

# Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (*coi1*) mutation occurs through two distinct mechanisms

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## Summary

A new allele of the coronatine-insensitive locus (*COI1*) was isolated in a screen for *Arabidopsis thaliana* mutants with enhanced resistance to the bacterial pathogen *Pseudomonas syringae*. This mutant, designated *coi1-20*, exhibits robust resistance to several *P. syringae* isolates but remains susceptible to the virulent pathogens *Erysiphe* and cauliflower mosaic virus. Resistance to *P. syringae* strain *PstDC3000* in *coi1-20* plants is correlated with hyperactivation of *PR-1* expression and accumulation of elevated levels of salicylic acid (SA) following infection, suggesting that the SA-mediated defense response pathway is sensitized in this mutant. Restriction of growth of *PstDC3000* in *coi1-20* leaves is partially dependent on *NPR1* and fully dependent on SA, indicating that SA-mediated defenses are required for restriction of *PstDC3000* growth in *coi1-20* plants. Surprisingly, despite high levels of *PstDC3000* growth in *coi1-20* plants carrying the salicylate hydroxylase (*nahG*) transgene, these plants do not exhibit disease symptoms. Thus resistance to *P. syringae* in *coi1-20* plants is conferred by two different mechanisms: (i) restriction of pathogen growth via activation of the SA-dependent defense pathway; and (ii) an SA-independent inability to develop disease symptoms. These findings are consistent with the hypotheses that the *P. syringae* phytotoxin coronatine acts to promote virulence by inhibiting host defense responses and by promoting lesion formation.

**Keywords:** salicylic acid, symptom development, tolerance, *nahG*.

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## Introduction

In response to microbial attack, plants activate a complex series of general defense responses that are believed to inhibit colonization of plant tissue by micro-organisms (Heath, 2000). These inducible defenses include a rapid oxidative burst; accumulation of elevated levels of the endogenous signaling compounds salicylic acid (SA) and jasmonic acid (JA); induction of several pathogenesis-related (PR) genes; and production of antimicrobial phytoalexins and lytic enzymes (Felix *et al.*, 1999; Glazebrook *et al.*, 1997; Hammond-Kosack and Jones, 1996; Lamb *et al.*, 1989). These defenses are also often

induced in response to infection by virulent pathogens. However, in these interactions induction of defenses responses occurs at a relatively late stage of infection and, although this does not prevent disease development, appears to be important in limiting pathogen aggressiveness (Glazebrook *et al.*, 1997; Jakobek *et al.*, 1993). Thus it is likely that successful plant pathogens evade or actively inhibit induction of host defenses to facilitate colonization of plant tissues. Presumably the ability to avoid detection, or to suppress activation of defense responses normally induced upon microbial attack, are traits that may distin-

guish successful plant pathogens from non-pathogenic organisms (Alfano and Collmer, 1996; Felix *et al.*, 1999; Lamb *et al.*, 1989). However, the pathogen virulence factors that are involved in these processes, and how they function to modify host defense mechanisms, are not well understood (Alfano and Collmer, 1996).

One example of a virulence factor that may promote parasitism by inhibiting host defenses is the bacterial phytotoxin coronatine (COR). COR is produced by several strains of the bacterial pathogen *Pseudomonas syringae* and has been demonstrated to contribute to virulence of *P. syringae* (Bender *et al.*, 1999; Moore *et al.*, 1989). *Pseudomonas syringae* mutants that do not produce COR exhibit a reduction in both growth and disease symptom development on several plant species, including *Arabidopsis thaliana* (Bender *et al.*, 1987; Budde and Ullrich, 2000; Mittal and Davis, 1995). The mode of action of COR in contributing to pathogen virulence is not well understood. However, based on both structural similarities and its effects on plant tissue, COR has been proposed to function as a molecular mimic of methyl jasmonate (MeJA), an endogenous plant hormone involved in defense signaling (Bender *et al.*, 1999; Feys *et al.*, 1994; Reymond and Farmer, 1998). Both MeJA and COR induce similar responses in plants, including inhibition of root elongation in *A. thaliana*, accumulation of anthocyanin, production of ethylene, and leaf senescence (Bender *et al.*, 1999).

In a series of experiments aimed at addressing the role of COR in virulence of *P. syringae*, Mittal and Davis (1995) found that a mutant of *P. syringae* pv. *tomato* strain DC3000 impaired in COR biosynthesis exhibited reduced growth and symptom production on *A. thaliana*. They also demonstrated that this reduced virulence was correlated with an enhanced ability to elicit expression of defense-related genes. These findings led Mittal and Davis (1995) to hypothesize that COR is important in early stages of infection by *P. syringae*, and may be involved in suppression of general host defenses that normally serve to inhibit colonization of plant tissue. However, the molecular basis for how COR modulates host defenses has not been explored.

The identification and characterization of *A. thaliana* mutants that are insensitive to COR is likely to provide insight into the molecular basis of COR virulence activity (Feys *et al.*, 1994). A series of COR-insensitive (*coi*) mutants was isolated on the basis that they exhibited normal root elongation in the presence of 50  $\mu$ M COR. Interestingly, the *coi* mutants defined a single locus, *COI1*, and were also shown to be insensitive to MeJA (Feys *et al.*, 1994). The *coi1-1* mutant has been extensively studied and has been shown to exhibit several additional phenotypes, including a defect in pollen development that renders it male sterile and resistance to the bacterial pathogen *P. syringae* pv.

*atropurpurea* (Feys *et al.*, 1994). Further evidence that the JA-signaling pathway is impaired in the *coi1-1* mutant stems from studies showing that mutant plants do not express several JA-responsive genes upon application of MeJA (Benedetti *et al.*, 1995; Benedetti *et al.*, 1998; Feys *et al.*, 1994), or after infection with the fungal pathogen *Alternaria* (Penninckx *et al.*, 1996; Thomma *et al.*, 1998). The *COI1* gene has been isolated and encodes a predicted protein that contains an F-box motif and a series of leucine-rich repeats, suggesting that the COI1 protein may be involved in targeting proteins for polyubiquitination and degradation (Xie *et al.*, 1998). The mechanism(s) underlying resistance to *P. syringae* in the *coi1-1* mutant has not been investigated. Presumably this resistance is due, at least in part, to the fact that mutant plants are insensitive to the virulence factor COR. Investigation of the molecular basis of resistance in *COI1* mutant plants would provide valuable insight into the mechanism of COR function, and could also provide clues as to how a mutant that is defective in a host defense response-signaling pathway confers pathogen resistance.

To identify plant genes that govern susceptibility to virulent pathogens, we carried out a screen for *A. thaliana* mutants with enhanced disease resistance to *P. syringae* (Boch *et al.*, 1998; G. Kalinowski, M. Verbsky and B. Kunkel, unpublished results). One of the mutants isolated in this screen defines a new allele of *COI1* and was thus designated *coi1-20*. To better understand the mechanism(s) underlying resistance in the *coi1-20* mutant, we investigated the molecular basis of resistance in this line. Here we demonstrate that resistance to *P. syringae* strain PstDC3000 in the *coi1-20* mutant is correlated with the hyper-activation of SA synthesis and SA-mediated defense responses upon infection. These results suggest that the SA-signaling pathway is sensitized in the *coi1-20* mutant such that defenses are rapidly induced in response to infection by the virulent PstDC3000 strain. We also show that *coi1-20* plants carrying mutations in the SA-signaling pathway allow high levels of PstDC3000 growth but, unexpectedly, do not develop disease symptoms. These findings suggest that the enhanced resistance phenotype of the *coi1* mutant is mediated via two different mechanisms: (i) sensitization of an SA-dependent defense-signaling pathway responsible for restricting pathogen growth; and (ii) an SA-independent impairment in disease symptom formation.

## Results

To identify genes that govern susceptibility to *P. syringae*, we conducted a screen for *A. thaliana* mutants that exhibit reduced disease symptoms upon infection with the *P. syringae* pv. *tomato* strain PstDC3000. Approximately 17 500  $M_2$  plants derived from ethylmethane sulfonate

(EMS)-mutagenized seeds of *A. thaliana* ecotype Columbia (Col-0) were screened, and 32 mutant lines that exhibited enhanced resistance in the  $M_3$  generation were isolated. One mutant, designated *coi1-20*, exhibited strong resistance to *PstDC3000* (Figure 1), and was chosen for further analysis. As this mutant was male sterile it was propagated by fertilizing mutant flowers with pollen from wild-type plants, and was maintained as a heterozygous line. Several additional mutants exhibiting enhanced disease resistance that have been isolated in this screen are described elsewhere (Boch *et al.*, 1998; G. Kalinowski, M. Verbsky and B. Kunkel, unpublished results).

#### Genetic analysis of *coi1-20*

To determine the genetic basis of the disease resistance phenotype in the *coi1-20* mutant, we crossed the mutant to wild-type Col-0 plants. As summarized in Table 1, the  $F_1$  progeny from this cross were susceptible to *PstDC3000* and were self-fertile, indicating that both enhanced resistance to *PstDC3000* and male sterility in *coi1-20* plants are caused by a recessive mutation(s). The  $F_1$  plants were allowed to self-pollinate, the resulting  $F_2$  progeny were assayed for disease resistance and fertility, and resistance to *PstDC3000* was observed to segregate as a recessive, single-gene trait (Table 1). Male sterility co-segregated with disease resistance in these plants. In over 200 *coi1-20*  $F_2$  progeny scored from a cross between *coi1-20* and *COI1* plants (Table 1 and data not shown) we did not detect any recombinant plants in which resistance to *PstDC3000* and the male sterile phenotype were separated. We also noted a third phenotype, an upright growth habit in which the rosette leaves were held in a more vertical position, such that the angle between the petiole and the main axis of the plant was smaller than observed in wild-type plants, co-segregated with the enhanced resistance and male sterile phenotypes among the  $F_2$  progeny. This upright phenotype appeared to become more pronounced after *PstDC3000* inoculation. These results indicate that the three phenotypes associated with the *coi1-20* mutation are conferred by a defect at a single locus or by mutations at tightly linked loci.

This unusual combination of male sterility and enhanced disease resistance phenotypes is reminiscent of those reported for coronatine-insensitive (*coi1*) mutants (Feys *et al.*, 1994). In addition to exhibiting male sterility and enhanced resistance to *P. syringae*, *coi1* mutants display a marked insensitivity to both the bacterial phytotoxin COR and the plant hormone MeJA (Feys *et al.*, 1994). To determine whether resistance in our *A. thaliana* mutant is due to a mutation at the *COI1* locus, we conducted genetic mapping experiments, direct phenotypic comparisons and complementation analysis with the *coi1-1* mutant isolated by Feys *et al.* (1994).

The *COI1* gene is located on the south arm of chromosome 2 between the visible markers *as* and *cer8* (Xie *et al.*, 1998). To determine the map position of our mutation we crossed the *coi1-20* mutant to wild-type plants from the ecotype Landsberg *erecta* (Ler; Table 1).  $F_2$  progeny from this cross were used to map *coi1-20* relative to a molecular marker (m429) known to be tightly linked to *COI1* (Konieczny and Ausubel, 1993; [http://www.arabidopsis.org/maps/CAPS\\_Chr2.html](http://www.arabidopsis.org/maps/CAPS_Chr2.html)). In our mapping population of 30  $F_2$  homozygous *coi1-20* mutant plants (Table 1 and data not shown) we found no recombinants that separated the *coi1-20* mutation from m429, demonstrating that *coi1-20* maps to the *COI1* region of chromosome 2.

Feys *et al.* (1994) previously reported that the *coi1-1* mutant is resistant to infection by the plant pathogen *P. syringae* pv. *atropurpurea*. To determine if the *coi1-1* and *coi1-20* mutants exhibit similar levels of resistance to our *P. syringae* isolate, we subjected self-progeny from *coi1-1* and *coi1-20* heterozygous plants to infection with *PstDC3000* and monitored appearance of disease symptoms. Both the *coi1-1* and *coi1-20* segregating families gave rise to approximately 25% resistant individuals that exhibited few or no visible disease symptoms (Table 1). The degree of resistance exhibited by the two mutants was indistinguishable.

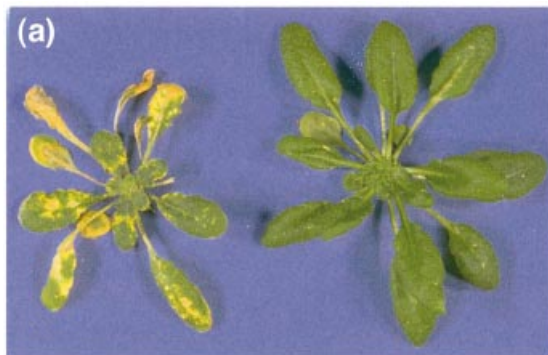
The sensitivity of the *coi1-20* mutant to COR and MeJA was assayed and compared to that of the *coi1-1* mutant by germinating segregating families of the mutants on Murashige and Skoog (MS) (Murashige and Skoog, 1962) plates containing 1  $\mu$ M COR or 10  $\mu$ M MeJA. Both segregating populations gave rise to approximately 25% COR- or MeJA-insensitive seedlings as measured by root elongation 6 days following germination (Feys *et al.*, 1994; see Experimental procedures). These results indicate that, like *coi1-1*, *coi1-20* exhibits resistance to both COR and MeJA.

Allelism tests between *coi1-1* and *coi1-20* revealed that the two mutants do not complement one another, indicating that the lines carry mutations at the same locus (Table 1). Thus, given our findings that: (i) the *coi1-1* and *coi1-20* mutations map to the same position on chromosome 2; (ii) both mutant lines exhibit resistance to *PstDC3000* and are insensitive to COR and MeJA; and (iii) the two mutants fail to complement one another, we conclude that the mutant we isolated based on its enhanced disease resistance phenotype defines a new allele of *COI1*. Thus we have assigned this mutant the designation *coi1-20*. The molecular nature of the *coi1-20* allele is not presently known. However, based on our observation that the COR- and MeJA-insensitive phenotypes of *coi1-20* are indistinguishable from those of *coi1-1* – a mutation that results in a truncated protein that appears to abolish JA-mediated signaling (Feys *et al.*, 1994; Xie

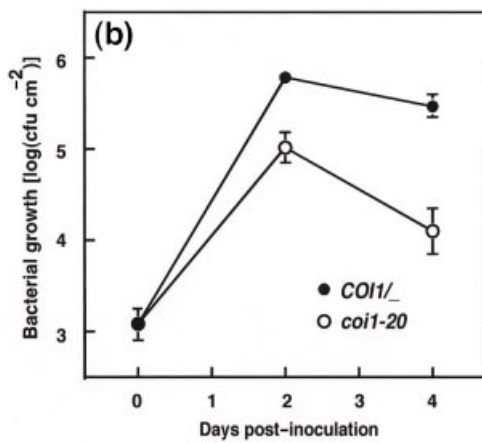
*etal.*, 1998) – it is likely that *coi1-20* also represents a strong loss of function allele.

*coi1-20* confers resistance to multiple strains of *P. syringae*

To determine whether enhanced resistance conferred by the *coi1-20* mutation is specific to *PstDC3000*, we inoculated *coi1-20* plants with two additional virulent strains of



*COI1/\_*                      *coi1-20*



*coi1-20*                      *COI1/\_*

Figure 1. Legend on facing page.



*COI1/\_ nahG*                      *coi1-20 nahG*



*COI1/\_ npr1-1*                      *coi1-20 npr1-1*



*JAR1*                                      *jar1-1*



Wild type                                      *fad3-2 fad7-2 fad8*

Figure 3. Legend on facing page.

*P. syringae* and observed that the mutant plants failed to develop disease symptoms following inoculation with either *P. syringae* pv. *tomato* strain *Pst3455* or *P. syringae* pv. *maculicola* strain *Psm m4* (data not shown). These observations are consistent with the finding by Feys *et al.* (1994) that *coi1-1* is resistant to *P. syringae*, and suggest that resistance in the *coi1-20* line is not due to gain of a novel capacity to specifically detect infection by *PstDC3000*, but rather appears to be conferred by enhanced resistance to several different virulent strains of *P. syringae*.

To determine if resistance in *coi1-20* plants was associated with restricted growth of *PstDC3000* within the plant,

**Table 1.** Genetic analysis of *coi1-20*

Cross	Generation	Number of plants			$\chi^2_{1:3}$
		R <sup>a</sup>	S <sup>a</sup>		
<i>coi1-20</i> × <i>COI1</i> (Col-0)	F <sub>1</sub> <sup>b</sup>	0	3 <sup>c</sup>	0.07	
	F <sub>2</sub>	32 <sup>c</sup>	91 <sup>c</sup>		
<i>coi1-20</i> × <i>COI1</i> (Ler)	F <sub>1</sub>	ND <sup>d</sup>	ND	0.31	
	F <sub>2</sub>	15	38		
<i>coi1-1/COI1</i> <sup>e</sup>	self progeny	25	53	2.07	
		Number of plants			
<i>coi1-20/coi1-20</i> × <i>coi1-1/COI1</i> <sup>e</sup>	F <sub>1</sub>	Sterile <sup>f</sup>	Fertile	0.09 <sup>g</sup>	
		5	6		

<sup>a</sup>Plants were inoculated by dipping in bacterial suspensions of *P. syringae* strain *PstDC3000* containing the surfactant Silwet L-77, and were scored 4–5 days after inoculation. R, resistant plants exhibiting no disease symptoms; S, susceptible plants with disease symptoms consisting of individual water-soaked lesions and chlorosis.

<sup>b</sup>Data presented for second back-cross of *coi1-20* to wild-type Col-0 (*COI1*).

<sup>c</sup>All resistant plants were sterile; all susceptible plants were fertile.

<sup>d</sup>ND, not determined.

<sup>e</sup>*coi1-1* mutant described by Feys *et al.* (1994).

<sup>f</sup>F<sub>1</sub> plants were allowed to flower and were scored for fertility.

<sup>g</sup> $\chi^2$  value for 1 : 1 segregation.

growth of the pathogen in *coi1-20* leaf tissue was monitored over several days. As shown in Figure 1(b), growth of *PstDC3000* was significantly reduced in *coi1-20* plants, obtaining a final concentration of only 10<sup>4</sup>–10<sup>5</sup> cfu cm<sup>-2</sup>. This was in marked contrast to the higher levels of bacterial growth observed in wild-type, susceptible siblings of *coi1-20* where *PstDC3000* reached a final concentration of approximately 10<sup>6</sup> cfu cm<sup>-2</sup> (Figure 1b).

*coi1-20* plants also exhibited normal macroscopic tissue collapse indicative of the hypersensitive response (HR) when inoculated with high levels of *PstDC3000* expressing the avirulence genes *avrRpm1* or *avrB* (data not shown). Thus the *coi1-20* mutation does not impair the basic ability of *A. thaliana* plants to mount an HR when challenged by an avirulent *P. syringae* strain. These results also demonstrate that *RPM1*-mediated pathogen recognition is functional in *coi1-20* mutant plants.

*coi1-20* plants do not exhibit enhanced resistance to virulent fungal or viral pathogens.

To determine whether the *coi1-20* mutant exhibited enhanced resistance against other virulent pathogens of *A. thaliana*, we examined its response to the fungal powdery mildew pathogen *Erysiphe cichoracearum* UCSC1, and the viral pathogen cauliflower mosaic virus (CaMV) (Adam and Somerville, 1996; Leisner and Howell, 1992; Melcher, 1989). Segregating F<sub>2</sub> plants from a cross of *coi1-20* to Col-0 were challenged with virulent *E. cichoracearum* UCSC1, and scored for disease symptoms 6 and 10 days following inoculation. As is summarized in Table 2, 100% of the plants exhibited extensive white, powdery fungal growth on leaf surfaces (Figure 1c). In two of these experiments, individual plants were allowed to flower following scoring of disease symptoms, in order to provide positive identification of *coi1-20* homozygotes based on the male sterile phenotype. As expected, approximately 25% of the F<sub>2</sub> plants were *coi1-20* mutants (Table 2). Thus both *coi1-20* homozygotes and their wild-

**Figure 1.** *coi1-20* plants exhibit enhanced resistance to *P. syringae* strain *PstDC3000*.

(a) Disease symptoms exhibited by wild-type (*COI1*/\_\_\_; left) and *coi1-20* (right) plants 4 days following inoculation with *PstDC3000*. Plants were inoculated by dipping into bacterial suspensions containing the surfactant Silwet L-77.

(b) Growth of *PstDC3000* in *coi1-20* (○) and wild-type (*COI1*/\_\_\_; ●) leaf tissue. Data points represent the means of three independent determinations ± SEM. Similar results were obtained in more than three independent experiments.

(c) Disease symptoms exhibited by *coi1-20* (left) and wild-type (*COI1*/\_\_\_; right) plants 10 days following inoculation with *E. cichoracearum* UCSC1. In all experiments, self-progeny from *coi1-20/COI1* heterozygous plants were inoculated and assayed for disease resistance. *coi1-20* homozygous plants were identified by scoring for male sterility. Data from phenotypically wild-type siblings (*coi1-20/COI1* and *COI1/COI1*; designated *COI1*/\_\_\_) were collected in the same experiments, and are presented as controls.

**Figure 3.** Disease symptoms caused by *PstDC3000* on various salicylic acid (SA) and jasmonic acid (JA) signaling mutants.

Disease symptoms exhibited by (a) *COI1*/\_\_\_ *nahG* (left) and *coi1-20 nahG* (right); (b) *COI1*/\_\_\_ *npr1-1* (left) and *coi1-20 npr1-1* (right); (c) wild-type Col-0 (*JAR1*; left) and Col-0 *jar1-1* (right); and (d) wild-type (*fad3-2 fad7-2 FAD7*/\_\_\_ (left) and *fad3-2 fad7-2 fad8* plants (right) 4 days following inoculation with *PstDC3000*. Plants were inoculated by dipping into bacterial suspensions containing the surfactant Silwet L-77.

**Table 2.** Inoculation of *coi1-20* plants with virulent *Erysiphe* and CaMV isolates

Pathogen	Genotype <sup>a</sup>	Disease phenotype <sup>c</sup>		
		+	-	Total scored
<i>E. cichoracearum</i>				
Exp. 1	<i>coi1-20/COI1</i> seg <sup>b</sup>	12	0	12
	<i>COI1</i>	14	0	14
Exp. 2	<i>coi1-20</i>	25	0	109
	<i>COI1/</i>	84	0	
Exp. 3	<i>coi1-20</i>	17	0	69
	<i>COI1/</i>	52	0	
CaMV				
Exp. 1	<i>coi1-20/COI1</i> seg <sup>b</sup>	45	0	45
	<i>COI1</i>	39	1	40
Exp. 2	<i>coi1-20/COI1</i> seg <sup>b</sup>	30	1	31
	<i>COI1</i>	11	0	11

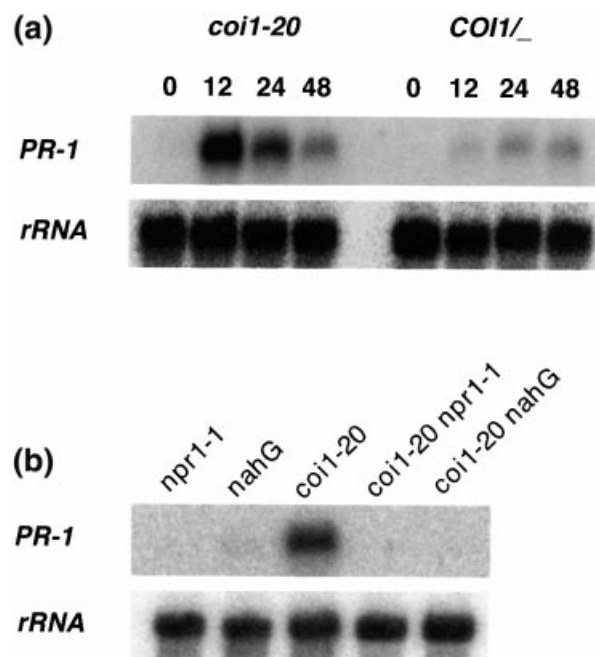
<sup>a</sup>Plant genotypes were determined by allowing self progeny from *coi1-20/COI1* heterozygous plants to flower and scoring for sterility. The segregating families were comprised of approximately 25% *coi1-20* and 75% wild-type, fertile individuals (*coi1-20/COI1* and *COI1/COI1*; indicated as *COI1/*).

<sup>b</sup>*coi1-20/COI1* seg, self progeny from *coi1-20/COI1* heterozygous plants. The *COI1* genotype was not determined in these experiments, and could not be obtained for CaMV-infected plants as they died prior to flowering.

<sup>c</sup>Disease phenotype: +, plant exhibited typical disease symptoms; -, no disease symptoms evident. Disease phenotypes were determined by visual observation. For *E. cichoracearum*-infected plants, diseased plants were characterized by dense fungal growth and abundant sporulation. For CaMV-infected plants, diseased plants exhibited mosaic symptoms on the rosette leaves.

type siblings appeared to be equally susceptible to *E. cichoracearum*.

Segregating  $F_2$  plants from a cross of *coi1-20* to Col-0 were challenged with the virulent CaMV isolate CM1841 and scored for disease symptoms daily from the point of inoculation until 42 days post-inoculation (dpi). No delay in disease progression was observed in any of the segregating  $F_2$  plants relative to the wild-type controls, and all the  $F_2$  individuals essentially exhibited typical systemic symptoms of viral pathogenesis (Table 2). If the *coi1-20* plants did exhibit altered responses to CaMV, we would have expected to see approximately 25% of the plants exhibiting either more or less severe symptoms after infection. These results indicate that, although the *coi1-20* mutant is strongly resistant to virulent strains of *P. syringae*, it exhibits no enhanced resistance to the virulent fungal or viral pathogens tested. Norman-Setterblad *et al.* (2000) have recently reported that *coi1-1* plants are extremely susceptible to *Erwinia carotovora*, a bacterial pathogen not known to produce COR. Thus resistance in *coi1* plants may be limited to bacterial pathogens that rely on COR as a key virulence factor.

**Figure 2.** *PR-1* mRNA expression in *coi1-20* plants.

(a) Time course of *PR-1* expression in *coi1-20* and wild-type (*COI1/*) plants after inoculation with *PstDC3000*. Self-progeny from *coi1-20/COI1* heterozygous plants were inoculated, and total RNA was prepared from tissue harvested at the indicated times (hours) after inoculation. *coi1-20* homozygous plants were identified by scoring for male sterility. Phenotypically wild-type siblings (*coi1-20/COI1* and *COI1/COI1*; designated *COI1/*) were included as controls.

(b) *PR-1* expression in *npr1-1*, *nahG*, *coi1-20*, *coi1-20 npr1-1* and *coi1-20 nahG* plants 12 h after inoculation with *PstDC3000*. Approximately 3  $\mu$ g of total RNA was loaded for each sample. The blots were first probed with *PR-1*, followed by hybridization with an rDNA probe as a loading control. Similar results were obtained in a second, independent experiment.

#### *coi1-20* resistance to *PstDC3000* is correlated with rapid induction of *PR-1*

To investigate the molecular basis of enhanced resistance to *P. syringae* in *coi1* mutants, we monitored expression levels of *PR-1*, a pathogenesis-related gene often used as a marker for SA-dependent host defense responses (Baker *et al.*, 1997; Lamb *et al.*, 1989), in infected *coi1-20* plants. *coi1-20* plants and their wild-type siblings were infiltrated with *PstDC3000* and assayed for *PR-1* expression at various times after infection. As is shown in Figure 2, the level of expression of *PR-1* was undetectable in leaves of mature *coi1-20* plants harvested immediately after infiltration with *PstDC3000* (time 0, see Experimental procedures). Thus *coi1-20* does not constitutively express *PR-1* and does not belong to the constitutive expresser of PR (*cpr*) class of disease resistance mutants (Figure 2a). However, *coi1-20* mutant plants responded to *PstDC3000* infection by rapidly and strongly inducing *PR-1* expression by 12 h post inoculation (Figure 2a). In contrast, expres-

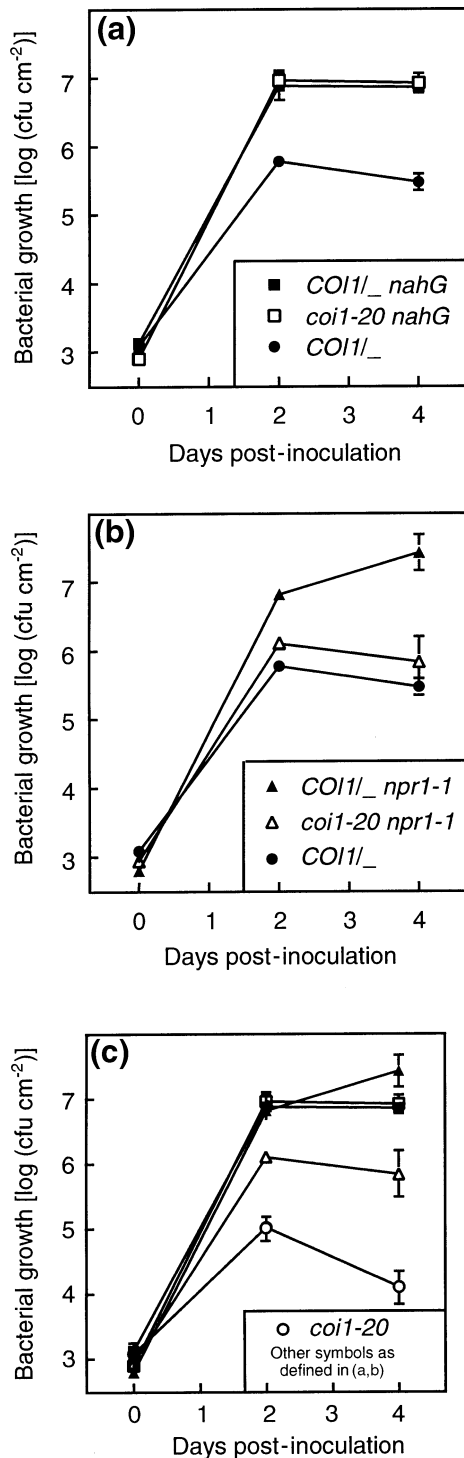
sion of *PR-1* was barely detectable in the susceptible, wild-type plants, even at 48 h post inoculation with *PstDC3000*. The rapid induction of *PR-1* in *coi1-20* plants is consistent with the enhanced resistance to *PstDC3000* exhibited by the mutant. These results also suggest that *coi1-20* plants are sensitized to infection with *PstDC3000* and respond by

rapidly inducing defense responses following pathogen attack. These results, taken in conjunction with the observations that *coi1* mutants are insensitive to COR and that *P. syringae* mutants that do not produce COR exhibit reduced virulence on *A. thaliana* (Mittal and Davis, 1995; D. Brooks, A. Kloeck and B. Kunkel, unpublished results), are consistent with the hypothesis that COR promotes virulence of *P. syringae* by inhibiting induction of host defense response upon pathogen attack (Mittal and Davis, 1995), and suggest that COR may function by modulating the SA-dependent defense pathway.

*Rapid induction of PR-1 expression in coi1-20 plants on infection with PstDC3000 is dependent on SA signaling*

As *PR-1* belongs to the systemic acquired resistance (SAR) class of defense-related genes that are induced in response to elevated SA levels in the plant, we examined the role of SA and SA-dependent signaling pathways in resistance exhibited by the *coi1-20* mutant. To generate *coi1-20* lines that are impaired for SA signaling, *coi1-20* was crossed to an *A. thaliana* *Ler* transgenic line carrying the *nahG* gene (Bowling *et al.*, 1994) and to a *Col-0* line carrying a mutation at the *NPR1* locus (Cao *et al.*, 1994). The *nahG* transgenic line carries a bacterial salicylate hydroxylase transgene which converts SA to the inactive product catechol, thus preventing accumulation of SA (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). The *npr1-1* mutant line carries a mutation that disrupts SA-dependent activation of defense-related transcripts (Cao *et al.*, 1994). Both lines are extremely susceptible to infection with *P. syringae* (Cao *et al.*, 1994; Delaney *et al.*, 1994).

*PR-1* expression in *coi1-20*, *coi1-20 npr1-1* and *coi1-20 nahG* plants was monitored by RNA blot analysis 12 h after inoculation with *PstDC3000*. *coi1-20* plants strongly expressed *PR-1* 12 h post-inoculation, whereas the *coi1-20 npr1-1* and *coi1-20 nahG* plants failed to express detectable *PR-1* message (Figure 2b). These results indicate that the rapid induction of *PR-1* expression (and presumably other defense responses) in *coi1-20* plants infected with *PstDC3000* is dependent on both SA and *NPR1*.

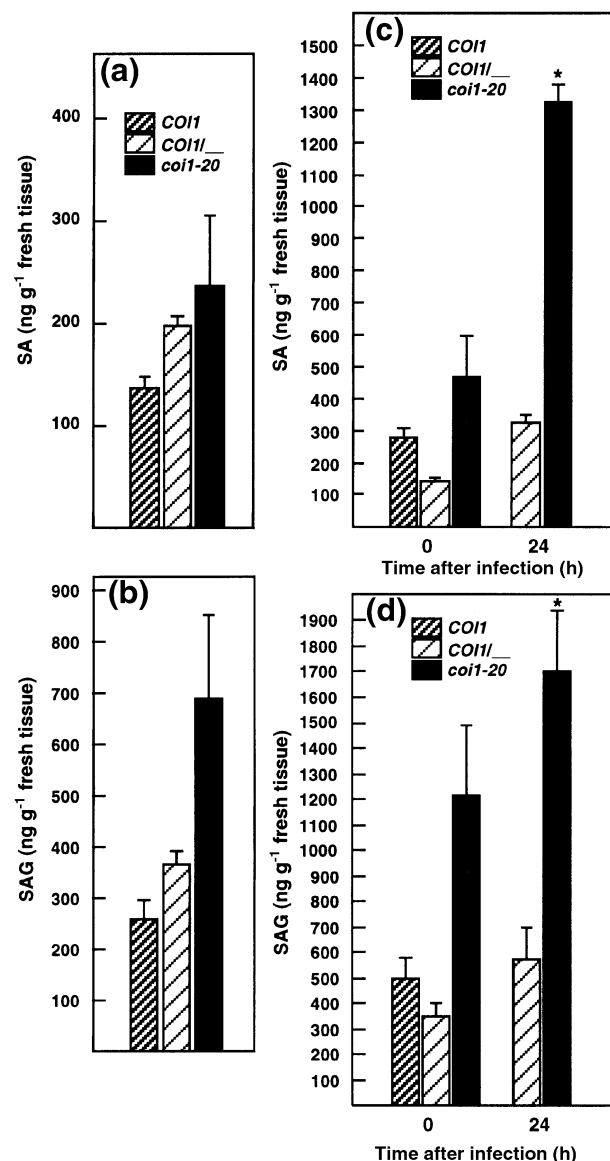


**Figure 4.** Restriction of growth of *PstDC3000* in *coi1-20* is dependent on salicylic acid (SA).

(a) Growth of *PstDC3000* in *coi1-20 nahG* (□), *COI1/ nahG* (■), and wild-type (*COI1/*; ●) leaf tissue.

(b) Growth of *PstDC3000* in *coi1-20 npr1-1* (△), *COI1/ npr1-1* (▲) and *COI1/* (●) leaf tissue.

(c) Combined *PstDC3000* growth data from (a,b) presented for direct comparison. Data from *coi1-20* plants (○) are provided for reference. All growth data presented were obtained from experiments carried out in parallel. Data points represent the means of three independent determinations ± SEM. Similar results were obtained in a second, independent experiment.



**Figure 5.** *coi1-20* plants accumulate elevated levels of salicylic acid (SA) in response to *PstDC3000* infection. Free SA (a) and SA glucoside (SAG) (b) levels were monitored in uninfected Col-0 (*COI1*), *COI1/*\_ and *coi1-20* plants. There was no significant difference in SA or SAG levels between *coi1-20* and Col-0 plants ( $P = 0.28$  for SA and  $P = 0.124$  for SAG in unpaired *t*-tests). SA (c) and SAG (d) levels were monitored in Col-0 (*COI1*), *COI1/*\_ and *coi1-20* plants just prior to inoculation (0 h), and in *COI1/*\_ and *coi1-20* plants 24 h after inoculation with *PstDC3000*. There was no significant difference in SA or SAG levels between uninfected *coi1-20* and *COI1/*\_ plants ( $P = 0.12$  for SA and  $P = 0.09$  for SAG in unpaired *t*-tests). In (c) the asterisk indicates that SA levels were significantly higher in *coi1-20* plants 24 h after inoculation with *PstDC3000* than in their wild-type siblings (*COI1/*\_;  $P < 0.001$  in unpaired *t*-tests); in (d) the asterisk indicates that SAG levels were significantly higher in *coi1-20* plants 24 h after inoculation with *PstDC3000* than in their wild-type siblings (*COI1/*\_;  $P = 0.01$  in unpaired *t*-tests). The SA and SAG values represent the mean of three or four replicate samples  $\pm$  SEM. Similar results were obtained in a second independent experiment.

#### Analysis of the requirement for SA in *coi1-20* mediated resistance to *PstDC3000*

To assess the contribution of SA-mediated defenses to resistance to *PstDC3000* in *coi1-20*, development of disease symptoms and pathogen growth on *coi1-20 nahG* and *coi1-20 npr1-1* plants and their *nahG* (*COI1/COI1 nahG* or *coi1/COI1 nahG*) or *npr1* (*COI1/COI1 npr1* or *coi1/COI1 npr1*) siblings were monitored following inoculation with *PstDC3000*. As illustrated in Figure 3(a,b), both double-mutant lines appeared to be resistant to *PstDC3000*, and exhibited only slight chlorosis and few or no individual water-soaked lesions. In contrast, *npr1-1* and *nahG* plants were extremely susceptible to *PstDC3000*, and developed severe chlorosis and numerous water-soaked lesions.

To determine if the phenotypic resistance was associated with restriction of pathogen growth in these lines, *PstDC3000* was infiltrated into leaves of *coi1-20 nahG*, *coi1-20 npr1-1*, and their corresponding wild-type siblings (*COI1/COI1 npr1* or *coi1/COI1 npr1* and *COI1/COI1 nahG* or *coi1/COI1 nahG*), and bacterial growth monitored over a 4 day period. Surprisingly, *coi1-20 nahG* plants allowed very high levels of bacterial growth that were essentially indistinguishable from those observed in *nahG* plants (Figure 4a). The *coi1-20 npr1-1* plants also supported high levels of bacterial growth, obtaining a level similar to that normally observed in wild-type plants (Figure 4b), but that was intermediate to levels observed in *coi1-20* and *npr1-1* plants (Figure 4c). Thus the restriction of *PstDC3000* growth in *coi1-20* mutant plants appears to be completely dependent on SA and partially dependent on *NPR1*.

These high levels of bacterial growth were unexpected, given the lack of disease symptoms on *coi1-20 nahG* and *coi1-20 npr1* plants infected with *PstDC3000* (Figure 3a,b). These results suggest that resistance to *PstDC3000* in *coi1-20* may involve two distinct mechanisms; an SA-dependent mechanism that limits pathogen growth, and an SA-independent mechanism that affects symptom development. However, the fact that the *coi1 nahG* line was generated in a mixed genetic background (e.g. by crossing Col-0 *coi1-20*  $\times$  *Ler nahG*) should be taken into consideration when interpreting the results of the bacterial growth experiments for the *coi1 nahG* plants.

#### *coi1-20* plants accumulate elevated levels of SA in response to infection with *PstDC3000*

The repression of *coi1*-mediated restriction of bacterial growth by the *nahG* transgene suggests that an SA-dependent signaling pathway is critical in mediating

some aspects of resistance to *PstDC3000* in the *coi1-20* mutant, and that this pathway has become either sensitized or partially de-repressed in the mutant. We envision two possible ways in which SA signaling could be altered in the *coi1-20* plants. One hypothesis is that *coi1-20* mutants are more sensitive to SA, such that even a small increase in SA levels (e.g. on infection with *PstDC3000*) results in unusually rapid and strong induction of SA-dependent defense responses. Alternatively, the *coi1-20* mutant could produce elevated levels of SA, either constitutively or upon infection with *PstDC3000*.

To address the possibility that *coi1-20* mutant plants might exhibit increased sensitivity to SA or similar chemical inducers of defense responses, we examined their response to benzothiadiazol (BTH), a synthetic analog of SA that has been shown to induce the SAR signal transduction pathway in *A. thaliana* (Lawton *et al.*, 1996). We used RNA blot experiments to monitor the induction of *PR-1* expression in *coi1-20* and wild-type plants following application of BTH. Both *coi1-20* and wild-type plants exhibited identical induction kinetics and responsiveness to a range of doses of BTH (50, 100 and 200  $\mu\text{M}$ ; data not shown). These results suggest that *coi1-20* mutant plants do not exhibit a significant increase in sensitivity to BTH, which suggests that sensitivity to SA is also likely to be unaltered.

To test the second hypothesis, we monitored SA levels in the *coi1-20* mutant. As shown in Figure 5, in uninoculated plants the mean levels of both free SA and SA glucoside (SAG) were higher in mutant tissue than in wild-type plants (Figure 5a,b; 0 h time points in Figure 5c,d). However, although we observed elevated levels of SA and SAG in uninoculated *coi1-20* plants in four independent experiments (Figure 5 and data not shown), these levels were not significantly higher than those observed in wild-type plants ( $P > 0.05$  in unpaired *t*-tests for all experiments). In contrast, within 24 h following inoculation with *PstDC3000*, both SA and SAG levels increased to significantly higher levels in *coi1-20* plants than in wild-type plants (Figure 5c,d). These results are consistent with our observations that the SA-signaling pathway is sensitized in the *coi1-20* mutant (Figure 2), and suggest that this occurs, at least in part, at the level of SA synthesis.

Our observation that mean SA levels were slightly elevated in uninoculated *coi1-20* plants may indicate that the levels of SA and SAG in uninoculated *coi1* plants are more highly variable than observed in wild-type plants. Although this may result in slightly higher than normal amounts of SA in uninoculated plants, these levels are apparently insufficient to induce constitutive expression of *PR-1* (Figure 2). However, slightly elevated levels of SA in *coi1-20* plants could serve to potentiate activation of the defense response-signaling pathway on infection with *PstDC3000* (Shirasu *et al.*, 1997).

#### Other *A. thaliana* mutants defective in JA production or signaling are susceptible to *PstDC3000*

To investigate whether the enhanced resistance to *PstDC3000* is specific for *coi1* mutants, or alternatively whether this phenotype is associated with a general defect in JA signaling, we assayed resistance to *PstDC3000* in two other JA-related mutants, the *fad3-2 fad7-2 fad8* triple mutant (McConn and Browse, 1996), and *jar1-1* (Staswick *et al.*, 1992). The *fad3-2 fad7-2 fad8* line is deficient in several fatty acid desaturase activities and is incapable of producing linolenic acid, the lipid precursor of JA, and is therefore unable to accumulate JA (McConn and Browse, 1996). Like the *coi1* mutants, *fad3-2 fad7-2 fad8* is male sterile. However, this mutant is able to respond to JA (McConn *et al.*, 1997), and is presumably sensitive to COR as well. The *jar1-1* mutant, which was isolated in a screen for mutants exhibiting decreased sensitivity to JA (Staswick *et al.*, 1992), is deficient in JA-induced expression of a subset of vegetative storage proteins, but is male fertile, and retains a significant degree of sensitivity to JA and COR when scored in root elongation assays (Staswick *et al.*, 1992; V. Joardar, G. Kalinowski and B. Kunkel, unpublished results). The *jar1-1* mutant has been reported to be susceptible to infection by *PstDC3000* (Pieterse *et al.*, 1998). However, studies investigating resistance to *P. syringae* in the *fad* triple mutant have not been reported.

The *fad3-2 fad7-2 fad8* and *jar1-1* mutants were infected with *PstDC3000* and scored for disease symptoms. Unlike the *coi1-20* mutant, both the *fad3-2 fad7-2 fad8* and *jar1-1* plants were fully susceptible to infection by *PstDC3000*, and exhibited numerous water-soaked lesions and extensive chlorosis (Figure 3c,d). Thus among the JA-signaling mutants tested, resistance to *PstDC3000* appears to be unique to *COI1* mutants.

#### Discussion

In a screen for *A. thaliana* mutants with enhanced disease resistance we isolated a new allele of *COI1*, *coi1-20*. Consistent with previous finding by Feys *et al.* (1994), this mutant exhibits strong resistance to *P. syringae*, consisting of the complete absence of visible disease symptoms and restriction of bacterial growth within mutant leaf tissue (Figure 1a,b). *coi1-20* mutant plants do not exhibit any significant alteration in their responses to the virulent fungal pathogen *E. cichoracearum* (Figure 1c; Table 2) and the viral pathogen CaMV, suggesting that the enhanced resistance exhibited by the *coi1-20* mutant could be limited to phytopathogenic pseudomonads. These findings are interesting given that in other studies the *coi1-1* mutant exhibited increased susceptibility to several fungal plant pathogens including *Pythium* sp. (Vijayan *et al.*, 1998), *Alternaria brassicicola* (Thomma *et al.*, 1998), and *Botrytis*

*cinerea* (Thomma *et al.*, 1998), as well as to *Erwinia carotovora* (Norman-Setterblad *et al.*, 2000), a macerating bacterial pathogen. *Alternaria brassicicola*, *B. cinerea* and *Pythium* sp. are not usually capable of causing disease on healthy *A. thaliana* plants, and resistance against all of these pathogens is thought to be controlled by JA-mediated defense responses (Dong, 1998; Staswick *et al.*, 1992). Therefore it is not surprising that the *coi1-1* mutant, with its insensitivity to JA, exhibits increased susceptibility to these pathogens. However, it is somewhat counter-intuitive that *coi1* mutants exhibit strong resistance to *P. syringae*, a pathogen known to be controlled by SA-dependent defense responses (Dong, 1998). This finding prompted us to examine more closely the role of SA-dependent signaling in *coi1-20* resistance to *PstDC3000*.

We have demonstrated that resistance to *PstDC3000* in *coi1-20* is correlated with enhanced induction of *PR-1* expression on infection with *PstDC3000* (Figure 2a). Although *coi1-20* plants do not constitutively express *PR-1*, expression of this gene is much more rapidly and strongly induced following infection with *PstDC3000* than is observed in wild-type plants. These findings suggest that in the *coi1-20* mutant, the SA-mediated defense pathway is sensitized to respond to infection by *PstDC3000*. Other *A. thaliana* disease resistance mutants that exhibit enhanced activation of defense responses upon pathogen invasion have been reported (Frye and Innes, 1998; Vogel and Somerville, 2000), but none that we are aware of exhibits as rapid and strong a response as that shown by *coi1-20*.

As further evidence that SA-mediated defenses are required for *coi1-20* resistance to *PstDC3000*, we found that *coi1-20 nahG* and *coi1-20 npr1-1* double mutants do not express *PR-1* in response to *PstDC3000* (Figure 2b) and support elevated levels of pathogen growth compared to *coi1-20* plants (Figure 4). In fact, both *coi1-20 nahG* plants and *nahG* plants allow indistinguishably high levels of pathogen growth, suggesting that the ability to restrict growth of *PstDC3000* is fully dependent on SA. In contrast, *coi1-20 npr1-1* plants allowed intermediate levels of pathogen growth (less than *npr1-1* but more than the *coi1-20* mutant), suggesting that SA signaling activates other defense pathways in addition to those regulated by *NPR1*. Evidence for *NPR1*-independent defense responses has also been reported in studies of *cpr6*, *ssi1* and *acd6* (Clarke *et al.*, 1998; Rate *et al.*, 1999; Shah *et al.*, 1999). However, although the *npr1-1* mutant appears to completely lack *NPR1* function (Cao *et al.*, 1994); X. Dong, personal communication), the fact that *npr1-1* is a missense mutation (Cao *et al.*, 1997), rather than a true null allele, must be taken into consideration when interpreting these results.

The rapid and strong activation of defense responses in *coi1-20* is correlated with a significantly greater increase in

SA levels on infection by *PstDC3000* than is observed in wild-type plants (Figure 5). These findings support our conclusion that resistance to *PstDC3000* in *coi1* plants is due, in part, to enhanced signaling through the SA-dependent defense pathway, and further suggest that this is mediated through increased SA synthesis. Based on these results, we hypothesize that *COI1* is involved in modulation of SA signaling in response to infection by *PstDC3000*.

In addition to playing a role in resistance to *P. syringae*, we found that *COI1* also affects symptom development. Despite the extensive bacterial growth supported by the *coi1-20 nahG* and *coi1-20 npr1-1* double mutants, neither line develops significant disease symptoms (Figure 3a,b). These results indicate that the wild-type *COI1* allele is required for development of visible signs of disease regardless of the levels of pathogen growth, and are consistent with the hypothesis that the plant host itself contributes actively to the production of disease symptoms (Greenberg *et al.*, 2000; Morel and Dangl, 1997). In the light of these findings, resistance to *PstDC3000* in *coi1-20* appears to be conferred by two different mechanisms: (i) restriction of pathogen growth via an SA-dependent defense mechanism; and (ii) an SA-independent inability to develop disease symptoms.

The finding that the *coi1-20 nahG* plants can support extremely high levels of bacteria without showing any visible disease symptoms, a phenotype often referred to as tolerance, is quite remarkable. Tolerance to bacterial pathogens in *A. thaliana* has been reported in only a few cases (Bent *et al.*, 1992; Buell and Somerville, 1995; Buell and Somerville, 1997; Tsuji *et al.*, 1991). For example, on infection with virulent strains of *P. syringae*, the ethylene-insensitive *ein2* mutant supports high levels of bacterial growth, yet it exhibits only mild disease symptoms (Bent *et al.*, 1992). The mechanism underlying tolerance in these plants is not understood. However, the fact that both *ein2* and *coi1* plants exhibit reduced symptoms and are impaired in JA-responsive gene induction (Penninckx *et al.*, 1996) suggests that both the ethylene- and JA-signaling pathways are involved in disease symptom development.

The molecular basis of *P. syringae*-induced disease lesion formation is not well understood. Several reports in the literature indicate that the *P. syringae* phytotoxin COR contributes to the formation of lesions on tomato, *A. thaliana* and soybeans (Bender *et al.*, 1987; Budde and Ullrich, 2000; Mittal and Davis, 1995). This hypothesis is strengthened by our findings that COR-insensitive *coi1-20 nahG* and *coi1-20 npr1* plants show no significant signs of disease despite high levels of pathogen growth. Ethylene has also been demonstrated to be important in later stages of *P. syringae* disease development, where it promotes

lesion expansion and chlorosis (Bent *et al.*, 1992; Lund, 1998).

What is the molecular basis of the enhanced defense response exhibited in the *coi1-20* mutant upon infection with *P. syringae*? In addition to being insensitive to coronatine, *coi1* mutants are blocked in JA signaling (Benedetti *et al.*, 1995; Penninckx *et al.*, 1996), raising the possibility that it is the defect in JA signaling that results in enhanced SA signaling. There is mounting evidence that the SA- and JA-mediated defense pathways are antagonistic, with signaling activity in one pathway having an inhibitory effect on the other (Dong, 1998; Felton *et al.*, 1999; Niki *et al.*, 1998; Shah *et al.*, 1999; Vidal *et al.*, 1997). However, while SA antagonism of the JA pathway has been clearly substantiated (Doares *et al.*, 1995; Dong, 1998; Shah *et al.*, 1999), evidence for the converse has been limited (Felton *et al.*, 1999; Niki *et al.*, 1998). The recent demonstration that JA signaling modulates an SA-dependent, ozone-induced cell death response in *A. thaliana* provides perhaps the best evidence to date that increased signaling through the JA pathway attenuates SA-signaling events (Rao *et al.*, 2000).

Our finding that the *coi1-20* mutant exhibits elevated SA signaling after infection with *P. syringae* is consistent with the hypothesis that the JA-signaling pathway negatively regulates induction of SA-dependent defenses. However, we found that other *A. thaliana* mutants defective in either JA production (*fad3-2 fad7-2 fad8*) or perception (*jar1-1*) exhibited wild-type susceptibility to *Pst*DC3000 (Figure 3c,d), suggesting that resistance to *P. syringae* is not a common feature of all mutants that affect JA signaling. Rather, this may be specific to *coi1* mutants, which are unique in that they appear to be completely impaired in perception of MeJA and COR. Thus we hypothesize that the enhanced resistance phenotype of *coi1* plants is due primarily to the fact that these mutants are insensitive to COR.

It is likely that the enhanced resistance phenotype of the *coi1* mutants provides insight into the role of COR in promoting virulence of *P. syringae*. Our data – taken in conjunction with observations that *P. syringae* mutants that do not produce COR exhibit reduced virulence on *A. thaliana* (Mittal and Davis, 1995; D. Brooks, A. Kloeck and B. Kunkel, unpublished results) – are consistent with the hypothesis that COR promotes virulence of *P. syringae* by inhibiting host defense responses (Budde and Ullrich, 2000; Mittal and Davis, 1995). We propose that COR accomplishes this by acting in a *COI1*-dependent manner to interfere with SA signaling. The ability to inhibit or delay activation of SA-dependent host defense responses is likely to be an important trait of pathogenic bacteria, as this may provide a window of opportunity during which the pathogen can colonize the host tissue. The molecular

mechanisms through which COR achieves this are not understood, and are currently under investigation.

## Experimental procedures

### Bacterial strains and plasmids

The bacterial pathogen strains *Pseudomonas syringae* pv. *tomato* (*Pst* DC3000, *Pst* 3455) and *P. syringae* pv. *maculicola* m4 have been described previously (Debener *et al.*, 1991; Whalen *et al.*, 1991). *Pseudomonas syringae* strains were cultured at 28°C in King's B medium (King *et al.*, 1954) containing 50 µg ml<sup>-1</sup> rifampicin. The fungal pathogen *Erysiphe cichoracearum* UCSC1 was maintained as described previously (Vogel and Somerville, 2000). The viral pathogen CaMV strain CM1841 (Gardner *et al.*, 1981) was maintained on turnip (*Brassica rapa* 'Just Right') or in lyophilized turnip tissue stored at 4°C. (Schoelz *et al.*, 1986).

### Plant material, growth conditions and inoculation procedures

The *coi1-20* mutant was isolated from a population of *M*<sub>2</sub> plants derived from EMS-mutagenized seed of a Columbia (Col-0)/Nossen (No-0) hybrid *A. thaliana* line carrying the *rps2-201C* mutation (Boch *et al.*, 1998). The *coi1-20* plants used in the experiments described here were derived from self-fertilized seed harvested from a heterozygous *F*<sub>2</sub> *coi1-20/COI1* individual generated from a second back-cross of *coi1-20 rps2-201C* to *Col-0 rps2-201C* (Kunkel *et al.*, 1993) or from a second back-cross of *coi1-20 rps2-201C* to wild-type Col-0 plants. As the presence or absence of the *rps2* mutation in the *coi1-20* plants is not relevant to the experiments described in this work, for the sake of clarity we have chosen not to refer to the *RPS2* allele present in each plant line. As *coi1* plants are male sterile, *coi1-20* mutant lines were maintained as heterozygotes.

The *Ler nahG* transgenic line (Bowling *et al.*, 1994) and the *Col-0 npr1-1* mutant (Cao *et al.*, 1994) were obtained from Scott Bowling and Xinnian Dong (Duke University). The *coi1-1* mutant line (Feys *et al.*, 1994) was obtained from John Turner (University of East Anglia). The *jar1-1* (Staswick *et al.*, 1992) and the *fad 3-2 fad7-2 fad8* (McConn and Browse, 1996) mutant lines were obtained from Paul Staswick (University of Nebraska) and John Browse (Washington State University), respectively. *Arabidopsis thaliana* plants were grown from seed in growth chambers under an 8 h photoperiod at 24°C.

*Pseudomonas syringae* inoculation of plants was carried out by dipping entire leaf rosettes of 3–5-week-old plants into bacterial suspensions of 2–4 × 10<sup>8</sup> colony forming units (cfu) ml<sup>-1</sup> containing 0.02% of the surfactant Silwet L-77 (OSi Specialties Inc., Danbury, CT, USA) and 10 mM MgCl<sub>2</sub> as previously described (Kunkel *et al.*, 1993). Bacterial growth within leaf tissue was monitored as described by Whalen *et al.* (1991).

*Arabidopsis thaliana* plants were inoculated with *E. cichoracearum* using settling towers as previously described (Vogel and Somerville, 2000). CaMV virions were partially purified from infected turnip leaves according to Schoelz *et al.* (1986) to concentrate the inoculum. The inoculum was applied to glass rods, then rubbed onto *A. thaliana* leaves that had been lightly dusted with carborundum.

For bacterial growth experiments in *coi1-20* plants, 15–20 progeny derived from self-fertilized seed harvested from a heterozygous *coi1-20/COI1* individual were infiltrated with

*Pst*DC3000 suspensions of  $1 \times 10^5$  cfu ml<sup>-1</sup>. Data were collected from each individual inoculated plant over a 4-day period. *coi1-20* homozygous plants were identified based on their resistance and male-sterile phenotypes, and data from individual *coi1-20* plants were then pooled. Data from the phenotypically wild-type plants (*coi1-20/COI1* and *COI1/COI1*) were also pooled and presented as controls in these experiments.

#### Genetic analysis and mapping

The *coi1-20 rps2-201C* mutant was crossed to both Col-0 *rps2-201C* (Kunkel *et al.*, 1993) and wild-type Col-0 to determine the genetic basis of resistance. Data from the cross to Col-0 are presented in Table 1. The F<sub>2</sub> progeny from the cross of the *coi1-20 rps2-201C* mutant to Col-0 *rps2-201C* segregated in a ratio of 97 resistant plants to 29 susceptible plants ( $\chi^2_{3:1} = 0.27$ ;  $P > 0.5$ ). Complementation tests between *coi1-1* (Feys *et al.*, 1994) and *coi1-20* were conducted by fertilizing homozygous *coi1-20* plants with pollen from heterozygous *coi1-1/COI1* plants. Non-complementation between *coi1-1* and *coi1-20* was established by the appearance of male-sterile, *Pst*DC3000-resistant F<sub>1</sub> progeny (*coi1-20/coi1-1*) occurring in a 1 : 1 ratio with fertile, susceptible (*coi1-20/COI1*) F<sub>1</sub> plants (Table 1). The phenotypically wild-type F<sub>1</sub> plants were allowed to self-pollinate, and gave rise to approximately 25% male-sterile, resistant progeny.

Genetic linkage analysis using co-dominant cleaved amplified polymorphic DNA sequences (CAPS) (Konieczny and Ausubel, 1993) was performed utilizing progeny from the cross between *coi1-20* and *Ler* plants (Table 1). Genomic DNA was isolated from leaf tissue of F<sub>2</sub> plants from this cross according to the procedure of Tai and Tanksley (1990), with modifications as described by Kunkel *et al.* (1993).

#### Coronatine, methyl jasmonate and BTH assays

The sensitivity of *coi1-20* mutants to COR and MeJA was assayed by germinating segregating families of the mutants on Murashige and Skoog (MS) (Murashige and Skoog, 1962) plates containing 1 µM coronatine or 10 µM MeJA (Feys *et al.*, 1994). Homozygous *coi1-20* plants were identified by their root-elongation phenotype 6 days following germination on either COR or MeJA MS plates. Putative *coi1-20* mutants were verified by transferring the seedlings to soil, growing them to maturity, and scoring for male sterility.

*Arabidopsis thaliana* plants were sprayed to run-off with 50, 100 or 200 µM benzo(1,2,3)thiadiazole-7-cabothioic acid (BTH, Novartis Inc., Research Triangle Park, NC, USA) dissolved in distilled H<sub>2</sub>O (Lawton *et al.*, 1996). BTH-treated plants were grown in a separate growth chamber, and tissue samples were harvested and frozen in liquid nitrogen at various times following treatment. Tissue samples were stored at -80°C until *coi1-20* mutants could be identified by scoring for male sterility. *coi1-20* and wild-type (*coi1-20/COI1* and *COI1/COI1*) tissues were pooled and saved for RNA isolation and RNA blot analyses.

#### Generation of *coi1-20 npr1-1* and *coi1-20 nahG* double mutants

*coi1-20 npr1-1* and *coi1-20 nahG* double mutants were generated using homozygous *coi1-20* mutant plants as the female parent in crosses with *npr1-1* and *nahG* plants. F<sub>2</sub> lines homozygous for *npr1-1* or *nahG* and heterozygous for *coi1-20* were identified by screening F<sub>3</sub> progeny using seedling assays. *npr1* homozygous plants become bleached when grown in the presence of SA (Cao

*et al.*, 1994; Cao *et al.*, 1998). This assay facilitated the identification of *coi1/COI1 npr1/npr1* lines that gave rise to approximately 25% bleached, MeJA-insensitive seedlings when germinated on MS plates containing 125 nM SA, 10 µM MeJA. *coi1/COI1 nahG/nahG* lines were identified as giving rise to approximately 25% kanamycin-resistant, MeJA-insensitive seedlings on MS plates containing 50 µg ml<sup>-1</sup> kanamycin, 10 µM MeJA, utilizing the linked kanamycin resistance marker to select for the presence of the *nahG* transgene (Bowling *et al.*, 1994).

#### RNA isolation and Northern analysis

Total RNA was isolated from *A. thaliana* leaf tissue using the RNeasy Plant RNA isolation kit (Qiagen, Chatsworth, CA, USA). RNA gel-blot analysis was carried out according to Sambrook *et al.* (1989). Total RNA (2–3 µg) was loaded in each lane. Hybridization probes were prepared using the Prime-it II kit (Stratagene, La Jolla, CA, USA). The *A. thaliana* cDNA corresponding to the *PR-1* gene was used as a probe (Uknes *et al.*, 1992). As a loading standard, a 3.7 kb *Eco*RI fragment from the 10 kb genomic region carrying *A. thaliana* rRNA genes was used (Vongs *et al.*, 1993). The RNA blots were analyzed using X-ray film or a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

For RNA analysis of inoculated *coi1-20* plants, 15–20 progeny derived from self-fertilized seed harvested from a heterozygous *coi1-20/COI1* individual were infiltrated with *Pst*DC3000 suspensions of  $1 \times 10^5$  cfu ml<sup>-1</sup>. Tissue from individual plants was harvested at various times post-infiltration and flash-frozen in liquid nitrogen. Tissue samples were stored at -80°C until *coi1-20* homozygous plants could be identified based on their male sterile phenotypes. Tissue from at least three homozygous *coi1-20* mutants was pooled for each time point and used for RNA isolation. Tissue from phenotypically wild-type plants (*coi1-20/COI1* and *COI1/COI1*) tissues were also pooled and subject to RNA blot analyses.

#### SA analyses

Progeny derived from self-fertilized seed harvested from a heterozygous *coi1-20/COI1* individual were inoculated with *Pst*DC3000 by dipping into suspensions of  $2-4 \times 10^8$  cfu ml<sup>-1</sup> containing 0.02% Silwet L-77. Tissue from individual plants was harvested just prior to inoculation (time 0) and at 12 and 24 h post-infection. Plants were scored for disease symptoms at 4 dpi, then placed in the greenhouse to flower. Tissue samples were stored at -80°C until *coi1-20* homozygous plants could be verified by scoring for male sterility. Tissue from the resistant, male sterile plants was combined to generate three or four pools of *coi1-20* tissue for each time point. Tissue from phenotypically wild-type siblings (*coi1-20/COI1* and *COI1/COI1*) was combined to generate three or four pools of *COI1/COI1* tissue for each time point. SA and SAG were extracted, and levels assayed as described by Bowling *et al.* (1994).

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