The Coronatine Toxin of *Pseudomonas syringae* Is a Multifunctional Suppressor of *Arabidopsis* Defense

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**INTRODUCTION**

Successful bacterial pathogens overcome plant immune responses. Contributions to defense suppression come from phytotoxins and type III effectors, which are bacterial proteins injected into host cells via the type III secretion system (Büttner and Bonas, 2002; He et al., 2004; Chisholm et al., 2006). Coronatine (COR) is a phytotoxin produced by several strains of *Pseudomonas syringae*, including *P. syringae* pv tomato strain DC3000 (Pto). Two moieties, coronafacic acid and coronamic acid, are conjugated by an amide linkage to form COR (Brooks et al., 2004), which is the predominantly active molecule in plant tissues (Uppalapati et al., 2005). COR is a structural and functional mimic of JA-Ile, the bioactive conjugate of jasmonic acid (JA) and Ile (Fonseca et al., 2009). JA signaling regulates plant growth and development and also plays essential roles in plant defense (Ballaré, 2011). The F-box protein CORONATINE INSENSITIVE1 (COI1) is a key component of the JA signaling pathway (Feys et al., 1994; Xie et al., 1998). JA-Ile interacts with a complex of COI1 and a jasmonate ZIM-domain (JAZ) transcriptional repressors protein and promotes the SCF**COI1** ubiquitin E3 ligase complex to induce proteasome-mediated degradation of the JAZ protein (Thines et al., 2007; Sheard et al., 2010). JA-responsive gene expression is activated by elimination of inhibitory JAZ proteins (Chini et al., 2007). Remarkably, COR is ~1000 times more active than JA-Ile, in vitro, at stabilizing interactions between JAZ proteins and tomato (*Solanum lycopersicum*) COI1 (Katsir et al., 2008).

COR promotes multiple aspects of *P. syringae* virulence, including reopening of stomata to facilitate invasion, proliferation in the apoplast, and development of disease symptoms (Bender et al., 1987, 1999; Brooks et al., 2004, 2005; Cui et al., 2005; Melotto et al., 2006; Uppalapati et al., 2008; Freeman and Beattie, 2009; Ishiga et al., 2009). The reduced virulence of Pto mutants unable to produce COR (hereafter referred to as Ptocor-) correlates with enhanced defense responses of the plant. The defense suppressing activity of COR, as well as endogenous JA conjugates, is at least partially dependent on their ability to antagonize salicylic acid (SA) signaling via COI1 activation. COR inhibits SA accumulation by differentially regulating the transcription of genes involved in its biosynthesis and metabolism (Zheng et al., 2012). COI1 mutant plants, which display enhanced resistance against Pto and other biotrophs, accumulate more SA at early stages of Pto infection (Kloek et al., 2001). Also, the impaired growth of Ptocor- is restored in SA signaling–deficient mutant plants (Brooks et al., 2005; Melotto et al., 2006; Zeng and He, 2010). COR also may function independent of suppressing SA signaling. The COR-dependent symptoms induced by Pto are not restored when Ptocor- infects tomato plants deficient in SA signaling (Brooks et al., 2005; Uppalapati et al., 2005). Also, pathogen-associated molecular pattern (PAMP)-induced callose deposition in *Arabidopsis thaliana* roots, which does not require SA signaling, is suppressed by COR (Millet et al., 2010).

COR may promote bacterial virulence through regulation of secondary metabolism. When *Arabidopsis* is challenged by Pto, COR affects the expression of genes involved in the synthesis of anthocyanin as well as Trp- and Met-derived glucosinolates (Thilmony et al., 2006). Trp-derived indole glucosinolates contribute to the elicitation of *Arabidopsis* defenses in response to PAMPs and non-host-adapted fungi, with the hydrolysis of...
4-methoxy-indol-3-ylmethylglucosinolate (4MI3G) by the atypical myrosinase, PENETRATION2 (PEN2), playing a key role (Bednarek et al., 2009; Clay et al., 2009). PEN2 is required for callose deposition induced by flg22 (a peptide PAMP from bacterial flagellin) in the cotyledons of liquid-grown Arabidopsis seedlings, and, notably, exogenous JA suppresses the response (Clay et al., 2009).

In addition to COR, Pto also depletes three effector functions to promote its virulence. HopM1 and AvrE1 are key type III effector genes within the conserved effector locus (CEL), a region flanking the genes encoding the structural components of the type III pilus (Alfano et al., 2000). A Pto mutant lacking the CEL (PtoΔCEL) displays reduced virulence on tomato and Arabidopsis (DebRoy et al., 2004; Badel et al., 2006). PtoΔCEL grows less well and induces stronger cell wall reinforcement, as measured by callose deposition, than wild-type Pto on Arabidopsis plants competent in SA signaling. These defenses are suppressed by either HopM1 or AvrE1, indicating that HopM1 and AvrE1 proteins suppress SA-dependent defense (DebRoy et al., 2004). Curiously, despite the ability of COR to suppress SA signaling, PtoΔCEL still elicits SA-dependent defense. Thus, in the context of a PtoΔCEL infection, COR may promote virulence independent of suppressing SA signaling.

Here, we analyzed defense responses elicited when Pto or Pto mutants lacking the CEL, unable to produce COR, or both are introduced into the leaves of soil-grown Arabidopsis plants with compromised defense signal transduction, including single and multiple mutants disrupting SA production, SA signaling, JA perception, and glucosinolate metabolism (see Supplemental Table 1 online). To focus on postinvasive defense, the experiments were done following infiltration of bacteria into the interior of plant leaves. Our results indicate that the activity of COR can be obscured by partially overlapping functions of type III effectors of the CEL. By examining the activity of COR in the PtoΔCEL strain, we show that, in addition to suppressing SA signaling, COR also suppresses callose deposition and promotes bacterial growth in a manner independent of suppressing SA signaling. We show that COR inhibits accumulation of an indole glucosinolate involved in the callose response. Additionally, we show that COR inhibits callose deposition in a col1 mutant, indicating a COI1-independent function of COR. These findings significantly extend our understanding of the relationship between COR and type III effectors in suppressing plant immunity and indicate that COR is a multifunctional toxin that likely has a plant target(s) in addition to COI1.

RESULTS

COR Suppresses SA-Dependent and SA-Independent Defense Responses

COR suppresses SA accumulation via COI1 activation (Kloek et al., 2001; Uppalapati et al., 2005, 2007), and yet the PtoΔCEL mutant, which produces COR, elicits SA-dependent defense responses that promote callose deposition and restrict bacterial growth (DebRoy et al., 2004). To understand the effect of COR on SA-dependent and SA-independent defense responses against Pto, we examined the interaction of Arabidopsis with Pto, PtoΔCEL (Alfano et al., 2000), Ptocor- (Brooks et al., 2004), and a PtoΔCEL cor- double mutant (see Supplemental Figure 1A online).

We first measured expression of the PATHOGENESIS RELATED1 (PR-1) gene, which is a marker of SA-dependent defense. PR1 transcript and protein accumulation was monitored after infiltration of Columbia-0 (Col-0) plants with a high-titer suspension of all four strains (see Supplemental Figure 2 online). While all four strains induced significantly more PR-1 transcript than buffer, Pto and PtoΔCEL induced less transcript than Ptocor- and PtoΔCEL cor-. Additionally, PtoΔCEL cor- induced more PR-1 protein accumulation than did PtoΔCEL after 48 and 72 h. Collectively, these data confirm previous findings that COR suppresses Pto-induced PR-1 expression, an effect that has been attributed to COR mimicking JA-ile and antagonizing SA signaling. Free SA levels were not higher after challenge with the COR-deficient strains than the COR-producing strains (see Supplemental Figure 3 online). Thus, COR may suppress Pto-induced PR-1 expression independent of suppressing SA levels.

Next, we examined the elicitation of callose in wild-type Col-0 and plants deficient in SA production and SA signaling (Figures 1A and 1B). SA-induction deficient-2 (sid2) encodes an isochorismate synthase required for defense-associated SA production (Wildermuth et al., 2001). In sid2 mutant plants, neither Pto nor any of our mutant strains induced detectable levels of SA (see Supplemental Figure 3 online). Nonexpressor of pathogenesis-related genes 1 (NPR1) encodes a protein that makes key contributions to SA signaling, including the activation of PR-1 expression (Cao et al., 1997). PtoΔCEL elicited high levels of callose deposition in Col-0 plants and reduced levels in sid2 and npr1 mutant plants, consistent with the findings of DebRoy et al. (2004) in other Arabidopsis mutant plants that do not accumulate SA (eds5 mutant and nahG expressing). Thus, although COR suppresses SA-dependent PR-1 expression elicited by PtoΔCEL, it fails to suppress SA-dependent callose deposition.

COR did suppress SA-independent callose deposition during Pto infection. Ptocor- elicited more callose than Pto in Col-0 and sid2 plants (Figure 1B). Thus, the inhibition of callose deposition by COR cannot be accounted for by suppression of SA signaling. This observation was reinforced by the observation that PtoΔCEL cor- elicited more callose than PtoΔCEL in sid2 and npr1 plants. Thus, COR suppresses a pathway that contributes to callose deposition in plants deficient in SA accumulation or signaling.

To determine if the increased callose response against COR-deficient strains of Pto resulted from their inability to produce COR, we tested for complementation by exogenous COR. PtoΔCEL or PtoΔCEL cor- alone or in combination with various concentrations of COR were infiltrated into the leaves of sid2 plants (Figure 1C). The increased callose deposition elicited by PtoΔCEL cor- relative to PtoΔCEL was inhibited by exogenous COR in a dose-dependent manner, with partial suppression by 0.3 μM COR and full suppression by 3 and 30 μM COR. Thus, COR suppresses callose deposition in response to Pto.

The results for PR-1 expression and callose induction indicate that COR suppresses both SA-dependent and SA-independent Arabidopsis defense responses. Next, we measured the contribution of COR to Pto growth in both wild-type and SA...
signaling–deficient plants. Col-0, sid2, and npr1 plants were infiltrated with a low-titer inoculum of Pto or Ptocor-, and bacterial numbers were measured after 4 d (Figure 1D). Consistent with earlier reports that COR promotes Pto growth when the bacteria are directly infiltrated into the apoplast at a low concentration (Zeng and He, 2010), we found that Ptocor- grew to lower levels than Pto in Col-0. And, as expected, sid2 and npr1 plants showed enhanced disease susceptibility (eds) relative to Col-0 plants. Notably, Pto grew to significantly higher levels than Ptocor- in sid2, npr1, and sid2 npr1 mutant plants. (Figure 1D; see Supplemental Figure 4B online). Thus, COR promotes Pto growth in SA signaling–deficient plants.

**COR and Type III Effectors of the CEL Make Overlapping Contributions to the Virulence of Pto**

We also measured the growth of PtoΔCEL and PtoΔCEL cor- in wild-type and SA signaling–deficient plants. The Ptocor- and PtoΔCEL single mutant strains grew to similar levels to one another and both strains grew significantly less than Pto in both Col-0 and SA signaling–deficient mutants (Figure 1D). The PtoΔCEL cor- double mutant strain consistently grew less than either the PtoΔCEL or Ptocor- single mutant. Although not supported by analysis of variance (ANOVA) on the composite data set in Figure 1D, the reduced growth of PtoΔCEL cor- relative to PtoΔCEL was significant (P < 0.05) by two-tailed t test in 11 of 12 comparisons of the two bacteria in Col-0, sid2, or npr1 from the four biological replicates comprising the data. Thus, COR and type III effectors from the CEL promote bacterial growth in the apoplastic space in a manner that (1) is at least partially independent of suppressing SA signaling and (2) is overlapping but not entirely redundant.

DebRoy et al. (2004) found that HopM1 suppresses SA-dependent defense signaling elicited by PtoΔCEL. Our results indicate that (1) both SA-dependent and SA-independent pathways contribute to callose elicitation by PtoΔCEL and (2)
COR suppresses the SA-independent pathway. To examine the defense suppressing activity of HopM1 in the context of our findings, we analyzed strains of PtoΔCEL and PtoΔCEL coi- expressing HopM1 from a plasmid, called PtoΔCEL (HopM1) and PtoΔCEL coi- (HopM1), respectively. Callose induction by and growth of these strains were measured in Col-0 and sid2 plants. HopM1 suppressed callose deposition induced by PtoΔCEL and PtoΔCEL coi- in Col-0 as well as that induced by PtoΔCEL coi- in sid2 (Figure 2A). Consistent with the findings of DebRoy et al. (2004), PtoΔCEL (HopM1) grew to higher levels than PtoΔCEL (Figure 2B). Though this conclusion is not supported by ANOVA, the differences in Col-0 and sid2 were significant (P < 0.05) by two-tailed t test. Notably, we also observed that PtoΔCEL coi- (HopM1) grew significantly better than PtoΔCEL coi- in both plant backgrounds. The ability of HopM1 to suppress callose deposition and enhance bacterial growth in sid2 plants indicates that its virulence activity is not mediated by suppression of SA signaling per se.

COI1 Suppresses SA-Dependent and SA-Independent Defense Responses Effective against Pto

We examined the role of COI1 in Arabidopsis defense against Pto and activity of COR. We tested if suppression of callose deposition by COR is dependent on COI1 (Figure 3A). Ptocor-elicted more callose than did Pto in wild-type Col-0. However, although overall levels of callose were elevated, Pto and Ptocor-elicted similar amounts of callose in coi1 plants. These results indicate that the ability of COR to suppress callose induction by Pto is dependent on COI1 (at least in plants with intact SA signaling; see below). Furthermore, these results indicate that, even if the bacteria do not produce COR, COI1-dependent signaling suppresses the levels of callose deposition, likely by responding to increases in plant-derived JA conjugates. Callose levels are similar in Col-0 and coi1 plants following challenge with PtoΔCEL and PtoΔCEL coi-, indicating that the lack of COR or the coi1 mutation does not significantly increase callose deposition beyond the high level induced by PtoΔCEL in Col-0.

Next, we tested the role of COI1 in mediating the growth enhancement provided by COR to Pto. Consistent with other coi1 alleles (Feys et al., 1994; Kloek et al., 2001), we observed that coi1-16 plants displayed an enhanced disease resistance (edr) phenotype against wild-type Pto (Figure 4C; see Supplemental Figure 4B online). The significant reduction in growth of Ptocorrelative to Pto in Col-0 plants was not apparent in coi1, and, similarly, growth of PtoΔCEL coi- was not reduced relative to PtoΔCEL in coi1 plants (Figure 4C; see Supplemental Figure 4B online). Thus, COR fails to enhance the growth of Pto in coi1 plants, indicating that, in SA signaling–competent plants, COR requires COI1 to promote Pto growth.

The edr phenotype of coi1 mutant plants to Pto is hypothesized to result from elevated SA signaling due to the lack of inhibitory crosstalk from COI1 (Feys et al., 1994; Kloek et al., 2001). However, our findings that COR also suppresses SA-independent defense raises the possibility that COI1 suppresses both SA-dependent and SA-independent defense responses. To explore this possibility, we generated sid2 coi1 and npr1 coi1 double mutant and sid2 npr1 coi1 triple mutant plants. We observed that each of our Pto strains induced elevated SA accumulation in coi1 relative to Col-0 and that this increase was statistically significant for all but wild-type Pto (see Supplemental Figure 3 online). No SA accumulation is detected in the sid2 coi1 double mutant, excluding the possibility that the coi1 mutation stimulates SId2-independent SA production. Thus, the sid2 and sid2 coi1 mutants are SA accumulation deficient in response to the examined Pto strains.

Pto strains grew better in multiple mutant plants deficient in both SA and JA signaling than in coi1 plants deficient in just JA signaling (Figure 4C; see Supplemental Figure 4B online). Thus, COI1-mediated suppression of SA signaling does contribute to restricting Pto growth. However, Pto strains grew less well in the SA and JA signaling–deficient plants than in sid2, npr1, and sid2 npr1 plants only deficient in SA signaling (Figures 1D and 4C; see Supplemental Figure 4B online). Thus, the edr phenotype of

Figure 2. HopM1 Promotes Bacterial Virulence in SA Signaling–Deficient Plants.

(A) Quantification of callose deposits following infiltration of the indicated strains into Col-0 and sid2 leaves. Shown are the mean and se of combined data from two independent biological replicates.

(B) Growth of the indicated strains 4 d after inoculation into Col-0 and sid2 plants. The dashed line indicates the starting inoculum of bacteria. Shown are the mean and se of four biological replicates. Different letter types indicate significant differences (P < 0.05) by one-way ANOVA and Tukey HSD test of comparisons between the indicated bacterial strains on individual plant genotypes.
COR inhibits the PEN2-dependent branch of a pathway contributing to callose deposition in response to Pto

The atypical myrosinase PEN2 (Bednarek et al., 2009) is required for flg22-induced callose deposition in the cotyledons and roots of liquid-grown Arabidopsis seedlings (Clay et al., 2009; Millet et al., 2010). However, the pen2 mutant did not differ from wild-type Col-0 in flg22-induced callose deposition in the leaves of soil-grown plants (see Supplemental Figure 5A online), although we did reproduce the finding that flg22-induced callose deposition is dependent on pen2 and is not dependent on sid2 in liquid-grown seedlings (see Supplemental Figure 5B online). In the liquid-grown seedling assay, pretreatment with exogenous SA rescued callose deposition in pen2 mutant plants (Clay et al., 2009). Based on these results, we hypothesize that (1) in the liquid-grown seedling assay, diffusion of endogenous SA renders the plants effectively SA accumulation deficient and thus dependent on PEN2; and (2) in soil-grown plants, the PAMP-induced accumulation of SA (Tsuda et al., 2008) permits callose deposition in the pen2 mutant background.

We further hypothesized that COR suppresses callose deposition elicited by PtoΔCEL in SA signaling–deficient plants by targeting the PEN2-dependent pathway. To examine the relationship between COR, PEN2-dependent signaling, and SA-dependent signaling, we compared callose deposition induced by PtoΔCEL and PtoΔCEL cor- in wild-type, sid2, pen2, and sid2 pen2 mutant plants (Figure 5A). In Col-0 and pen2 mutant plants, callose deposition was strong in response to both strains. In sid2 single mutant plants, callose deposition was strong in response to PtoΔCEL cor- and was reduced in response to PtoΔCEL. In sid2 pen2 double mutant plants, callose deposition was reduced in response to both PtoΔCEL and PtoΔCEL cor-. Thus, the high level of callose elicited by PtoΔCEL cor- in sid2 mutant plants is PEN2 dependent and COR suppresses the PEN2-dependent pathway. Interestingly, PtoΔCEL cor- elicited more callose than PtoΔCEL in the sid2 pen2 double mutant plants. This result indicates that the pen2 mutant is not completely penetrant, perhaps due to other myrosinases that can substitute for PEN2, and that COR suppresses the residual activity in the pen2 mutant background.

COR Suppresses Indole Glucosinolate Metabolism Upstream of PEN2

Next, we sought to ascertain the effect of COR on the PEN2-dependent pathway. Pathogen or PAMP elicitation induces expression of numerous enzymes, including cytochrome P450s, that mediate the multistep conversion of Trp into 4MI3G (Bednarek et al., 2009; Clay et al., 2009). PEN2 then hydrolyzes 4MI3G into an as yet unidentified substrate that is crucial for the eventual deposition of callose. 4MI3G levels do not fluctuate significantly following defense elicitation in wild-type plants, presumably because it is efficiently processed by PEN2. However, 4MI3G levels increase following elicitation in pen2 mutant plants. We reasoned that COR would prevent the accumulation of 4MI3G in pen2 mutant plants if it functions upstream of PEN2 enzyme activity, but not if it inhibits PEN2 activity or downstream events. Infiltration of flg22, PtoΔCEL, or PtoΔCEL cor- each elicited
elevated accumulation of 4MI3G in pen2 mutant plants relative to Col-0 (Figure 5B). Notably, in pen2, PtoΔCEL cor- elicited significantly more 4MI3G than did PtoΔCEL. Also, exogenous COR (3 μM) suppressed 4MI3G accumulation elicited by PtoΔCEL cor-. The precursor indol-3-ylmethylglucosinolate (I3G) is first hydroxylated then converted by a methyltransferase into 1-methoxy-indol-3-ylmethylglucosinolate (1MI3G) and 4MI3G (Pfalz et al., 2011). Infiltration with fig22 also induced elevated accumulation of 1MI3G in pen2 relative to Col-0 plants (see Supplemental Figure 6A online). And similar to 4MI3G, 1MI3G accumulation induced by PtoΔCEL cor- was suppressed by exogenous COR. Though not supported by ANOVA, this difference was significant (P < 0.05) by two-tailed t test. Levels of I3G in pen2 plants did not vary significantly with any of the tested infiltrations (see Supplemental Figure 6B online). Collectively, these data indicate that COR suppresses glucosinolate metabolism upstream of 1MI3G and 4MI3G accumulation.

We sought to determine the relationship between SA signaling and the suppression of glucosinolate metabolism by COR. In each of three biological replicates, fig22 elicited significantly higher levels of 4MI3G and 1MI3G in pen2 than sid2 pen2 plants (composite data shown in Figure 5B and Supplemental Figure 6A online). This observation, along with the elicitation of SA by fig22 (Tsuda et al., 2008), indicates that SA promotes indole glucosinolate metabolism. On the contrary, PtoΔCEL elicited similar levels of 4MI3G and 1MI3G in both pen2 and sid2 pen2. Thus, COR may limit glucosinolate metabolism upstream of PEN2 function by inhibiting SA signaling. However, COR also suppresses glucosinolate metabolism independent of suppressing SA signaling. In sid2 pen2 plants, accumulation of 4MI3G and 1MI3G induced by PtoΔCEL cor- was suppressed by exogenous COR. Though not supported by ANOVA, this conclusion is in both cases significant (P < 0.05) by two-tailed t test. Thus, in SA signaling–deficient plants, COR suppresses both glucosinolate metabolism and callose deposition.

COR might suppress 4MI3G and 1MI3G accumulation by preventing the expression of enzymes required for glucosinolate metabolism. In roots, COR suppresses the expression of MYB51
(Millet et al., 2010), which is an R2R3 MYB family transcription factor that promotes the expression of numerous cytochrome P450 monooxygenases involved in glucosinolate metabolism (Gigolashvili et al., 2007) and is required for PAMP-induced callose deposition in liquid-grown seedlings (Clay et al., 2009). CYP79B2 and CYP79B3 encode cytochrome P450 monooxygenases that convert Trp into indol-3ylacetaldoxime (Hull et al., 2000). CYP83B1 and CYP81F2 participate in the multistep conversion of indol-3ylacetaldoxime into 4MI3G (Bak et al., 2001; Bednarek et al., 2009). PAMP-induced expression of CYP79B2, CYP79B3, and CYP83B1, but not CYP81F2, are MYB51 dependent in liquid-grown seedlings (Bednarek et al., 2009; Clay et al., 2009). In soil-grown plants, PtoΔCEL induced expression of all five genes between approximately threefold and ~10-fold relative to a buffer infiltration (Figure 6). The complex effect of COR on expression of these genes was revealed by comparing PtoΔCEL to PtoΔCEL cor-. Relative to PtoΔCEL cor-, PtoΔCEL induced less expression of MYB51, comparable expression of CYP83B1 and CYP81F2, and increased expression of CYP79B2 and CYP79B3. Thus, bacterially produced COR has variable effects on the expression of genes involved in indole glucosinolates metabolism. MYB51 may act as a master regulator responsible for the ultimate reduction in 4MI3G accumulation (Clay et al., 2009), but how it does so remains unclear.

**PEN2** is important for antifungal defense (Bednarek et al., 2009). To test whether **PEN2** contributes to defense against Pto, we tested bacterial growth in Col-0, sid2, pen2-1, and sid2 pen2-4 mutant plants (Figure 5C). The growth of Pto, Ptocor-, PtoΔCEL, and PtoΔCEL cor- does not differ significantly between Col-0 and pen2, indicating that **PEN2** does not play a major role in defense against these strains of Pto. Interestingly, the eds phenotype of sid2 pen2 relative to pen2, which is apparent for Pto, Ptocor-, and PtoΔCEL, is not apparent for PtoΔCEL cor-. Thus, **PEN2** contributes to the susceptibility of SA-deficient plants to Pto severely compromised by the lack of key type III effectors from the CEL and an inability to produce COR.

![Figure 5. COR Inhibits Indole Glucosinolate Accumulation Upstream of PEN2.](image)
DISCUSSION

Our results support a model integrating the contributions of SA signaling, JA signaling, and indole glucosinolate metabolism and the suppressive activities of COR to Arabidopsis defense against Pto (Figure 7). COR and type III effectors from the CEL both make significant contributions to suppressing callose and promoting Pto growth. Although these virulence activities are largely overlapping with respect to bacterial growth, COR and HopM1 differ in their modes of action. COR suppresses callose deposition mediated by a PEN2-dependent pathway but cannot block callose deposition in SA signaling–competent plants. HopM1, on the other hand, suppresses callose deposition mediated through the SA-dependent and PEN2-dependent pathways. Thus, HopM1 does not suppress SA signaling per se, but instead may suppress a signaling step upstream or downstream of the requirements for SA and PEN2.

COR suppresses SA signaling. The ability of COR to suppress SA-dependent PR-1 expression has been shown here and elsewhere (Kloek et al., 2001) and is thought to occur through suppression of SA accumulation by activated COI1 (Spoel and Dong, 2008). However, SA measurements did not reveal increased SA accumulation in response to COR-deficient strains. Furthermore, COR fails to suppress SA-mediated callose deposition elicited by PtoΔCEL. Thus, differences may exist between the quantitative requirement for and qualitative nature of SA signaling in callose deposition versus PR-1 expression. SA signaling promotes various aspects of Arabidopsis defense against P. syringae, including activation of PR genes by NPR1 in association with TGA transcription factors and genes involved in vesicle secretion by NPR1 through TL1 elements in their promoters (Fan and Dong, 2002; Wang et al., 2005). Notably, vesicle secretion is required for efficient cell wall fortification, including callose deposition (Assaad et al., 2004; Hardham et al., 2007). Thus, the activation of secretion by SA could contribute to its support of callose deposition, perhaps downstream or independent of PEN2. The reduced accumulation of 4MI3G and 1MI3G following PAMP elicitation in sid2 pen2, compared with pen2, indicates that SA signaling also supports callose deposition by promoting indole glucosinolate metabolism upstream of PEN2. This activity of SA may result from its ability to suppress the accumulation of JA, which, similar to COR, may antagonize 4MI3G accumulation. 4MI3G accumulation was not compromised in SA signaling–deficient plants in response to aphid feeding, perhaps because JA signaling is already strongly induced (Kim and Jander, 2007).

COR also suppresses Arabidopsis defense independent of suppressing SA signaling. The enhanced callose deposition elicited by and the reduced growth of COR-deficient strains was apparent in SA signaling–deficient plants. In fact, the contribution of COR to suppressing callose induction by PtoΔCEL is only apparent in SA signaling–deficient mutants. Defense suppressing activities of COR in SA signaling–deficient mutant plants are apparent in both COI1 and coi1 backgrounds. Of particular note is the ability of COR to suppress callose deposition and promote the growth of P. syringae in coi1 plants. An activity of COR, other than targeting COI1, has not been described. One possibility is that, in functioning as a JA mimic, COR stimulates COI1-independent
responses. JA induces COI1-independent transcriptional re-programming in Arabidopsis (Devoto et al., 2005). Alternatively, COR may have a function other than acting as a JA mimic. The coronamic acid component of COR is a structural mimic of ACC and COR has been shown to elicit de novo production of ethylene from Met (Ferguson and Mitchell, 1985; Kenyon and Turner, 1992). Ethylene signaling contributes to the expression of MYB51 and plays a key role in callus deposition (Clay et al., 2009; Millet et al., 2010). Thus, it is reasonable to speculate that COR perturbs ethylene signaling.

COR regulates secondary metabolism. Glucosinolates are a class of thioglucosides produced by the Capparales, including Arabidopsis, which play significant roles in defense against insects and microorganisms (Halkier and Gershenzon, 2006; Bednarek et al., 2009; Clay et al., 2009). Trp-derived indole glucosinolates, including 4MI3G, are key in defense against microorganisms. Our previous findings indicate that COR activates the expression of CYP79B2 and CYP79B3, the gene products of which convert Trp into indol-3-ylacetaldoxime (Zhao et al., 2002; Thilmont et al., 2006). In another interesting result, spraying Arabidopsis leaves with 5 μM COR induces the expression of ST5a, which synthesizes I3G (Piotrowski et al., 2004), which is subsequently converted by CYP81F2 and indole glucosinolate methyltransferases into 4MI3G and 1MI3G (Bednarek et al., 2009; Pfalz et al., 2011). We observed that bacterially produced and/or exogenous COR suppressed MYB51 expression 6 h and 4MI3G and 1MI3G accumulation upstream of the PEN2 myrosinase 9 h after bacterial challenge. Thus, the effects of COR on gene expression and glucosinolate metabolism are complex.

SA signaling and COI1-dependent signaling are antagonistic to one another (Sopel and Dong, 2008). Here, we demonstrate that, in addition to antagonizing one another, both regulate Arabidopsis defense against P. syringae independent of the other. The eds phenotype of SA signaling mutants is still apparent in the coi1 background, and the cfr phenotype of the coi1 mutant is still apparent in SA signaling mutant backgrounds. The virulence activity of COR can be mediated in three ways: (1) activation of COI1 to suppress SA signaling, (2) activation of COI1 to suppress SA-independent defense responses, and (3) through an unknown mechanism that is independent of targeting COI1 and may be independent of mimicking JA. Thus, COR is a multifunctional suppressor of plant immunity.

METHODS

Plants

Arabidopsis thaliana plants used in this work were of the Col-0 ecotype and were grown under 8 h (115 μmol m⁻² s⁻¹), 23°C days, and 16 h, 16°C nights. The following mutants were used: sid2-2 has a deletion from amino acid 439 to 455 (Wildermuth et al., 2001); npr1-1 has a mutation of conserved His into Tyr at amino acid 334 (Cao et al., 1997); coi1-16 has a mutation of Leu to Phe at amino acid 245 (Ellis and Turner, 2002); pen2-4 has a mutation of Gly to Asp at amino acid 150 (Westphal et al., 2008); pen2-1 has a mutation at amino acid 48 Trp to stop codon (Lipka et al., 2005). The original line carrying coi1-16 also harbored the pen2-4 mutant allele (Westphal et al., 2008). The coi1-16 and pen2-4 single mutants and the various double and triple mutants described in the work were produced by crossing and marker-assisted selection (see Supplemental Figures 1B to 1E and Supplemental Tables 1 and 2 online).

Bacteria

Pto and mutant strains were grown at 28°C on King’s B (KB) plates containing the appropriate antibiotics for selection. Mutant strains used are PtoΔCEL (Alfano et al., 2000) and PtoΔcor- (DB4G3) (Brooks et al., 2004). To construct the PtoΔCEL cor- double mutant, homologous recombination was used to introduce the cfa6 mutation into PtoΔCEL. Plasmid pDB29, which is a derivative of pRK415 (Tc) containing a 7.8-kb genomic fragment from strain DB4G3 including the 3.2-kb Tn5 insertion in cfa6 and 4.5-kb of flanking DNA (cfa6::Tn5 uidA Km') (Brooks et al., 2004), was transformed into PtoΔCEL (Sm/Sp). Selection of kanamycin and tetracycline resistance clones isolated cells carrying pDB29. These were then propagated for 4 d in liquid KB media (diluting the saturated culture 1:1000 into fresh media every day) containing kanamycin (50 μg/mL) but lacking tetracycline to allow: (1) homologous recombination between the wild type, chromosomal cfa6, and plasmid-borne, cfa6::Tn5 and (2) subsequent loss of the pRK415 plasmid. The final bacterial culture was diluted and plated on KB containing kanamycin. Individual clones were then replica plated onto Sp Km and Sp Km Tc to identify Sp Km' Tc' clones as candidate pRK415-free double mutants. PCR screening of individual clones for insertion of Tn5 into cfa6 identified the PtoΔCEL cor- mutant. Primers used for screening are shown in Supplemental Table 2 online. The plasmid pORF43, which carries HopM1-ShcM (DebRoy et al., 2004), was transformed into PtoΔCEL and PtoΔCEL cor- by electroporation to generate PtoΔCEL (HopM1) and PtoΔCEL cor- (HopM1).

Bacterial growth assays were conducted by infiltrating 10⁵ colony-forming units (CFU)/mL (OD₆₀₀ = 0.0002) in 10 mM MgCl₂ into the underside of the leaves of 5-week-old plants using a needleless 1cc syringe. After the infiltrated leaves were dry, plants were returned to the growth room. After 4 d, nine leaf discs were separated into three technical replicates containing three leaf discs each. The bacterial titer in each technical replicate was determined by grinding leaf discs to homogeneity in 10 mM MgCl₂, serially diluting the samples in a 96-well plate, and transferring with a multiplating tool onto KB plates with appropriate selection (Kim and Mackey, 2008). Colonies were counted and used to calculate the mean CFU/cm² for each treatment, and the values were log transformed. The log-transformed means from individual biological replicates, as single data points, were then combined from multiple independent biological replicates and used to calculate the mean and standard error. Minitab 16 statistical software was used to determine significant differences by one-way ANOVA and Tukey honestly significant difference (HSD) test (P < 0.05), either between bacteria in the same plant background or between plants infiltrated with the same bacterial strain. Where indicated by brackets, the two-tailed t test was used to compare the indicated pairs of treatments.

Protein Immunoblotting

Five-week-old leaves of Col-0 plants were hand-infiltrated with 10⁶ CFU/mL (OD₆₀₀ = 0.2) of bacteria in 10 mM MgCl₂. After infiltrated leaves were dry, the plants were covered with the transparent lid for the reminder of the experiment. Protein extracts were prepared and quantified as previously described (Kim et al., 2005). Samples containing 20 μg of protein were resolved on 12% SDS-PAGE gels, transferred to polyvinylidene difluoride membrane, and blotted with Anti-PR-1 sera (Kleibenstein et al., 1999) at 1:10,000.

Callose Staining

Callose detection was conducted as previously described (Kim and Mackey, 2008). Briefly, leaves of 4-week-old plants were hand-infiltrated
with 10^6 CFU/mL of bacteria in 10 mM MgCl₂ or 30 μM fig22. At 15 h after infiltration, leaves were collected, cleared with lacticophenol, washed with 50% ethanol and then with water, stained with 0.01% aniline blue dissolved in 150 mM K₂HPO₄, pH 9.5, mounted on slides in 50% glycerol, and examined with a Nikon Eclipse 80i epifluorescent microscope. At least four to five individual leaves were analyzed for each treatment. Images were captured from the similar middle area of each leaf, and the number of callose deposits was calculated using ImageJ software (http://rsweb.nih.gov/ij). Except where noted in Supplemental Figure 5 online, similar results for all callose experiments were observed in three or more independent biological replicates. Statistical analyses of log-transformed data were by one-way ANOVA and Tukey HSD test (P < 0.05) using Minitab 16 statistical software.

Callose staining of 10-d-old liquid-grown seedlings was done after seedlings were exposed to 1 μM fig22 or water for 18 h. Cotyledons were cleared and stained as described (Clay et al., 2009).

Quantitative Real-Time PCR

MYB51, CYP79B2, CYP79B3, CYP83B1, CYP81F2, and PR1 transcript levels were measured using quantitative real-time PCR (qRT-PCR). Leaves of 5-week-old Col-0 plants were hand-infiltrated with buffer or 10^6 CFU/mL of PtoΔCEL or PtoΔCEL cor. At 6 h after infiltration, leaves were frozen in liquid nitrogen and ground by mortar and pestle. Total RNA was prepared using the Plant RNeasy Mini Prep Kit (Qiagen). One microgram of total RNA was treated with DNaseI (Invitrogen) and then cDNA synthesis was done using the reverse transcription system (Promega). For individual biological replicates, each cDNA sample was tested by three technical replicates. qRT-PCR reactions were set up using IQ SYBR green supermix (Bio-Rad) and run in an iQ5 real-time PCR detection system (Bio-Rad). Gene expression data were analyzed using the ΔCT (cycle threshold) method with the relative quantification to ACTIN2 as the reference gene. The specificity of PCR products was verified on 1.5% agarose gels and by melt curves in the iQ5. Primers used for RT-PCR are shown in Supplemental Table 3 online. Statistical analysis for the combined data from biological replicates was done using one-way ANOVA and Tukey HSD test (P < 0.05) by Minitab 16 statistical software.

SA Extraction and Quantification

Leaves of 5-week-old plants were hand-infiltrated with buffer or 10^6 CFU/mL of bacteria in 10 mM MgCl₂. At 15 h after infiltration, leaves were collected and ground in liquid N₂ and 0.2 g of grindate was extracted twice in 6% grade methanol, vortexed well, and spiked with 30 μL of 1.25 mM sinigrin (Sigma-Aldrich) as an internal standard. After incubating at 75°C for 15 min to deactivate myrosinases, the extraction mixture was centrifuged at 15K rpm for 10 min at 4°C and 400 μL of supernatant was collected. 4MI3G, 1MI3G, and 13G were detected as previously described (Cataldi et al., 2007). Then, 30-μL samples were run on liquid chromatograph–mass spectrometer (Varian) in a negative ion electrospray mode. The samples were separated by HPLC fitted with a C18-A column (A2001150X046). The mobile phases were A - water and B - 90% acetonitrile (Fisher Scientific) at room temperature. Column linear gradient was 0 to 18 min, 85% A, 15% B; 18 to 25 min, 45% A, 55% B; 25 to 28 min, 100% B; 28 to 30 min, 85% A, 15% B, with a flow rate of 300 μL/min. The internal standard sinigrin, 4MI3G, 1MI3G and 13G were observed as mass-to-charge ratios of 358, 477, 477, and 447, respectively. For all four compounds, the retention times as well as masses of daughter ions match published values (Cataldi et al., 2007). Statistical analysis for the combined data from three biological replicates was done using one-way ANOVA and Tukey HSD test (P < 0.05) by Minitab 16 statistical software.

Supplemental Data

The following materials are available in the online version of this article:

Supplemental Figure 1. DNA Analysis of Mutant Bacteria and Plants.

Supplemental Figure 2. COR Inhibits PR-1 Expression.

Supplemental Figure 3. SA Accumulation after Bacterial Infiltration.

Supplemental Figure 4. COR Promotes Bacterial Virulence in sid2 npr1 Plants Independent of Targeting COI1.

Supplemental Figure 5. PEN2 Is Not Required for fig22-Induced Callose Deposition in Soil-Grown Plants.

Supplemental Figure 6. COR Inhibits Indole Glucosinolate Accumulation Upstream of PEN2.

Supplemental Table 1. Phentotype of Arabidopsis Types Used in This Study.

Supplemental Table 2. Primers Used for Screening Mutant Plants and Bacteria.

Supplemental Table 3. Primers Used for qRT-PCR.

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X.G. and D.M. designed the research. X.G. and A.G. performed the research. X.G., A.G., J.C., and D.M. analyzed data. X.G. and D.M. wrote the article.

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