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Virulence systems of *Pseudomonas syringae* pv. *tomato* promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway

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Summary

***Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) causes bacterial speck disease on tomato. The pathogenicity of *Pst* DC3000 depends on both the type III secretion system that delivers virulence effector proteins into host cells and the phytotoxin coronatine (COR), which is thought to mimic the action of the plant hormone jasmonic acid (JA). We found that a JA-insensitive mutant (*jai1*) of tomato was unresponsive to COR and highly resistant to *Pst* DC3000, whereas host genotypes that are defective in JA biosynthesis were as susceptible to *Pst* DC3000 as wild-type (WT) plants. Treatment of WT plants with exogenous methyl-JA (MeJA) complemented the virulence defect of a bacterial mutant deficient in COR production, but not a mutant defective in the type III secretion system. Analysis of host gene expression using cDNA microarrays revealed that COR works through *Jai1* to induce the massive expression of JA and wound response genes that have been implicated in defense against herbivores. Concomitant with the induction of JA and wound response genes, the type III secretion system and COR repressed the expression of pathogenesis-related (*PR*) genes in *Pst* DC3000-infected WT plants. Resistance of *jai1* plants to *Pst* DC3000 was correlated with a high level of *PR* gene expression and reduced expression of JA/wound response genes. These results indicate that COR promotes bacterial virulence by activating the host's JA signaling pathway, and further suggest that the type III secretion system might also modify host defense by targeting the JA signaling pathway in susceptible tomato plants.**

Keywords: jasmonic acid, *Pseudomonas syringae*, coronatine, type III secretion, bacterial speck disease, salicylic acid.

Introduction

Pseudomonas syringae pv. *tomato* strain DC3000 (*Pst* DC3000), which has recently been sequenced by The Institute for Genomic Research (TIGR), is a model organism for molecular studies of plant–pathogen interactions (Buell *et al.*, 2003; Katagiri *et al.*, 2002; Preston, 2000). In nature, this pathogen causes bacterial speck disease on tomato, and it can also infect *Arabidopsis* and *Brassica* species (Whalen *et al.*, 1991; Zhao *et al.*, 2000). A key factor in the pathogenicity of *Pst* DC3000 and many other plant and animal bacteria is the type III secretion system that delivers virulence effector proteins into host cells (Bonas and Lahaye, 2002; Collmer *et al.*, 2000; Cornelis and van Gijsegem, 2000; Galan and Collmer, 1999; He, 1998; Jin *et al.*, 2003; Staskawicz *et al.*, 2001). *P. syringae hrp*/*hrc* mutants, which lack a functional type III secretion system, lose their ability to elicit the hypersensitive response (HR) in non-host plants and pathogenicity in host plants. A recent genome-wide search has led to identification of over 30 effector proteins in *Pst* DC3000 that are secreted by the Hrp type III secretion system (Boch *et al.*, 2002; Fouts *et al.*, 2002; Guttman *et al.*, 2002; Petnicki-Ocwieja *et al.*, 2002; Zwiesler-Vollick *et al.*, 2002). Although the avirulence functions of some effector proteins (e.g. *avrPto*) have been described in detail (Kim *et al.*, 2002; Tang *et al.*, 1996),

the role of effectors in virulence is not well understood. Moreover, mutations in individual effector genes usually have little or no effect on bacterial virulence. These findings demonstrate the difficulties of using bacterial genetics as a sole tool to study the virulence functions of type III effectors in plants and illustrate a need for defining the global effects of type III effectors on host physiology using plant genetic and genomic approaches.

In addition to type III effectors, *Pst* DC3000 also produces another virulence factor, the phytotoxin coronatine (COR; Bender *et al.*, 1999). COR is a non-host-specific, chlorosis-inducing polyketide produced by several pathovars of *P. syringae*. Studies in tomato have shown that COR increases the severity of disease symptoms by promoting lesion expansion and bacterial growth (Bender *et al.*, 1987; Penaloza-Vazquez *et al.*, 2000). In *Arabidopsis*, COR plays an important role in the early stages of infection, and may promote virulence by suppressing the expression of host genes involved in pathogen defense (Kloek *et al.*, 2001; Mittal and Davis, 1995). It has also been noted that COR acts as a structural and functional analog of jasmonic acid (JA) and related signaling compounds (collectively referred to here as JAs) such as methyl-JA (MeJA) and 12-oxo-phytodienoic acid (12-OPDA), the C_{18} precursor of JA/MeJA (Bender *et al.*, 1999; Feys *et al.*, 1994; Lauchli and Boland, 2003; Weiler *et al.*, 1994). For example, a broad spectrum of physiological processes including chlorosis, ethylene emission, tendril coiling, inhibition of root elongation, volatile production, and biosynthesis of various stress-related compounds are induced by both COR and JAs (Benedetti *et al.*, 1995; Boland *et al.*, 1995; Feys *et al.*, 1994; Greulich *et al.*, 1995; Haider *et al.*, 2000; Lopukhina *et al.*, 2001; Palmer and Bender, 1995; Weiler *et al.*, 1994). Although it is widely accepted that the JA signaling pathway plays a central role in plant defense against herbivores and some fungal pathogens (Kunkel and Brooks, 2002; Liechti and Farmer, 2002; Walling, 2000; Wasternack and Hause, 2002), relatively little is known about how the interaction of this pathway with COR is related to the virulence effects of the phytotoxin.

Identification of the *Arabidopsis coi1* mutant that is insensitive to both JAs and COR further supports the notion that COR and JA act through a common signaling pathway (Feys *et al.*, 1994). The *COI1* gene encodes an F-box protein that participates in ubiquitin-dependent protein degradation, which presumably regulates the abundance of proteins that control the expression of JA/COR-responsive genes (Devoto *et al.*, 2002; Turner *et al.*, 2002; Xie *et al.*, 1998; Xu *et al.*, 2002). *coi1* mutant lines of *Arabidopsis* show elevated resistance to *P. syringae* infection, and both symptom development and multiplication of bacterial populations are severely compromised (Feys *et al.*, 1994; Kloek *et al.*, 2001). Furthermore, increased levels of salicylic acid (SA) and hyperexpression of the pathogenesis-related (*PR*) gene *PR-1* were observed in *coi1* plants inoculated with *Pst* DC3000 (Kloek *et al.*, 2001). A role for SA in *coi1*-mediated resistance is further supported by the observation that *coi1* plants expressing the *nahG* gene, which encodes the SA-degrading enzyme salicylate hydroxylase, failed to restrict bacterial growth. In addition to its requirement for efficient bacterial multiplication, COI1 is also essential for normal symptom development induced by *Pst* DC3000, but in an SA-independent manner (Kloek *et al.*, 2001).

Jasmonate signaling mutants of tomato (*Lycopersicon esculentum*) provide potentially valuable tools to study the role of JA and COR in bacterial speck disease in tomato. Several mutants that fail to synthesize antiherbivore defensive proteins such as proteinase inhibitors (PIs) and polyphenol oxidase (PPO) in response to wounding have been shown to be deficient in either JA synthesis or JA perception (Howe and Ryan, 1999; Li *et al.*, 2001, 2002a, 2003). In this paper, we demonstrate that a JA-insensitive mutant (*jai1*) of tomato is unresponsive to COR and highly resistant to infection by *Pst* DC3000. Evaluation of bacterial growth, symptom development, and the expression of defense-related genes using a custom cDNA microarray showed that *Pst* DC3000 employs COR to coordinately activate JA/wound response genes and repress SA-response genes in tomato. Interestingly, treatment of wild-type (WT) plants with MeJA rescued the virulence defect of the COR^- mutant, demonstrating that COR and MeJA are functionally exchangeable in promoting bacterial virulence in tomato. Severe attenuation of *Pst* DC3000 multiplication in infiltrated *jai1* leaves contrasted with the modest reduction in growth of COR-defective bacteria in WT leaves, suggesting that virulence factors other than COR may also be interacting with the *Jai1* signaling pathway to promote disease. Evidence is presented to indicate that the type III secretion system might work together with COR to modulate JA- and SA-response genes, thereby promoting disease development in tomato.

Results

jai1 *plants are insensitive to the* P. syringae *phytotoxin coronatine*

Previous studies have shown that treatment of tomato leaves with COR, as well as JA and MeJA, induces the expression of PIs that play a role in defense against insects (Feys *et al.*, 1994; Palmer and Bender, 1995; Pautot *et al.*, 2001; Zhao *et al.*, 2001). To determine whether COR-induced PI expression requires a functional JA response pathway, we determined the capacity of exogenous COR to induce accumulation of the serine PI, PI-II, in *spr2* and *jai1* plants that are defective in JA biosynthesis and JA signaling, respectively (Li *et al.*, 2002a, 2003). Application of various amounts of COR to the lower leaf of WT plants in the two-leaf stage

Table 1 Proteinase inhibitor II accumulation in various tomato genotypes in response to coronatine

	PI-II μg ml^{-1} of leaf juice					
	WT		*spr2*		*jai1*	
COR[a] (ng per leaflet)	Local	Systemic	Local	Systemic	Local	Systemic
0	ND[b]	ND	ND	ND	ND	ND
0.1	27 ± 9	ND	20 ± 6	ND	ND	ND
1	46 ± 4	ND	36 ± 5	ND	ND	ND
10	62 ± 6	45 ± 6	33 ± 6	30 ± 10	ND	ND
100	67 ± 15	73 ± 4	50 ± 10	62 ± 10	ND	ND

[a]Various amounts of pure COR (dissolved in 0.1 M NH_4HCO_3) were applied in a 2-μl volume to the adaxial surface of three leaflets on the lower leaf of WT, *spr2*, and *jai1* plants that contained two fully expanded leaves and a third emerging leaf. Control plants were treated with 2 μl of 0.1 M NH_4HCO_3. PI-II levels were determined 24 h after COR or buffer application. For each concentration of COR tested, three leaflets from the lower treated leaves (local) and upper untreated leaves (systemic) were used to measure the PI-II levels using a radial immunodiffusion assay. Values represent the mean PI-II concentration of three plants per COR concentration ± SD. The experiment was repeated three times, and similar results were obtained.
[b]ND, not detectable, below the detection limit of the assay (approximately 5 μg PI-II per milliliter of leaf juice).

resulted in PI-II accumulation in the treated tissue (local response; Table 1). This response was dose dependent within the range of 0.1–10 ng COR per leaflet, but appeared to saturate at COR concentrations between 10 and 100 ng per leaflet. Treatment of *spr2* leaves with COR also induced PI-II accumulation, albeit to levels that were lower than those in WT plants. At COR concentrations of 10 ng per leaflet and greater, PI-II protein also accumulated in upper untreated leaves (systemic response) of WT and *spr2* plants. In contrast, COR did not induce local or systemic PI-II accumulation in *jai1* plants, even when applied at relatively high concentrations (e.g. 100 ng per leaflet). We also observed that COR concentrations of 50 ng ml^{-1} (0.1 ng per leaflet) or greater caused chlorosis on WT and *spr2* leaves 3 days after the treatment, but did not cause chlorosis on *jai1* leaves (data not shown). These results demonstrate that COR-induced PI-II expression and chlorosis in tomato leaves require the *Jai1*-dependent signal transduction pathway, but does not depend strictly on JA biosynthesis.

Susceptibility of tomato to Pst *DC3000 requires a functional JA response pathway*

The insensitivity of *jai1* plants to exogenous COR suggested that the mutant might have increased resistance to *P. syringae*. To test this hypothesis, 3-week-old WT and *jai1* plants were infiltrated with *Pst* DC3000 and monitored for symptom development and *in planta* bacterial growth. Five days after inoculation, WT plants showed typical bacterial speck disease symptoms, including necrotic lesions surrounded by chlorosis. By contrast, *jai1* plants exhibited no detectable disease symptoms (Figure 1a). Disease symptoms on WT leaves were correlated with relatively high levels of bacterial growth *in planta* during a 3-day period following infiltration. Bacterial growth in *jai1* leaves at the end of the time course was approximately 200-fold less than that in WT plants (Figure 1b). The enhanced resistance of *jai1* plants to *Pst* DC3000 infection indicates that the *Jai1* gene product plays an important role in the susceptibility of tomato to *Pst* DC3000.

To determine whether the increased resistance to *Pst* DC3000 is specific for *jai1*, or whether this phenotype reflects a general defect in the JA response pathway, we evaluated pathogen resistance in several other tomato mutants that are altered in the JA response pathway. Infiltration of the s*pr2* and *def1* JA biosynthetic mutants (Howe *et al.*, 1996; Li *et al.*, 2003) with the pathogen resulted in the development of typical bacterial speck symptoms, the severity and timing of which were comparable to symptoms observed in WT plants (data not shown). Consistent with this observation, the rate of bacterial growth in *spr2* (Figure 1b) and *def1* (data not shown) leaves was not significantly different from that in WT leaves. These results indicate that susceptibility of tomato to *Pst* DC3000 depends on a functional *Jai1* signaling pathway but does not strictly require JA biosynthesis. Pathogenicity assays were also conducted with a *35S::prosystemin* transgenic line that constitutively expresses wound- and JA-responsive genes in a *Jai1*-dependent manner (Li *et al.*, 2001; McGurl *et al.*, 1994). Following infiltration with *Pst* DC3000, symptom development and bacterial growth in *35S::prosystemin* leaves were comparable to those in WT plants (data not shown). Thus, constitutive activation of the *Jai1* signaling pathway in *35S::prosystemin* leaves does not significantly affect the virulence of *Pst* DC3000.

Loss of function of Jai1 *has a greater effect on disease development than does loss of COR production by* P. syringae

To determine the relative contribution of *Jai1*, COR, and the type III secretion system to *Pst* DC3000 pathogenicity, we

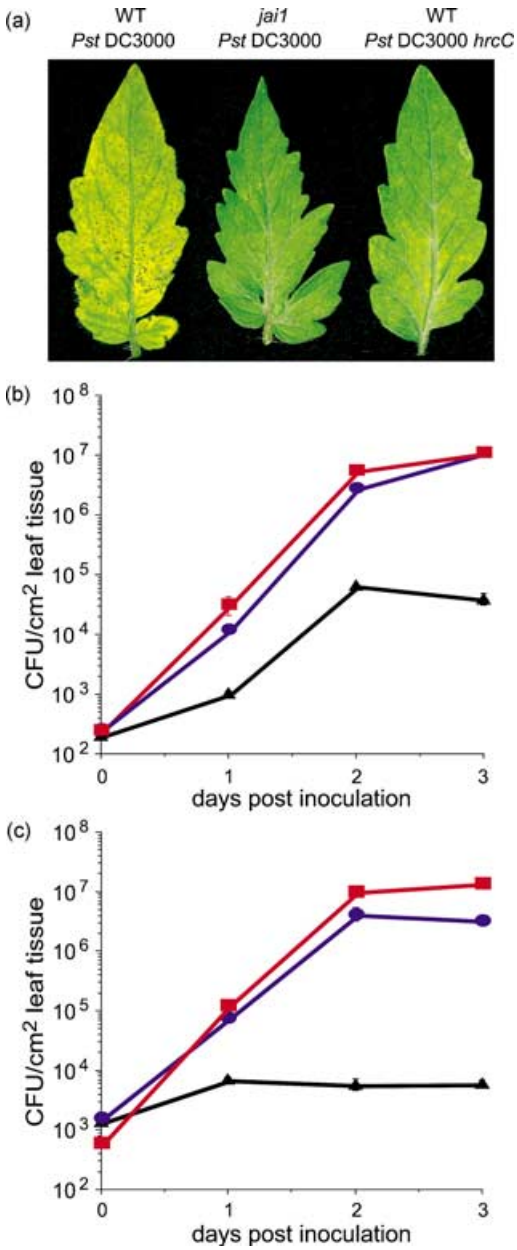


Figure 1. *jai1* plants are resistant to *Pst* DC3000.
(a) Necrotic specks and diffuse chlorosis caused by *Pst* DC3000 in a WT tomato leaf (left), but not in a *jai1* mutant leaf (center) or in a WT leaf inoculated with the *hrcC* mutant (right). Pictures were taken 5 days after vacuum-infiltration with bacterial suspensions containing 1×10^5 CFU ml^{-1}.
(b) Growth of *Pst* DC3000 in WT (squares), *spr2* (circles), and *jai1* (triangles) plants.
(c) Growth of *Pst* DC3000 (squares), *Pst* DC3118 COR$^-$ (circles), and *Pst* DC3000 *hrcC* (triangles) in WT plants. Data points represent means of three replicates ± SD. Similar results were obtained in two independent experiments.

assayed symptom development and bacterial growth of the *hrcC* type III secretion mutant and a COR$^-$ mutant (*Pst* DC3118) in WT and *jai1* plants. The *hrcC* mutant grew only two- to fivefold in WT leaves 3 days after inoculation, representing an approximately 10 000-fold reduction relative to *Pst* DC3000 (Figure 1c). The non-pathogenic phenotype of the *hrcC* mutant was similar to that reported for the *Pst* DC3000 *hrpS* and *hrpA* mutants (Roine *et al.*, 1997). As expected, *hrcC* bacteria did not cause disease on *jai1* plants (data not shown). The COR$^-$ mutant when infiltrated into WT tomato leaves induced small necrotic lesions without chlorosis, as reported previously for a COR$^-$ mutant of another *Pst* strain (Bender *et al.*, 1987). Growth of COR$^-$ bacteria was comparable to *Pst* DC3000 2 days post-inoculation but, by day 3, decreased between 5- and 20-fold relative to the growth of *Pst* DC3000 (Figure 1c). When inoculated onto *jai1* plants, the COR$^-$ mutant grew to similar levels as *Pst* DC3000 and did not produce visible symptoms (data not shown). Therefore, loss of the *Jai1*-dependent signaling pathway had a much greater effect on bacterial multiplication and symptom production than did loss of COR production by the pathogen. These results suggest that bacterial virulence factors other than COR may also be working through *Jai1* to contribute to disease development.

Exogenous jasmonate complements loss of COR production

The insensitivity of *jai1* plants to both COR and JA, together with the enhanced resistance of this tomato mutant to DC3000, suggested that COR promotes virulence by activating the host's JA signaling pathway. To further test this hypothesis, WT plants were inoculated with the COR$^-$ mutant and then exposed to volatile MeJA for 3 days. Results from three independent experiments showed that leaves of MeJA-treated plants supported 8- to 50-fold more bacterial growth than leaves from mock-treated plants (Figure 2). At 3 days post-inoculation, infected leaves that were treated with MeJA exhibited both specks and chlorosis, similar to typical bacterial speck disease symptoms. In contrast, significant chlorosis was not observed in mock-treated control plants inoculated with the COR$^-$ bacteria. Experiments conducted with DC3000 and the *hrcC* mutant showed that multiplication of these strains and symptom development were not significantly affected by treatment of plants with MeJA (data not shown). These results demonstrate that activation of the host JA signaling pathway is necessary and sufficient to complement the virulence defect of a COR$^-$ bacterial strain, and provide direct support for the hypothesis that the COR toxin contributes to virulence by mimicking the action of endogenous jasmonate.

Transcriptional profiling of host gene expression

To gain insight into the roles of and relationships between *Jai1*, COR, and type III effectors in disease development in

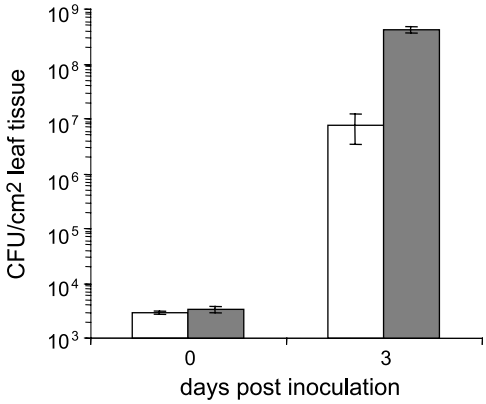


Figure 2. Treatment of tomato plants with MeJA complements the virulence defect of a COR$^-$ mutant of *P. syringae*.
Two different sets of 3-week-old WT tomato plants (experimental and control) were vacuum infiltrated with *Pst* DC3118 COR$^-$ bacteria. Inoculated plants were allowed to dry for 3 h under ambient conditions, and then sampled for bacterial growth (day 0). Immediately after sampling, one set of plants (MeJA-treated, filled bars) was placed into an enclosed Lucite box containing vaporous MeJA. The second set of plants (mock control, open bars) was placed into a separate box containing an equivalent amount of ethanol. The boxes were placed together in a growth chamber (same light and temperature conditions used for initial growth of the plants) for 3 days. On day 3, the boxes were opened and leaf disk samples were harvested for bacterial growth assays. The data show the mean ± SD of at least three replicates per sample, and are representative of three independent experiments.

tomato, we profiled the expression of host genes using a custom cDNA microarray. The microarray slide used for these experiments comprised 607 tomato cDNAs that represent approximately 500 unique genes involved in various aspects of pathogen defense, lipid biosynthesis and signaling, wound- and JA-induced antiherbivore defense, signal transduction, and cellular metabolism. This microarray was therefore enriched for defense-related genes. A complete list of these clones is provided in Supplementary Material (Table S1). Microarray analysis was used to examine host gene expression using the three experimental comparisons shown in Figure 3(a): (i) inoculation of both WT and *jai1* plants with *Pst* DC3000 (experiment 1); (ii) inoculation of WT plants with *Pst* DC3000 and the COR$^-$ mutant (experiment 2); and (iii) inoculation of WT plants with *Pst* DC3000 and the *hrcC* mutant (experiment 3). Total RNAs for sample labeling were isolated from leaf tissue 24 h after inoculation. This time point was chosen because it represents a stage of the interaction when *Pst* DC3000 grows rapidly within host tissues but prior to the onset of disease symptoms (Figure 1a). Moreover, both JA/wound response genes and *PR* genes are expressed at this time point (van Kan *et al.*, 1992; Li *et al.*, 2003).

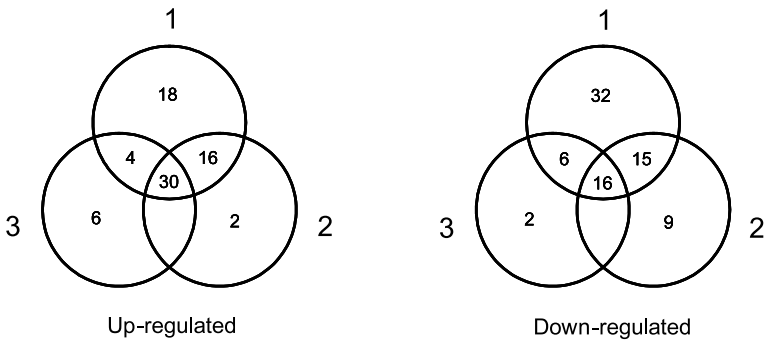
By means of microarray analysis, we identified 156 genes that were differentially regulated at least twofold in one or more of the three experimental comparisons. Among these genes, 76 were up-regulated and 80 were down-regulated (Figure 3b; Table S1). The largest effect on gene expression, in terms of both the number of regulated genes and the expression ratio, was observed between *Pst* DC3000-inoculated WT and *jai1* samples (experiment 1). Comparison of WT plants inoculated with *Pst* DC3000 and the *hrcC* mutant (experiment 3) revealed the least amount of differential gene expression (Table S2). As depicted in Figure 3(b), however, there was significant overlap in the gene expression profiles between the three experiments: 87 genes were differentially regulated in at least two of the three experimental comparisons (Table S2), whereas the remaining 69 genes were differentially regulated in a single comparison (Table S3). The former set of 87 overlapping genes included 50 induced genes and 37 repressed genes, a

Figure 3. Effect of *Jai1*, COR, and the type III secretion system on differential gene expression in the tomato–*P. syringae* interaction.
(a) The pathogen strains and host genotypes used in each of the three experimental comparisons are indicated. For each comparison, the test treatment is listed first, followed by the control treatment.
(b) Venn diagrams of the number of overlapping or non-overlapping genes that were induced (left; expression ratio > 2.0) or repressed (right; expression ratio < 0.5) in the three experimental comparisons. Numbers outside the circles indicate the experimental comparison described in (a). Expression data for all genes on the array are listed in Table S1. Expression data for the 87 genes that were differentially regulated (50 induced, 37 repressed) in at least two of the three experimental comparisons are listed in Table S2.

(a)

Experiment	Pathogen Strain(s)	Tomato genotype(s)
1	*Pst* DC3000	wild-type/*jai1*
2	*Pst* DC3000/COR$^-$	wild-type
3	*Pst* DC3000/*hrcC*	wild-type

(b)



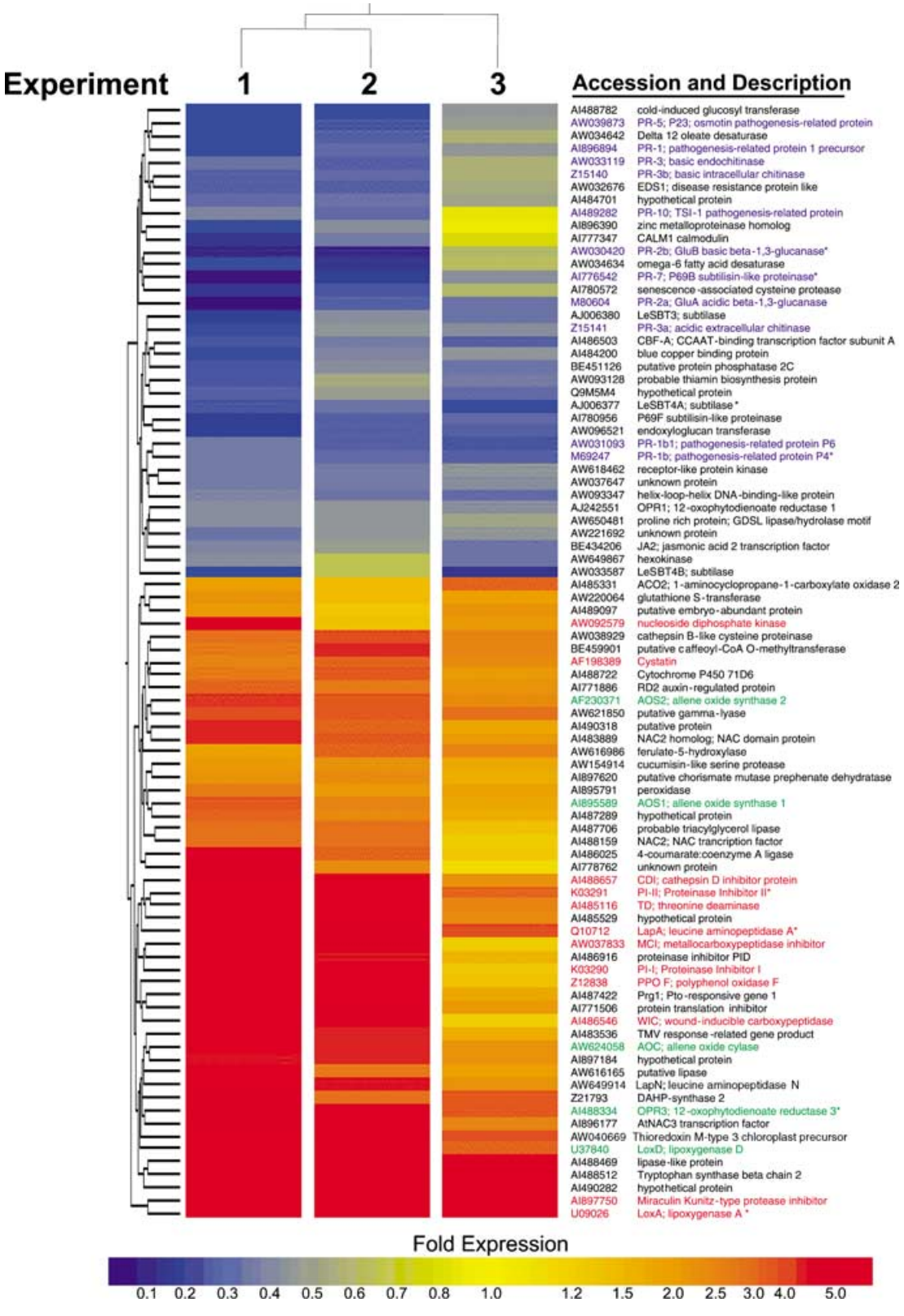
subset of which (30 and 16, respectively) were differentially regulated in all three experiments (Figure 3b). Cluster analysis of the 87 overlapping genes (Figure 4) showed that the overall expression profile of experiment 1 (*Pst* DC3000 on WT/*jai1* plants) was more similar to that of experiment 2 (*Pst* DC3000/COR$^-$ mutant on WT plants) than to that of experiment 3 (*Pst* DC3000/*hrcC* mutant on WT plants). This finding indicates that COR plays a major role in modulating the expression of *Jai1*-dependent genes in susceptible tomato plants. However, the overall similarities in gene expression patterns observed between experiments 1 and 3 suggest that the type III secretion system also directly or indirectly contributes to *Jai1*-dependent changes in host gene expression. Thus, COR and the type III secretion system may coordinately target the *Jai1*-dependent pathway(s) in this plant–pathogen interaction.

The microarray analysis revealed clear differences in the expression profile of genes involved in pathogen defense-related functions compared to genes associated with responses to JA and wounding (Figure 4). For example, transcripts encoding known JA/wound response proteins (annotated in red text) accumulated to higher levels in *Pst* DC3000-infected WT leaves than in *Pst* DC3000-infected *jai1* leaves or WT leaves inoculated with the *hrcC* and COR$^-$ mutants (Table S2). Included within this group are genes encoding a broad range of PIs (PI-I, PI-II, cathepsin D inhibitor (CDI), metallocarboxypeptidase inhibitor (MCI), cystatin, miraculin), proteases such as leucine amino peptidase (LAP) and wound-inducible carboxypeptidase (WIC), and other proteins implicated in antiherbivore defense (e.g. PPO). *Pst* DC3000 infection also resulted in *Jai1*-dependent expression of genes encoding the JA biosynthetic enzymes lipoxygenase (LoxD), allene oxide synthase (AOS1 and AOS2), allene oxide cyclase (AOC), and 12-OPDA reductase (OPR3; annotated in green text), as well as other enzymes involved in lipid metabolism (i.e. the 9-LOX, LoxA, and several putative lipases). Concomitant with the induction of JA/wound response genes, genes encoding several PR proteins were expressed at a lower level in *Pst* DC3000-infected WT leaves as compared to leaves inoculated with the *hrcC* and COR$^-$ mutants, or to *Pst* DC3000-infected *jai1* leaves (Figure 4; annotated in blue text). One of these repressed genes, *PR-7*, encodes the subtilisin-like protease also known as P69B. Interestingly, genes encoding several other members (SBT3, SBT4A, SBT4B, and P69F) of this protease family were also repressed in leaves inoculated with *Pst* DC3000 as compared to leaves inoculated with the *hrcC* or COR$^-$ mutants. These experiments point to the general conclusion that susceptibility of tomato to *Pst* DC3000 is associated with induction of JA/wound response genes and concomitant repression of *PR* genes, and these changes in gene expression depend on the ability of COR and also apparently the Hrp type III secretion system to interact with the host JA signaling pathway.

RNA blot hybridization was used to confirm the steady-state transcript level of eight genes whose expression was shown by microarray analysis to be induced (*PI-II*, *LapA*, *LoxA*, and *OPR3*) or repressed (*PR-1b*, *PR-2b*, *PR-7*, and *SBT4A*) by *Pst* DC3000 compared to the *hrcC* or COR$^-$ mutant. In general, these results were in good agreement with the expression ratios obtained from the microarray data (Figure 5). For example, RNA blot analysis showed that *PI-II*, *LapA*, *LoxA*, and *OPR3* mRNA levels were relatively low in control WT and *jai1* plants, and were induced by a *Jai1*-dependent pathway 24 h after inoculation with *Pst* DC3000. These four genes also showed significant induction in WT plants inoculated with the *hrcC* mutant, but exhibited little or no induced expression in WT plants inoculated with the COR$^-$ mutant. These results confirm that the expression of JA/wound response genes after infection with *Pst* DC3000 is *Jai1* dependent, and largely results from the action of COR. The observation that mRNA levels for some JA/wound response genes (e.g. *LoxA*) were lower in *hrcC* than in *Pst* DC3000-challenged WT leaves provides additional evidence that the type III secretion system plays a role in up-regulating the expression of at least some JA-response genes.

RNA blot analysis showed that the pathogen defense-related genes *PR-1b*, *PR-2b*, *PR-7* (also known as *P69B*), and *SBT4A* were expressed at low or undetectable levels in uninfected WT and *jai1* plants (Figure 5). Typical of *PR* genes, the steady-state level of these transcripts increased in WT plants in response to *Pst* DC3000 infection. Interestingly, all four genes were expressed to even higher levels in *Pst* DC3000-infected *jai1* plants. This finding suggests that one or more virulence effectors produced by *Pst* DC3000 acts through *Jai1* to repress pathogen defense-related genes in susceptible tomato plants. Consistent with this, the expression levels of *PR-1b*, *PR-2b*, *PR-7*, and *SBT4A* were greater in WT plants inoculated with the *hrcC* and COR$^-$ mutants than in *Pst* DC3000-infected WT plants. This finding agrees with the microarray data and suggests that

Figure 4. Cluster analysis of genes that are differentially regulated in the tomato–*P. syringae* interaction. GENESPRING^{TM} software was used to analyze the expression of 87 genes that are differentially regulated in at least two of the three experimental comparisons (numbers above the columns) described in Figure 3. The display depicts the average ratios from three biological replicates for each of the three comparisons. Expression ratios were calculated as test/control (Figure 3a). The GENESPRING tree illustrating the relationship between the three experimental comparisons is shown at the top, and a tree indicating the relationships between each of the individual genes is shown on the left. The Accession number and brief description of the corresponding gene or gene homolog in the cluster is listed on the right. Red text indicates genes that have previously been shown to be up-regulated by JA and/or wounding. Green text denotes JA/wound responses genes encoding enzymes involved in JA biosynthesis. Blue text denotes known SA-regulated *PR* genes. The asterisk (*) indicates genes that were selected for further analysis by RNA blot hybridization (Figure 5). The color bar represents the scale of the fold change in gene expression within the gene tree.


Experiment
1
2
3
Accession and Description
AI488782 cold-induced glucosyl transferase
AW039873 PR-5; P23; osmotin pathogenesis-related protein
AW034642 Delta 12 oleate desaturase
AI896894 PR-1; pathogenesis-related protein 1 precursor
AW033119 PR-3; basic endochitinase
Z15140 PR-3b; basic intracellular chitinase
AW032676 EDS1; disease resistance protein like
AI484701 hypothetical protein
AI489282 PR-10; TSI-1 pathogenesis-related protein
AI896390 zinc metalloproteinase homolog
AI777347 CALM1 calmodulin
AW030420 PR-2b; GluB basic beta-1,3-glucanase*
AW034634 omega-6 fatty acid desaturase
AI776542 PR-7; P69B subtilisin-like proteinase*
AI780572 senescence-associated cysteine protease
M80604 PR-2a; GluA acidic beta-1,3-glucanase
AJ006380 LeSBT3; subtilase
Z15141 PR-3a; acidic extracellular chitinase
AI486503 CBF-A; CCAAT-binding transcription factor subunit A
AI484200 blue copper binding protein
BE451126 putative protein phosphatase 2C
AW093128 probable thiamin biosynthesis protein
Q9M5M4 hypothetical protein
AJ006377 LeSBT4A; subtilase *
AI780956 P69F subtilisin-like proteinase
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AW031093 PR-1b1; pathogenesis-related protein P6
M69247 PR-1b; pathogenesis-related protein P4*
AW618462 receptor-like protein kinase
AW037647 unknown protein
AW093347 helix-loop-helix DNA-binding-like protein
AJ242551 OPR1; 12-oxophytodienoate reductase 1
AW650481 proline rich protein; GDSL lipase/hydrolase motif
AW221692 unknown protein
BE434206 JA2; jasmonic acid 2 transcription factor
AW649867 hexokinase
AW033587 LeSBT4B; subtilase
AI485331 ACO2; 1-aminocyclopropane-1-carboxylate oxidase 2
AW220064 glutathione S-transferase
AI489097 putative embryo-abundant protein
AW092579 nucleoside diphosphate kinase
AW038929 cathepsin B-like cysteine proteinase
BE459901 putative caffeoyl-CoA O-methyltransferase
AF198389 Cystatin
AI488722 Cytochrome P450 71D6
AI771886 RD2 auxin-regulated protein
AF230371 AOS2; allene oxide synthase 2
AW621850 putative gamma-lyase
AI490318 putative protein
AI483889 NAC2 homolog; NAC domain protein
AW616986 ferulate-5-hydroxylase
AW154914 cucumisin-like serine protease
AI897620 putative chorismate mutase prephenate dehydratase
AI895791 peroxidase
AI895589 AOS1; allene oxide synthase 1
AI487289 hypothetical protein
AI487706 probable triacylglycerol lipase
AI488159 NAC2; NAC trancription factor
AI486025 4-coumarate:coenzyme A ligase
AI778762 unknown protein
AI488657 CDI; cathepsin D inhibitor protein
K03291 PI-II; Proteinase Inhibitor II*
AI485116 TD; threonine deaminase
AI485529 hypothetical protein
Q10712 LapA; leucine aminopeptidase A*
AW037833 MCI; metallocarboxypeptidase inhibitor
AI486916 proteinase inhibitor PID
K03290 PI-I; Proteinase Inhibitor I
Z12838 PPO F; polyphenol oxidase F
AI487422 Prg1; Pto-responsive gene 1
AI771506 protein translation inhibitor
AI486546 WIC; wound-inducible carboxypeptidase
AI483536 TMV response-related gene product
AW624058 AOC; allene oxide cylase
AI897184 hypothetical protein
AW616165 putative lipase
AW649914 LapN; leucine aminopeptidase N
Z21793 DAHP-synthase 2
AI488334 OPR3; 12-oxophytodienoate reductase 3*
AI896177 AtNAC3 transcription factor
AW040669 Thioredoxin M-type 3 chloroplast precursor
U37840 LoxD; lipoxygenase D
AI488469 lipase-like protein
AI488512 Tryptophan synthase beta chain 2
AI490282 hypothetical protein
AI897750 Miraculin Kunitz-type protease inhibitor
U09026 LoxA; lipoxygenase A *
Fold Expression
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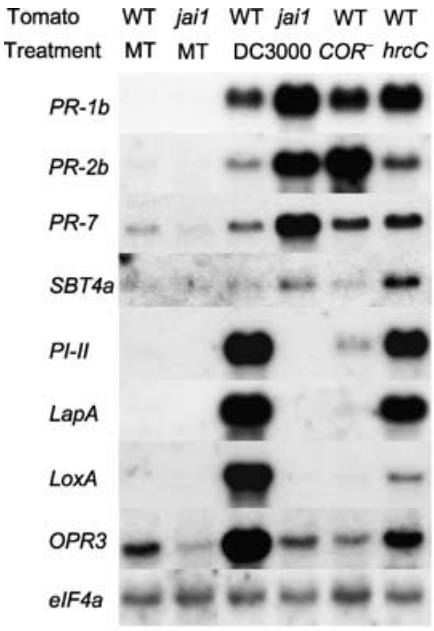

	WT/*jai1* DC3000	WT DC3000/*COR*⁻	WT DC3000/*hrcC*
PR-1b	0.41	0.35	0.32
PR-2b	0.17	0.16	0.67
PR-7	0.14	0.23	0.47
SBT4a	0.28	0.39	0.24
PI-II	71.23	10.07	3.24
LapA	48.48	16.75	3.70
LoxA	73.67	33.79	10.56
OPR3	5.16	5.51	3.62
eIF4a	1.03	1.02	1.30

Figure 5. RNA blot hybridization of eight differentially regulated genes identified by microarray analysis.
WT and *jai1* plants were either mock-treated (MT) or inoculated with the bacterial (1×10^5 CFU ml^{-1}) strain indicated. Total RNA was isolated from leaves 24 h post-inoculation. Aliquots of total RNA (5 μg) were analyzed by blot hybridization for accumulation of transcripts corresponding to the indicated genes (left). Pathogenesis-related genes *PR-1b*, PR-*2b*, *PR-7* (also known as *P69B*); *SBT4a*, tomato subtilase; *PI-II*, proteinase inhibitor II; *LapA*, leucine aminopeptidase; *LoxA*, lipoxygenase A; *OPR3*, 12-OPDA reductase; *eIF4a*, eukaryotic initiation factor 4a gene (as a loading control). Values in the accompanying table (right) indicate the average expression ratio from the three biological replicates for the corresponding gene as determined by microarray analysis.

repression of *PR* and other pathogen defense response genes is caused by the coordinate and *Jai1*-dependent action of the Hrp type III secretion system and COR.

Discussion

jai1 *impairs COR-mediated signaling and enhances resistance to* Pst *DC3000*

The phytotoxin COR of *P. syringae* has been implicated as an important virulence factor in several diseases caused by *P. syringae* (Bender *et al.*, 1999). As the virulence function of COR has been correlated with its ability to mimic the action of JA and related octadecanoid signaling compounds, we conducted experiments to investigate the role of COR in the interaction of *Pst* DC3000 with tomato genotypes that are impaired in the JA response pathway. In addition to confirming previous studies showing that exogenous COR induces local and systemic expression of PIs in tomato plants (Table 1; Feys *et al.*, 1994; Palmer and Bender, 1995; Zhao *et al.*, 2001), we demonstrate here that a JA signaling mutant (*jai1*) of tomato is defective in COR-induced PI expression. The inability of exogenous COR to cause chlorosis on *jai1* leaves suggests that the mutation does not specifically affect PI expression, but rather abrogates the broad effects of the phytotoxin. Thus, like the *COI1* gene of *Arabidopsis*, *Jai1* is required for responsiveness of tomato to both JAs (Li *et al.*, 2002a) and COR (this study). In contrast to *jai1*, *spr2* plants were responsive to applied COR. As the *spr2* mutation abolishes the function of an omega-3 fatty acid desaturase that is required for JA biosynthesis (Li *et al.*, 2003), this finding indicates that JA and other oxylipins derived from trienoic fatty acids are not required for COR-induced PI expression. We note, however, that the level of PI-II in *spr2* plants treated with subsaturating doses of COR was significantly less than that in WT plants (Table 1). This observation suggests that COR may induce the biosynthesis of JA in tomato leaves, which is consistent with the results from the DNA microarray analysis (see below). With regard to the systemic response, the results are keeping with the idea that COR, like JA, can be transported from the site of application to distal tissues where it activates the JA signaling pathway leading to PI expression (Li *et al.*, 2002a; Zhao *et al.*, 2001). The ability of virulent strains of *P. syringae* to induce systemic expression of PIs and PPO in tomato plants (Pautot *et al.*, 1991; Stout *et al.*, 1999; Thipyapong and Steffens, 1997) suggests that COR-induced systemic signaling may be an important part of the virulence strategy of this pathogen.

The insensitivity of *jai1* plants to exogenous COR was accompanied by the absence of *Pst* DC3000-induced disease symptoms and a severe (aproximately 200-fold) reduction in the growth of the pathogen (Figure 1). The enhanced resistance and COR-insensitive phenotype of *jai1* plants is thus very similar to that of the *coi1* mutant of *Arabidopsis*. Experiments are currently in progress to determine whether *Jai1* encodes the tomato ortholog of COI1 or another component of the JA signaling pathway. In contrast to *jai1*, tomato plants that are either attenuated (*spr2* and *def1*) or enhanced (*35S::prosystemin*) in their capacity to synthesize JA remain susceptible to *Pst* DC3000, as is the case for JA biosynthetic mutants of *Arabidopsis* (Kloek *et al.*, 2001).

Significantly, treatment of WT plants with MeJA increased the virulence of the COR$^-$ mutant (Figure 2), indicating that activation of the JA signaling pathway is necessary and sufficient to complement a deficiency in COR production. Taken together, the most straightforward interpretation of these results is that COR, functioning as a JA analog, promotes susceptibility by activating the host's JA signaling pathway. Because of the structural similarity of COR to JAs (Lauchli and Boland, 2003; Weiler *et al.*, 1994), it can be hypothesized that COR exerts its effects by interacting with components of the JA perception apparatus.

We observed that the contribution of COR to *Pst* DC3000 virulence was subtle, manifested as loss of tissue chlorosis and a slight reduction in bacterial multiplication following infiltration of bacteria (Figure 1). Previous work in *Arabidopsis* and tomato with COR deficient mutants using dipping or spraying inoculation techniques revealed significant impairments in both growth and symptom development; however, when bacteria were infiltrated directly into host leaves (the method used in our study), more modest effects were observed (Mittal and Davis, 1995; Penaloza-Vazquez *et al.*, 2000). This phenotype contrasts with the severe attenuation of bacterial multiplication observed in tomato *jai1* or *Arabidopsis coi1* leaves inoculated with virulent strains of *P. syringae* (Figure 1c; Feys *et al.*, 1994; Kloek *et al.*, 2001), and suggests that virulence factors other than COR may be interacting with the host JA signaling pathway to promote disease.

Regulation of JA/wound response genes in the Pst *DC3000–tomato interaction*

In contrast to our detailed understanding of the mechanism of resistance to bacterial speck disease (Kim *et al.*, 2002; Mysore *et al.*, 2002; Tang *et al.*, 1996), relatively little is known about the molecular basis of host susceptibility to virulent strains of *P. syringae*. In an effort to broadly examine host processes that are altered during the susceptible response, we used cDNA microarray analysis to identify genes that are differentially expressed during the tomato–*Pst* DC3000 interaction. Of the approximately 500 genes represented on the microarray, we identified 50 genes that were induced by *Pst* DC3000 in a *Jai1*-dependent manner. This number clearly reflects a bias on the array of JA/wound-responsive genes (e.g. *Pl*s). Nevertheless, the results demonstrate that susceptibility of tomato to *Pst* DC3000 is correlated with massive induction of this class of genes. Experiments performed using mutants of *P. syringae* showed that increased expression of JA/wound-responsive genes depended largely on the action of COR, although the involvement of other elicitors cannot be excluded. In this context, it is noteworthy that many JA/wound response genes were expressed to higher levels in *Pst* DC3000-infected WT leaves than in *hrcC*-infected leaves. In particular, the expression of the JA-regulated *LoxA* gene encoding a 9-lipoxygenase (A. Itoh and G.A. Howe, unpublished; Beaudoin and Rothstein, 1997) was greatly reduced in leaves inoculated with the *hrcC* mutant as compared to the *Pst* DC3000-inoculated leaves (Figure 5). Thus, the type III secretion system also appears to influence the induction of some, but not all, JA/wound response genes.

Many of the *Jai1*-dependent genes that were up-regulated in *Pst* DC3000-infected plants are associated with wound-induced defense responses to herbivores. For example, we observed strong induction of genes encoding PIs (PI-I, PI-II, CDI, MCI, miraculin, and cystatin) and PPOs that are thought to impair digestive processes and nutrient acquisition in some lepidopteran pests (Ryan, 2000). These results confirm previous reports showing that *P. syringae* pv. *tomato* and COR activate the expression of PIs and PPO in tomato leaves (Palmer and Bender, 1995; Pautot *et al.*, 1991; Thipyapong and Steffens, 1997). We also observed *Jai1*- and COR-dependent expression of JA/wound response genes encoding LAP and WIC. Several researchers have suggested that these proteases may be involved in mobilizing intracellular pools of amino acids to support the selective synthesis of PIs during the wound response (Chao *et al.*, 1999; Moura *et al.*, 2001; Pautot *et al.*, 1993, 2001). *Pst* DC3000 infection also increased the expression of genes encoding biosynthetic enzymes for JA (*LoxD*, *AOS1*, *AOC*, and *OPR3*) and ethylene (*ACO2* and *ACO3*), both of which are required for wound-induced PI expression in tomato (O'Donnell *et al.*, 1996). It is therefore possible that the ethylene and JA pathways synergistically interact to promote disease. A role for ethylene in host susceptibility is supported by the observation that pathogen invasion and COR stimulate ethylene production (Greulich *et al.*, 1995; Kenyon and Turner, 1992), and that ethylene-insensitive *Arabidopsis* and tomato mutants have attenuated pathogen-induced disease symptoms (Bent *et al.*, 1992; Lund *et al.*, 1998). Induction of JA biosynthetic genes in *Pst* DC3000-infected plants supports recent work showing that these transcripts, together with JA, coordinately accumulate in wounded tomato leaves (Strassner *et al.*, 2002). It will be interesting to determine whether induction of JA biosynthetic genes in *Pst* DC3000-infected tomato leaves is correlated with increased levels of JA, which may serve to amplify the expression of JA/wound response genes. Nevertheless, the results obtained using *spr2* plants indicate that activation of JA/wound response genes by *Pst* DC3000 is mediated primarily by COR, and that host susceptibility to the pathogen does not strictly require JA biosynthesis.

The well-established role of the JA/wound response pathway in the protection of tomato against herbivores (Li *et al.*, 2002b; Ryan, 2000; Walling, 2000) raises the question of why this defense system is activated by the COR toxin. The structural and functional similarity of COR to JA, together

with our results showing that COR activates the signaling pathway leading to expression of JA/wound responsive genes, strongly suggests that this is part of the overall virulence strategy of the pathogen. One hypothesis is that COR-induced expression of antiherbivore defenses reflects a strategy of the pathogen to protect its growth habitat against destruction by herbivores. At the physiological level, COR-induced expression of PI and other wound response proteins may promote virulence by depleting metabolic resources and protein synthesis capacity that are normally used to mount a successful defense against the pathogen. This explanation is consistent with the increased virulence of COR$^-$ bacteria in MeJA-treated WT plants (Figure 2). It is also in agreement with a growing body of evidence that the JA signaling pathway for antiherbivore defense antagonizes defense responses against pathogens (Felton and Korth, 2000; Kunkel and Brooks, 2002; Stout *et al.*, 1999; Thaler *et al.*, 2002). In this context, a prediction of our results is that COR-mediated induction of the JA/wound signaling pathway will promote enhanced resistance of tomato to herbivores; this may explain the previous observation that tomato leaves infected with *P. syringae* are less susceptible to insect feeding (Bostock *et al.*, 2001; Stout *et al.*, 1999).

Of course, we cannot exclude the possibility that some JA/wound response proteins play a role in pathogen defense. For example, overexpression of PPO in transgenic tomato plants increases PPO-catalyzed phenolic oxidation and restricts the pathogenicity of *P. syringae* (Li and Steffens, 2002). As a result of the highly reactive nature of their products and their induction in response to both pathogens and wounding (Constabel *et al.*, 1995; Thipyapong and Steffens, 1997), PPOs may function in tomato as a general line of defense against both insects and pathogens.

Regulation of SA/pathogen defense-related genes

Plant defense responses to pathogen attack typically involve the concerted activation of SA-regulated genes encoding PR and other proteins related to pathogen defense (Delaney *et al.*, 1994; van Kan *et al.*, 1992; van Loon and van Strien, 1999; Mysore *et al.*, 2002). Tomato contains at least eight distinct families of *PR* genes, each of which can be divided into subgroups based on the isoelectric point and molecular weight of the corresponding proteins (van Loon and van Strien, 1999). We observed that the steady-state level of *PR* transcripts was significantly lower in *Pst* DC3000-treated WT plants that are susceptible to the pathogen than in *Pst* DC3000-treated *jai1* plants that show enhanced resistance (Figures 4 and 5). Among the *PR* genes that were repressed in this comparison were *PR-1b*, *PR-1b1*, *PR-2a*, *PR-2b*, *PR-3*, and *PR-7* (*P69B*). Because all of these genes are known to be induced by SA (Jordá *et al.*, 1999; van Kan *et al.*, 1995; Meichtry *et al.*, 1999), it can be hypothesized that SA levels in *Pst* DC3000-treated *jai1* plants are higher than SA levels in infected WT plants, as was shown to be the case for similar experiments conducted in *Arabidopsis* (Kloek *et al.*, 2001). Although additional work is needed to test this hypothesis in the tomato system, the microarray results reported herein support the general conclusion that COR promotes virulence by suppressing the expression of pathogen defense-related genes (Kloek *et al.*, 2001; Mittal and Davis, 1995). Our results broaden this concept, however, by showing that the primary target of COR is the JA signaling pathway, and that activation of JA/wound response genes may also contribute to susceptibility.

Microarray experiments designed to compare the effects of *Pst* DC3000 and the *hrcC* mutant (experiment 3) extend this knowledge by showing that the type III secretion system also appears to play a role in suppressing *PR* gene expression in tomato. Several *PR* genes (*PR1b*, *PR2b*, and *PR-7*) were in fact induced in WT plants by *Pst* DC3000, but the absolute level of induction was significantly greater in WT plants inoculated with the *hrcC* and COR$^-$ mutants (Figure 5). The most straightforward interpretation of these results is that both COR and type III effector proteins attenuate SA-dependent pathogen defense responses. The ability of both virulence systems (COR and type III effector proteins) to suppress putative host defense responses requires *Jai1*, and thus is consistent with the general concept of antagonism between the JA and SA signaling pathways (Kunkel and Brooks, 2002; Thaler *et al.*, 2002). In the case of the tomato–*Pst* DC3000 interaction, it can be proposed that one or more components of the JA signaling pathway are specifically targeted by pathogen-derived molecules.

At this point, we cannot rule out the possibility that the effect of the type III secretion system on gene expression may be indirect. The *hrcC* mutant strain used in this study contains a polar mutation that inactivates *hrcC*, *hrpT*, and *hrpV* and is unable to produce a functional type III secretion system. Penaloza-Vazquez *et al.* (2000) observed that the *hrcC* mutant produced significantly higher amounts of COR than the WT strain *in vitro*. The overproduction of COR in the *hrcC* mutant was because of a loss of function of the negative regulator *hrpV* (Penaloza-Vazquez *et al.*, 2000). However, the *hrpS* mutation, which opposes the effect of the *hrpV* mutation, also increased COR production *in vitro* (Penaloza-Vazquez *et al.*, 2000), suggesting a more complicated interaction between the type III secretion system and COR biosynthesis. Despite the potential overproduction of COR by the *hrcC* mutant, we show here that many JA/wound response genes were expressed at 2- to 13-fold lower levels in *hrcC*-infected leaves than in *Pst* DC3000-infected WT leaves (Table S2), suggesting that either the *hrcC* mutant-inoculated leaves contained a lower level of COR than the DC3000-inoculated leaves and/or, as we favor, that the type III secretion system also contributes to the

regulation of these genes. In support of the latter possibility, it is important to note that despite the overall qualitatively similar patterns of gene regulation by COR and the type III secretion system, the degree of regulation of each gene was often different. For example, expression of some genes (e.g. *PR1*) was similar in WT leaves inoculated with either the COR$^-$ or the *hrcC* mutant, whereas the expression of many others (*PR2b*, *SBT4a*, *PI-II*, *LapA*, and *OPR3*) was significantly different between the two treatments (Figure 5, lanes WT/COR$^-$ and WT/*hrcC*). This result suggests overlapping, but not identical, effects of COR and the type III secretion system.

Within the group of 37 genes that were repressed in *Pst* DC3000-infected WT plants (experiment 1) were five genes (*P69B/PR-7*; *P69F*; *LeSBT3*; *LeSBT4A*; *LeSBT4B*) that encode members of a large family of subtilisin-like serine proteases (Meichtry *et al.*, 1999). Some members (e.g. *P69B/PR-7*) of this family are expressed in tomato leaves in response to *Pst* DC3000 and SA, and thus it has been suggested that they serve a function in pathogen defense (Jordá *et al.*, 1999; Tornero *et al.*, 1996). Like many PR proteins, subtilases are secreted into the apoplastic space in which *Pst* DC3000 grows (Jordá *et al.*, 1999; Meichtry *et al.*, 1999). It is conceivable that this and perhaps other extracellular proteases comprise a host defense system that attacks proteinaceous components, such as the Hrp secretion apparatus, that are essential for pathogen virulence. This hypothesis would be consistent with the notion that *Pst* DC3000 promotes virulence by suppressing the expression of these extracellular proteins.

In summary, our results suggest a general model for the role of Jai1, the Hrp type III secretion system, and COR in the tomato–*Pst* DC3000 interaction (Figure 6). The model indicates that *Pst* DC3000 uses both the phytotoxin COR and Hrp-dependent effector proteins as virulence factors. Although very little is known about the macromolecular target(s) of COR within the host cell (Zhao *et al.*, 2001), our results demonstrate that the toxin activates the JA/wound response pathway in a manner that is dependent on *Jai1* but independent of endogenous JA. This conclusion implies that COR biosynthesis has evolved in a manner that has selected for the capacity of the molecule to function as an agonist of the JA receptor. Effector proteins, on the other hand, are translocated into the host cell via the type III secretion system. This virulence system may contribute to the increased expression of some JA/wound response genes such as *LoxA*. Induction of JA/wound response genes alone is likely not sufficient to increase the susceptibility of tomato to *Pst* DC3000 infection or the development of disease symptoms. Rather, virulence of the pathogen also depends on the ability of COR and likely also the Hrp secretion system to repress the expression of pathogen defenses (e.g. *PR* genes). The role of *Jai1* in enhancing susceptibility and promoting genome-wide changes in gene expression shows that multiple virulence systems of *Pst* DC3000 appear to target the host JA signaling pathway. This working model provides a framework for future studies to determine the virulence function of the type III secretion system and COR in susceptible tomato plants.

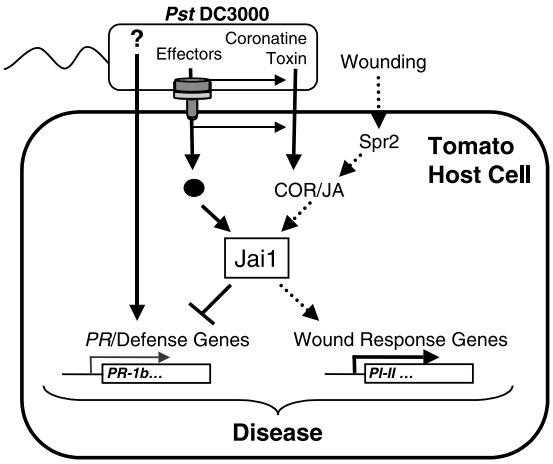

Figure 6. Proposed model for the role of the host JA signaling pathway, COR, and the type III secretion system in the tomato–*Pst* DC3000 interaction. *Pst* DC3000 produces the phytotoxin COR, which mimics the action of JA. COR works through Jai1 to coordinately repress (hammerhead) the expression of pathogenesis-related defense genes (e.g. *PR-1b*) and induce (arrowhead) the expression of JA/wound response genes (e.g. *PI-II*). Wound-induced defense responses to herbivores require Spr2-dependent biosynthesis of JA, which also works through Jai1 to activate expression of PI-II and other JA/wound response genes. The virulence function of some bacterial effector proteins (filled circle), which are delivered to the plant cell through the type III secretion apparatus, may also require Jai1 to suppress *PR* gene expression and activate JA/wound response genes. In addition, components of the type III effector system may have an indirect effect on COR production or action (horizontal arrows from type III effector protein to COR). Unknown bacterial factors (**?**) induce *PR* expression independent of Jai1 and COR.

Experimental procedures

Plant material and treatments

Tomato (*L. esculentum* cv. Castlemart) was used as the WT cultivar for all experiments. Seed for the sterile *jai1-1* mutant was obtained from a segregating population as described by Li *et al.* (2001). Seed for all other tomato lines was obtained as described by Li *et al.* (2002a). Plants were grown and maintained in Jiffy peat pots (Hummert International, St Louis, USA) in a growth chamber under 16 h light (200 μE m^{-2} sec^{-1}) at 25°C and 8 h dark at 20°C. Application of purified COR to tomato plants and the source of COR were described by Zhao *et al.* (2001), with the exception that tomato plants were 18 days old at the time of treatment. For experiments involving MeJA treatment, plants were exposed to 2.5 μl of vaporous MeJA (Bedoukian Research, DanBury, CT, USA) in an enclosed 8L Lucite box as previously described by Li and Howe (2001).

Quantification of PI-II protein levels was performed using a radial immuno-diffusion assay (Ryan, 1967). Briefly, a 5-μl aliquot of expressed leaf juice was placed into a well (0.5 mm diameter) of an agar plate (2% (w/v) Noble agar, 0.9% (w/v) NaCl, 20 mM Tris,

pH 8.5) containing 1% (v/v) polyclonal antiserum obtained from a goat that was immunized with tomato PI-II. One day later, the diameter of the immunoprecipitate ring that results from the antibody–antigen interaction was measured and used to calculate the amount of PI-II per milliliter of leaf juice. Based on a standard curve obtained using purified PI-II, the detection limit of the assay was estimated to be about 5 μg PI-II per milliliter of leaf juice.

Bacterial strains, inoculum preparation, and plant inoculation

Pst DC3000 and the mutant strains *Pst* DC3000 *hrcC* (formerly the *hrpH* mutant; Yuan and He, 1996) and *Pst* DC3118 COR$^-$ (Ma *et al.*, 1991; Moore *et al.*, 1989) were grown in a low-salt liquid Luria Bertani (LB) medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract and 5 g l^{-1} NaCl, pH 7.0) at 28°C. Antibiotics were added to LB at the following concentrations (μg ml^{-1}): chloramphenicol, 34; kanamycin, 50; and rifampicin, 100. Bacterial suspensions were prepared as previously described and were adjusted to 1×10^5 colony-forming units (CFU) ml^{-1} ($OD_{600} = 0.0002$) in sterile distilled water containing 0.004% of the surfactant Silwet L-77 (Osi Specialties, Friendship, WV, USA; Katagiri *et al.*, 2002). Three-week-old tomato plants with three to four true leaves were used for inoculation. Bacterial suspensions were vacuum-infiltrated into the leaves as previously described by Katagiri *et al.* (2002). We monitored bacterial growth within leaf tissue by grinding six leaf disks (0.6 cm^2) per sample and plating dilutions of the ground material on LB media with the appropriate antibiotics (Katagiri *et al.*, 2002). Three replicate samples were taken for each treatment over a 4-day period. Each *in planta* growth experiment was independently conducted at least three times.

cDNA microarray analysis

The cDNA microarray was constructed using an existing collection of tomato cDNA clones (Strassner *et al.*, 2002) plus additional tomato expressed sequence tag (EST) clones obtained from the Clemson University Genomics Institute (Clemson, SC, USA). The identity of each clone was verified by single pass DNA sequencing at the MSU Genomics Technology Support Facility. cDNA inserts were amplified by polymerase chain reaction (PCR) in a 100-μl reaction volume using pBluescript SK(–) primers T3 and T7. PCR products were precipitated with ethanol and re-suspended in 25 μl of 3× SSC (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate). One microliter of PCR product was analyzed on a 1% agarose gel to verify the effectiveness of the PCR step and the presence of a single PCR product. These DNA samples were printed onto amine-coated glass slides (Telechem, Sunnyvale, CA, USA) using an Omnigridder robot (Gene Machines, San Carlos, CA, USA) equipped with four ArrayIt chipmaker pins (Telechem). Each DNA sample was printed in triplicate on each slide, and the array was printed in a 12 × 14 format containing 12 subarrays (four subarrays printed three times per slide). At the bottom of each subarray were included cDNAs representing eight negative control genes: green fluorescent protein (AF078810), neomycin phosphotransferase II (V00618), β-glucuronidase (*uidA*), luciferase (X65316), human globin (NM_000518), *Bacillus thuringiensis* cry1AC (U89872), phosphinothricin acetyltransferase (X17220), and hygromycin B phosphotransferase (K01193); and 10 genes used as spiking controls for data normalization: B-cell receptor protein (AF126021), myosin heavy chain (X13988), myosin light chain2 (M21812), insulin-like growth factor (X07868), FLJ10917 fis (AK001779), HSPC170 (AF161469), tyrosine phosphatase, β2 microglobin (NM_004048), phosphoglycerate kinase (NM_000291), and G10 homolog (U11861). A complete list of cDNAs spotted on the microarray slide is given in Table S1. Blocking of printed slides was performed using the recommended protocol from Telechem.

Total RNA (100 μg) was isolated from tomato leaves as previously described by Howe *et al.* (1996), and further purified according to the RNAeasy kit cleanup protocol (Qiagen, Valencia, CA, USA). Labeled probes for microarray analysis were generated by direct incorporation of Cy3- or Cy5-conjugated deoxy UTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) during reverse transcription. Tomato RNA was combined with 2 μl of the spiking control mixture (containing approximately equal amounts of mRNA for each of the 10 spiking control genes listed above) and 3 μl (6 μg) oligo-$dT_{23}V$ (Invitrogen, Carlsbad, CA. USA) in a total volume of 16.5 μl and incubated at 70°C for 10 min. The mixture was chilled on ice for 5 min, followed by the addition of 2 μl of FluoroLink Cy3- or Cy5-dUTP, 3 μl of 0.1 M DTT, 6 μl of 5× first-strand buffer, 0.5 μl of 50× dNTPs mix (25 mM dATP, dCTP, dGTP, and 9 mM dTTP), and 2 μl of Superscript II reverse transcriptase (Invitrogen). Reactions were incubated at 42°C for 120 min. RNA was hydrolyzed by adding 0.5 μl of RNase A (10 mg ml^{-1}) and 0.25 μl of RNase H (Invitrogen) at 37°C for 30 min. The Cy3- and Cy5-labeled cDNA probes were purified separately on a Microcon YM-30 filter (Millipore, Bedford, MA, USA) and then further purified using a PCR purification kit (Qiagen). The Cy3- and Cy5-labeled probes were then combined, concentrated, and re-suspended in 4 μl of 10 mM EDTA, pH 8.0. The labeled probes were denatured at 95°C for 10 min; 30 μl SlideHyb buffer 1 (Ambion, Austin, TX, USA) was added to the denatured probes. The mixture was hybridized to slides at 54°C in a slide hybridization chamber (Ambion) for 16–20 h. After hybridization, slides were washed two times in 2× SSC, 0.5% SDS for 5 min at 65°C, in 0.1× SSC, 0.2% SDS for 5 min, and in 0.1× SSC for 5 min at room temperature. Washed slides were dried by centrifugation at 300 ***g*** for 5 min and then scanned with an Affymetrix 428 Array Scanner.

Spot intensities were quantified using AXON GENEPIX PRO 3 image analysis software (Axon, Foster City, CA, USA). Ratio data were extracted and normalized to the set of spiking controls as follows. A normalization factor (NF) was calculated as the sum of the mean intensity of the Cy5 channel (F635mean) divided by the sum of the mean intensity of the Cy3 channel (F532mean) of all spiking control spots in which the proportion of pixels greater than one SD above the average background intensity was greater than 0.65. This procedure thus adjusted the ratio of the majority of spiking controls to as close to 1.0 as possible. The ratio of individual test spots was then calculated as F635mean × NF/F532mean. NF values typically ranged between 0.9 and 1.05. Cluster analysis was performed using GENESPRING™ 5.0.3 software (Silicon Genetics, Redwood City, CA, USA). Cluster analysis of the three experimental comparisons was conducted by means of GENESPRING™ Experiment Tree software to measure similarity using standard correlation with the default settings. We constructed the GENESPRING™ Gene Tree by measuring similarity using the distance default settings.

The reproducibility of differential gene expression was assessed as follows. For each of the three experimental comparisons described in Figure 3, three biological replicate RNA samples were used for hybridization. Thus, a total of nine hybridization experiments were performed (Table S1). In one of the three replicates, labeling of the two RNA samples with Cy5 or Cy3 deoxy UTP was reversed (relative to the other two experiments) to avoid potential dye-related differences in labeling efficiency. When analyzing the data from each hybridization experiment, the ratios of the three

replicate spots for each clone on the array were averaged. Only those genes having an expression ratio >2.0 or <0.5 in at least two biological replicates were counted as being differentially regulated. For these 156 genes, the final expression ratio was calculated as the average ratio from the three biological replicates (Table S1). Additional information concerning microarray experiments, including the raw data for all hybridization experiments, is available at http://www.prl.msu.edu/howe.shtml

RNA isolation and blot analysis

Total RNA was isolated from tomato leaves as previously described by Howe *et al.* (1996). The RNA was evaluated on denaturing agarose gels, and the RNA concentration was determined by absorbance at 260 nm. RNA blot hybridizations were performed as described by Li *et al.* (2002a), with an *eIF4A* cDNA probe as a loading control.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ1895/TPJ1895sm.htm

Table S1 A complete list of cDNA microarray clones with average expression ratios (±SE) for all nine hybridization experiments

Table S2 List of 87 genes that were differentially regulated in at least two experimental comparisons

Table S3 List of 69 genes that were differentially regulated in only one of the three experimental comparisons

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