11-11-2003

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The Plant Journal (2004) **37**, 589–602 doi: 10.1111/j.1365-313X.2003.01986.x

Activation of a COI1-dependent pathway in *Arabidopsis* by *Pseudomonas syringae* type III effectors and coronatine

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Received 20 September 2003; revised 10 November 2003; accepted 11 November 2003.
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Summary

Gram-negative bacteria use a variety of virulence factors including phytotoxins, exopolysaccharides, effectors secreted by the type III secretion system, and cell-wall-degrading enzymes to promote parasitism in plants. However, little is known about how these virulence factors alter plant cellular responses to promote disease. In this study, we show that virulent *Pseudomonas syringae* strains activate the transcription of an *Arabidopsis* ethylene response factor (*ERF*) gene, *RAP2.6*, in a coronatine insensitive 1 (COI1)-dependent manner. A highly sensitive *RAP2.6* promoter-firefly luciferase (*RAP2.6-LUC*) reporter line was developed to monitor activities of various bacterial virulence genes. Analyses of *P. syringae* pv. *tomato* DC3000 mutants indicated that both type III secretion system and the phytotoxin coronatine are required for *RAP2.6* induction. We show that at least five individual type III effectors, avirulence B (AvrB), AvrRpt2, AvrPphB, HopPtoK, and $AvrPphE_{Pto}$, contributed to *RAP2.6* induction. Gene-for-gene recognition was not involved in *RAP2.6* induction because plants lacking *RPM1* and *RPS2* responded normally to AvrB and AvrRpt2 in *RAP2.6* expression. Interestingly, the role of coronatine in *RAP2.6* induction can be partially substituted by the addition of *avrB* in DC3000, suggesting that AvrB may mimic coronatine. These results suggest that *P. syringae* type III effectors and coronatine act by augmenting a COI1-dependent pathway to promote parasitism.

Keywords: ERF, *Pseudomonas*, virulence, type III effectors, coronatine, jasmonates.

Introduction

The recent completion of genome sequences for several phytopathogenic bacteria, including *Xanthomonas campestris*, *Ralstonia solanacerum*, and *Pseudomonas syringae,* uncovered a large number of genes with a putative role in bacterial pathogenesis. For example, the *P. syringae* pv. *tomato* DC3000 contains 276 genes encoding potential virulence determinants such as type III secretion system (TTSS), effector proteins secreted by TTSS, cell-wall-degrading enzymes, enzymes required for the biosynthesis of the phytotoxin coronatine, exopolysaccharide alginate, and receptors responsible for sensing the host environment (Buell *et al.*, 2003).

A major challenge that follows the exciting breakthrough brought about by genome sequencing is to identify the function of these virulence factors *in planta*. In particular, type III effector genes that are thought to play a crucial role in bacterial virulence and host range determination often encode proteins of unknown function. Many plant bacterial effectors were first identified as avirulence (Avr) proteins that specify resistance (*R*) gene-mediated disease resistance (White *et al.*, 2000). The biochemical basis of type III effector virulence function is only beginning to emerge for a handful of effectors. For example, *P. syringae* effectors AvrB, AvrRpm1, AvrPto, and AvrRpt2 confer virulence function on plants lacking cognate resistance genes (Ashfield *et al.*, 1995; Chen *et al.*, 2000; Ritter and Dangl, 1995; Shan *et al.*, 2000b). AvrRpt2 also suppresses defense responses specified by AvrRpm1–RPM1 interaction (Chen *et al.*, 2000; Reuber and Ausubel, 1996; Ritter and Dangl, 1996). AvrRpt2 appears to be a cysteine protease whose presence triggers the degradation of the RIN4 protein (Axtell and Staskawicz, 2003; Axtell *et al.*, 2003; Mackey *et al.*, 2003). *Pseudomonas* effectors that suppress gene-for-gene resistance also include VirPphA and AvrPphF from *P. syringae* pv.

phaseolicola and AvrPtoB from DC3000 (Abramovitch *et al.*, 2003; Jackson *et al.*, 1999; Tsiamis *et al.*, 2000). AvrPto appears to function as a virulence factor by suppressing host genes involved in callose deposition (Hauck *et al.*, 2003). AvrPto, AvrB, and AvrRpm1 target plant plasma membrane through myristoylation (Nimchuck *et al.*, 2000; Shan *et al.*, 2000a). At least AvrPto requires myristoylation for the virulence function (L. Shan and X.T., unpublished results). However, the majority of type III effectors do not produce measurable virulence effects. Consequently, their role in bacterial parasitism has been difficult to study.

Many *P. syringae* strains, such as *P. syringae* pv. *glycinea, P. syringae* pv. *maculicola,* and *P. syringae* pv. *tomato,* also produce the phytotoxin coronatine. In DC3000, type III effectors and coronatine biosynthetic genes are regulated in a coordinated fashion (Boch *et al.*, 2002), but TTSS *per se* is not required for coronatine secretion (Penaloza-Vazquez *et al.*, 2000). Coronatine contributes to virulence in *P. syringae* pv. *tomato* by promoting bacterial growth and chlorosis in the plant (Mittal and Davis, 1995). Coronatine consists of two moieties, coronafacic acid (CFA) and coronamic acid (CMA). They are structurally similar to jasmonic acid (JA) and aminocyclopropane carboxylic acid (ACC), the immediate precursor of ethylene, respectively (reviewed by Bender *et al.*, 1999). Coronatine is known to mimic JA (Feys *et al.*, 1994), and it may also mimic ethylene because of the similarity of CMA to ACC (Ferguson and Mitchell, 1985). JA and ethylene often act synergistically in response to wounding or pathogen infection (reviewed by Kunkel and Brooks, 2002). Coronatine-producing *Pseudomonas* bacteria may manipulate the host signaling pathways through these hormones to promote parasitism. However, early molecular events involved in this process remain poorly understood.

Here, we describe the use of a transgenic reporter line carrying the *RAP2.6* promoter fused with the firefly luciferase gene *LUC* to investigate host cellular activities modulated by DC3000 type III effectors and coronatine. *RAP2.6* (At1g43160) is an *Arabidopsis* ethylene response factor (ERF) family transcription factor (Okamuro *et al.*, 1997) that is strongly induced by virulent *P. syringae* strains. The induction of *RAP2.6* largely depended on a coronatine insensitive 1 (COI1)-mediated pathway. An intact TTSS, a number of type III effectors, and coronatine are all required for the *RAP2.6* induction by the wild-type DC3000 bacterium. Bacterial mutants unable to produce coronatine or defective in TTSS poorly induce the *RAP2.6* promoter. The coronatine and TTSS mutants fully complemented each other to activate the *RAP2.6-LUC* reporter when co-inoculated into plants, suggesting that type III effectors and coronatine acted coordinately to induce *RAP2.6* expression. However, the addition of an exogenous effector gene, *avrB*, allowed DC3000 to induce *RAP2.6* in the absence of coronatine, suggesting that AvrB acted, at least in part, to mimic coronatine. The results support a view that bacterial type III effectors and coronatine target the COI1 pathway to promote parasitism.

Results

Association of RAP2.6 *expression with disease susceptibility*

A number of *Arabidopsis ERF* genes are induced upon pathogen infection (Chen *et al.*, 2002; Onate-Sanchez and Singh, 2002). One of these *ERF* genes, *RAP2.6*, was induced strongly by two virulent strains, DC3000 and *P. syringae* pv. *maculicola* ES4326 (R. Warren, P.H., and J.Z., unpublished results). Interestingly, microarray analysis indicated that *RAP2.6* was more responsive to virulent bacteria in the *npr1* and *pad4* mutants and *NahG* transgenic plants (Chen *et al.*, 2002). These plants are enhanced in disease susceptibility because of a deficiency in salicylic acid (SA)-mediated defense. These experiments examined gene expression at a relatively late time point (30 h) after infection. To further understand the potential role of *RAP2.6* expression in plant–bacterial interactions, we examined the *RAP2.6* transcripts in the wild type Columbia (Col-0), the resistant mutant *cpr5-1* (Bowling *et al.*, 1997) and the susceptible mutant *npr1-1* (Cao *et al.*, 1994) at earlier time points after

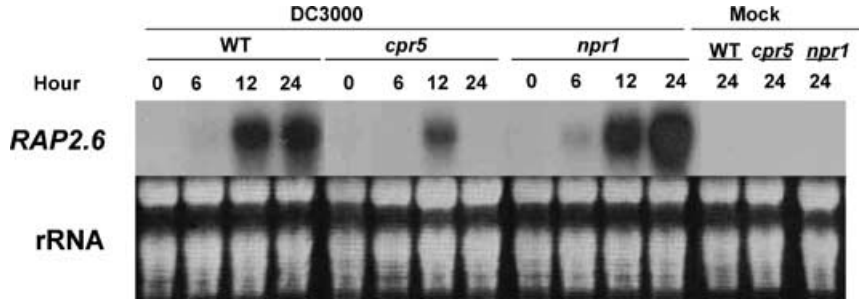


Figure 1. Association of *RAP2.6* expression with disease susceptibility.
Wild-type Col-0 (WT), *cpr5-1*, and *npr1-1* plants were infiltrated with 10 mM $MgCl_2$ (mock), or *P. syringae* pv. *tomato* DC3000 at 2×10^6 cfu ml^{-1}, and RNA was isolated from tissues collected before (0 h) or after (6, 12, and 24 h) infiltration. The ethidium bromide gel pictures indicate equal loading of RNA samples. The Northern experiments were independently repeated two times with similar results.

inoculation with DC3000. As shown in Figure 1, *RAP2.6* transcripts were induced 12 h after inoculation in all plants tested. The transcript level was the lowest in *cpr5-1* plants, and it further declined at 24 h. In contrast, *npr1* plants showed the greatest *RAP2.6* transcript level at 24 h. Thus, the expression of *RAP2.6* in response to DC3000 appeared to be positively correlated with disease susceptibility and negatively with disease resistance of plants. *cpr5* and *npr1* are known to be altered in SA accumulation in plants during bacterial infection. The latter may suppress *RAP2.6* expression through SA/JA antagonism, a possibility that remains to be tested.

The RAP2.6 *promoter is bacterial inducible*

To better monitor the *RAP2.6* gene activation in response to *Pseudomonas* infection, we developed a reporter transgenic line by fusing a 2-kb fragment upstream of the *RAP2.6* open-reading frame to the reporter gene *LUC*. The resulting construct, *RAP2.6-LUC,* was transformed into *Arabidopsis* Col-0, and 20 independent transgenic lines were obtained. To determine if the transgenic plants confer bacterial-induced *LUC* expression, we inoculated the plants with *P. syringae* pv. *maculicola* ES4326 (*avrB*). Previous experiments had indicated that this strain gave a strong and early induction of *RAP2.6* transcripts (Figure 2a). The *RAP2.6* transcript level reached a maximum at 6 h after bacterial inoculation, and it was not significantly affected by the bacterial concentration (from 2×10^8 to 2×10^6 cfu ml^{-1}). Mock-inoculated plants did not show an induction at 6 h, but a transient induction was observed 1 h after infiltration (data not shown). Figure 2(b) shows the induction of *LUC* transcripts in three independent transgenic lines after mock and bacterial infiltration. The *LUC* transcripts increased dramatically 6 h after pathogen infiltration in all transgenic lines compared with mock infiltration, which is consistent with the induction of the endogenous *RAP2.6* gene. Lines 13 and 14 that contained multiple copies of transgene (data not shown) displayed a stronger induction of the reporter gene than did line 12 that contained a single-copy transgene. All three lines also displayed a strong bacterial-inducible luciferase activity. Figure 2(c,d) shows bacterial-induced luciferase activity in line 12, as determined by *in vivo* luminescence imaging and luminometer assay. These results demonstrated that the *RAP2.6* promoter is pathogen inducible. The homozygous line 12 was thus chosen for all luciferase experiments described below.

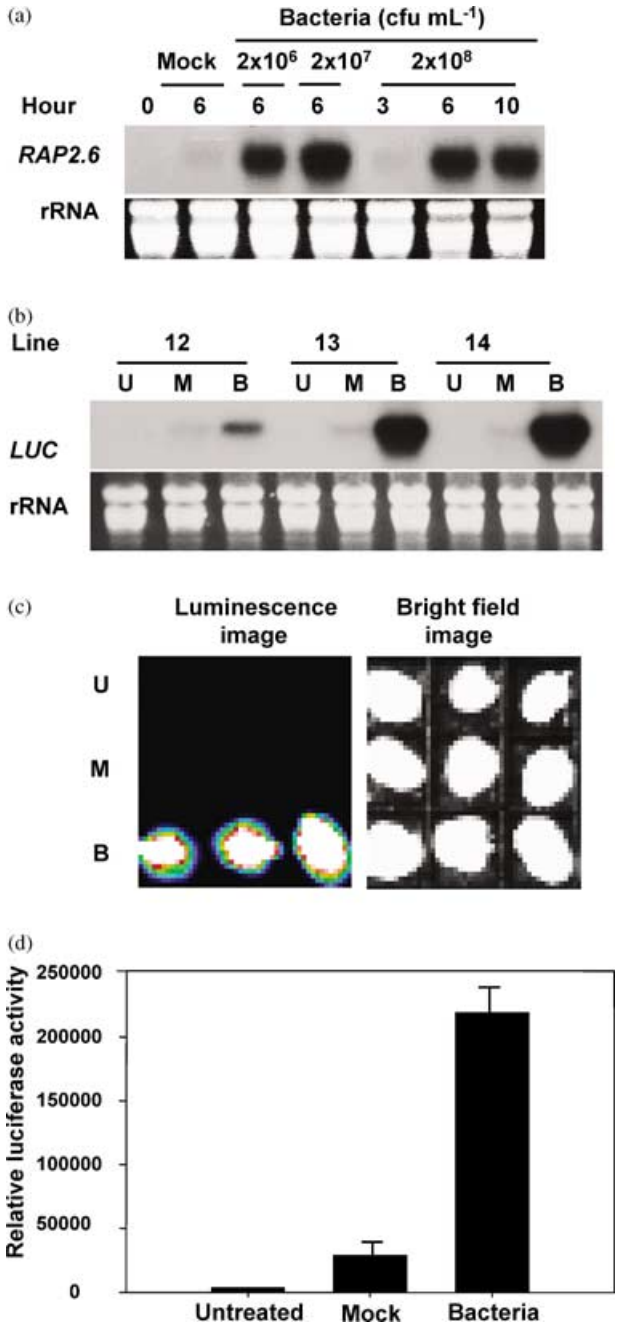


Figure 2. The *RAP2.6* promoter is induced by bacterial infection.
(a) Wild-type Col-0 plants were vacuum-infiltrated with *P. syringae* pv. *maculicola* ES4326 (*avrB*) at the indicated concentrations, and tissues were collected at different times for RNA isolation. Control plants were infiltrated with 10 mM $MgCl_2$ (mock).
(b) *RAP2.6-LUC* transgenic plants (Lines 12, 13, and 14) were inoculated with water (M) or ES4326 (*avrB*) (B) at 2×10^7 cfu ml^{-1}. RNA was isolated from tissues collected before (U) or 6 h after inoculation, and the RNA blot was hybridized with a radiolabeled *LUC* DNA fragment.
(c) A pseudo-color luminescence image (left) and bright-field image (right) of untreated transgenic leaves (U), or leaves 6 h after inoculation with water (M) or ES4326 (*avrB*) (B).
(d) Relative luciferase activity after inoculation with water (mock) or 2×10^7 cfu ml^{-1} ES4326 (*avrB*) (bacteria). Luciferase assays were performed 6 h after treatment. Each data point consisted of four leaves. Error bars indicate SEs. The ethidium bromide gel pictures in panels (a) and (b) indicate equal loading of RNA samples. The experiments were repeated four times with similar results.

Association of RAP2.6 *promoter activity with* P. syringae *pathogenicity*

There are over 40 pathovars of *P. syringae* classified according to their host range among different plant species (Hirano and Upper, 1990). Only certain strains of *P. syringae* pv. *tomato* and *P. syringae* pv. *maculicola* are known to infect *Arabidopsis* (Davis *et al.*, 1989). To test whether the *RAP2.6* promoter activity was correlated with bacterial pathogenicity, we inoculated the *RAP2.6-LUC* transgenic plant with different *P. syringae* strains that are either highly virulent or non-pathogenic on *Arabidopsis*. DC3000 is a highly virulent pathogen on *Arabidopsis* (Davis *et al.*, 1989; Dong *et al.*, 1991); *P. syringae* pv. *tomato* strain T1, *P. syringae* pv. *tabaci* R11528 race 0, and *P. syringae* pv. *phaseolicola* NPS3121 are non-pathogenic on *Arabidopsis* (Davis *et al.*, 1989; Lu *et al.*, 2001; Yu *et al.*, 1993; P.H. and J.Z., unpublished results). DC3000 bacteria dramatically induced *LUC* at 12 h after inoculation (Figure 3a). The *RAP2.6* promoter activity increased further at 24 h and was about 20-fold higher than that in mock-inoculated leaves. A strong induction of luciferase was also observed when plants were inoculated with another virulent strain, ES4326 (see Figure 5). In contrast, the three non-pathogenic strains showed no significant *LUC* induction throughout the entire experiment. No bacterial growth in the plant was detected for any of the strains in the first 12 h (Figure 3b), indicating that the induction of *LUC* by DC3000 was not a result of bacterial growth. Overall, the results indicate that the *RAP2.6* promoter activity was correlated with the pathogenicity of the *P. syringae* strains used.

DC3000 virulence mutants are severely reduced in RAP2.6 *promoter activation*

To further test if the *RAP2.6* promoter activity could be used to detect bacterial virulence, we inoculated *RAP2.6-LUC* transgenic plants with DC3000 mutants *hrcC*$^-$, *ΔCEL*, and COR$^-$. *hrcC* encodes an outer membrane protein of the TTSS, and its mutation abolishes pathogenicity (Yuan and He, 1996). *CEL* is a conserved effector locus in the *hrp* pathogenicity island and is required for pathogenicity on tomato (Alfano *et al.*, 2000). The COR$^-$ mutant is unable to produce coronatine (Ma *et al.*, 1991) and shows reduced bacterial growth in the plant when inoculated by dipping or spraying (Penaloza-Vazquez *et al.*, 2000). The loss of pathogenicity in *hrcC*$^-$ and *ΔCEL* was confirmed by measuring the bacterial growth on *Arabidopsis* plants (Figure S1). *ΔCEL* bacterial growth was reduced by approximately 30-fold, whereas the growth of the *hrcC*$^-$ mutant was reduced by at least 100-fold compared with the wild-type DC3000 strain. The COR$^-$ mutant and the wild-type DC3000 did not differ in bacterial growth when inoculated by syringe infiltration (Figure S1). This is consistent with the previous report that the COR$^-$ mutant display reduced bacterial growth only when plants are inoculated by dipping or spraying, but not by infiltration (Mittal and Davis, 1995; Penaloza-Vazquez *et al.*, 2000). Nonetheless, the COR$^-$ mutant failed to cause chlorosis in the plant in our assay, indicating reduced virulence. Figure 4(a) shows that all three mutants were poor inducers of the *RAP2.6* promoter, with the COR$^-$ mutant showing the least induction. Interestingly, the *hrcC*$^-$ mutant showed slightly higher induction of the *RAP2.6* promoter than the *ΔCEL* and COR$^-$

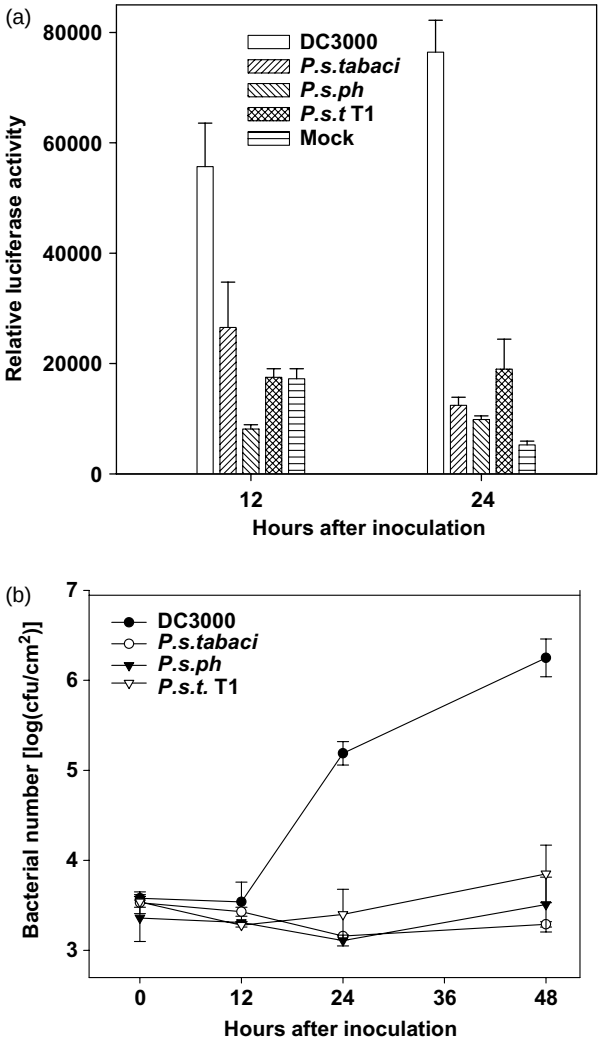
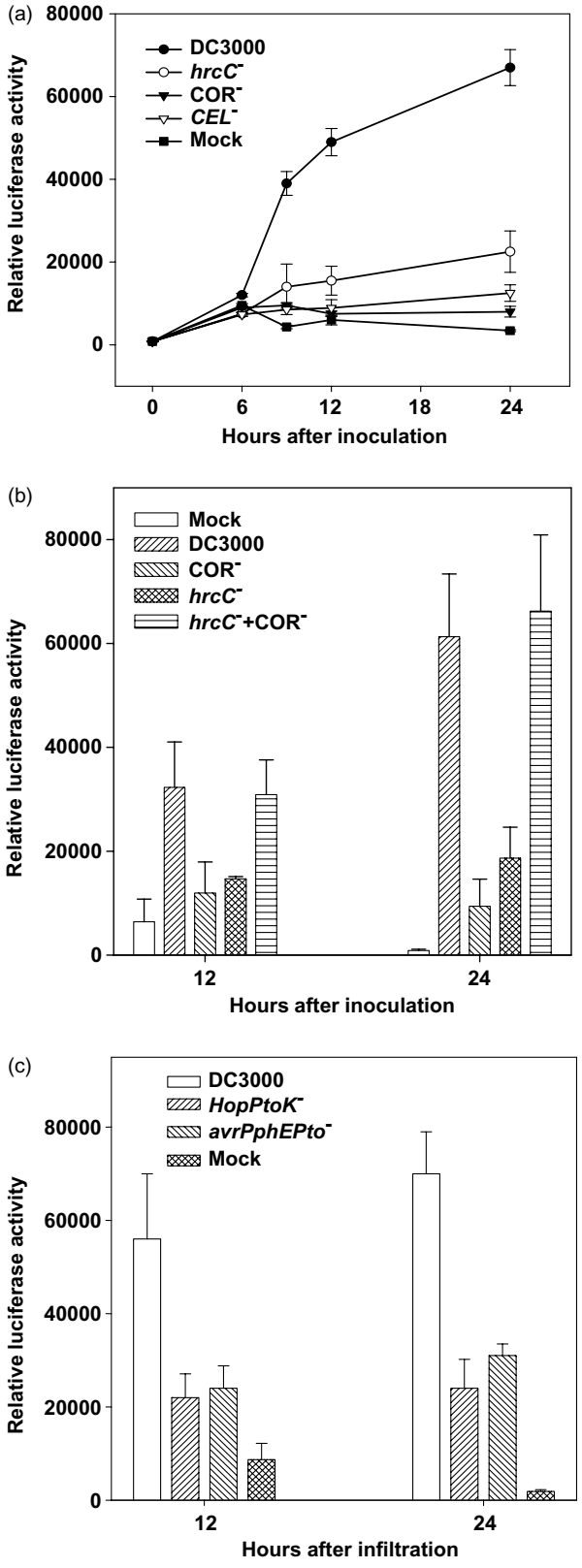


Figure 3. Association of *RAP2.6* promoter activity with *P. syringae* pathogenicity.
(a) Leaves were collected 12 and 24 h after inoculation, and the luciferase activity was analyzed by using a luminometer.
(b) Bacterial growth in the plant. Each data point consisted of four replicates. Error bars indicate SEs. The experiments were repeated four times with similar results.
RAP2.6-LUC transgenic plants were hand-injected with water (mock), *P. syringae* pv. *tomato* DC3000 (DC3000), *P. syringae* pv. *tomato* T1 (*P. s. t.* T1), *P. syringae* pv. *tabaci* R1152 race 0 (*P. s. tabaci*), and *P. syringae* pv. *phaseolicola* NPS3121 (*P. s. ph.*) at the concentration of 2×10^6 cfu ml^{-1}.



mutants. The *hrcC*$^-$ mutant appears to overproduce coronatine (Penaloza-Vazquez *et al.*, 2000), whereas the *CEL* deletion is not known to affect coronatine production. The requirement of both *hrcC* and coronatine for *RAP2.6* induction suggests that the phytotoxin and type III effectors acted together to modulate host cellular activities. We further tested this possibility by co-inoculating plants with the *hrcC*$^-$ and COR$^-$ mutant strains. Figure 4(b) clearly shows that the two mutant strains complemented each other in bacterial mixing experiments, and the *RAP2.6* promoter activity was completely restored, suggesting that coronatine and TTSS effectors modulate the same pathways in plants.

The RAP2.6 *promoter activity is modulated by individual effector genes in DC3000*

The requirement of TTSS and *CEL* in the induction of *RAP2.6* prompted us to examine the role of individual effector genes in *RAP2.6* induction. There are at least 36 experimentally confirmed Hrp outer proteins (Hops) in DC3000, most of which are type III effectors (Collmer *et al.*, 2002). However, the virulence function for most effectors examined to date remains unclear. To determine if the *RAP2.6* promoter could be used to monitor effector activities in plants, we tested Campbell insertion (single crossover) mutants for 10 individual DC3000 effector genes for *RAP2.6* inducibility (J.R.A., manuscript submitted). These include *avrPphE*$_{Pto}$, *avrPpiB1*$_{Pto}$, *avrPpiB2*$_{Pto}$, *hopPsyA*$_{Pto}$, *hopPtoD1, hopPtoD2, hopPtoC, hopPtoK, hopPtoJ*, and *hopPtoF.* The generation of these mutants will be published separately. None of these mutants exhibited observable changes in bacterial growth or disease symptoms on *Arabidopsis* plants (Figure S2; L. Shan and X.T., unpublished results). However, the *hopPtoK*$^-$ and *avrPphE*$_{Pto}^-$ mutants were weakened in *RAP2.6* activation, and the luciferase inducibility was reduced by 60–70% compared with that of the wild-type DC3000 strain at 12 and 24 h after inoculation (Figure 4c). Mutations in *hopPsyA*$_{Pto}$, *hopPtoD1*, and

Figure 4. DC3000 virulence mutants are severely reduced in *RAP2.6* promoter activation.
(a) *hrcC*, *CEL*, and coronatine synthesis are essential for *RAP2.6* activation. Luciferase activity of *RAP2.6* plants was measured after inoculation with water (mock), DC3000, and mutant bacteria *hrcC*$^-$, *ΔCEL*, and COR$^-$ at 2×10^6 cfu ml^{-1}. Leaves were collected at the indicated time points and analyzed by using a luminometer. Each data point consisted of four leaves.
(b) *hrcC*$^-$ and COR$^-$ co-inoculation restores *RAP2.6* inducibility. *RAP2.6-LUC* plants were co-inoculated with the two mutants at 10^6 cfu ml^{-1} per strain. Error bars indicate SEs. The experiments were repeated four times with similar results.
(c) The *RAP2.6* promoter activity reports the activity of individual DC3000 effector genes. *RAP2.6-LUC* transgenic plants were hand-injected with water (mock), DC3000, and the *hopPtoK*$^-$ and *avrPphE*$_{Pto}^-$ mutants at 2×10^6 cfu ml^{-1}. Luciferase activity was measured at 12 and 24 h after inoculation. Each data point consisted of at least three leaves. Error bars indicate SEs. The experiments were repeated three times with similar results.

hopPtoD2 had a smaller, but reproducible, reduction in *RAP2.6* promoter activity (data not shown). The remaining effector mutants were identical to the wild-type DC3000 in *RAP2.6* activation.

avr *genes accelerate the activation of the* RAP2.6 *promoter*

As mentioned above (Figure 2a), *P. syringae* pv. *maculicola* bacteria carrying the *avrB* gene, a type III effector gene isolated from *P. syringae* pv. *glycinea,* induced the *RAP2.6* transcripts in plants earlier than the strain without *avrB*. We further tested DC3000 strains carrying heterologous *avr* genes for *RAP2.6* promoter activation. At 6 h after inoculation, DC3000 strains carrying *avrB*, *avrRpt2*, or *avrPphB* strongly induced *RAP2.6-LUC* expression, while DC3000 without an added *avr* gene did not induce *LUC* until 9 h (Figure 5a). DC3000 (*avrRps4*) showed a marginal, but reproducible, induction of *LUC.* ES4326 carrying *avrB* also strongly induced *LUC* at 6 h. The small effect of *avrRps4* may be a result of gene redundancy caused by the endogenous *hopPtoK* that is highly homologous to *avrRps4* (Collmer *et al.*, 2002). Among the four avirulence genes tested, *avrB* was the most potent in *RAP2.6* promoter activation. At the later time points (9–24 h), the LUC activity in DC3000 (*avrB*)-inoculated plants declined, while it steadily increased in DC3000-inoculated plants (Figure 5b). The maximum induction upon DC3000 (*avrB*) infection at 6 h was nearly two times of that induced by DC3000 at 24 h (Figure 5b).

R *gene-specified resistance is not required for* avrB-*mediated* RAP2.6 *induction*

RAP2.6-LUC transgenic plants were constructed in the Col-0 ecotype that carries the cognate *R* genes for *avrB, avrRpt2, avrPphB,* and *avrRps4* (Gassmann *et al.*, 1999; Innes *et al.*, 1993; Kunkel *et al.*, 1993; Warren *et al.*, 1998). Thus, the enhanced *RAP2.6* expression could be a result of gene-for-gene interaction. However, the primary function of *avr* genes is thought to promote parasitism. In fact, many of the cloned *avr* genes are known to possess a measurable virulence function in plants (White *et al.*, 2000). Therefore, the enhanced induction of the *RAP2.6* promoter equally could be caused by the virulence effect of the *avr* genes. We tested whether the early induction of the *RAP2.6* gene by *avr* genes is because of the gene-for-gene-based resistance reaction. *P. syringae* pv. *phaseolicola* NPS3121 is a non-host strain on *Arabidopsis* and does not induce the hypersensitive response (HR; Lu *et al.*, 2001; Yu *et al.*, 1993). However, heterologous *avr* genes introduced into NPS3121 can be recognized by corresponding *R* genes in *Arabidopsis* plants to initiate typical HR resistance reaction. We transferred *avrB*, *avrRpt2*, *avrPphB*, and *avrRps4* into NPS3121 and inoculated them in the *RAP2.6-LUC* transgenic plants. Similar to DC3000 (*avrB*), NPS3121 *(avrB*) induced an HR 5–8 h after inoculation (Figure 5c). NPS3121 (*avrRpt2*) and NPS3121 *(avrPphB*) induced an HR 16–20 h after inoculation. The *RPS4* gene in Col-0 is weak and does not show a detectable HR in response to the *avrRps4* gene (Gassmann *et al.*, 1999). None of the *avr* genes in NPS3121 had a significant effect on *RAP2.6* promoter activation over the course of 24 h (Figure 5b). These results indicated that the recognition of the *avr* genes by corresponding *R* genes was not responsible for the induction of the *RAP2.6* promoter.

We further tested the requirement of gene-for-gene resistance in the *avrB*-mediated induction of *RAP2.6* transcripts in the *rps3/rpm1* and *ndr1* mutants by using Northern analysis (Figure 6a). The *rps3/rpm1* mutant carries a mutation in the *RPM1* gene and is unable to initiate the *avrB*-specified disease resistance (Bisgrove *et al.*, 1994; Innes *et al.*, 1993). NDR1 is an important signaling component essential for resistance mediated by several *R* genes, including *RPM1* (Century *et al.*, 1995). Similar to wild-type Col-0 plants, a strong *avrB*-dependent *RAP2.6* induction was observed in both *rps3/rpm1* and *ndr1* plants 6 h after bacterial inoculation (Figure 6a). In contrast, *RAP2.6* transcripts in DC3000-inoculated plants were indistinguishable from the mock-inoculated plants at 6 h. The results demonstrate that *avrB* induced *RAP2.6* expression independent of the *RPM1* resistance function.

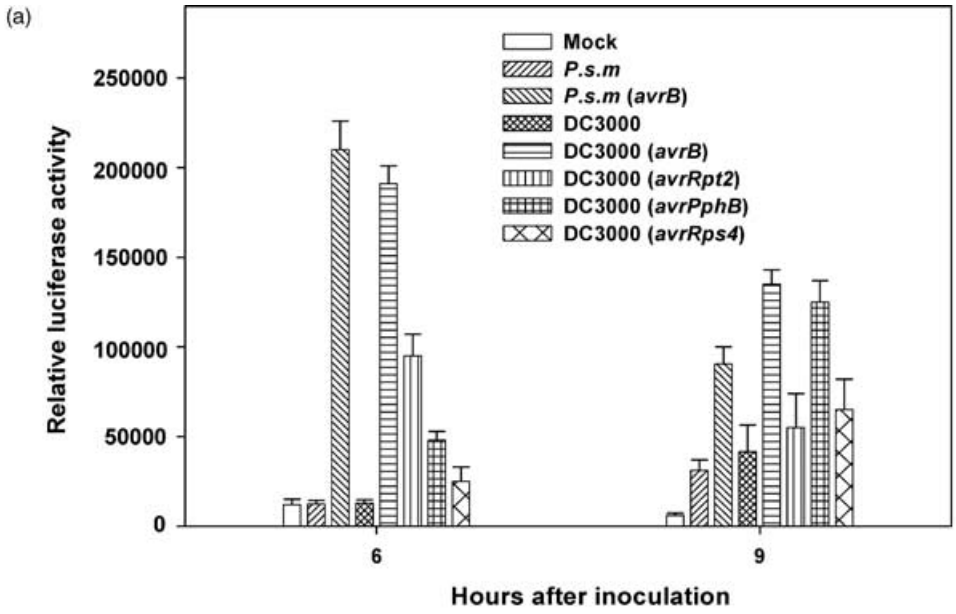
Similarly, we tested if *RPS2* is required for the enhanced *RAP2.6* expression by *avrRpt2. rps2* mutant plants (Yu *et al.*, 1993) were dip-inoculated with DC3000 bacteria with or without *avrRpt2*. Figure 6(b) shows that the presence of *avrRpt2* in DC3000 enhanced the *RAP2.6* expression 2–3 days after inoculation. *rps2* plants expressing the

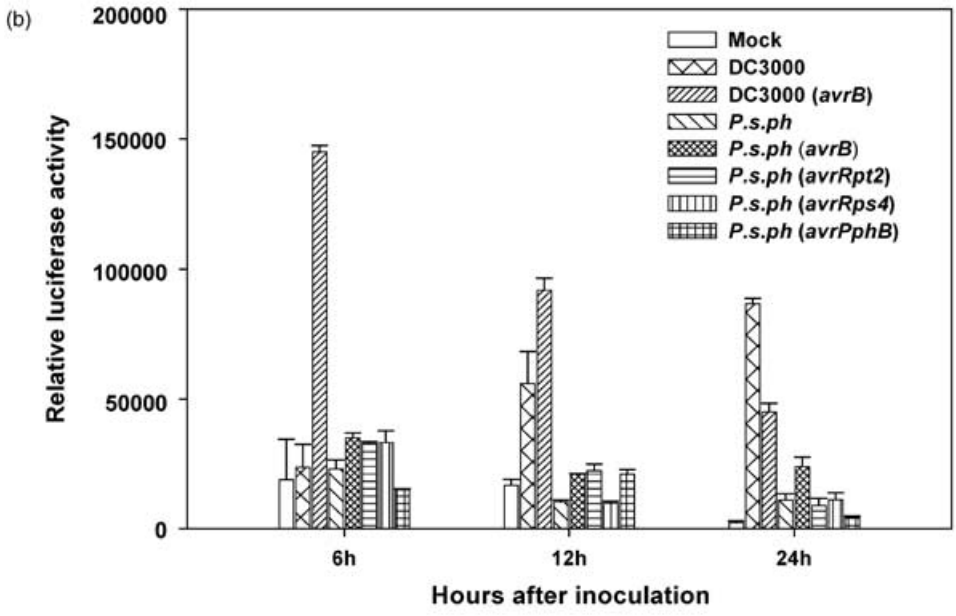
Figure 5. *avr* genes accelerate *RAP2.6* activation.

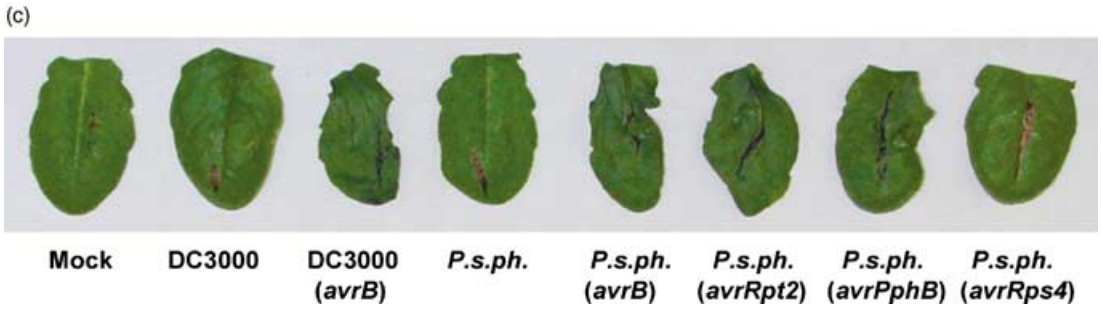
(a) *avr* genes in DC3000 and ES4326 accelerate *RAP2.6* expression. *RAP2.6-LUC* transgenic plants were hand-injected with water (mock), DC3000, DC3000 (*avrB*), DC3000 (*avrRpt2*), DC3000 (*avrPphB*), DC3000 (*avrRps4*), *P. syringae* pv. *maculicola* ES4326 (*P. s. m)*, or *P. s. m.* (*avrB*) at 2×10^6 cfu ml^{-1}. Luciferase activity was determined by using a luminometer at 6 and 9 h after inoculation. Each data point consisted of at least three leaves. Error bars indicate SEs. The experiments were repeated three times with similar results.

(b) *avr* genes in *P. syringae* pv. *phaseolicola* do not activate *RAP2.6* promoter. *RAP2.6-LUC* transgenic plants were hand-injected with water (mock), or 2×10^6 cfu ml^{-1} of DC3000, DC3000 (*avrB*), *P. syringae* pv. *phaseolicola* NPS3121 (*P. s. ph.*), *P. s. ph.* (*avrB*), *P. s. ph.* (*avrRpt2*), *P. s. ph.* (*avrPphB*), or *P. s. ph.* (*avrRps4*). Luciferase activity was determined as in (a). The experiments were repeated three times with similar results.

(c) *avr* genes in *P. syringae* pv. *phaseolicola* induce HR. Plants were inoculated with 1×10^8 cfu ml^{-1} bacteria (same strains as in (b) and photographed 20 h after inoculation.


(a)
Mock
P.s.m
P.s.m (avrB)
DC3000
DC3000 (avrB)
DC3000 (avrRpt2)
DC3000 (avrPphB)
DC3000 (avrRps4)
Relative luciferase activity
250000
200000
150000
100000
50000
0
6
9
Hours after inoculation


(b)
Mock
DC3000
DC3000 (avrB)
P.s.ph
P.s.ph (avrB)
P.s.ph (avrRpt2)
P.s.ph (avrRps4)
P.s.ph (avrPphB)
Relative luciferase activity
200000
150000
100000
50000
0
6h
12h
24h
Hours after inoculation


(c)
Mock
DC3000
DC3000 (avrB)
P.s.ph.
P.s.ph. (avrB)
P.s.ph. (avrRpt2)
P.s.ph. (avrPphB)
P.s.ph. (avrRps4)

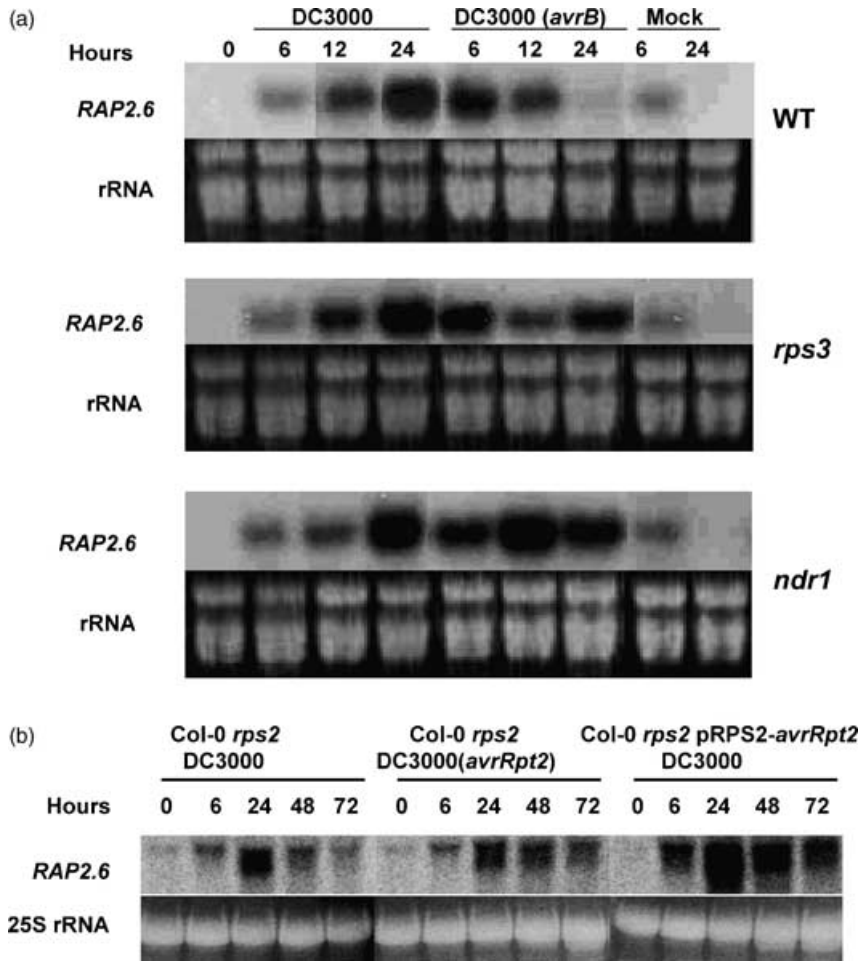


Figure 6. Gene-for-gene resistance is not required for *RAP2.6* induction.
(a) The *RPM1*-specified resistance is not required for *RAP2.6* induction by *avrB*. Wild-type Col-0 (WT), *rps3*, and *ndr1* plants were infiltrated with 10 mM $MgCl_2$ (mock), DC3000, or DC3000 (*avrB*) at 2×10^6 cfu ml^{-1}, and RNA was isolated at the indicated times.
(b) *RPS2* is not required for *RAP2.6* induction by *avrRpt2*. Four- to 5-week-old Col-0 *rps2-101C* plants and Col-0 *rps2-101C* plants carrying the *avrRpt2* transgene under the control of the *RPS2* promoter were dip-inoculated with the indicated bacteria at 5×10^8 cfu ml^{-1} in 10 mM $MgCl_2$ and 0.02% Silwet L-77; leaves were harvested at the indicated time for Northern blot analysis. The Northern blots were hybridized to the *RAP2.6* cDNA probe. The ethidium bromide gel pictures indicate equal loading of RNA samples. The experiments were repeated three times with similar results.

avrRpt2 transgene display enhanced susceptibility to DC3000 (Chen *et al.*, 2000). When inoculated with DC3000 bacteria, these plants showed much greater *RAP2.6* induction than non-transgenic *rps2* plants (Figure 6b). These results demonstrated that *avrRpt2* also enhanced *RAP2.6* expression independent of *RPS2* function.

Interestingly, the strong induction of *RAP2.6* by DC3000 (*avrB*) at 6 h was followed by a dramatic decline, and the transcripts reduced to the basal level after 24 h (Figure 6a). This was not a result of general transcriptional cessation upon HR induction because the expression of PR1 was dramatically induced at 12–48 h (Figure S3). A decline of LUC activity was also seen in *RAP2.6-LUC* plants inoculated with DC3000 (*avrB*) following the strong induction at the 6-h time point (Figure 5). In contrast, a slower but steady induction over the course of 24 h was observed following the DC3000 inoculation in both the Northern analysis and the LUC reporter assay (Figures 5 and 6). The decline of *RAP2.6* expression between 6 and 24 h was dependent on the gene-for-gene resistance because the decline was blocked in both *rps3/rpm1* and *ndr1* plants (Figure 6a). These results were repeatedly observed and suggest that the *avrB-RPM1*-mediated resistance actively suppressed the *RAP2.6* expression, reinforcing the notion that the expression of *RAP2.6* gene is negatively modulated by disease resistance.

Requirement of the JA pathway in RAP2.6 *induction*

The requirement of coronatine production for *RAP2.6* induction by DC3000 suggested the involvement of JA and ethylene signaling because the phytotoxin is thought to mimic both hormones (Bender *et al.*, 1999). An earlier study using microarrays indicated that the JA-signaling mutant *coi1* and ethylene-signaling mutant *ein2* showed partial *RAP2.6* transcript induction 30 h after *P. syringae* pv. *maculicola* inoculation (Chen *et al.*, 2002). It was not clear whether JA and ethylene signaling are required for the bacterial-induced *RAP2.6* expression at earlier time points that are more relevant to bacterial virulence. We examined *RAP2.6* transcript levels in DC3000-inoculated wild-type, *coi1* (Feys *et al.*, 1994), and *ein2-1* (Guzmann and Ecker, 1990) plants. The wild-type plants showed a strong induction of *RAP2.6* 12 h after inoculation. In contrast, the *coi1* plants did not accumulate any detectable transcripts, while

the *ein2* plants showed reduced transcripts (Figure 7a). *P. syringae* pv. *maculicola* also strongly induced *RAP2.6* in wild-type plants 9 and 18 h after inoculation (Figure 7b). In *coi1* plants, *RAP2.6* transcripts were not detectable at 9 h and were only partially induced at 18 h. The *ein2* mutant showed reduced *RAP2.6* transcripts at both time points. The absolute requirement of *COI1* for the bacterial-induced *RAP2.6* expression at early hours prior to bacterial multiplication suggests that the JA signaling is critical for early activities of bacterial virulence factors in host cells.

We next tested if the *avrB*-dependent *RAP2.6* induction also required *COI1* and *EIN2*. As shown in Figure 7(c), while the *RAP2.6* transcripts were strongly induced at 6 h in an *avrB*-dependent manner, they were abolished in *coi1* and reduced in *ein2,* indicating that the AvrB effector required both JA and ethylene pathways to fully activate the *RAP2.6* expression.

JA, ethylene, and SA weakly activate RAP2.6 *promoter*

The apparent involvement of the JA and ethylene pathways in bacterial-induced *RAP2.6* expression prompted us to test if these phytohormones alone were able to induce the *RAP2.6* promoter. Figure 7(d) shows that application of methyl jasmonic acid (MeJA) or ACC clearly activated the promoter within 6 h. The maximal induction occurred at 12 h for MeJA and 24 h for ACC. Interestingly, SA also activated the promoter to a similar level. However, the *RAP2.6* promoter activity induced by these hormones was only about 20% of that induced by bacterial inoculation. This is consistent with the previous microarray study showing that JA only marginally induced the *RAP2.6* transcripts 2–12 h after treatment (Chen *et al.*, 2002).

AvrB enhances RAP2.6 *expression in the absence of coronatine*

The results described above suggested a crucial role of coronatine in *RAP2.6* induction. We therefore tested if coronatine is also required for the enhanced *RAP2.6* expression mediated by *avrB.* The original *avrB* plasmid, which is kanamycin resistant, was modified by integrating a tetracycline resistance transposon and introduced into DC3000 and the COR$^-$ mutant strain DC3682, which is kanamycin resistant. The transposon insertion rendered the plasmid somewhat less effective in *RAP2.6* induction. Nevertheless, the DC3000 strain carrying the new *avrB* plasmid was capable of inducing *RAP2.6-LUC* at 6 h. Surprisingly, the COR$^-$ strain carrying *avrB* repeatedly gave identical LUC induction at 6 h (Figure 8), demonstrating that coronatine is not needed for the AvrB-dependent enhancement of *RAP2.6* expression.

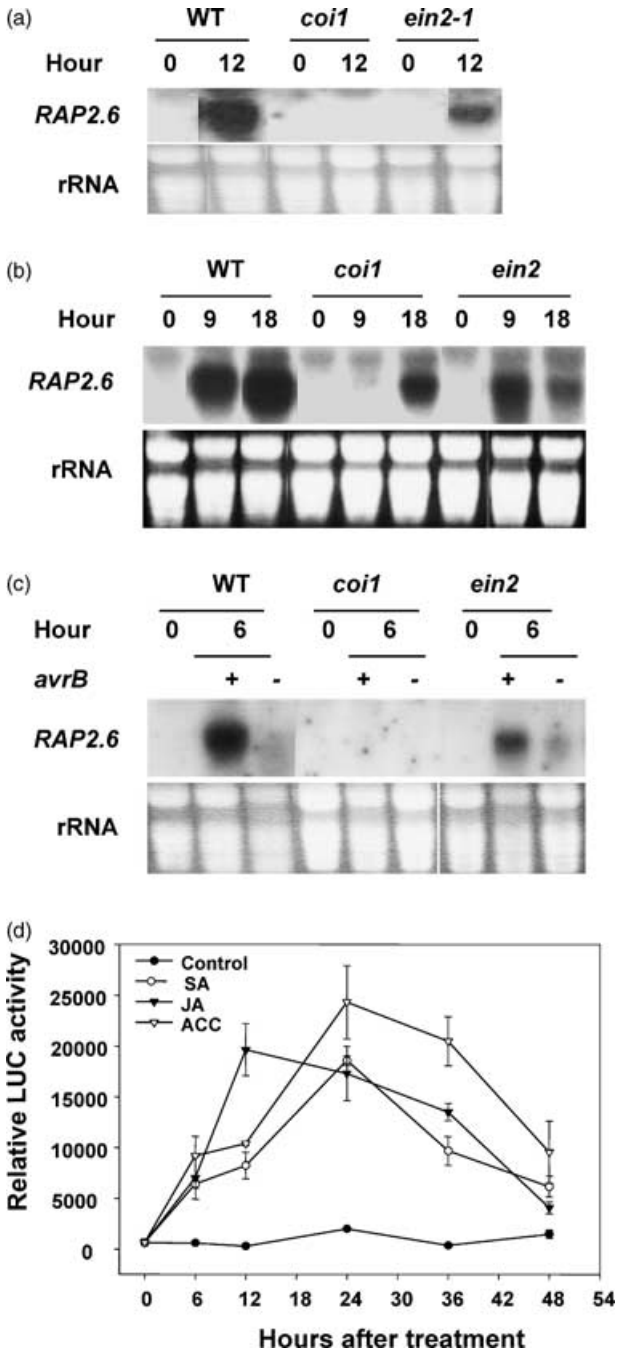


Figure 7. Requirement of JA and ethylene pathways in *RAP2.6* induction.
(a) *RAP2.6* induction in response to DC3000.
(b) *RAP2.6* induction in response to *P. syringae* pv. *maculicola* ES4326.
(c) *RAP2.6* induction in response to DC3000 with (+) or without (–) *avrB*. Wild-type Col-0 (WT), *coi1*, and *ein2* plants were infiltrated with the indicated bacteria at 2×10^6 cfu ml^{-1}, and RNA was isolated at the indicated times after inoculation. The Northern blots were hybridized with the *RAP2.6* cDNA probe. The ethidium bromide gel pictures indicate equal loading of RNA samples. The Northern experiments were repeated two times with similar results.
(d) Exogenous JA, ethylene, and SA weakly activate *RAP2.6* promoter. *RAP2.6-LUC* plants were dipped in a 0.015% Silwet 77 solution containing 0.5 mM SA, 0.5 mM ACC, or 50 μM MeJA. The luciferase activity was measured at the indicated times with a luminometer. Each data point consisted of four leaves. Error bars indicate SEs. Similar results were obtained in two independent experiments.

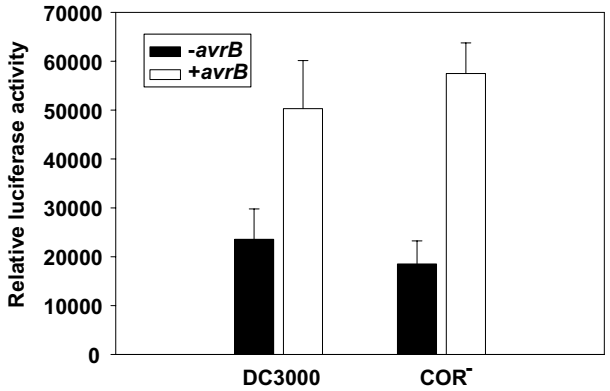


Figure 8. AvrB enhances *RAP2.6* expression independent of coronatine. *RAP2.6-LUC* transgenic plants were infiltrated with the DC3000 and COR$^-$ mutant strains with or without the *avrB* gene at 2×10^6 cfu ml^{-1}. Relative luciferase activity was measured 6 h after infiltration. The experiment was repeated four times with similar results.

Discussion

In this report, we demonstrate that the *RAP2.6-LUC* reporter gene was a reliable indicator for activities associated with virulence factors defined by at least five DC3000 mutants, including those defective in the biosynthesis of the phytotoxin coronatine, TTSS, and several effector genes. In addition, *RAP2.6-LUC* also reported the activity of three *avr* genes, *avrB, avrRpt2,* and *avrPphB*, when added into DC3000. At least the *avrB*-mediated *RAP2.6* activation was independent of gene-for-gene resistance, suggesting that the activation is related to the virulence activity. Furthermore, we suggest that the JA-signaling pathway plays a large role at the early stages of pathogenesis and that at least some type III effectors require the JA-signaling pathway for function.

Traditionally, bacterial virulence or plant susceptibility is measured by visual disease symptoms and bacterial multiplication in the plant, often monitored at a late stage of disease development, and is difficult to quantify. The use of the *RAP2.6-LUC* reporter provided a highly sensitive and reliable measurement of bacterial activity in the plant that can be easily visualized at the early hours during infection, demonstrating its utility in bacterial pathogenesis studies. The ability to monitor the early cellular activities of single bacterial effectors with ease will significantly facilitate the molecular dissection of their functions in the host cell.

RAP2.6 belongs to the ERF family of plant-specific transcription factors, many of which are pathogen inducible at the transcript level (Gu *et al.*, 2000; Onate-Sanchez and Singh, 2002; Thara *et al.*, 1999; Zhou *et al.*, 1997; R. Warren and J.Z., unpublished results). Several members of the ERF family, including the tobacco Tsi1 and tomato Pti4, Pti5, and Pti6, play a positive role in plant defense against *P. syringae* (Gu *et al.*, 2002; He *et al.*, 2001; Park *et al.*, 2001). Overexpression of these genes enhances defense gene expression and disease resistance to *Pseudomonas* bacteria. However, not all ERFs function positively in bacterial resistance. For example, the *Arabidopsis ERF1* gene appears to play a negative role in resistance to *Pseudomonas* bacteria. Overexpression of *ERF1* enhances susceptibility to *P. syringae,* although it increases resistance to fungal pathogens (Berrocal-Lobo *et al.*, 2002). The function of *RAP2.6* in disease susceptibility remains to be determined, but its expression appeared to be associated positively with disease susceptibility and negatively with disease resistance. The *RAP2.6* expression was stronger in the *npr1* and *pad4* mutants that are more susceptible to DC3000 than wild-type plants (Figure 1; Chen *et al.*, 2002). Interestingly, *P. syringae* pv. *tabaci* that was unable to induce *RAP2.6* transcripts in wild-type plants strongly induced *RAP2.6* in the *nho1* mutant (data not shown). *nho1* compromises *Arabidopsis* resistance to several non-host *Pseudomonas* bacteria including *P. syringae* pv. *tabaci* (Kang *et al.*, 2003; Lu *et al.*, 2001). Conversely, the *cpr5* mutant that is more resistant to DC3000 than wild-type plants showed diminished *RAP2.6* transcript induction during infection. Furthermore, the *RAP2.6* expression was suppressed upon the activation of gene-for-gene resistance. We have shown recently that plants activate *NHO1* gene expression for general resistance, but virulent bacteria actively suppress this to promote parasitism (Kang *et al.*, 2003). While the biological function of *RAP2.6* is yet to be tested genetically, it is intriguing to speculate that the activation of *RAP2.6* may be also modulated by bacteria to promote parasitism.

It is possible that the activation of the *RAP2.6* promoter reflects stress or damage caused by virulent bacteria. However, several lines of evidence argue against this. The *RAP2.6* promoter activation occurred prior to bacterial multiplication. DC3000 activated the *RAP2.6* promoter as early as 9 h after inoculation. The presence of *avrB, avrRpt2,* and *avrPphB* accelerated this induction by at least 3 h. The bacterial multiplication, however, did not occur in the first 12 h after inoculation (Figure 3b), and the disease symptoms did not develop until 3–4 days after DC3000 inoculation at the concentration used. While it had the greatest impact on *RAP2.6* induction, the coronatine mutation had smaller effects on disease severity than did the *hrcC*$^-$ or Δ*CEL* mutation. Of the 14 individual effector genes tested, only one (*avrRpt2*) had a visible virulence effect on *Arabidopsis* (Chen *et al.*, 2000), but at least five were able to stimulate the *RAP2.6* promoter when expressed in DC3000. Therefore, the activation of the *RAP2.6* promoter did not appear to be a result of bacterial growth or disease symptom development. Instead, it likely reflects the early cellular activity associated with certain virulence factors.

Coronatine plays an important role in *RAP2.6* induction. The COR$^-$ mutant was completely unable to induce *RAP2.6,* indicating that coronatine production was essential for the

wild-type DC3000 to activate *RAP2.6* transcription. This may explain why both DC3000 and ES4326 strongly induced *RAP2.6,* whereas *P. syringae* pv. *tabaci* and *P. syringae* pv. *phaseolicola* failed to do so. The first two strains produce coronatine, whereas the latter two do not (Bender *et al.*, 1999).

However, coronatine production by itself may not be sufficient to induce *RAP2.6* because *P. syringae* pv. *tomato* strain T1 that is fully capable of producing coronatine was unable to activate the *RAP2.6* promoter. Similarly, the *hrcC*$^{-}$ mutant produced more coronatine than does the wild-type DC3000 (Penaloza-Vazquez *et al.*, 2000), but it induced very poorly the *RAP2.6* promoter. These data suggested that, in addition to coronatine, type III effectors were essential for *RAP2.6* activation. This was further demonstrated by the complementation of the *COR*$^{-}$ and *hrcC*$^{-}$ mutants for *RAP2.6* activation. Alternatively, these strains did not make sufficient amount of coronatine because of the inability to grow in *Arabidopsis*. Interestingly, the requirement of coronatine for the wild-type DC3000 to induce *RAP2.6* can be obviated by the addition of *avrB,* suggesting that the latter may function, at least in part, to mimic the effects of coronatine in the host cell.

The role of type III effectors in *RAP2.6* induction was directly supported by the study of five individual effector genes, including *avrPphE*$_{Pto}$*, hopPtoK, avrB, avrRpt2*, and *avrPphB.* AvrPphB is a cysteine protease that cleaves the *Arabidopsis* protein kinase PBS1, and that is required for HR on RPS5-resistant plants (Shao *et al.*, 2002, 2003). AvrRpt2 also appears to act as a cysteine protease to trigger resistance in RPS2-resistant plants (Axtell *et al.*, 2003). AvrPphE and AvrB do not have detectable virulence function in *Arabidopsis* but are known to function as virulence factors in bean and soybean plants, respectively (Ashfield *et al.*, 1995; Stevens *et al.*, 1998). The *ΔCEL* mutant contains a deletion spanning at least six genes, *avrE,* ORF2, *hopPtoM*, *ShcM, hrpW*, and *hopPtoA1*, encoding one harpin and three effectors. (Alfano *et al.*, 2000; Badel *et al.*, 2003; Collmer *et al.*, 2002). *hopPtoM* is responsible for lesion formation on *Arabidopsis* and partially accounts for the *ΔCEL* phenotype (Badel *et al.*, 2003). The biochemical functions for the other effectors tested in this study, however, remain unknown. All of these effectors contributed to *RAP2.6* induction. At least *avrB* and *avrRpt2* did not require gene-for-gene recognition for *RAP2.6* induction, suggesting a connection to virulence activities of the effectors. These findings are reminiscent of the RPS2-independent degradation of the RIN4 protein mediated by AvrRpt2 (Axtell and Staskawicz, 2003; Axtell *et al.*, 2003; Mackey *et al.*, 2003) and the RPS5-independent cleavage of the PBS1 kinase by AvrPphB (Shao *et al.*, 2003).

It should be pointed out that none of the effectors tested in this report was sufficient for *RAP2.6* induction on its own. Direct expression of AvrRpt2 in *Arabidopsis* did not result in constitutive *RAP2.6* expression. Similarly, AvrB, AvrRpt2, AvrPphB, and AvrRps4 delivered by *P. syringae* pv. *phaseolicola* were insufficient to induce *RAP2.6.* It is possible that the presence of other, compatible effectors or coronatine was necessary for *RAP2.6* induction by these effectors. This is different from AvrPto that, when overexpressed in the plant, induces/suppresses plant gene expression on its own (Hauck *et al.*, 2003). Perhaps, the difference can be explained by different expression levels of the effector genes.

The JA-signaling pathway may be an important avenue for *Pseudomonas* bacteria to promote parasitism. Consistent with a role of JA in susceptibility, the JA-insensitive mutant *coi1* is significantly more resistant to *Pseudomonas* bacteria (Feys *et al.*, 1994; Kloek *et al.*, 2001). We showed recently that COI1 is also required by DC3000 bacteria to actively suppress the *NHO1* gene expression, which is necessary for resistance to non-host *Pseudomonas* bacteria (Kang *et al.*, 2003). Type III effectors and coronatine are likely to interact with the JA- and ethylene-signaling pathways to alter plant cellular activities. Indeed, the type III and coronatine-dependent *RAP2.6* induction required *COI1* and, to a lesser extent, *EIN2.* Taken together, the results support that different effectors and cornatine coordinately augment the JA- and ethylene-mediated pathways to promote parasitism.

Experimental procedures

Plants, bacterial strains, and inoculation

Arabidopsis plants used in this study were wild-type ecotype Col-0 and mutants *rpm1, ndr1, rps2-101C, rps2-101C* with *pRPS2-avrRpt2* transgene, and *coi1-1* (Century *et al.*, 1995; Chen *et al.*, 2000; Feys *et al.*, 1994; Innes *et al.*, 1993; Yu *et al.*, 1993). Homozygous *coi1* plants were identified by using a CAPs marker, as described by Xie *et al.* (1998). All plants were grown in a growth chamber at 21°C with a 10 h per day photoperiod for 35 days. Wild-type *P. syringae* strains used in this study included: *P. sringae* pv. *tomato* DC3000 (Cuppels, 1986); *P. syringae* pv. *tomato* T1 (Ronald *et al.*, 1992); *P. syringae* pv. *maculicola* ES4326 (Dong *et al.*, 1991); *P. syringae* pv. *phaseolicola* NPS3121 (Lindgren *et al.*, 1986); and *P. syringae* pv. *tabaci* R11528 (Willis *et al.*, 1988). DC3000 mutants included: DC3682 (COR^{-}; Ma *et al.*, 1991); *hrcC*$^{-}$(Yuan and He, 1996); *ΔCEL* (Alfano *et al.*, 2000); *avrPphE*$_{Pto}$*, avrPpiB1*$_{Pto}$,, and *avrPpiB2*$_{Pto}$; *hopPsyA*$_{Pto}$*, hopPtoD1*, and *hopPtoD2*; *hopPtoC, hopPtoK, hopPtoJ*, and *hopPtoF* (J.R.A., manuscript submitted). Avirulence genes, *avrB*, *avrRpt2*, *avrPphB*, and *avrRps4* (Gassmann *et al.*, 1999; Innes *et al.*, 1993; Warren *et al.*, 1998; Whalen *et al.*, 1991), were also incorporated into DC3000, ES4326, and NPS3121 as indicated. To introduce *avrB* into DC3682, a tetracycline *R* gene was inserted into the plasmid carrying *avrB* by using EZ::TN^{TM} < TET-1 > insertion kit (Epicentre, Madison, WI, USA). Bacteria were grown overnight at room temperature in King's B medium containing the appropriate antibiotics, precipitated, washed two times, and diluted to the desired concentration with 10 mM $MgCl_2$ for plant inoculation prior to Northern analysis. For luciferase activity assay and bacterial growth assay, the bacteria were diluted to 2×10^6 cfu ml^{-1} in H_2O supplemented with

0.1 mM luciferin and syringe-infiltrated into leaves. Each data point consisted of at least three replicates. All experiments have been repeated at least two times with similar results.

Construction of RAP2.6-LUC *and* Arabidopsis *transformation*

The *RAP2.6* promoter fragment was PCR-amplified by using the primers, 5′-AAAAGCTTCCTAGATACATACAGCGAG-3′ and 5′-AAGATATCTTGCGGTGGTAGACAAGTTG-3′, and Col-0 genomic DNA as the template. The promoter fragment was digested with *Hin*dIII and *Eco*RV and placed upstream of the firefly luciferase gene *LUC* (Promega, Madison, WI, USA). The chimeric DNA was inserted between *Hin*dIII and *Sac*I sites of pBI121 to form the *pRAP2.6-LUC* construct. This construct was introduced into *Agrobacterium tumefaciens* strain LBA4404 and transformed into Col-0 by floral dip (Clough and Bent, 1998). The transgene copy number was determined by Southern blot and segregation ratio of kanamycin resistance in T_2 seedlings.

CCD imaging and luminometer assay for Luciferase activity assays

Arabidopsis leaves were sprayed with 1 mM of the luciferase substrate luciferin supplemented with 0.01% Triton X-100. The leaves were kept in the dark for 6 min before luminescence images were captured in 1-min exposures by using an LN/CCD low-light imaging system (RoperScientific, Trenton, NJ, USA) and the WINVIEW imaging software.

Quantitative luciferase assay was performed by using Luciferase Assay System (Promega, Madison, WI, USA) and a microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA) following the manufacturer's instructions.

RNA blot analysis and phytohormone treatment

Leaf tissue was collected either before or after inoculation at the indicated time points. RNA extraction and RNA gel blot analysis were performed as described by Goldsbrough *et al*. (1990). The SA, JA, and ACC treatments were carried out according to Ton *et al*. (2002).

Acknowledgements

The authors thank Drs Frank White and Scot Hulbert for critical review of the manuscript and Douglas Baker for excellent technical assistance. The authors are also grateful to Drs Diane Cuppels and Carol Bender for providing DC3682, and Dr Alan Collmer for sharing results prior to publication. B.N.K. was supported by a USDA grant (2002-35319-12728). J.R.A. and X.T. were supported by a NSF Plant Genome grant (DBI0077622). J.M.Z. was supported by a NSF grant (MCB9808701). This is a contribution of Kansas Agriculture Experimental Station (#03-397-J).

Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ1986/TPJ1986sm.htm

Figure S1. Bacterial growth assay of DC3000, *COR*$^-$, *hrcC*$^-$, and *ΔCEL* mutants on Col-0 plants.

Figure S2. Bacterial growth assay of DC3000, *hopPtoK*$^-$, and *avrPphR*$_{Pto}^-$ mutants on Col-0 plants.

Figure S3. *PR1* expression in *Arabidopsis* plants upon bacterial infection. Col-0 plants were infiltrated with DC3000 and DC3000(*avrB*) at 2×10^6 cfu ml^{-1}. RNA was isolated from leaves at the indicated times, and RNA blots were hybridized with *PR1* probe. Ethidium bromide gel picture indicate equal loading of RNA.

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