TECHNICAL ADVANCE

Pathogenicity of *Pseudomonas syringae* pv. *tomato* on Tomato Seedlings: Phenotypic and Gene Expression Analyses of the Virulence Function of Coronatine

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Bacterial speck disease, which is caused by *Pseudomonas syringae* pv. *tomato*, is an economically important disease on tomato. In the present study, we show that *P. syringae* pv. *tomato* DC3000 is a pathogen of tomato seedlings, an aspect of pathogen biology that has not been previously investigated. This resulted in the development of a virulence assay on tomato seedlings that has several advantages over labor-intensive foliar assays, including a shorter growth and incubation period, ease of inoculation and handling, and rapid generation of larger sample sizes per experiment. The utility of this assay was investigated by exploring the virulence function of coronatine (COR) on tomato seedlings. Using the COR⁻ mutant DB29 and a MAPMAN display of transcript data from TOM1 microarrays, COR-dependent expression of genes involved in secondary metabolism, polyamine biosynthesis, reactive oxygen species homeostasis, and the novel transcription factor *SINAC2* were identified. Furthermore, during pathogenesis, genes involved in photosynthetic light reactions and the Calvin-Benson cycle were strongly repressed by COR. In conclusion, we show that *P. syringae* pv. *tomato* infects tomato seedlings and that COR is required for virulence in seedlings. The seedling assay can be used in high-throughput screens for the identification of molecular targets for COR and for the identification of genes involved in pathogenesis.

Additional keywords: chlorosis, jasmonic acid, phytohormone, senescence, *Solanum lycopersicum*.

Bacterial speck disease, which is caused by *Pseudomonas syringae* pv. *tomato*, is an economically important disease of tomato. *P. syringae* pv. *tomato* causes necrotic lesions on the

leaves, stems, and fruit of tomato plants (Goode and Sasser 1980). Fruit lesions are slightly raised and vary in size from tiny flecks to visible lesions approximately 3 mm in diameter (Goode and Sasser 1980). Fruit infection can lead to infestation of tomato seed, and *P. syringae* pv. *tomato* can survive on seeds, especially inside cavities present on the seed surface (Devash et al. 1980). Seedlings from infested seeds either develop visible disease symptoms or remain symptomless; in the latter case, asymptomatic plants grown under high relative humidity contain massive populations of the pathogen, thus serving as a reservoir for future infections (McCarter et al. 1983). Although several reports have suggested that *P. syringae* pv. *tomato* is seed-borne (Bashan et al. 1982) and can spread through tomato transplant fields (Gitaitis 1990), these aspects of disease etiology and pathogen biology are not well understood.

Bacterial speck disease of tomato is largely regarded as a foliar disease (Preston 2000), and studies on the *P. syringae* pv. *tomato*–tomato interaction have primarily been conducted in the leaves of 3- to 4-week-old tomato plants. Foliar assays generally require high relative humidity to enable pathogen entry and development of typical disease symptoms following dip or spray inoculation (Lund et al. 1998; Peñaloza-Vázquez et al. 2000; Uppalapati et al. 2007; Zhao et al. 2003). Unfortunately, these assays require extensive growth-chamber or greenhouse space and are labor intensive. Thus, the development of reliable seedling assay for *P. syringae* pv. *tomato*–tomato interactions could reduce both the time and space needed for virulence assays.

The foliar lesions induced by *P. syringae* pv. *tomato* are generally surrounded by a yellow chlorotic halo due to production of the phytotoxin coronatine (COR) (Bender et al. 1987, 1999). The COR molecule has an unusual structure and consists of two components: coronafacic acid (CFA), a polyketide, and coronamic acid (CMA), an ethylcyclopropyl amino acid derived from alloisoleucine (Couch et al. 2004; Ichihara et al. 1977; Parry et al. 1994; Vaillancourt et al. 2005). Both the CFA and CMA components of COR were shown to contribute to the development of wild-type symptoms in tomato foliage (Uppalapati et al. 2007). COR plays several important roles during *P. syringae* pv. *tomato* pathogenesis. Recent studies in *Arabidopsis* demonstrate that COR facilitates the entry of *P. syringae* pv. *tomato* into foliar tissue by suppressing the clo-

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sure of stomata, a basal defense response induced upon microbial attack (Melotto et al. 2006). COR is also required for the growth and persistence of *P. syringae* pv. *tomato* in the foliage of tomato, *Arabidopsis*, and *Brassica* spp. (Bender et al. 1987; Brooks et al. 2004; Elizabeth and Bender 2007; Mittal and Davis 1995; Penaloza-Vazquez et al. 2000; Uppalapati et al. 2007).

P. syringae pv. *tomato* DC3000 is a model strain for investigating plant-microbe interactions due to its genetic tractability and pathogenicity on tomato, *Arabidopsis*, and *Brassica* spp. (Cuppels 1986; Elizabeth and Bender 2007; Whalen et al. 1991). Genetic studies using defined CFA⁻ and CMA⁻ mutants of *P. syringae* pv. *tomato* DC3000 indicated that the intact COR molecule must be produced for maximal virulence on *Arabidopsis*, tomato, and *Brassica* spp. (Brooks et al. 2004, 2005; Elizabeth and Bender 2007; Uppalapati et al. 2007). However, it is not known whether *P. syringae* pv. *tomato* DC3000 infects tomato seedlings and if COR functions to promote virulence in seedling tissue.

Using mutants defective in COR biosynthesis, it has been demonstrated that COR activates jasmonic acid (JA) biosynthesis and JA-mediated signaling and also functions to suppress salicylic acid (SA)-mediated defense responses in tomato (Uppalapati et al. 2005, 2007; Zhao et al. 2003). However, unlike *Arabidopsis*, COR has additional roles in virulence that are SA independent (Uppalapati et al. 2007). Furthermore, purified COR elicits visible chlorosis on tomato leaves (Palmer and Bender 1996; Uppalapati et al. 2005) but not on *Arabidopsis* (Bent et al. 1992). These results suggest that COR may function differently in tomato and *Arabidopsis*.

In the present study, we describe a simple, inexpensive seedling-based assay that expedites the analysis of *P. syringae* pv. *tomato*–tomato interactions. Our results indicate that *P. syringae* pv. *tomato* DC3000 is a pathogen of tomato seedlings, an aspect of *P. syringae* pv. *tomato* DC3000 biology that has not been previously investigated. We also demonstrate the use of a tomato seedling assay to study *P. syringae* pv. *tomato* virulence and to identify host signaling pathways targeted by COR. Our results show that COR functions as an important virulence factor in tomato seedling disease development. Using the well-defined COR-defective mutant DB29 and a tomato microarray (TOM1), we identified several genes that may function in bacterial speck of tomato in a COR-dependent manner.

RESULTS AND DISCUSSION

A seedling assay to study

P. syringae pv. *tomato* DC3000–tomato interactions.

To obtain seedlings for the experiments, tomato (cv. Glamour) seeds were surface-sterilized and germinated in the dark. At 4 days postgermination, seedlings containing 2- to 3-cm-long hypocotyls were selected and the first pair of cotyledons was used for bacterial inoculation (Fig. 1A). Seedlings were inoculated by flooding MS (Murashige and Skoog) agar plates with a bacterial suspension until the seedlings were completely submerged in inoculum. The seedlings were exposed to the bacterial suspension for 2 to 3 min with gentle mixing, a process that did not dislodge or wound the seedlings. The bacterial suspension was then discarded, and inoculated tomato seedlings were incubated in a growth room with a 12-h photoperiod.

To study the effect of inoculum concentration on symptom development, tomato seedlings were immersed in three different concentrations of *P. syringae* pv. *tomato* DC3000 (OD₆₀₀ = 0.01, 0.1, or 1.0). Seedlings exposed to the highest concentration of bacteria (OD₆₀₀ = 1.0; 2×10^8 to 5×10^8 CFU/ml) exhibited severe necrotic symptoms within 24 h (data not shown) and were dead by 3 days postinoculation (dpi). Thus, an OD₆₀₀ = 1.0 was deemed lethal for studying disease progression. However, seedlings inoculated with bacteria at an OD₆₀₀ = 0.1 (2×10^7 to 5×10^7 CFU/ml) exhibited discrete necrotic lesions on the hypocotyl and cotyledons at 3 and 5 dpi, respectively (Fig. 1A). Closer inspection of *P. syringae* pv. *tomato* DC3000-inoculated seedlings indicated that the cotyledons showed extensive chlorosis (Fig. 2A), which is a hallmark of bacterial speck disease in foliar tissue; consequently, bacteria concentrated to an OD₆₀₀ = 0.1 was used in all subsequent experiments.

In addition to symptom development, the virulence of *P. syringae* pv. *tomato* DC3000 is generally assessed by measuring bacterial growth in planta (Glazebrook et al. 1997; Tornero and Dangl 2001). In inoculated tomato seedlings, *P. syringae* pv. *tomato* DC3000 multiplied approximately 1,000-fold within the first 4 dpi, with the bacterial population remaining relatively constant for the rest of the assay period (Fig. 2G). These results are similar to the bacterial growth curves observed in tomato and *Arabidopsis* leaves of mature plants (Katagiri et al. 2002; Uppalapati et al. 2007; Zhao et al. 2003).

As described above, the inoculation procedure involved immersing the entire seedling in a bacterial suspension and then

Fig. 1. A seedling assay for the analysis of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) interactions with tomato. **A**, Disease phenotype of seedlings immersed in a bacterial suspension of *P. syringae* pv. *tomato* DC3000. Photograph was taken 7 days postinoculation (dpi). Mock-inoculated seedlings were immersed in sterile distilled H₂O containing 0.025% Silwet. **B**, Anatomical regions of tomato seedlings. **C**, Spatial distribution of *P. syringae* pv. *tomato* DC3000 in tomato seedlings inoculated by immersion in a bacterial suspension (optical density at 600 nm = 0.1). The population of *P. syringae* pv. *tomato* DC3000 in tomato seedling cotyledons, hypocotyls, nodes, and roots was sampled by dissecting seedling leaves, pooling samples, and measuring the population at 3 dpi. Vertical bars indicate the standard error for three independent experiments.

evaluating virulence. To better understand the spatial distribution of *P. syringae* pv. *tomato* DC3000 in inoculated tomato seedlings, the internal population of *P. syringae* pv. *tomato* DC3000 was measured in cotyledons, hypocotyls, nodes, and roots (Fig. 1B and C). *P. syringae* pv. *tomato* DC3000 colonized the cotyledons, hypocotyls, and the nodal regions of the seedlings at high inoculum densities ($>10^8$ CFU per gram of

tissue) (Fig. 1C). However, the population of bacteria recovered from roots was significantly lower (approximately 10^6 CFU per gram of tissue), which is consistent with the absence of obvious disease symptoms in tomato seedling roots (Fig. 1A). Thus, *P. syringae* pv. *tomato* DC3000 preferentially colonizes the aerial portions of tomato seedlings, which is consistent with its identity as a foliar pathogen. However, it is important

Fig. 2. Coronatine (COR) functions as a virulence factor in the pathogenesis of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) on tomato seedlings. **A** and **B**, Disease phenotype of seedlings immersed in a suspension of *P. syringae* pv. *tomato* DC3000, **C** and **D**, the COR⁻ mutant DB29, or **E** and **F**, sterile distilled water. **A**, **C**, and **E**, Intact inoculated seedlings on Murashige and Skoog agar plates. **B**, **D**, and **F**, Disease phenotype on individual cotyledons. Arrows in panel **D** show the location of small pinpoint necrotic lesions in DB29-inoculated seedlings; **E**, scale bar = 1 cm. Photographs were taken 7 days postinoculation. **G**, Population of *P. syringae* pv. *tomato* DC3000 and DB29 in tomato seedlings and **H**, seedling leaves (cotyledons). Bacterial populations were obtained by surface-sterilizing inoculated seedlings or cotyledons and then, plating dilutions to selective media 0, 1, 3, 5, and 7 (G) or 0, 2, 4, and 7 (H) dpi. Vertical bars indicate the standard errors for five independent experiments.

to note that *P. syringae* pv. *tomato* DC3000 infected roots of *Arabidopsis* seedlings in liquid cultures (Bais et al. 2005).

To study the utility of tomato seedling assays to effectively identify the genes involved in pathogenesis, we used several well-characterized virulence mutants. Our results using vacuum infiltration of tomato seedlings with different *P. syringae* pv. *tomato* DC3000 mutants agreed well with previous results obtained by foliar inoculation of tomato plants (Supplementary Table S4; Supplementary Results; Wangdi 2007). In the current study, the seedling assay was investigated in detail by exploring the virulence function of COR on tomato seedlings.

COR functions as a virulence factor in tomato seedlings.

Previous studies using leaves of 3- to 4-week-old tomato plants indicated that the phytotoxin COR plays an important role in the pathogenicity of *P. syringae* pv. *tomato* DC3000 (Bender et al. 1987; Mittal and Davis 1995; Peñaloza-Vázquez et al. 2000; Uppalapati et al. 2007; Zhao et al. 2003). To further evaluate the utility of the tomato seedling assay and to assess whether COR functions in the virulence of *P. syringae* pv. *tomato* DC3000 on tomato seedlings, tomato seedlings were inoculated with *P. syringae* pv. *tomato* DC3000 and the COR⁻ mutant DB29 (Brooks et al. 2004). *P. syringae* pv. *tomato* DC3000 caused severe necrosis on hypocotyls and cotyledons (Figs. 1A and 2A), and diffuse chlorosis developed at later stages (Fig. 2B). However, DB29-inoculated seedlings appeared relatively healthy (Fig. 2C); when examined closely, tiny necrotic lesions were observed on some of the cotyledons (Fig. 2D, arrows). Chlorosis was not observed on DB29-inoculated seedlings, which is consistent with the absence of COR production. The population of *P. syringae* pv. *tomato* DC3000 and DB29 in inoculated tomato seedlings was compared over a 7-day period (Fig. 2G). The population of DB29 was significantly lower than *P. syringae* pv. *tomato* DC3000 at 2 dpi and was approximately 1 to 1.5 logs lower than *P. syringae* pv. *tomato* DC3000 at 5 to 7 dpi (Fig. 2G). Interestingly, the growth of DB29 was more impaired in seedling leaves (cotyledons) as compared with the population in whole seedlings (Fig. 2H). It is tempting to speculate that COR is required for successful colonization in tomato cotyledons. Thus COR is important for the multiplication, persistence, and disease symptom development of *P. syringae* pv. *tomato* DC3000 in tomato seedlings, which has not been previously reported.

Use of the tomato seedling assay to identify host signaling pathways targeted by COR.

Recent studies suggest that COR has multiple virulence functions. In *Arabidopsis* for example, it has been demonstrated that COR facilitates the entry of *P. syringae* pv. *tomato* into foliar tissue by suppressing the closure of stomata (Melotto et al. 2006). COR was also shown to contribute to *P. syringae* pv. *tomato* DC3000 pathogenicity in *Arabidopsis* by stimulating auxin levels (Kunkel et al. 2004). However, unlike *Arabidopsis*, COR has additional roles in virulence that are SA-independent (Uppalapati et al. 2007). Purified COR elicits visible chlorosis on tomato leaves (Palmer and Bender 1996; Uppalapati et al. 2005), which is not observed in *Arabidopsis*. Interestingly, Thilmony and associates (2006) reported that in *Arabidopsis*, type III secretion system (T3SS) effectors but not COR were involved in the suppression of the photosynthetic light reaction and genes in the Calvin-Benson cycle. Thus COR may function differently in tomato and *Arabidopsis*. Using a small set (approximately 500) of unique tomato genes and a genetically undefined COR⁻ mutant, Zhao and associates (2003) provided evidence for the COR-dependent suppression of SA-mediated defense responses during pathogenesis in tomato. Furthermore, using purified COR on tomato leaves, we have shown that COR

targets multiple phytohormone pathways and also targets the JA pathway in tomato (Uppalapati et al. 2005). It is important to note that our previous study (Uppalapati et al. 2005) was conducted in the absence of the pathogen; thus, we could have missed several processes that are related to pathogenesis. For example, suppression of the SA pathway by COR was not observed when using purified COR (Uppalapati et al. 2005). However, a comprehensive list of tomato signaling pathways targeted by *P. syringae* pv. *tomato* DC3000 in a COR-dependent manner is not available, and the precise mode of action of COR during pathogenesis remains unclear.

To identify novel signaling pathways targeted by COR, we compared the expression profiles of tomato seedlings inoculated with *P. syringae* pv. *tomato* DC3000 and DB29 24 h postinoculation (hpi) using TOM1 (Alba et al. 2004). The TOM1 cDNA microarray contains 12,899 expressed sequence tag (EST) clones representing 8,500 independent tomato genes (Alba et al. 2004). When a twofold cutoff ($P < 0.05$) relative to the gene expression in DB29-inoculated tissues was used, 774 and 424 genes belonging to different functional groups were identified as induced or repressed, respectively, in a COR-dependent manner. A complete list of COR-regulated genes is presented as supplementary data (Tables S1 to S3). Here, we discuss some of the genes that were previously shown to be regulated in foliar assays and other COR-regulated genes that have not been previously identified in tomato or *Arabidopsis*.

In the current study, we used a recently developed MAPMAN ontology conversion of the TOM1 microarray (Thimm et al. 2004; Urbanczyk-Wochniak et al. 2006) to obtain a global view of COR-regulated genes in various metabolic processes (Fig. 3; Supplementary Figs. S2 and S3). It is noteworthy that several genes involved in amino-acid metabolism were differentially expressed in response to COR (Fig. 3A, amino acids). Interestingly, genes involved in the catabolism of the serine-glycine group of amino acids were down-regulated, whereas several genes involved in biosynthesis of the same group of amino acids were strongly induced by COR (Fig. 3A, amino acids). Similarly, several genes involved in the biosynthesis of N₂-transport amino acids belonging to the glutamine-asparagine family were induced by COR (Fig. 3A, amino acids). Bashan and associates (1986) demonstrated that *P. syringae* pv. *tomato* can utilize free amino acids as a nitrogen source and can grow well in serine, glycine, threonine, or glutamine. Furthermore, during pathogenesis, tomato leaves accumulate high levels of asparagine and glutamine, and the application of asparagine or glutamine to tomato leaves increased disease severity (Bashan et al. 1986). Interestingly, our microarray results provided a role for COR in regulating amino-acid metabolism during pathogenesis. When considered in the context of the physiological studies conducted by Bashan and associates (1986), we propose that COR-induced amino-acid changes help the pathogen multiply to higher levels and contribute to symptom development in tomato.

A MAPMAN conversion of TOM1 data also allowed a direct comparison of results obtained in tomato with COR responses reported in *Arabidopsis* (Thilmony et al. 2006). Unlike *Arabidopsis*, many genes involved in the photosynthetic light reaction, Calvin-Benson cycle, and tetrapyrrole synthesis were strongly repressed by COR in tomato (Fig. 3A; Supplementary Table S2). In tomato, several genes involved in reactive oxygen species (ROS) homeostasis were strongly induced by COR (Fig. 3A; genes numbered 1 through 7). One of the hallmarks of bacterial speck is the formation of necrotic lesions surrounded by prominent chlorotic halos (Bender et al. 1987; Gnanamanickam et al. 1982). However, the significance of COR-induced chlorosis in disease development is not known. Based on the differential expression of Calvin-Benson cycle genes and ROS, it is

Fig. 3. MAPMAN illustration of TOM1 microarray data showing the genes involved in different metabolic processes targeted by coronatine (COR). **A**, Global transcriptional changes in tomato seedlings inoculated by flooding with *Pseudomonas syringae* pv. *tomato* DC3000 and COR⁻ mutant DB29 were compared in order to identify COR-regulated genes. **B**, A MAPMAN illustration showing stress-related genes. Genes that are numbered include: 1 = thioredoxin (TOM1 ID: 1.1.9.2), 2 = tetrathiocoredoxin (TOM1 ID: 3.1.10.4), 3 = dehydroascorbate reductase (TOM1 ID: 4.1.10.19), 4 = glutathione reductase (TOM1 ID: 6.4.6.14), 5 = an unknown protein (TOM1 ID: 2.3.13.16), 6 = glutathione peroxidase (TOM1 ID: 5.4.11.13), 7 = L-ascorbate oxidase (TOM1 ID: 4.3.15.21), 8 = pathogenesis-related (PR)-1 (TOM1 ID: 2.3.10.15); 9 = PR-23 (TOM1 ID: 7.2.20.16); 10 = a PR-5-like protein (TOM1 ID: 6.3.1.19); 11 = a PR protein (TOM1 ID: 2.2.14.17), 12 = a disease-resistance protein (TOM1 ID: 3.2.17.12), 13 = a systemin precursor (TOM1 ID: 5.4.9.7); and 14 = a hypothetical protein (TOM1 ID: 2.3.11.13). Red indicates upregulation, and green downregulation.

tempting to speculate that COR-induced effects on photosynthesis and the Calvin cycle may lead to ROS generation and promote cell death in a COR-dependent manner. In support of this, preliminary results suggest that COR-induced effects on photosynthetic machinery result in the accumulation of ROS in a light-dependent manner, which leads to necrosis in bacterial speck disease of tomato (data not shown).

COR targets multiple phytohormone pathways in tomato seedlings.

To gain a better understanding of tomato seedling responses to *P. syringae* pv. *tomato*, the effects of COR on phytohormone pathways was analyzed. In general, COR targeted multiple phytohormone pathways (JA, ethylene [ET], auxin) in tomato seedlings, which is similar to previous reports (Thilmony et al. 2006; Uppalapati et al. 2005; Zhao et al. 2003). Our transcriptome analysis identified both known and novel genes associated with COR. In a COR-dependent manner, *P. syringae* pv. *tomato* DC3000 induced genes involved in JA biosynthesis, including lipoxygenase (*LOXD*), allene oxide cyclase (*AOC*), and 12-oxo-phytodienoate reductase 3 (*OPR3*). Furthermore, many JA and wound-inducible genes, such as proteinase inhibitor (*PI*) and

polyphenol oxidase (*PPO*) were also up-regulated in a COR-dependent manner during pathogenesis in tomato seedlings. Northern blot analyses further confirmed that the expression of JA pathway genes (*LOXD*, *OPR3* and *PI-II*) were induced in seedlings inoculated with *P. syringae* pv. *tomato* DC3000 within 12 hpi but not in seedlings inoculated with DB29 (Fig. 4A).

Threonine deaminase (*TD*), a gene involved in the biosynthesis of isoleucine (Ile), was strongly induced by *P. syringae* pv. *tomato* DC3000 in a COR-dependent manner (Fig. 4A). A recent study has shown that *TD*-induced Ile forms conjugates with JA, resulting in JA-Ile (Kang et al. 2006). Previously, a *jar1* mutant of *Arabidopsis* (jasmonate resistance 1, which was unresponsive to JA-induced root inhibition) showed altered physiological responses to purified COR but the same level of susceptibility to *P. syringae* pv. *tomato* DC3000 as wild-type plants (Laurie-Berry et al. 2006). However, COR is shown to mimic JA-Ile in bioassays (Krumm et al. 1995). It remains unclear whether COR-induced *TD* was responsible for the JA-Ile-like activity.

Recently, *AtJAZ1* and *SlJAZ1* (Thines et al. 2007) and *AtJAZ3* (Chini et al. 2007), which belong to the JAZ protein family, were identified as repressors of jasmonate-responsive tran-

Fig. 4. Expression profiles of jasmonic acid (JA)-inducible and salicylic acid (SA)-dependent genes in tomato seedlings in response to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), COR⁻ mutant DB29, or water as a control (Cont.). **A**, Expression profiles are presented for JA-inducible lipoxygenase (*LOXD*), 12-oxophytodienoate reductase 3 (*OPR3*), proteinase inhibitor II (*PI-II*), and threonine deaminase (*TD*). **B**, Expression profiles are presented for SA-dependent pathogenesis-related genes 1b (*PR1b*), 2a (*PR2a*), and 2b (*PR2b*). Total RNA (10 µg) was loaded, and the ethidium bromide-staining of rRNA shows equal quality and quantity of each samples.

scription factors in *Arabidopsis* and tomato, respectively. Although *SIJAZ1* is not represented in the TOM1 microarray, our transcriptome analysis identified a COR-induced tomato clone, cLER1112, which showed high homology to *AtJAZ2*. Although the functional analysis of *SIJAZ2* in COR-induced responses has not been conducted, our results show the potential of TOM1 arrays in identifying new targets for COR.

COR functions to suppress SA-mediated defense in tomato seedlings.

We further investigated whether tomato seedlings respond to *P. syringae* pv. *tomato* DC3000 by mounting SA defense responses and whether COR functions as a suppressor of SA responses in seedlings. In tomato seedlings, COR downregulated several genes involved in biotic stress and defense, including

Fig. 5. Coronatine (COR) induces genes involved in polyamine biosynthesis during pathogenesis. **A**, Overview of ethylene and polyamine biosynthesis. The values represent the fold changes from TOM1 expression data ($\log_2$ value). **B**, Expression profiles of selected genes in tomato seedlings in response to *Pseudomonas syringae* pv. *tomato* DC3000, COR⁻ mutant DB29, or water as a control (Cont.). Expression profiles using Northern blot analysis are presented for *S*-adenosylmethionine synthetase 2 (*SAM2*), 1-aminocyclopropane-1-carboxylate oxidase (*ACO1*), arginase, spermidine synthase (*SPDS*), spermine synthase (*SPMS*), and *S*-adenosylmethionine decarboxylase (*SAMDC*). Total RNA (10 μ g) was loaded, and the ethidium bromide-staining of rRNA shows equal quality and quantity of each samples.

Fig. 6. Coronatine (COR) induces *SINAC2* and other genes belonging to jasmonic acid and salicylic acid pathways. **A**, Expression profiles of bHLH-type transcription factor (*bHLH*), MAP kinase 4 (*MPK4*) gene, and a NAC-type transcription factor (*SINAC2*) in tomato seedling leaves flood-inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 or the COR⁻ mutant DB29. The expression profile of α -tubulin and ethidium bromide-staining of rRNA were used to show the quality and quantity of cDNA and RNA, respectively. **B**, Fold induction (24 h posttreatment [hpt]) relative to the water controls of *SINAC2* in tomato seedling leaves treated individually with 200 pmol of COR, coronafacic acid (CFA), coronamic acid (CMA), or methyl jasmonic acid (MeJA). **C**, Fold induction (24 hpt) relative to the water controls of *SINAC2* (24 hpi) in tomato seedling leaves flood-inoculated individually with *P. syringae* pv. *tomato* DC3000, CMA⁻ (AK7E2), CFA⁻ (DB4G3), or the CMA⁻CFA⁻ (DB29) mutant. **D**, Phylogenetic tree based on the amino-acid sequences of NAC domain proteins of tomato NAC (LeNAC/SGN-U313171, named as *SINAC2*). Phylogenetic tree was constructed using the UPGMA program of Genetyx-Mac (Software Development, Tokyo). The names of proteins are indicated at the right of the tree with the accession number from the protein database or code number from the genome database.

11 pathogenesis-related (PR) genes (e.g. *PR1*, *PR2*, *PR3*, *PR5*, and *PR23*; Fig. 3B, numbers 8, 9, and 10). Interestingly, several genes encoding biotic stress-related proteins, including two unknown PR-related proteins (Fig. 3A; numbers 11 and 12), systemin (Fig. 3A; number 13), and a hypothetical protein (Fig. 3A; number 14), were induced by COR. To confirm the microarray results and follow the kinetics of *PR* gene expression, we monitored the expression of *PR1a*, *PR2a*, and *PR2b* in seedlings inoculated with *P. syringae* pv. *tomato* DC3000 and DB29. The results clearly demonstrated that *P. syringae* pv. *tomato* DC3000 suppresses the expression of *PR* genes during pathogenesis relative to DB29 (Fig. 4B). The results further demonstrated differential expression patterns of *PR* genes during disease development (Fig. 4B). For example, the expression of *PR1b* and *PR2a* was strongly suppressed for the first 24 hpi in tissues inoculated with *P. syringae* pv. *tomato* DC3000 but not in tissues inoculated with the COR⁻ mutant DB29 (Fig. 4B). However, the expression of *PR2b* was initially (12 hpi) induced after inoculation with either *P. syringae* pv. *tomato* DC3000 or DB29 (Fig. 4B). Interestingly, at 24 hpi, the COR-producing strain DC3000 suppressed the expression of a basic *PR* gene, *PR2b* (Fig. 4B). Basic *PR* genes are generally induced by ET signaling (Brederode et al. 1991). However, Zhao and associates (2003) have shown that both the T3SS and COR suppress *PR2b*. Taken together, these results suggest a complex cross-talk of ET and JA-mediated signaling pathways during bacterial speck disease development in tomato.

COR differentially regulates genes involved in polyamine metabolism.

One novel finding of the present work is that *P. syringae* pv. *tomato* DC3000 targets polyamine biosynthesis in a COR-dependent manner during pathogenesis. The expression of arginase, an enzyme potentially involved in polyamine (putrescine) biosynthesis via ornithine (Fig. 5A) was significantly induced in seedling tissues inoculated with *P. syringae* pv. *tomato* DC3000 but not DB29 (Fig. 5A and B). Furthermore, genes involved in the biosynthesis of spermidine, spermine, and putrescine, including *S*-adenosylmethionine decarboxylase (*SAMDC*), spermidine synthase (*SPDS*) (Fig. 5A), spermine synthase (*SPMS*) (Fig. 5B) arginine decarboxylase and arginase (Fig. 3A, number 15) were strongly induced in a COR-dependent manner during pathogenesis. These results indicate that COR from *P. syringae* pv. *tomato* DC3000 induces polyamine biosynthetic genes.

The role of polyamines in compatible plant-pathogen interaction is unclear. Previously, Chen and associates (2004) demonstrated the induction of arginase in response to COR and *P. syringae* pv. *tomato* DC3000 infection in tomato. Arginase is an important enzyme in polyamine biosynthesis, and therefore, Chen and associates (2004) speculated a role for polyamines in wound healing. Our results on expression of *SAMDC*, *SPDS*, *SPMS*, and arginine decarboxylase (Fig. 5) present further evidence for polyamine biosynthesis during pathogenesis. Interestingly, during plant interactions with biotrophic fungal pathogens, polyamines were suggested to play a role in maintaining metabolic activity or juvenility of plant tissues surrounding infection sites (Walters 2003). These metabolically active sites can then act as a source of nutrients for biotrophs. Based on our results, it is tempting to speculate that COR may play an important role in maintaining metabolically active cells in areas surrounding the infection site by inducing polyamine levels during the early stages of pathogenesis.

COR modulates expression of *SINAC2* and regulatory genes in the SA and JA pathways.

Microarray analysis revealed that COR from *P. syringae* pv. *tomato* DC3000 upregulates several genes that function in

cross-talk between the JA-ET-SA and JA-ET signaling pathways (Anderson et al. 2004; Lorenzo et al. 2004). These genes included *LeMPK4*, *LeHHLH* (Fig. 6A), and *LeMYB4*. Interestingly, *AtMPK4* has been implicated in negative regulation of SA-mediated defense responses in *Arabidopsis* (Petersen et al. 2000); whereas, the homologs of *LeHHLH* in *Arabidopsis* (*AtHHLH*, *AtMYC2/JIN1*) act as key regulators of JA and ET-SA cross-talk. *AtMYC2/JIN1* mutants are known to be resistant to biotrophic pathogens (Laurie-Berry et al. 2006; Nickstadt et al. 2004). Based on these results, it is tempting to speculate that the COR-induced tomato ortholog of *AtMPK4* and *AtMYC2/JIN1* may function to suppress SA-mediated defense responses during *P. syringae* pv. *tomato* DC3000 pathogenesis in tomato.

Furthermore, we identified *SINAC2*, a NAC domain transcription factor (TF) that is specifically induced by COR (Fig. 6). To further test the specificity of *SINAC2* expression, we conducted a more sensitive real-time reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) analysis (Fig. 6B and C). Interestingly, at equimolar concentrations (200 pmole), COR induced a fivefold higher induction of *SINAC2* than methyl jasmonic acid (MeJA) (Fig. 6B). Furthermore, CFA and CMA (the components of COR) had no significant effect on *SINAC2* expression (Fig. 6B). Similarly, only the wild-type *P. syringae* pv. *tomato* DC3000 but not the CMA⁻ (AK7E2), CFA⁻ (DB4G3), or CFA⁻ (DB29) mutants induced the expression of *SINAC2* (Fig. 6C), suggesting that *SINAC2* expression is specifically regulated by COR during pathogenesis.

NAC domain TF are widespread in plant genomes, and more than 160 NAC domain-containing TF have been identified in *Arabidopsis* and rice (Olsen et al. 2005). Several NAC TF are implicated in biotic stress and hormone responses (Olsen et al. 2005). For example, ATAF2 is shown to act as a repressor of *PR* genes in *Arabidopsis* (Delessert et al. 2005) and HvNAC6 is involved in fungal resistance (Jensen et al. 2007). Phylogenetic analysis revealed that *SINAC2* is a unique NAC-domain TF and belongs to a different subfamily from SIJA2 (*Solanum lycopersicum* JA2, a JA-inducible protein) and ATAF2 (Fig. 6D). However, *SINAC2* showed a close phylogenetic relationship with *AtNAP*, an *Arabidopsis* gene encoding NAC family TF (Fig. 6D) (Guo and Gan 2006). Interestingly, *AtNAP* was shown to be specifically induced during senescence but not by other phytohormones including MeJA (Guo and Gan 2006). Previously, in a comparative study of COR- and MeJA-induced responses on tomato leaves, we demonstrated that MeJA (100 μ M) does not elicit chlorosis at the application site and induced a strong expression of *SINAC2* on tomato leaves (Uppalapati et al. 2005). Taken together with the results presented in this study (Fig. 6), it is tempting to speculate that COR-induced *SINAC2* may play an important role in the severe chlorotic phenotype induced by COR but not by MeJA on tomato leaves.

Conclusions.

Foliar assays have been used to screen for host resistance of tomato to *P. syringae* pv. *tomato* (Pilowsky and Zutra 1982; Salmeron et al. 1994) and for isolating *P. syringae* pv. *tomato* mutants with impaired virulence (Cuppels 1986). Boch and associates (2002) and Brooks and associates (2004) screened transposon mutants of *P. syringae* pv. *tomato* DC3000 for impaired virulence on *Arabidopsis* using a dip-inoculation method. Although these methods for assessing the virulence of *P. syringae* have been established, they are not rapid, high-throughput methods that facilitate the screening of large numbers of mutants. Thus the aim of this work was to develop a seedling assay that could be used in both virulence screens and in gene expression studies. To our knowledge, our assay is a

novel approach to study *P. syringae* pv. *tomato* DC3000 infection of tomato seedlings, and the assay parameters were optimized to improve efficiency, consistency, and reproducibility. The method described here can be effectively used for high-throughput screens and has several advantages over foliar assays, including a shorter growth and incubation period (5 weeks vs. 7 to 10 days), ease of inoculation and handling (4 to 5 min vs. 20 to 30 min per sample), and rapid generation of larger sample sizes per experiment. Tornero and Dangel (2001) previously described a high-throughput bacterial quantification method using young (14-day-old) plants grown in pots. Our assay is performed on seedlings in petri dishes, an approach that requires less labor, space, and maintenance than plants grown in pots. In our hands, high-throughput estimation of bacterial populations by shaking the seedlings in a buffer, as described by Tornero and Dangel (2001) for *Arabidopsis*, was less reliable for tomato seedlings (data not shown). Homogenization of the tomato seedling was required for reliable measurement of bacteria in the apoplast (Figs. 1C and 2G and H). The method we adapted to homogenize seedlings in an Eppendorf tube using a motorized pestle also reduced labor and saved time. One of the advantages of the seedling assay over foliar assays is the low experimental variation between replicate experiments (Fig. 2G and H). Furthermore, when our seedling assay is combined with the recently reported luminescence quantification assay of luxCDABE-tagged *P. syringae* (Fan et al. 2007) or GFPuv-labeled bacteria (Wang et al. 2007), it may be possible to identify virulence phenotypes that have subtle effects.

In conclusion, our assay is a novel approach to study *P. syringae* pv. *tomato* DC3000 infection of seedlings and to dissect interactions with tomato. We have established that the seedling assay could be used effectively for the identification of genes involved in pathogenesis by using some of the well-characterized virulence mutants. Furthermore, we have demonstrated that COR is required for virulence in tomato seedlings. Using seedling assays and a MAPMAN display of TOM1 microarrays data, we identified additional host pathways targeted by COR in tomato that have not been previously reported.

MATERIALS AND METHODS

Plant material, bacterial isolates, and bacterial inoculation.

Tomato seeds of *Solanum lycopersicum* cv. Glamour were obtained from Stokes Seeds, Inc. (Buffalo, NY, U.S.A.). Seedlings were grown on MS medium (0.8% agar) with Gamborg vitamins (PhytoTechnologies Laboratories, Shawnee Mission, KS, U.S.A.). After 4 days of germination in darkness at 28°C, usually seedlings had 2- to 3-cm-long hypocotyls and two seedling leaves (cotyledons). In general, a period of 4 days was sufficient to obtain seedlings suitable for inoculation; however, it is important to mention that the incubation period could vary depending on the cultivar and conditions used.

P. syringae pv. *tomato* DC3000 and mutants AK7E2 (CFA⁺ CMA⁻; *cmaA*::Tn5), DB4G3 (CFA⁻ CMA⁺; *cfa6*::Tn5), and DB29 (CFA⁻ CMA⁻; *cfa6-cmaA* double mutant; Brooks et al. 2004) were grown at 28°C on King's B liquid medium (KB) (King et al. 1954) for 36 to 48 h. Antibiotics were added to media in the following concentrations (μg/ml): rifampicin, 100; kanamycin, 25; and spectinomycin, 25. Prior to inoculation, bacteria were suspended in 1 ml of sterile distilled H₂O. To study the effect of inoculum concentration on symptom development, three different concentrations (optical density at 600 nm [OD₆₀₀] = 0.01, 0.1 and 1.0) of *P. syringae* pv. *tomato* DC3000 were used.

Tomato seedlings were inoculated by a flooding method in which the MS agar plate was flooded with bacterial cells as

described below. The bacterial suspension (OD₆₀₀ = 0.1) consisted of sterile distilled H₂O containing 0.025% Silwet L-77 (OSi Specialties, Inc., Danbury, CT, U.S.A.). To achieve uniform inoculation, the bacterial suspension was dispensed into petri dishes until the seedlings were completely submerged and the seedlings were exposed to bacterial cells for 2 to 3 min with gentle mixing. After the inoculum was discarded, petri dishes containing inoculated seedlings were sealed with parafilm and were incubated at 24°C with light intensity of 100 to 150 μE m⁻² s⁻¹ and a 12 h-light, 12-h dark photoperiod. Seedlings were observed at 1, 2, 4, and 7 dpi for symptom development. Another set of seedlings was flooded with sterile water that served as mock control. In each experiment, 10 to 15 seedlings were evaluated, and each experiment was repeated at least three times.

Bacterial growth in seedlings was measured by determining the internal bacterial population at several timepoints after inoculation. Prior to sampling, seedlings were surface-sterilized with 15% H₂O₂ for 3 min to eliminate epiphytic bacteria and then were washed with sterile distilled H₂O. Seedlings were then homogenized in sterile distilled H₂O, and serial dilutions were plated onto KB medium containing the appropriate antibiotics. The bacterial population at 0 dpi was estimated from seedlings harvested 1 hpi. It is important to note that H₂O₂ may not kill all the epiphytic bacteria, and therefore, our internal population counts might include a small number of epiphytic bacteria.

RNA extraction and microarray analysis.

Total RNA isolation, purification, and TOM1 microarray analyses were done using the methods described earlier (Uppalapati et al. 2005). Briefly, total RNA was purified from tomato seedlings inoculated with *P. syringae* pv. *tomato* DC3000 and DB29 for 24 h, using TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Total RNA was extracted from 10 inoculated seedlings, and following isolation, the total RNAs were pooled to represent one biological replicate. Three independent experiments were carried out on three different days. Total RNA was treated with DNase I (Invitrogen) to eliminate genomic DNA and was further concentrated using Microcon YM100 filters (Millipore Corp., Bedford, MA, U.S.A.).

Approximately 50 μg of denatured total RNA from three biological replicates was used to synthesize cDNA with an oligo d(T) primer, which was tagged with either Cy3- or Cy5-specific 3DNA capture sequence (Genisphere Inc., Hartfield, PA, U.S.A.). Total RNA (50 μg) and tagged oligo d(T) (1 μl) primer were mixed in a final volume of 10 μl with RNase-free water, were heat-denatured at 80°C for 10 min, and were cooled on ice. For reverse transcription, master mix (8 μl) containing 2 μl of 10× Superscript RT buffer (Invitrogen), 1 μl of RNase-OUT (Invitrogen), 1 μl of 10 mM dNTP (Invitrogen), 2 μl of 0.1M dithiothreitol (Invitrogen), and 2 μl of 25 mM MgCl₂ (Invitrogen) was prepared and mixed with denatured total RNA and oligo d(T). Each sample was heated at 42°C for 3 min, and 2 μl of Superscript III RT (Invitrogen) was added. The RT reaction was incubated at 42°C for 3 h, was supplemented with 3.5 μl of stop solution, containing 0.5 M NaOH and 50 mM EDTA, and then, was incubated at 65°C for 10 min to stop the RT reaction and hydrolyze template RNA. For neutralization of cDNA mix, 5 μl of 1 M Tris-HCl (pH 7.5) was added and 6.6 μl of Cy3- or Cy5-labeled cDNA was used for hybridization.

TOM1 arrays were obtained from the Center for Gene Expression Profiling, Boyce Thompson Institute, Cornell University (Ithaca, NY, U.S.A.). For the hybridization reaction, 6.6 μl of tagged cDNA, 1.5 μl of salmon sperm DNA at a concentra-

tion of 1 µg/µl (Invitrogen), 2 µl of oligo d(T) blocker (Genisphere), and 1 µl of high-end differential enhancer (Genisphere) were added to 41.3 µl of high sodium dodecyl sulfate (SDS) hybridization buffer (Genisphere). A total of 60 µl of mixture was incubated initially at 80°C for 10 min and then at 60°C for 20 min before applying to prewarmed slides. A cover glass was placed on top of the glass slide to uniformly distribute the hybridization solution. The slides were then incubated at 60°C in a water bath for 16 h in a Corning hybridization chamber (Corning Inc., Acton, MA, U.S.A.). The three posthybridization washes were follows: i) at 60°C for 15 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% SDS; ii) at room temperature in 2× SSC; and iii) at room temperature in 0.2× SSC. Then, the samples were fixed in 95% ethanol for 2 min and were dried by centrifugation at 1,000 × g for 2 min. The hybridized cDNA was incubated with Cy3 and Cy5 3DNA reagent and was incubated for 3 h as explained above. The slides were washed and dried as described above.

The slides were analyzed with ScanArray 3000 (GSI Lumonics, Billerica, MA, U.S.A.) and the spots were quantified using GenePix Pro 4.0 (Axon Instruments, Inc., Union City, CA, U.S.A.). Preprocessing of data was accomplished using GenePix Autoprocessor. The data was normalized using global locally weighted linear regression normalization (Yang et al. 2002). A candidate list of differentially expressed genes was then generated using a 5% false discovery rate (FDR) and >2-fold change between treatments. We used moderated *t* statistics and associated *P* values after FDR in the LIMMA package of the Bioconductor software package. A total of three biological replicate experiments were conducted for each samples. In compliance with MIAME guidelines, raw data (before normalization) and normalized data with the mean ratios for COR-regulated genes, along with a sample of the scanned image, are presented in Supplementary Figures S4 and S5. Furthermore, all the data will be submitted to the Tomato expression database (Cornell University).

MAPMAN conversion of TOM1 microarray data.

The average mean ratios of normalized data were imported into the MAPMAN software, which converts the data values to a false-color scale and associates a gene to a relevant metabolic function and places them on an appropriate metabolic pathway (Thimm et al. 2004; Usadel et al. 2005). The overview figures and functional categorization of COR-regulated genes were prepared using mapping file version Ses_TOM1 Sep06 as described (Urbanczyk-Wochniak et al. 2006).

Northern blot, semiquantitative RT-PCR, and real-time qPCR analyses.

Total RNA was purified from tomato seedling leaves treated with water (control) or inoculated with *P. syringae* pv. *tomato* DC3000 or DB29. Total RNA was extracted using TRIzol (Invitrogen), and 15 treated or inoculated seedling leaves were pooled to represent one biological replicate. Three independent experiments were carried out on three different days. Total RNA (10 µg) was denatured and fractionated by electrophoresis on a 1% agarose gel and, then, was blotted onto nylon membranes (Amersham BioScience, Uppsala, Sweden). Digoxigenin (DIG)-labeled DNA probes were made with DIG PCR labeling mix (Boehringer Mannheim, Mannheim, Germany), using gene-specific forward and reverse primer sets. Hybridization was performed at 65°C overnight in a solution containing 50 mM Church buffer (pH. 7.2) (Church and Gilbert 1984), 5× SSC, 0.1% laurylsarcosine, 7% SDS (wt/vol), and 2% blocking solution (Boehringer Mannheim). Final washes were at 65°C in a solution containing 0.5× SSC and 0.1% SDS. Hybridized mRNAs were detected with anti-

DIG antibody conjugated with alkaline phosphatase (Boehringer Mannheim) and its chemiluminescent substrate CDP-Star (Boehringer Mannheim).

Semiquantitative RT-PCR was conducted with cDNA generated using methods described previously (Uppalapati et al. 2005). For semiquantitative RT-PCR, 1 µl of first-strand cDNA (diluted 1:50 in reaction buffer) was amplified using *Taq* DNA polymerase (Promega). To check for equal amounts of cDNA in each reaction, PCR was performed with primers specific for the gene encoding α -tubulin 4 (Mysore et al. 2002).

SINAC2 was quantified using real-time qPCR, based on the primers designed after the EST information on TOM1, using methods described previously (Mysore et al. 2002; Uppalapati et al. 2005). Melt-curve analysis was performed to monitor primer-dimer formation and to check amplification of gene-specific products. The average threshold cycle (C_T) values, calculated from triplicate samples, were used to determine the fold expression relative to the controls. Primers specific for α -tubulin 4 were used to normalize small differences in template amounts. Data analysis including normalization and standard deviations were calculated using Gene Expression Macro, ver 1.1 (Bio-Rad, Philadelphia) with the algorithms described by Vandesompele and associates (2002). Data quantification and analysis was performed using iCycler software (ver. 3.06.6070) and Gene Expression Macro (ver 1.1) (Bio-Rad). Average C_T values calculated from triplicate samples were used to determine the fold expression relative to the controls. Primers specific for α -tubulin 4 were used to normalize small differences in template amounts.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Bioconductor's LIMMA software: www.bioconductor.org
 Cornell University's Tomato expression database: ted.bti.cornell.edu
 Oklahoma State University Microarray Core Facility's GenePix Autoprocessor (GPAP) software: darwin.biochem.okstate.edu/gpap3