The plant signal salicylic acid shuts down expression of the *vir* regulon and activates quormone-quenching genes in *Agrobacterium*

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Contributed by Eugene W. Nester, May 24, 2007 (sent for review April 13, 2007)

Agrobacterium tumefaciens **is capable of transferring and integrating an oncogenic T-DNA (transferred DNA) from its tumor-inducing (Ti) plasmid into dicotyledonous plants. This transfer requires that the virulence genes (*vir* regulon) be induced by plant signals such as acetosyringone in an acidic environment. Salicylic acid (SA) is a key signal molecule in regulating plant defense against pathogens. However, how SA influences *Agrobacterium* and its interactions with plants is poorly understood. Here we show that SA can directly shut down the expression of the *vir* regulon. SA specifically inhibited the expression of the *Agrobacterium virA/G* two-component regulatory system that tightly controls the expression of the *vir* regulon including the *repABC* operon on the Ti plasmid. We provide evidence suggesting that SA attenuates the function of the VirA kinase domain. Independent of its effect on the *vir* regulon, SA up-regulated the *attKLM* operon, which functions in degrading the bacterial quormone *N*-acylhomoserine lactone. Plants defective in SA accumulation were more susceptible to *Agrobacterium* infection, whereas plants overproducing SA were relatively recalcitrant to tumor formation. Our results illustrate that SA, besides its well known function in regulating plant defense, can also interfere directly with several aspects of the *Agrobacterium* infection process.**

two-component system | tumorigenesis | defense response | rhizosphere | plant–microbe interaction

Agrobacterium tumefaciens, a member of the α-*Proteobacteria*, can transfer and integrate an oncogenic T-DNA (transferred DNA) from its tumor-inducing (Ti) plasmid into dicotyledonous plants, leading to the formation of crown gall tumors (1). This unique ability to transfer DNA forms the basis of plant molecular genetics. T-DNA transfer requires activation of the *vir* regulon on the Ti plasmid (1). *vir* genes are activated at an acidic pH, pH 5.5–6.0, which approximates the pH of the rhizosphere (2). Under acidic conditions, plant phenolic compounds together with many monosaccharide components of the plant cell wall are recognized by the sensor protein of a two-component regulatory system, VirA. After autophosphorylation, the VirA protein transfers the phosphate to the transcriptional regulator, VirG, which then binds to a specific 12-bp sequence (*vir* box) upstream of each of the Ti plasmid-encoded *vir* operons and promotes their transcription (1, 3). The plant signals also activate the transcription of the *virA/G* regulatory system. The 30 known *vir* genes include a gene, *virD2*, encoding an endonuclease that recognizes and cleaves the border sequences of the T-DNA, thereby releasing a single-stranded DNA molecule. This DNA (the T-strand) is exported through a type IV secretion system encoded by the *virB* operon (1). In addition to the *vir* genes, the transcription of the *repABC* operon that controls the copy number of the Ti plasmid is also activated by the VirA/G system (4).

The VirA/G system has been studied extensively (1). VirA exists as a dimer whose formation is independent of plant signals. The VirA protein is composed of four domains: the periplasmic domain, which binds the sugar-binding protein ChvE with its associated sugars and also detects acidic pH; the linker domain, which most likely interacts directly with the phenolic signal, such as acetosyringone; the kinase domain, involved in the autophosphorylation of the conserved histidine moiety; and the C-terminal receiver domain, whose function is unclear (5, 6).

In addition to the Ti plasmid-encoded *vir* genes, a number of chromosomally encoded genes, termed *chv*, are also important for virulence (1, 3). These genes include *chvA* and *chvB*, which are required for the attachment of *Agrobacterium* to plant cells. One of the most intensively studied is *chvE* (1). The ChvE protein binds a number of different sugars in the rhizosphere and then interacts with the periplasmic region of the VirA protein. The ChvG/I two-component system is required for *virG* gene expression and for growth under acidic conditions (1). None of the chromosomally encoded *chv* genes has a *vir* box in its upstream sequences, nor are they regulated by the VirA/G system.

Salicylic acid (SA), a plant phenolic metabolite, is a key signal molecule in regulating plant defense in response to a wide variety of pathogens (7–9). Upon infection, SA triggers either a localized or systemic acquired resistance response in which the plant gains long-lived resistance to pathogens (10). Studies on SA function have been focused primarily on defense mechanisms within the plant (10). However, some evidence suggests that SA may also directly affect bacteria. SA was shown to down-regulate fitness and virulence factor production in *Pseudomonas aeruginosa* PA14 (7). At concentrations that did not inhibit growth, SA also affected bacterial attachment and biofilm formation in this organism (7).

In this work, we demonstrate that SA directly affects the *Agrobacterium* infection process by inhibiting the induction of *vir* genes at concentrations that have little effect on growth. This phenolic compound also induces the expression of a lactonase, which degrades the quormone *N*-acylhomoserine lactone. Mutants and transgenic *Arabidopsis* plants whose metabolism of SA is modified show the predicted alterations in susceptibility to infection by *Agrobacterium*.

Author contributions: Z.-C.Y., P.L., and E.W.N. designed research; Z.-C.Y., M.P.E., P.L., P.S., A.A.W., and E.R. performed research; Z.-C.Y. and K.K. analyzed data; and Z.-C.Y., L.M.B., A.N.B., and E.W.N. wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: IAA, indoleacetic acid; PR, pathogenesis-related; SA, salicylic acid; T-DNA, portion of the Ti (tumor-inducing) plasmid that is transferred to plant cells.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0704866104/DC1.

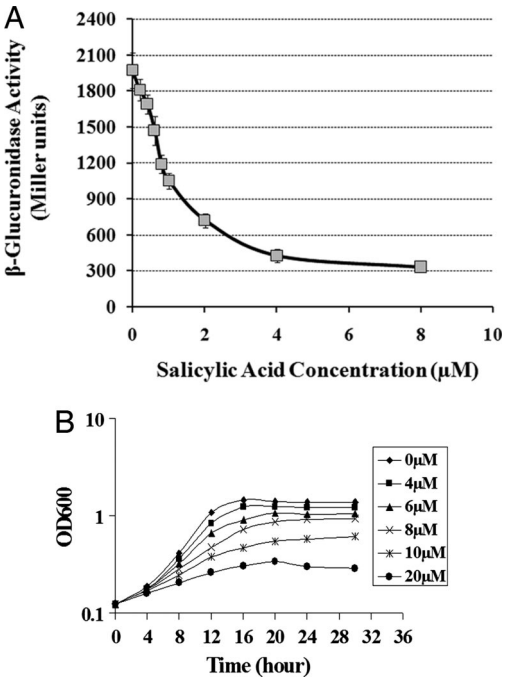

Fig. 1. SA inhibits *vir* gene induction and, at higher concentrations, bacterial growth. (*A*) Effect of SA on *Agrobacterium vir* gene expression. Expression of a plasmid-borne *virB1*::*gusA* transcriptional gene fusion in *Agrobacterium* C58 grown in induction medium with acetosyringone (100 μM), carbenicillin (100 μg/ml), and various concentrations of SA (0–8 μM) is shown. After 16 h, β-glucuronidase activity was measured as described in *Materials and Methods*. (*B*) Effect of SA on *Agrobacterium* growth. *Agrobacterium* growth under various concentrations of SA (0–20 μM) is shown. Cells were grown in acidified AB minimal medium (pH 5.5) supplied with various concentrations of SA. Readings at A_{600} (OD600) are the mean of three independent experiments.

Results

SA Inhibits *vir* Gene Expression. Numerous studies have shown that small molecules of plant origin play important roles in the interaction of *Agrobacterium* with plants. Phenolics, such as the many *vir* gene-inducing compounds, seem to be especially important in this regard (1). Indoleacetic acid (IAA), a plant hormone produced by T-DNA-encoded enzymes in transformed plants, can shut down *vir* gene induction (11). In the course of screening other phenolic compounds for their effect on *vir* gene induction, we tested SA, a phenolic compound important in plant defense. The expression of *vir* genes was compared in wild-type (WT) strain C58 grown in *vir* gene-inducing medium (pH 5.5) supplemented with varying concentrations of SA. The data in Fig. 1*A* show that SA significantly inhibited the expression of *vir* genes as monitored by assaying the promoter activity of a *virB1*::*gusA* transcriptional gene fusion. Two μM SA inhibited *virB1* expression by >50%, and 8 μM SA exerted ≈90% inhibition. Moreover, at higher concentrations (>10 μM), SA also inhibited *Agrobacterium* growth significantly (Fig. 1*B*). The inhibition of *vir* gene expression and growth occurred at an acidic pH (pH 5.5), but at pH 7, growth was not inhibited (data not shown). Because the rhizosphere is typically acidic at the site of *Agrobacterium*–plant interactions, these data suggest that SA, under biologically relevant conditions, can directly affect the interaction of *Agrobacterium* with the plant either by repressing *vir* gene expression or, at slightly higher concentrations, by inhibiting bacterial growth. The observation that the plotted data (Fig. 1*A*) fit nicely to a sigmoidal logistic model suggests that the interaction of SA with its target is a simple, single-site interaction with no cooperativity and is specific and not the result of nonspecific mechanisms.

The two-component regulatory system, VirA/G, controls the expression of the *vir* regulon including *virA*/*virG* itself (1). To explore whether other members of the *vir* regulon were also inhibited by SA, we examined its effects on the expression of *virA*, *virG*, *virD*, *virE*, *virH*, as well as *tzs* (another VirA/G-regulated gene) (3). We also examined the expression of *chvA*, *chvD*, *chvE*, *chvG*, and *chvI* genes with or without SA treatment. We found that SA inhibited the expression of all of these *virA*/*G*-regulated *vir* genes but not *chv* genes as monitored by transcriptional gene fusions (Table 1). It seems highly unlikely that the inhibition of *vir* gene expression was the result of internal acidification of the bacteria because SA has a pK_a of 2.97, whereas the induction medium was pH 5.5. Although the previous experiments were performed on the C58 strain, which induces the synthesis of the opine nopaline in tumors, the same results were also observed in strain A6, which induces octopine synthesis (data not shown). These results suggest that: (*i*) SA can shut down the transfer of T-DNA and virulence proteins by inhibiting the expression of the *vir* regulon but not *chv* genes, and (*ii*) this inhibition is mediated by repressing the expression of *virA*/*G* itself.

Table 1. Effects of SA on expression of *Agrobacterium vir* and *chv* genes

Gene fusion	AS, 0 μM	AS, 100 μM	AS, 100 μM SA, 8 μM
virD1::*gusA*	193	4,215	439
virE0::*gusA*	261	4,927	523
virH1::*gusA*	349	2,782	531
tzs::*gusA*	247	1,658	455
virA::*gusA*	430	1,894	784
virG::*lacZ*	134	470	210
chvA::*gusA*	1,704	1,688	1,722
chvD::*gusA*	1,770	1,902	1,686
chvE::*gusA*	1,732	1,626	1,695
chvG::*gusA*	660	601	692
chvI::*gusA*	1,754	2,028	1,790

Agrobacterium cells harboring the indicated fusion plasmids were grown for 16 h in induction medium (pH 5.5) with or without 8 μM SA. The β-galactosidase or β-glucuronidase activity was determined as described in *Materials and Methods*. AS, acetosyringone.

Several SA derivatives were also tested for their effects on the expression of *vir* genes. At concentrations of 10 μM, both acetyl-SA (aspirin) and methyl-SA inhibited the expression of the *virB1* gene, whereas salicylamide and benzoic acid had no effect (data not shown). We also tested catechol because SA is converted to catechol by salicylate hydroxylase (encoded by Atu1574) (12). Even at concentrations of 200 μM, catechol had no effect on the expression of *vir* genes, nor did it affect bacterial growth either at pH 5.5 or 7 (data not shown). Jasmonic acid, which also plays an important role in modulating plant defenses (10), had no effect on *vir* gene expresssion (data not shown).

Microarray Analysis of Cells Grown with SA. To gain a global view of the effect of SA on *Agrobacterium* gene expression, we compared the transcriptome of C58 cells cultured at pH 5.5 (+100 μM acetosyringone) in the presence or absence of 6 μM SA for 6 h. This concentration of SA had no significant effect on cell growth under the conditions in which the cells were grown for RNA isolation (see *Materials and Methods*). Microarray analysis revealed that the transcription of 49 genes was inhibited by SA at least 2-fold. These genes included the 30 *vir* regulon members previously identified as being under the control of the VirA/G system (Table 2). Interestingly, none of the *chv* genes such as *chvA*, *B*, *D*, and *E*, which are not regulated by the *virA*/*G* system nor induced by phenolic compounds, was affected by SA, nor was the expression of another two-component system important in virulence, *chvG*/*I*. These data confirmed the gene expression data obtained by using transcriptional gene fusions (Table 1) and established that SA inhibited the

Table 2. Selected list of microarray data for the *vir* regulon, *rep* genes, *chv* genes, and *attKLM* operon exhibiting altered gene expression in cells grown with salicylic acid (6 μM) for 7 h

Gene	Ratio, log2	Gene	Ratio, log2	Gene	Ratio, log2
tzs	−2.63	*virC2*	−1.39	*repA*	−1.25
virA	−1.25	*virD1*	−2.46	*repB*	−1.18
virB1	−3.48	*virD2*	−1.66		
virB2	−3.27	*virD3*	−2.16	*chvA*	0.21
virB3	−3.19	*virD4*	−1.35	*chvB*	0.21
virB4	−3.23	*virE0*	−2.66	*chvD*	0.07
virB5	−3.36	*virE1*	−3.08	*chvE*	−0.14
virB6	−2.06	*virE2*	−2.93	*chvH*	0.61
virB7	−1.82	*virE3*	−1.97	*chvG*	0.32
virB8	−1.68	*virF*	−1.94	*chvI*	−0.2
virB9	−2.21	*virG*	−0.98		
virB10	−2.89	*virH1*	−2.21	*attK*	1.68
virB11	−2.17	*virH2*	−2.28	*attL*	1.85
virC1	−1.19	*virK*	−2.54	*attM*	1.65

The ratio, log2, shows genes inhibited (negative) or induced (positive) by 6 μM SA.

expression of the *vir* regulon, but not other processes required for crown gall formation in which *chv* genes play essential roles (1, 3).

The microarray analysis also revealed that SA repressed the expression of the *repABC* operon, which encodes the Ti plasmid replication and partitioning machinery (Table 2). Previous studies showed that the expression of this operon is under dual regulation. It is a member of the *vir* regulon induced by acetosyringone (4) and is also induced by the quorum-sensing signal 3OC8-HSL (13). The induction of the *repABC* operon elevates the Ti plasmid copy number, thereby increasing the number of copies of T-DNA and genes encoding the T-DNA export machinery located on the Ti plasmid. This action could enhance T-DNA transfer as well as conjugative transfer of the Ti plasmid (4, 13). The observation that SA repressed the expression of this *repABC* operon suggests that this plant defense molecule can reduce the increase in copy number of the Ti plasmid associated with *vir* gene induction.

Microarray analysis further revealed that 36 genes were induced by SA at least 2-fold. Perhaps of greatest interest is the observation that SA stimulated the expression of the *attKLM* operon, which encodes a quormone degradation system (14). In *Agrobacterium*, the lactonase encoded by *attM* can hydrolyze the quorum-sensing signal 3OC8-HSL (14). Acylated homoserine lactones are a prevalent class of extracellular bacterial signals implicated in several important cell processes, including bacterial virulence (15). Recent studies have shown that γ-aminobutyric acid (GABA) and succinic semialdehyde activate the expression of the *attKLM* operon, resulting in quorum-sensing signal decay and reduced *Agrobacterium* virulence (16, 17). The microarray data were confirmed by demonstrating that 8 μM SA induced the expression of an *attKLM*::*lacZ* gene fusion (Fig. 2). Moreover, the SA-induced *attKLM* expression only occurred under acidic conditions and was not observed when cells were grown at pH 7 (Fig. 2). Acetosyringone did not affect the induction of the *attKLM* operon by SA (data not shown), which suggests that induction of this quormone-quenching system and inhibition of the *vir* regulon by SA are independent. The SA-activated expression of the *attKLM* operon is currently under investigation.

SA-Inhibited *vir* Gene Expression Can Be Rescued by Either *virA* or *virG* Mutants That Activate *vir* Gene Induction Independent of Acetosyringone. An *Agrobacterium virA* mutant (G665D) has been isolated that does not require acetosyringone and monosaccharides to activate the *vir* regulon (18). A point mutation in *virG* (N54D) also renders *vir* regulon transcription independent of a phenolic signal and monosaccharides as well as VirA (19, 20). Although both the

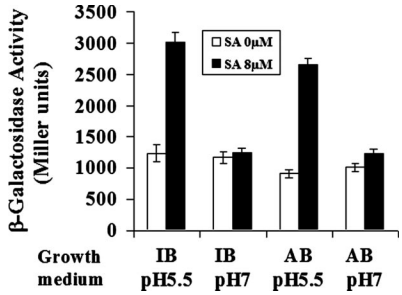

Fig. 2. Effect of SA on the expression of the *attKLM* operon. Cells harboring the *attKLM*::*lacZ* gene fusion (16) were grown for 20 h with 0 μM or 8 μM SA at the indicated pH. IB, inducing medium containing 100 μM acetosyringone; AB, inducing medium without acetosyringone.

virA (G665D) and *virG* (N54D) mutants activate *vir* gene induction constitutively, acidic conditions are still required for the induction. To gain insight into the site of inhibition of the *vir* regulon by SA, we assessed whether *vir* gene expression in cells containing either the *virA* (G665D) or *virG* (N54D) was still inhibited by SA. The data in Fig. 3 show that the inhibitory effect of SA on *vir* gene expression was completely abolished in cells containing either a *virA* (G665D) or *virG* (N54D) plasmid. These observations further support the previous conclusion that SA inhibits *vir* regulon expression through the *virA*/*G* regulatory system. In addition, these results implicate both acetosyringone and VirA in the SA-inhibited expression of the *vir* regulon because neither acetosyringone-independent (either *virA* G665D or *virG* N54D mutants) nor *virA*-independent (*virG* N54D mutant) *vir* gene expression was inhibited by SA (18–20).

SA-Inhibited *vir* Gene Expression Can Be Partially Overcome by Increased Levels of Acetosyringone. During *Agrobacterium*–plant host interactions, plant-released phenolic compounds such as acetosyringone interact with the sensory histidine kinase VirA to initiate the microbe–plant signaling process (1). To decipher the relationship between the plant-synthesized inhibitory signal molecule SA and the plant-released *vir* regulon-inducer acetosyringone, we assayed the degree of *vir* gene inhibition by SA when the levels of acetosyringone were modulated. Increasing the acetosyringone level significantly attenuated the inhibitory effects of SA on *vir* gene expression. With 4 μM SA, *vir* gene expression was inhibited >80% at 0.1 mM acetosyringone; in contrast, expression was only diminished 45% at 1.6 mM acetosyringone (Fig. 4). These data, together with the above observation that both acetosyringone-independent and *virA*-independent *vir* gene expression were not inhibited by SA, suggest that SA interferes with the activation of the VirA protein by acetosyringone.

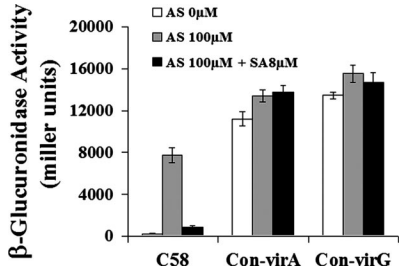

Fig. 3. Constitutively active *virA* or *virG* rescues *vir* gene expression inhibited by SA. Cells containing a WT *virA*/*virG* allele and a *virB1*::*gusA* fusion plasmid are indicated as C58. The constitutive *virA* plasmid (indicated as Con-virA) or constitutive *virG* plasmid (indicated as Con-virG) were introduced into the C58 strain harboring the *virB1*::*gusA* fusion plasmid (pFUS1). Cells were grown for 24 h with the indicated concentrations of SA or acetosyringone (AS), and the β-glucuronidase activity was measured as described in *Materials and Methods*.

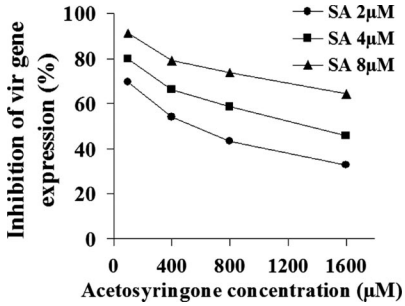

Fig. 4. Increasing acetosyringone attenuates the inhibition of *vir* gene expression by SA. Acetosyringone dose–response curves were obtained for cells grown in the absence or presence of the indicated concentrations of SA for 24 h, and the percentage inhibition in *virB1*::*gusA* expression was calculated. The data represent the averages of three independent determinations.

The VirA Kinase Domain Is the Site of SA Inhibition. The results described in the preceding two sections indicate that SA inhibits *vir* gene expression through an effect on the VirA protein, possibly by competing with acetosyringone for a binding site on VirA. To explore this hypothesis, we compared the effect of 10 μM SA on *vir* gene expression that depended on VirA derived from pTiA6 and certain sections of that protein (Table 3). The expression and stability of the VirA protein fragments were confirmed previously (5, 6). The signal-transducing activity of WT (full-length VirA), PLK (periplasmic, linker, and kinase domains), LK (linker and kinase domains), and K (kinase domain) were all inhibited by 10 μM SA, regardless of the presence of acetosyringone. In agreement with the findings of others (5, 6), our assays indicate that the linker domain is required for a significant response to acetosyringone; while showing significant activity in the absence of acetosyringone, the PLK and LK versions of VirA are strongly stimulated by acetosyringone. Importantly, 10 μM SA inhibited the *vir* gene expression that depended on the acetosyringone-independent kinase (K) domain to roughly the same extent as that driven by the acetosyringone-responsive LK fragment, and the percentage of inhibition was not significantly reduced by added acetosyringone. Thus, SA apparently hinders the function of the VirA kinase domain rather than the ability of the protein to detect the phenolic inducer through the linker domain.

Plants Overproducing SA Are More Resistant to *Agrobacterium* Infection, and Plants Defective in SA Accumulation Are More Susceptible. To assess the role of SA in conferring resistance to *Agrobacterium* infection *in planta*, we assayed tumor formation on mutant or transgenic *Arabidopsis* plants that have altered levels of SA and SA-mediated signaling. Mutant *cpr5-2* (22) and transgenic 35S-LOX2 (23) plants overaccumulate SA and express pathogenesis-related (PR) genes constitutively or in response to infection, respectively. Transgenic *nahG* plants, in contrast, convert endogenous SA to catechol, thereby reducing SA accumulation (24). SA-induced PR gene expression and systemic acquired resistance occur primarily through a signaling pathway involving the transcriptional activator NPR1. Mutant *npr1-1* plants are defective in this signaling and exhibit decreased PR gene expression (25), although they accumulate at least WT SA levels in response to infection by various pathogens (26). We tested whether SA levels and *NPR1*-dependent activation of systemic acquired resistance affected the ability of WT *Agrobacterium* (strains A208 and A348) to form tumors on root segments. At a bacterial cell concentration of 10^6 cells per ml, *cpr5-2* plants developed ≈8-fold fewer tumors than WT Col-0 plants, whereas *NahG* plants developed significantly more tumors than WT. Both *LOX2* and *npr1-1* plants showed a substantial decrease in tumor formation, although they were more susceptible than *cpr5-2* plants [Table 4 and supporting information (SI) Fig. 5].

To probe whether altered SA production affected the plant's susceptibility to tumorigenesis because of the inhibition of *vir* gene expression or whether it acted at the level of responses within the plant (or both), we tested the ability of cells containing the constitutively active VirA to restore tumor formation on the most resistant *Arabidopsis* line, the *cpr5-2* mutant (SI Fig. 5 and data not shown). Bacteria expressing the constitutive *virA* allele were highly virulent on the *cpr5-2* roots, whereas WT C58 cells were unable to incite a single tumor on this plant line (Table 5). Together, these results indicate that SA inhibits tumor formation by *Agrobacterium* through an *NPR1*-independent mechanism and that the inhibition may be attributable primarily to the ability of SA to prevent *vir* gene induction in the bacterium.

Discussion

It is well established that acetosyringone induces the expression of all *vir* genes and the Ti plasmid *repABC* operon (1, 4). Our observations that (*i*) SA significantly inhibited the expression of all *vir* genes and the Ti plasmid *repABC* operon; (*ii*) high levels of acetosyringone attenuated the SA-induced reduction in *vir* gene expression; and (*iii*) constitutively active *virA* or *virG* overcame the SA inhibition, led to the proposition that SA and acetosyringone play opposing roles during *Agrobacterium*–plant host interactions. SA interferes with the signaling process stimulated by acetosyringone, thereby blocking expression of the genes encoding the type IV secretion system and other virulence proteins required for tumorigenesis (1, 3). Previous studies have reported that at plant infection sites, SA accumulates to concentrations of 3–4 μM (27), approximately the level that we find affects *vir* regulon induction. Therefore, the SA concentration and the acidic conditions used in our study mimic the environment and are biologically relevant to the interaction of *Agrobacterium* with its plant hosts.

Although SA counteracts the virulence-promoting activity of acetosyringone, it appears to exert its effect at the kinase domain

Table 3. SA inhibits the function of the kinase domain of VirA

VirA construct	0 μM AS			100 μM AS		
	0 μM SA	10 μM SA	Average inhibition, %	0 μM SA	10 μM SA	Average inhibition, %
WT	97 (7)	70 (4)	28	3,310 (290)	2,037 (231)	38
PLK	2,746 (148)	788 (145)	71	6,045 (359)	2,437 (326)	60
LK	1,080 (59)	495 (27)	54	8,682 (324)	5,324 (346)	39
K	554 (36)	263 (25)	53	476 (36)	282 (32)	41

A136 cells expressed *virG* and all *virA* constructs from the constitutive PN25 promoter (21). VirA domains are indicated as WT, full-length *virA*; PLK, amino acids 1–711; LK, amino acids 285–711; and K, amino acids 426–711. Plasmid constructs also carry a *virB*::*lacZ* fusion and have been described previously (6). Assays of *vir* gene expression were performed after growth in acidified induction medium containing glucose and 0 or 100 μM acetosyringone with the indicated amounts of SA. The numbers in parentheses are the SD values of three independent samples.

Table 4. Tumor formation by *A. tumefaciens* on *A. thaliana* lines with altered SA

Arabidopsis line	Bacterial cell density in cocultivation, cells per ml			
	10^8	10^7	10^6	10^5
Col-0 (WT)	33% (n = 69)	33% (n = 51)	24% (n = 37)	3% (n = 37)
NahG	47% (n = 30)	48% (n = 54)	38% (n = 29)	5% (n = 38)
npr1-1	17% (n = 35)	2% (n = 42)	9% (n = 23)	0% (n = 23)
cpr5-2	22% (n = 36)	18% (n = 76)	3% (n = 40)	0% (n = 33)
Col-0 (WT)	34% (n = 61)		19% (n = 63)	
LOX2	9% (n = 171)		9% (n = 155)	

Root segments were cocultivated with WT strain A208. Values represent the percentage of root segments with tumors. n, no. of root segments assayed. All tumors were approximately the same size, and each root segment had at most one tumor, regardless of the plant line used.

of VirA rather than directly blocking signal perception (Table 3). Two VirA functions that depend on the kinase region are autophosphorylation at the conserved histidine at codon 474 and transfer of the phosphate from that histidine to VirG. *In vivo* studies have shown that although the histidine on an LKR (linker–kinase–receiver) fragment appears to be constitutively phosphorylated, movement of the phosphate from VirA to VirG requires the addition of acetosyringone (21, 28). Thus, SA probably does not compete with detection of the phenolic inducer but may inhibit the phosphate transfer reaction that depends on its presence. The capacity of acetosyringone to overcome SA-mediated inhibition of *vir* gene expression (Fig. 4) may be the result of acetosyringone-mediated stimulation of phosphotransfer activities of VirA and simply reflect the combined effects of SA and acetosyringone.

In addition to inhibiting the *vir* regulon, SA induced the expression of the quormone degradation system encoded by the *attKLM* (Fig. 2). Recent studies have shown that GABA and succinic semialdehyde activate the expression of *attKLM*, resulting in quorum-sensing signal decay and reduced *Agrobacterium* virulence (16, 17). Thus, in addition to its role in orchestrating defense responses within the plant, SA provides the plant with additional defense strategies against *Agrobacterium* and perhaps other quorum-sensing bacteria in the rhizosphere.

Recent studies have shown that SA-treated *P. aeruginosa* accumulates less endogenous quormone than nontreated cells (29), although the molecular mechanisms by which SA affects quormone levels have yet to be defined. SA also affects the ability of *Rhizobium* to nodulate host plants (8, 9); whether this effect is mediated through quormone degradation remains obscure, although evidence exists that *Rhizobium* quorum sensing plays an essential role during symbiosis (30, 31).

In addition to SA, plants secrete other small molecules that inhibit VirA/G function. Among grasses, defensive benzoxazinones, such as the exuded product 2-hydroxy-4,7-dimethoxybenzoxazin-3-one (HDMBOA), also inhibit the VirA/G system (32, 33). Recent work suggests that the *Agrobacterium vir* genes are subject to feedback control (11). Upon expression of the integrated T-DNA genes, IAA inhibits the transcription of the *vir* regulon, presumably to save energy and metabolic resources. However, both microarray and gene fusion studies revealed that IAA does not activate expression of the *attKLM* operon (Z.-C.Y. and E.W.N., unpublished results). SA may serve to reinforce the feedback activity of IAA on *vir* gene expression. Thus, in an intriguing twist of evolution, SA produced by plants may have been hijacked by *Agrobacterium* to serve the pathogen by shutting off *vir* gene expression once these genes have served their purpose. Microarray analysis carried out at pH 5.5 demonstrated that unlike SA and IAA, GABA did not affect expression of the *vir* regulon (Z.-C.Y. and E.W.N., unpublished results).

Unexpectedly, the *npr1-1* mutant was more resistant to *Agrobacterium* infection, even though *npr1* mutants exhibit increased susceptibility to a wide range of pathogens, and conversely, overexpression of *NPR1* in *Arabidopsis* improves resistance to several pathogens including *Pseudomonas syringae* (10). *NPR1* activates the WRKY transcription factors involved in SA-dependent upregulation of the defense-related PR genes (10). Our results indicate that *NPR1* is not essential for *Arabidopsis* to mount a successful defense against *Agrobacterium* infection, suggesting that SA functions primarily by inhibiting induction of the *vir* regulon. This conclusion is further supported by our discovery that a constitutively active VirA conferred on C58 cells the ability to infect the highly resistant mutant plant, *cpr5-2*, which overaccumulates SA (Table 5). The enhanced resistance of the *npr1-1* line is consistent with the observation that *npr1* mutants overexpress the *ICS1* gene required to synthesize and accumulate elevated levels of SA after infection relative to infected WT plants (10). Another potential explanation is that SA signaling also contributes *in planta* to resistance to *Agrobacterium* infection, but through an *NPR1*-independent pathway (10). It has been reported that *Agrobacterium* rapidly reduces the host systemic acquired resistance by downregulating PR gene expression and decreasing the free SA level by 40% within 1 h after infection (34). It is conceivable that this effect is mediated through *NPR1*; an inactivating mutation in *NPR1* would abrogate the pathogen's ability to disable the plant defenses, resulting in enhanced host resistance.

Taken together, our data suggest a model in which SA defends against *Agrobacterium* infection through direct effects on the transcription of the *vir* regulon and potentially also through an *NPR1*-independent (or partially *NPR1*-independent) signaling response that induces defense-related genes in the host. Such a combinatorial line of defense has also been implicated in *Arabidopsis* resistance to *Staphylococcus aureus* infection (26). The dramatic restoration of *Agrobacterium* virulence by a constitutively active VirA makes it highly unlikely that the level of SA produced by even the most resistant mutant is blocking tumorigenesis merely by affecting bacterial viability. However, at higher SA concentrations, inhibiting bacterial cell growth could be another strategy in the plant's defensive arsenal. *Agrobacterium* in turn appears to have evolved a mechanism to stymie the host defenses (34) and may even capitalize on the inhibitory effects of SA on *vir* gene expression to conserve

Table 5. Constitutive VirA restores tumor formation on *A. thaliana* lines with elevated salicylic acid content

Arabidopsis line	Bacterium	Bacterial cell density, cells per ml	
		10^8	10^6
Col-0 (WT)	C58	8% (n = 50)	9% (n = 32)
	Con-VirA	20% (n = 40)	16% (n = 50)
cpr5-2	C58	0% (n = 28)	0% (n = 31)
	Con-VirA	19% (n = 32)	10% (n = 10)

Values represent percentage of root segments with tumors. C58 is the WT strain; Con-VirA indicates C58 with a plasmid carrying the constitutive allele of *virA*. n, no. of root segments assayed.

its metabolic resources. This complex interplay between host and pathogen likely reflects an exquisite evolutionary balance, in which bacterial subversion of the host's intrinsic protection against disease counters the plant's multifaceted defense strategy mediated through SA.

Materials and Methods

Bacterial Strains, Growth Conditions, and Generating Transcriptional Gene Fusions. *A. tumefaciens* strain C58, whose genome has been sequenced (12), was used to assay the expression of *vir* genes and the *attKLM* operon. The cells were grown in either MG/L or AB minimal medium (35) with arabinose as a carbon source at 28°C. *A. tumefaciens* strains A208, A348, and C58 were used to assay virulence on *Arabidopsis thaliana* roots. A208 is an *Agrobacterium* isolate that is more virulent on roots of *A. thaliana* than C58. A348 contains the same genome components as C58 except for the Ti plasmid, which originated from strain A6. The *virB1* promoter was amplified and cloned into the pFUS1 vector to generate a *virB1*::*gusA* transcriptional gene fusion (36). Plasmid pWT160 (37) was used to measure *virG* expression. For generating *gusA* gene fusions to other *vir* and *chv* genes, a modified pJP2 *gusA* reporter vector was used (38). The primers used for amplifying all promoters are listed in SI Table 6. The reporter plasmids were introduced into *Agrobacterium* by electroporation.

Cell Culture and RNA Isolation. *Agrobacterium* C58 cells were grown overnight in MG/L and then washed in induction medium (35) three times and subcultured in 25 ml of induction medium (with 100 μM acetosyringone) at an initial A_{600} of 0.15, with or without 6 μM SA. After 7 h, the A_{600} for the SA-treated culture was $\approx$0.65, whereas the A_{600} for the nontreated culture was $\approx$0.7. Four milliliters of the cultures were mixed with 8 ml of RNA Protect bacteria reagent (Qiagen, Valencia, CA) and processed as recommended by the manufacturer. Total bacterial RNA was isolated by using the Qiagen mini-RNA isolation kit according to the manufacturer's protocol. For the IAA and GABA microarray analyses, IAA was added at 40 μM; GABA was added at 1 mM.

Assaying Gene Fusions. Cells harboring the gene fusion plasmids were grown overnight in MG/L supplemented with carbenicillin (100 μg/ml). Cells were washed with acidified AB minimal medium (pH 5.5) three times and inoculated into induction medium (pH 5.5) supplemented with 0.2% arabinose and 100 μM acetosyringone at an initial A_{600} of 0.1. Bacteria were incubated for 16–20 h and then assayed for β-galactosidase or β-glucuronidase activity as described previously (39). Unless otherwise indicated, the data presented represent the average of three independent determinations.

Microarray. Unique 60-mer oligonucleotides representing each of the 5,419 predicted ORFs were selected by using the Featurama program designed at the Institute for Systems Biology in Seattle. Each oligonucleotide was synthesized *in situ* on 2.5- $\times$ 7.5-cm glass slides by Agilent Technologies (Santa Clara, CA). Each microarray experiment represents four biological replicates. cDNA was generated from 30 μg of total RNA by using random hexamer primers and SuperScript II (Invitrogen, Carlsbad, CA). Aminoallyl-modified dUTP was incorporated into the cDNA at a ratio of 4:1 aa-dUTP:dTTP, and cDNA was labeled with Cy3 or Cy5 monoreactive dyes (Amersham, Piscataway, NJ). Arrays were hybridized and washed according to the manufacturer's instructions (Agilent publication G4140-90030). Data acquisition was performed by using an Agilent G2565AA microarray scanner and extracted by using Agilent feature extraction software.

Microarray Data Analysis. Initial data handling and visualization were done with the Matlab software (MathWorks, Natick, MA). All remaining data analysis was done in the R statistical computing environment by using the *samr* package in Bioconductor (40). Normalized data were analyzed to identify candidate differentially expressed genes. A *t* test statistic and a reference distribution were carried out.

Root Infection Assays. The *Arabidopsis* WT *Col-0* line and its derivatives *NahG* (24), 35S-*LOX2* (23), *npr1-1* (25), and *cpr5-2* (22) were used in the root infection assays, which were performed as described previously (41).

We thank Drs. Matt Parsek and Amy Schaefer for helpful discussions and Drs. David Lynn and Justin Maresh (Emory University, Atlanta, GA) for *virA* plasmids and for suggestions on plotting and interpreting Fig. 1*A*. Dr. Xinnian Dong (Duke University, Durham, NC) kindly provided the transgenic *Arabidopsis* line *nahG*. We thank the *Arabidopsis* Stock Center (Columbus, OH) for the other *Arabidopsis* lines. We also thank Cassie Sather for helping with microarrays. We are grateful to Drs. Stan Gelvin and Janis Bravo for advice on the root infection assays. This work was supported by National Institutes of Health (NIH) Grant GM032618 and a U.S.–Israel Binational Agricultural Research and Development Research (BARD) Grant IS-3622-04R (to E.W.N.), by National Science Foundation Grant MCB-0416471 (to L.M.B.), and by NIH Grant GM47369 and the Carolyn Hoff Lynch Term Professor Fund (to A.N.B.).

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