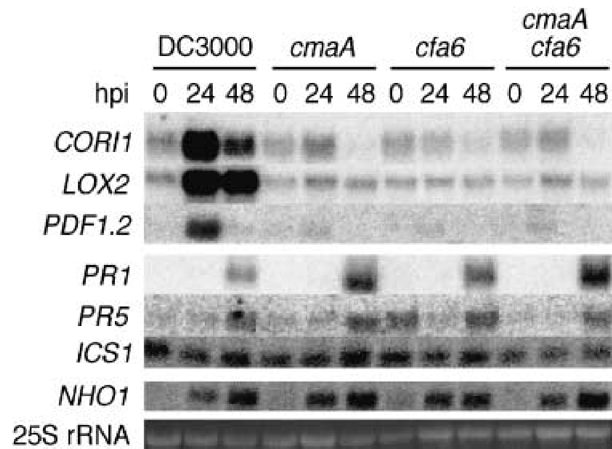

Fig. 3 Expression of *A. thaliana* defence-related genes after infection of *A. thaliana* with wild-type DC3000 and COR biosynthetic mutant strains. Plants were dip inoculated with 10 mM $MgCl_2$, 0.02% Silwet L77 and 5×10^8 cfu/mL of DC3000 or the indicated COR⁻ mutants. Total RNA was prepared from leaf tissue harvested at the indicated times after infection (hours post infection; hpi). Approximately 30 μg of total RNA was loaded for each sample. The resulting RNA blots were hybridized sequentially with the indicated *A. thaliana* gene probes: *LOX2* (*lipoxygenase 2*), *CORI1* (*coronatine-induced 1* encoding a predicted chlorophyllase), *PDF1.2* (*plant defensin 1*), *PR1* (*pathogenesis-related 1*), *PR2* (*pathogenesis-related 2*), *PR5* (*pathogenesis-related 5* encoding a thaumatin-like protein), *ICS1* (*isochorismate synthase 1*) and *NHO1* (*non-host resistance 1*). *PR2* results are not shown but were similar to those obtained for *PR5*. Ethidium bromide staining of 25S rRNA is shown as a loading control. Similar results were obtained in a minimum of three independent experiments. Although in some experiments we detected slightly elevated levels of *PR1* transcript in plants inoculated with various COR⁻ mutants, we observed no reproducible difference when compared with plants infected with wild-type DC3000 across all experiments.

Gene expression profiling studies have revealed that the exogenous application of JA, COR or CFA induce similar responses in tomato plants in the absence of pathogen (Uppalapati *et al.*, 2005). These findings suggest that COR and CFA function, at least in part, as jasmonate analogues within the plant. However, the roles of these molecules during pathogenesis have not been previously evaluated. To test the hypothesis that COR and/or CFA function as jasmonate analogues during *P. syringae* infection of *A. thaliana*, the expression of several jasmonate-responsive transcripts was monitored in plants that were dip-inoculated with either wild-type DC3000 (COR⁺) or the COR biosynthetic (COR⁻) mutants described above. Expression of three jasmonate-responsive genes, *AtCORI1*, *LOX2* and *PDF1.2*, was monitored by RNA blot analysis in these plants. *AtCORI1* encodes a predicted chlorophyllase that has previously been shown to be induced by jasmonate or coronatine treatment (Benedetti *et al.*, 1998). *LOX2* encodes a lipoxygenase involved in jasmonate biosynthesis that is also induced upon treatment with JA or wounding (Bell and Mullet, 1993; Bell *et al.*, 1995). The *PDF1.2* gene, which encodes a defensin, is induced by JA, ethylene and DC3000 infection (Block *et al.*, 2005; Penninckx *et al.*, 1998). All three genes were strongly induced within 24 h after infection with wild-type DC3000 (Fig. 3). In contrast, inoculation with the *cmaA*, *cfa6* or *cmaA cfa6* mutants did not result in significant induction of any of these jasmonate-responsive transcripts, indicating that COR is necessary for their induction in *P. syringae*-infected *A. thaliana* plants. Thus, COR functions as a jasmonate analogue during *P. syringae* infection of *A. thaliana*.

COR is required to overcome SA-dependent plant defences

Given the virulence defects of the DC3000 COR⁻ mutants (Figs 1 and 2), the observations that SA-dependent defences are hyperactivated in COR-insensitive *coi1* and *jin1* mutant plants (Kloek *et al.*, 2001; Zhao *et al.*, 2003; N. Laurie-Berry and B. Kunkel, unpublished data), and the finding that infection of tomato plants with a DC3000 COR⁻ mutant strain results in elevated expression of several defence-related *PR* genes (Zhao *et al.*, 2003), we hypothesized that COR is involved in the suppression of SA-dependent defences that normally serve to limit growth of *P. syringae* in *A. thaliana*. Thus, we predicted that the virulence of

the COR$^-$ mutants would be restored when inoculated on to plants compromised for SA-mediated defences. To examine this, DC3000 and the COR$^-$ mutants were inoculated on to *nahG* transgenic lines and *sid2-2* mutant plants, both of which fail to accumulate high levels of SA (Delaney *et al*., 1994; Dewdney *et al*., 2000; Nawrath and Metrauz, 1999).

Wild-type DC3000 multiplied to high levels (~10^{10} cfu per gram of leaf tissue) in *nahG* plants (Fig. 1B). Interestingly, although these lines are reported to exhibit enhanced susceptibility to virulent pathogens, we observed only slightly higher levels of bacterial growth in *nahG* plants than in the non-transgenic controls (Fig. 1A,B). The COR$^-$ mutants grew to similarly high levels on the *nahG* plants (Fig. 1B). Thus, COR is not required for high levels of bacterial growth in *nahG* SA-deficient transgenic plants. To determine whether increased levels of growth of the COR$^-$ mutants in the *nahG* transgenic *A. thaliana* plants resulted in a corresponding augmentation of disease symptom production, symptom development was monitored on the same set of plants used to assay bacterial growth. Inoculation of *nahG* transgenic plants with wild-type DC3000 resulted in an increase in the number of leaves exhibiting chlorosis and/or individual water-soaked lesions (IWSLs) relative to that elicited on the Col-0 parent (Fig. 2A,B). The increase in disease severity of *nahG* plants was most notable when plants were examined later than 4 dpi (data not shown). These findings are consistent with previous reports that *nahG* transgenic plants exhibit increased susceptibility to infection by *P. syringae* (Delaney *et al*., 1994). As described above, infection with the COR$^-$ mutants did not elicit disease symptom development on wild-type plants (Fig. 2A). By contrast, infection of *nahG* transgenic plants with any of the three COR$^-$ mutants resulted in the development of clearly visible disease symptoms, with a minimum of 10% of the infected leaves exhibiting both IWSLs and chlorosis (Fig. 2B). However, the overall severity of disease symptoms was reproducibly less than that induced by wild-type DC3000 on either Col-0 or *nahG* plants. Interestingly, the CMA$^-$ (*cmaA*) and CFA$^-$ (*cfa6*) mutants behaved somewhat differently on the *nahG* plants, as the *cmaA* mutant induced significantly more severe disease symptoms than the *cfa6* or the *cmaA cfa6* mutants (Fig. 2B). These results suggest that CFA may play a more significant role in promoting symptom formation than CMA in *nahG* plants.

The results of the infection experiments using the *nahG* transgenic plants suggest that COR is required to overcome SA-dependent defences that normally serve to limit *P. syringae* growth *in planta*. To confirm these findings, we utilized the *sid2-2* mutant, which carries a mutation in a gene encoding isochorismate synthase (*ICS1*, Wildermuth *et al*., 2001). This mutant is unable to convert chorismate to isochorismate, a key step in pathogen-induced SA biosynthesis. *A. thaliana* mutants defective in *ICS1* do not accumulate elevated levels of SA in response to infection by *P. syringae* and exhibit enhanced disease susceptibility to *P. syringae* pv. *maculicola* ES4326 (Nawrath and Metrauz, 1999).

In our assays, *sid2-2* plants did not reproducibly support higher levels of DC3000 growth than wild-type Col-0 (Figs 1D and 2C). However, multiplication of the COR$^-$ mutants was not impaired on *sid2-2* plants, and reached levels essentially equivalent to that observed for wild-type DC3000 (Fig. 1D). These results corroborate our observations of *nahG* plants infected with the COR$^-$ mutants, and support the hypothesis that COR is required to overcome SA-dependent defences.

The severity of disease symptoms caused by the COR$^-$ mutants was also increased on *sid2-2* plants, although not to the level observed with wild-type DC3000 or when these mutants were inoculated on to *nahG* plants (Fig. 2). Furthermore, when either wild-type or *sid2-2* plants were infected with DC3000, bright chlorotic haloes often surrounded the patches of water-soaked lesions, making the lesions stand out in stark contrast to the surrounding green tissue (data not shown). However, infection of *sid2-2* plants with the COR$^-$ mutants resulted in paler and more diffuse chlorosis at 4 dpi (data not shown). Thus, although the COR$^-$ mutants were able to multiply to essentially wild-type levels in these plants, they were unable to induce wild-type disease symptoms. These results indicate that COR production is also required for normal disease symptom development. Interestingly, on the *sid2-2* mutant plants we could not detect a significant difference in disease symptom severity elicited by the different COR$^-$ mutants.

COR does not inhibit transcript accumulation of known SA-dependent defence-related genes

Our finding that COR is required to overcome SA-dependent defences in *A. thaliana* (Fig. 1) is consistent with the hypothesis that production of COR results in suppression of SA-dependent defences during infection (Kunkel and Brooks, 2002; Zhao *et al*., 2003). Thus, we predicted that infection of *A. thaliana* with the DC3000 COR$^-$ mutants would result in enhanced expression of SA-dependent defence-related genes. To test this hypothesis we monitored accumulation of several *PR* transcripts in Col-0 plants infected with wild-type DC3000 or the COR$^-$ mutant strains. Consistent with previous studies, infection with DC3000 resulted in the induction of *PR1*, *PR2* and *PR5* within 48 h after dip inoculation (Fig. 3 and data not shown; Chen *et al*., 2004). We were unable to detect any reproducible or significant alteration in *PR* gene transcript levels or induction kinetics in plants infected with the COR$^-$ mutants (Fig. 3). Thus, unlike what has been reported for tomato (Zhao *et al*., 2003), we do not observe a correlation between the virulence activity of COR and the suppression of SA-dependent *PR* transcript accumulation in *A. thaliana*.

In several previous studies, *PR* transcript levels were not observed to correlate well with the degree of plant susceptibility

(e.g. see Chen *et al.*, 2004; Clarke *et al.*, 1998). Thus, these genes may not be appropriate for monitoring the effect of COR on SA-mediated defences. To gain further insight into how COR may function to modulate SA-dependent defence responses, we examined the expression of two additional *A. thaliana* defence-related genes, *ICS1* and *NHO1*, in response to infection with wild-type and COR-deficient strains. Both of these genes have been demonstrated to contribute to defence against *P. syringae.* As described above, *ICS1* encodes an isochorismate synthase involved in pathogen-induced SA biosynthesis (Nawrath and Metrauz, 1999; Wildermuth *et al.*, 2001). *NHO1* encodes a glycerol kinase involved in resistance against non-pathogenic strains of *P. syringae* (Kang *et al.*, 2003; Lu *et al.*, 2001). The *ICS1* transcript was reported to be induced in *A. thaliana* plants infiltrated with the wild-type, COR-producing *P. syringae* pv. *maculicola* strain ES4326 at 24 h post infection (Wildermuth *et al.*, 2001). However, in our experiments, dip inoculation with DC3000 did not result in induction of *ICS1* (Fig. 3). We observed a similar pattern of essentially constitutive *ICS1* expression in plants infected with the DC3000 COR$^-$ mutants (Fig. 3). Thus, COR does not appear to modulate the expression of this SA biosynthetic gene during infection with DC3000.

Kang *et al.* (2003) observed that *NHO1* was significantly induced in *A. thaliana* plants infiltrated with non-pathogenic *P. syringae* strains, but that this transcript did not accumulate after infiltration with DC3000. Furthermore, over-expression of *NHO1* in transgenic plants resulted in enhanced resistance to DC3000. Taken together, these data suggest an important role for *NHO1* in defence against microbial attack. Interestingly, the lack of induction of the *NHO1* transcript during infection by DC3000 appears to be dependent on the ability of the host to respond to COR (Kang *et al.*, 2003). This finding suggests that COR may contribute to DC3000 virulence by suppressing *NHO1* expression, and leads to the prediction that *NHO1* would be more strongly and/or rapidly induced upon infection with the COR$^-$ mutants than in response to infection with wild-type DC3000. Surprisingly, we observed that infection with wild-type DC3000 induced accumulation of *NHO1* transcripts within 24 h after dip inoculation (Fig. 3), suggesting that expression of this gene may be regulated differently in plant tissues subjected to different inoculation protocols. We did not observe any significant or reproducible alterations in *NHO1* transcript levels in plants inoculated with the COR$^-$ mutants, suggesting that COR does not promote virulence by suppressing *NHO1* expression (Fig. 3).

DISCUSSION

Plant pathogens deploy a variety of virulence factors that alter the physiology of their hosts and render plant tissue more suitable for pathogen growth and disease development (Alfano and Collmer, 1996; Kunkel and Chen, 2005; Nomura *et al.*, 2005). One such virulence factor, the *P. syringae* phytotoxin COR, is hypothesized to contribute to pathogen virulence by suppressing antimicrobial plant defences. Specifically, COR, a molecular mimic of one or more jasmonates, has been proposed to stimulate jasmonate signalling and jasmonate-mediated antagonism of SA-dependent defences in infected tissue, thereby providing *P. syringae* with an opportunity to colonize and grow to high levels within the plant (Kunkel and Brooks, 2002). In this study, we used a set of well-defined COR biosynthetic mutants of *P. syringae* pv. *tomato* strain DC3000 to explore the role of COR, CFA and CMA in *P. syringae* pathogenesis, and to test directly the hypothesis that COR (or its intermediates) functions to promote pathogen virulence by interfering with SA-dependent defences that normally serve to limit growth of *P. syringae*.

The intact COR molecule, but not its precursor CFA, functions as a jasmonate analogue during *P. syringae* infection of *A. thaliana*

Our gene expression studies (Fig. 3) demonstrate that COR produced by DC3000 during growth in *A. thaliana* induces several jasmonate-responsive genes in infected plants. These results are not unexpected, given that exogenous application of purified COR has been shown to induce jasmonate-responsive genes in many plants, including *A. thaliana* (Benedetti *et al.*, 1995; Feys *et al.*, 1994) and tomato (Uppalapati *et al.*, 2005; Weiler *et al.*, 1994; Zhao *et al.*, 2003). They are also consistent with findings by Zhao *et al.* (2003), who observed that in tomato the induction of several jasmonate- and wound-responsive genes upon infection with DC3000 results largely from the action of COR. Furthermore, our observation that *LOX2*, a jasmonate biosynthetic gene, is induced within 24 h after infection is consistent with previous observations that DC3000 infection results in accumulation of elevated levels of OPDA and JA in *A. thaliana* tissue (Block *et al.*, 2005; Schmelz *et al.*, 2003).

We were surprised to observe induction of jasmonate-responsive genes only in plants infected with wild-type bacteria synthesizing the intact COR molecule (Fig. 3). Given the structural similarity of the CFA component of COR with MeJA and OPDA (Lauchli and Boland, 2003; Weiler *et al.*, 1994), and given recent observations that treatment of tomato plants with exogenous CFA results in induction of many JA-responsive genes (Uppalapati *et al.*, 2005), we predicted that the CFA moiety would be responsible, at least in part, for the induction of jasmonate-dependent genes in *A. thaliana*. In other words, we expected that infection with a COR$^-$ mutant strain that produces CFA (e.g. the *cmaA* mutant AK7E2; Brooks *et al.*, 2004), would also induce expression of these genes. In contrast, our findings indicate that the intact COR molecule, but not its precursor CFA, functions to stimulate jasmonate signalling during *P. syringae* infection of *A. thaliana*. These findings, taken together with the fact that

production of the intact COR molecule is required for full virulence on *A. thaliana* (Fig. 1), also indicate that stimulation of jasmonate signalling in the plant plays a key role in *P. syringae* virulence.

It is presently unclear why only the intact COR molecule acts as a functional jasmonate analogue in infected *A. thaliana* tissue. One possibility is that the entire molecule is required for uptake and/or stability within plant cells. Alternatively, rather than functioning as a mimic of MeJA or OPDA, COR may function as an analogue of one or more naturally occurring JA–amino acid conjugates (Staswick and Tiryaki, 2004). The recent discovery that the *A. thaliana* JAR1 protein catalyses the formation of JA-isoleucine (JA-Ile), a jasmonate that is required for optimal jasmonate signalling in *A. thaliana* (Staswick and Tiryaki, 2004), has led to the hypothesis that COR may function as an analogue of JA-Ile to promote pathogenesis (Cui *et al*., 2005; N. Laurie-Berry and B. Kunkel, unpublished data).

COR promotes virulence by overcoming SA-dependent defences

Based on previous studies suggesting that the jasmonate and SA defence signalling pathways are antagonistic, it has been hypothesized that COR functions to promote *P. syringae* virulence by suppressing SA-dependent defences that normally limit the growth of *P. syringae* (Glazebrook *et al*., 2003; Kloek *et al*., 2001; Kunkel and Brooks, 2002; N. Laurie-Berry and B. Kunkel, unpublished data). To investigate this hypothesis we utilized our well-defined DC3000 COR^- mutants and two SA-deficient lines of *A. thaliana* to test whether the virulence of the COR biosynthetic mutants could be restored in plants compromised for SA-mediated defences. Our observation that the COR^- mutants, which are unable to grow to high levels in wild-type *A. thaliana* plants, grew to levels similar to those achieved by wild-type DC3000 on SA-deficient plants (Fig. 1) suggests that COR is required to overcome SA-dependent defences in *A. thaliana.* These findings, taken together with the observations that COR functions as a jasmonate analogue, support the model that COR functions to stimulate jasmonate signalling, which in turn antagonizes expression of SA-dependent defences within the plant. Thus, one result of COR production during infection appears to be suppression of host defences, thereby providing the pathogen with the opportunity to grow to high levels and ultimately to cause disease within the plant.

Zhao *et al*. (2003) reported that COR acts to suppress several SA-dependent defence-related genes in tomato, including *PR-1b* and *PR-2b.* Interestingly, in our analysis of plant defence gene expression, we did not reproducibly observe COR-mediated inhibition of known *A. thaliana* defence-related genes upon infection by *P. syringae*. Our analysis included several genes commonly used as reporters for SA-dependent defences (e.g. *PR1*, *PR2* and *PR5*) as well as two genes previously demonstrated to contribute to defence against virulent strains of *P. syringae* (*ICS1* and *NHO1*; Fig. 3). Several possible explanations exist for our inability to detect an effect of COR on the expression of these defence-related genes. It is important to note that in our experiments, which involved dip inoculation, the growth defect of the COR^- strains was more pronounced than in experiments where plants were inoculated by direct infiltration (Brooks *et al*., 2004; Zhao *et al*., 2003). Thus, in our experiments, the reduced number of COR^- bacteria present in the plant tissue could possibly explain why we did not observe enhanced expression of defence-related genes. Furthermore, it is plausible that an effect of COR on defence gene expression was not observed due to the presence of additional virulence factors that also act through the jasmonate signalling pathway to inhibit plant defences. This hypothesis is supported by the fact that some type III-secreted virulence effectors require a functional *COI1*-dependent jasmonate signalling pathway to promote host susceptibility (He *et al*., 2004; Zhao *et al*., 2003). Finally, our data may indicate that, in the context of infection with virulent *P. syringae*, the genes tested in our study may not be appropriate markers of SA-dependent pathogen defence (Chen *et al*., 2004; Clarke *et al*., 1998). Thus, the molecular mechanism(s) by which COR acts to overcome SA-dependent defences in *A. thaliana* remains unclear, and experiments to identify host responses that are modulated by COR are in progress.

During the course of our studies, Block *et al*. (2005) published a report investigating the hypothesis that COR functions to suppress SA-dependent defences. Their results suggest that COR functions primarily through an SA-independent mechanism to promote *P. syringae* virulence. These findings are unexpected, given the abundance of previous molecular and genetic data supporting the hypothesis that COR promotes pathogen virulence by stimulating jasmonate-mediated suppression of SA-dependent defences (Kloek *et al*., 2001; Kunkel and Brooks, 2002; Zhao *et al*., 2003). One possible explanation for the discrepancy between their results and those presented in our study is that Block *et al*. conducted their studies using a poorly defined COR-defective strain of DC3000 (strain DC3661; Moore *et al*., 1989). As this mutant has not been carefully characterized, nor has its virulence defect been shown to be due solely to the inability to synthesize COR, it is possible that this strain may possess additional defects impacting virulence.

COR is required to promote disease symptom development

The disease symptoms observed on *sid2* mutant *A. thaliana* plants infected with the DC3000 COR^- mutant strains were less severe than those observed for *sid2* plants infected with wild-type DC3000 (Fig. 2). Given that the level of *in planta* growth achieved by the COR-defective strains was equal to that of

DC3000 (Fig. 1), these data indicate that COR is also involved in the production of disease symptoms. These findings are consistent with the results of studies examining disease development in the *A. thaliana coi1* and *jin1* jasmonate signalling mutants, both of which exhibit reduced disease susceptibility to DC3000 (Kloek *et al.*, 2001; Nickstadt *et al.*, 2004; N. Laurie-Berry and B. Kunkel, unpublished data). In both cases the abolition of SA-mediated defences in the mutant plants restored the ability of the plants to support high levels of pathogen growth. However, although growth of DC3000 was restored to wild-type levels in *coi1 nahG* plants and in *jin1 sid2* plants, the plants did not develop normal levels of disease symptoms (Kloek *et al.*, 2001; N. Laurie-Berry and B. Kunkel, unpublished data). Thus, COR is also required for stimulating production of disease symptoms via a mechanism that functions independently of SA.

One possible mechanism by which COR may function to promote symptom development is by stimulating the synthesis of the plant hormone indole acetic acid (IAA, or auxin). Free IAA levels increased in tomato and *A. thaliana* plants infected with DC3000 (Kunkel *et al.*, 2004; O'Donnell *et al.*, 2003), and the exogenous application of COR induced the expression of several IAA-related genes in tomato (Uppalapati *et al.*, 2005). Several genes involved in either IAA synthesis or the release of free IAA from conjugated forms were induced when *A. thaliana* was infected with DC3000, and this response was stimulated by COR (Kunkel *et al.*, 2004). Furthermore, the exogenous application of the IAA analogue 1-naphthaleneacetic acid (NAA) enhanced disease symptoms in DC3000-infected *A. thaliana* plants (Kunkel *et al.*, 2004). Thus, COR-dependent stimulation of IAA production in plant tissue may promote disease severity; however, the mechanistic basis for this phenomenon is not well understood.

Do CFA and CMA play specific roles in pathogenesis?

It is surprising that we were unable to uncover clear roles for CFA and CMA in *P. syringae* virulence on *A. thaliana*. However, the *cmaA* and *cfa6* mutants elicited different disease symptoms on NahG plants (Fig. 2B), suggesting that CFA and CMA may function independently to modulate pathogenesis in other *P. syringae*–host interactions. Consistent with this hypothesis, recent studies on tomato and several crucifers indicate that CFA may contribute to disease symptom development, as infection with the DC3000 *cmaA* mutant resulted in mild necrosis and chlorosis of tomato and turnip leaves (S. R. Uppalapati and C. Bender, unpublished data; S. Elizabeth and C. Bender, unpublished). Furthermore, in experiments in which tomato plants were treated with exogenous CFA and CMA, both compounds exhibited biological activity and induced distinctly different sets of genes (Uppalapati *et al.*, 2005). These results suggest that CFA and CMA may play specific, albeit subtle, roles in pathogenesis of some host plants. Future studies involving more sensitive assays, including gene expression profiling and proteomics, may reveal additional roles for these molecules during DC3000 interactions with *A. thaliana* and other host plants.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids

P. syringae strains were grown on King's B medium (King *et al.*, 1954) or NYG medium (Daniels *et al.*, 1988) at 28 °C, and *E. coli* strains were maintained on LB medium (Sambrook *et al.*, 1989) at 37 °C. Antibiotics used for selection of *P. syringae* strains included (in μg/mL): rifampicin, 100; kanamycin, 25; and spectinomycin, 100. The antibiotic used for selection of *E. coli* strains was ampicillin (at 100 μg/mL).

Plant material and inoculation procedures

A. thaliana ecotype Columbia (Col-0), the Col-0 *sid2-2* mutant (Dewdney *et al.*, 2000) and Col-0 *nahG* plants (Reuber *et al.*, 1998) were used in this study. Plants were grown from seed in 3 × 3-inch plastic pots covered with nylon mesh, and maintained in growth chambers for 4–5 weeks under an 8-h photoperiod at 22 °C and 75% relative humidity. To assay for disease, *A. thaliana* plants were dip inoculated by immersing whole rosettes into bacterial suspensions ($\sim 5 \times 10^8$ cfu/mL) containing 0.02% Silwet L-77 (OSi Specialties Inc., Danbury, CT) and 10 mM $MgCl_2$ as described previously (Kunkel *et al.*, 1993), then returned to growth chambers and covered with clear plastic lids. Lids were removed 1 day after infection, and disease was monitored 3–5 days after inoculation. To determine levels of *P. syringae* bacteria within leaf tissue, individual rosette leaves were removed 0, 2 and 4 dpi and weighed. The leaves were then homogenized and dilutions plated on media containing the appropriate antibiotics and amphotericin B (2.5 μg/mL) to inhibit fungal growth. Leaves sampled on day 0 were surface sterilized in 15% H_2O_2 for 5 min and then rinsed twice with sterile water prior to homogenization and plating.

RNA extraction and analysis

At each time point approximately 0.5 g of leaf tissue was removed from infected *A. thaliana* rosettes, flash-frozen in liquid nitrogen and stored at –80 °C until RNA was extracted. The 0 h time point in dip inoculation studies represents tissue removed from plants ~30 min after inoculation, which allowed tissue to dry prior to freezing. A modified extraction technique based on the RNAgents Kit (Promega, Madison, WI) was employed as follows: a pestle and mortar were precooled with liquid N_2 and frozen leaves were homogenized to a fine powder. Extraction buffer (8 mL) was added, and the tissue was further homogenized. Total RNA (> 1 mg) was extracted from each 0.5 g sample of leaf

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tissue in this manner, and quantified by UV-spectroscopy. For gel blot analysis, 30 μg of total RNA for each sample was used following standard procedures (Sambrook *et al*., 1989). RNA blots were sequentially probed using DNA probes amplified by PCR and radio-labelled with the Redi-Prime Kit (Amersham North America, Piscataway, NJ). To generate probes corresponding to the *A. thaliana CORI1* mRNA, a *CORI1* cDNA fragment was amplified from pBBS::CORI1, which contains a 1.5-kb *Eco*RI–*Xho*I cDNA fragment from the *A. thaliana CORI1* gene (R. Thilmony and S. He, personal communication; Benedetti *et al*., 1996), using the T3 (5′-ATTAACCCTCACTAAAG-3′) and T7 (5′-GTAATACGACTCACTATAGGGC-3′) vector-based primers. The *LOX2* probe was amplified from a clone containing a 1.0-kb *Eco*RI–*Bam*HI cDNA fragment from the *A. thaliana LOX2* gene cloned into pZL1 (Bell and Mullet, 1993) using the M13FOR (5′-CCCAGTCACGACGTTGTAAAACG-3′) and M13REV (5′-AGCGGATAACAATTTCACACAGG-3′) vector-based primers. The *PDF1.2* probe was generated using a 0.5-kb *PDF1.2 A. thaliana* cDNA clone in pBluescript KS (+) and the T3 and T7 vector-based primers mentioned above. *A. thaliana PR1*, *PR2* and *PR5* probes were similarly amplified from cDNA clones corresponding to these genes (Uknes *et al*., 1992). A fragment hybridizing to the *A. thaliana NHO1* mRNA was amplified from genomic DNA using the following primers, which span the largest exon of the gene: NHO1FOR (5′-ATTGTCTGGATGGATGCTCGTACC-3)′ and NHO1REV (5′-CTGTCTCTAAGCCACTGAACAGCA-3′). These primers were designed with the use of the published *NHO1* gene complete sequence (Lu *et al*., 2001). The *ICS1* probe was prepared as previously described (Wildermuth *et al*., 2001).

Imaging of hybridization patterns was performed with a Personal FX phosphorimager (Bio-Rad, Hercules, CA). Blots were stripped prior to re-probing by boiling in 0.5% SDS for 15 s. Probe removal was confirmed by re-imaging of the stripped blot.

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