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Pleiotropic Phenotypes Caused by Genetic Ablation of the Receiver Module of the *Agrobacterium tumefaciens* VirA Protein

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The VirA protein of *Agrobacterium tumefaciens* is a transmembrane sensory kinase that phosphorylates the VirG response regulator in response to chemical signals released from plant wound sites. VirA contains both a two-component kinase module and, at its carboxyl terminus, a receiver module. We previously provided evidence that this receiver module inhibited the activity of the kinase module and that inhibition might be neutralized by phosphorylation. In this report, we provide additional evidence for this model by showing that overexpressing the receiver module in *trans* can restore low-level basal activity to a VirA mutant protein lacking the receiver module. We also show that ablation of the receiver module restores activity to the inactive VirA (Δ324-413) mutant, which has a deletion within a region designated the linker module. This indicates that deletion of the linker module does not denature the kinase module, but rather locks the kinase into a phenotypically inactive conformation, and that this inactivity requires the receiver module. These data provide genetic evidence that the kinase and receiver modules of VirA attain their native conformations autonomously. The receiver module also restricts the variety of phenolic compounds that have stimulatory activity, since removal of this module causes otherwise nonstimulatory phenolic compounds such as 4-hydroxyacetophenone to stimulate *vir* gene expression.

Agrobacterium tumefaciens is a gram-negative, rod-shaped, aerobic bacterium responsible for the crown gall disease of a variety of dicotyledonous plants. This species has provided a useful model to study interactions between bacterial pathogens and their hosts. *A. tumefaciens* incites tumors on susceptible hosts by transferring a fragment of oncogenic DNA from the Ti plasmid into the plant genome. Transfer is mediated by the products of approximately 25 *vir* genes, whose expression is positively regulated by the VirA and VirG proteins in response to diffusible chemical signals that originate from plant wound sites (8, 10, 20, 45, 46, 53). VirA and VirG are members of the family of two-component regulatory proteins (24, 29, 40, 47). VirA is a transmembrane sensory kinase that is autophosphorylated at a histidine residue and transfers this phosphoryl group to a conserved aspartic acid in VirG (12, 16, 17). Phospho-VirG activates transcription of at least 11 *vir* promoters (18, 21, 48). Three environmental stimuli are required to activate the VirA kinase: substituted phenolic compounds, monosaccharides, and extracellular acidity (3, 37, 38).

Sequence analysis and experimental results from several laboratories indicate that VirA contains one periplasmic module and three cytoplasmic modules (4, 27). The periplasmic region interacts with the chromosomally encoded monosaccharide-binding protein ChvE (3, 34, 35). The three cytoplasmic modules are designated the linker, kinase, and receiver modules. The kinase module resembles members of the family of two-component kinases, while the receiver module resembles the receivers of two-component response regulators. Genetic ablation of the periplasmic module, the receiver module, or both modules did not abolish stimulation by phenolic compounds (4). This suggests that the receptor for phenolic compounds may lie within the linker module or, less likely, the kinase module. Although the two membrane-spanning regions of VirA have been implicated in binding phenolic compounds, our deletion of the periplasmic module also removed both of these regions, indicating that the binding site must lie elsewhere. This deletion also removed half of an amphipathic α helix (residues 278 to 288) that was implicated in binding of phenolic compounds (10), indicating that this region, too, is dispensable for sensing phenolic compounds.

Evidence has been provided that phenolic compounds may not bind to VirA at all but rather may bind to one or two soluble, chromosomally encoded proteins designated P10 and P21 (22). Recently, it was shown that several naturally occurring alleles of *virA* are stimulated by different classes of phenolic compounds (23). This suggests that VirA itself contains the binding site for phenolic compounds, although it remains conceivable that phenolic compounds bind to phenolic compound-binding proteins and that different VirA proteins are able to recruit different phenolic compound-binding proteins.

The C-terminal receiver module, which is homologous to the amino-terminal half of VirG, is not essential for sensing any inducing stimulus. VirA(Δ712-829), a VirA mutant lacking the receiver module, exhibits greater kinase activity than the wild-type protein in the absence of phenolic compounds, suggesting that the receiver module negatively modulates VirA kinase activity. Inhibition by this domain may be neutralized by phosphorylation of a conserved aspartate residue of the receiver module (D-766), since alteration of this residue decreased kinase activity (4). Some VirA homologs, such as the ArcB and VsrB proteins, have similar receiver modules, and genetic ablation of these modules causes similar phenotypes (11, 14, 15).

Although the finding that removal of the receiver module increased kinase activity suggested that the receiver normally inhibits kinase activity, it remained possible that the receiver has some other function and that its removal altered the conformation of the kinase in such a way that it became hyperactive. We therefore sought to obtain additional evidence for our model by determining whether the receiver module could restore low-level kinase activity when provided as a separate protein. Such data would indicate that both the kinase and the

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli ES1578	*mutD5 ara-14 argE3*(Oc) *galK2 hisG4*(Oc) *kdgK51 lacY1 leuB6 mgl-51 mtl-1 rac rfbD1 rpsL31 supE44 thi-1 thr-1 tsx-33 xyl-5*	52
A. tumefaciens A136	C58, cured of pTiC58, Rf^r Nal^r	43
Plasmids		
pCH114	IncW origin, *virA* Ap^r Tet^r	4
pCH116	IncP origin, *virB-lacZ* P*lac-virG* Kan^r	4
pCH117	IncW, no *virA*, Ap^r Tet^r	4
pCH203	ColE1 origin, *virA*(Δ*11-283*) Ap^r	4
pCH225	IncW, *virA*(Δ*712-829*) Ap^r Tet^r	4
pCH235	ColE1, *virA*(*D766N*) Ap^r	4
pCH251	ColE1, P*lac-virA*(*712-829*) Ap^r	This study
pCH260	IncW, *virA* P*lac-virA*(*712-829*) Ap^r	This study
pCH263	IncW, *virA*(Δ*712-829*), P*lac-virA*(*712-829*) Ap^r	This study
pCH280	ColE1, PT7-6×His-*virA*(*712-829*)(*D766N*) Ap^r	This study
pCH283	ColE1, PT7-6×His-*virA*(*712-829*) Ap^r	This study
pCH325	ColE1, P*tac*-6×His-*virA*(*712-829*)(*D766N*) Ap^r	This study
pCH326	ColE1, P*tac*-6×His-*virA*(*712-829*) Ap^r	This study
pCH338	ColE1, P*tac*-6×His-*virA*(*815-829*) Ap^r	This study
pCH351	IncW, *virA* P*tac*-6×His-*virA*(*712-829*)(*D766N*) Ap^r	This study
pCH355	IncW, *virA*(Δ*712-829*) Ap^r P*tac*-6×His-*virA*(*712-829*)(*D766N*)	This study
pCH357	IncW, P*tac*-6×His-*virA*(*712-829*)(*D766N*) Ap^r	This study
pCH361	IncW, *virA* P*tac*-6×His-*virA*(*712-829*) Ap^r	This study
pCH365	IncW, *virA*(Δ*712-829*), P*tac*-6×His-*virA*(*712-829*) Ap^r	This study
pCH367	IncW, P*tac*-6×His-*virA*(*712-829*) Ap^r	This study
pCH381	IncW, *virA* P*tac*-6×His-*virA*(*815-829*) Ap^r	This study
pCH385	IncW, *virA*(Δ*712-829*) P*tac*-6×His-*virA*(*815-829*) Ap^r	This study
pCH387	IncW, P*tac*-6×His-*virA*(*815-829*) Ap^r	This study
pCH395	IncP, P*lac-virG*(*N54D*) Kan^r	This study
pCH136	IncW, *virA*(Δ*324-413*)(*G665D*) Ap^r Tet^r	This study
pCH169	IncW, *virA*(Δ*324-413*) Ap^r Tet^r	This study
pCH310	IncW, *virA*(Δ*324-413*)(Δ*712-829*) Ap^r Tet^r	This study
pET3.His	ColE1 origin, 6×His tag, Ap^r	5
pPR1068	pMAL-c2 derivative, *Nde*I at the start of MalE, Ap^r	New England Biolabs
pRSETA	ColE1, Ap^r	Invitrogen
pSW169	ColE1 origin, Ap^r, wild-type *virA*	50
pTZ18R	ColE1, Ap^r	U.S. Biochemicals
pUCD2	IncW origin, Ap^r Tet^r Kan^r Sp^r	7
pYF3a	IncP, *virG*(*N54D*) Tet^r	9

receiver modules fold into wild-type conformations when expressed as separate proteins. Further evidence that the kinase can fold autonomously was sought by determining whether the receiver deletion can restore activity to a phenotypically silent VirA protein that has an in-frame deletion within its linker module. Finally, after isolating two *virA* alleles that respond to a noninducing phenolic compound and discovering that they had mutations in their receiver modules, we asked whether removal of the receiver could influence the recognition of phenolic compounds.

MATERIALS AND METHODS

Bacterial strains, plasmids, and reagents. The bacterial strains and plasmids used in this study are listed in Table 1. Carbenicillin, tetracycline, kanamycin, *o*-nitrophenyl-β-D-galactoside, and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma (St. Louis, Mo.). Acetosyringone (AS), vanillin, acetovanillone, syringic acid, ferulic acid, 4-hydroxyacetophenone (HAP), 3,5-dimethoxyacetophenone (DMA), guaiacol, 4-hydroxybenzaldehyde, and 4-hydroxybenzoic acid were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). *p*-Coumaric acid was purchased from Sigma. Buffer-saturated phenol was purchased from Amresco (Solon, Ohio).

Overexpression of VirA receiver modules. To increase the expression level of the VirA receiver, we expressed the corresponding fragment of the *virA* gene by using the P*tac* promoter. Plasmids encoding proteins containing a tag of six histidine residues (6×His tag) fused to the VirA receiver were constructed by digesting pCH203 (wild-type *virA*) and pCH235 [*virA*(*D766N*)] (4) with *Eag*I and *Sma*I. The resulting fragments, encoding the VirA receiver modules, were treated with the Klenow fragment of DNA polymerase and ligated with pET.His (5) that was previously treated with *Nco*I and Klenow fragment, resulting in plasmids pCH283 and pCH280. These plasmids were then digested with *Nde*I and *Pst*I, and the resulting 0.8-kb fragments encoding the VirA receiver modules were ligated with a 5.3-kb *Nde*I-*Pst*I fragment of pPR1068 (a derivative of pMAL-c2) to make pCH325 and pCH326, respectively. Plasmid pCH326 encodes a P*tac*-regulated 6×His fusion of the wild-type receiver domain, whereas pCH325 encodes a P*tac*-regulated 6×His fusion of the D766N mutant receiver. A negative control plasmid, pCH338, encoding the 6×His tag fused to the last 15 amino acids of VirA, was constructed by digesting pCH325 with *Xho*I and *Eco*RV and treating the products with Klenow fragment and T4 DNA ligase.

Plasmid pCH325 was digested with *Bcl*I and ligated with the large fragment of *Bam*HI-digested pCH114 (which expresses wild-type *virA*), pCH117 (which expresses no *virA*) or pCH225 [which expresses *virA*(Δ*712-829*)] to create pCH351, pCH357, and pCH355, respectively. By the same strategy, plasmid pCH326 was introduced into pCH114, pCH117, or pCH225, creating pCH361, pCH367, and pCH365, respectively, while plasmid pCH338 was introduced into pCH114, pCH117, or pCH225 to make pCH381, pCH387, and pCH385, respectively.

We also placed the receiver module under the control of the P*lac* promoter by using oligonucleotide 5′-CACAGGAAACAGCTATGGGAAACGGGGAGAT TGT-3′ and the *virA*-encoding plasmid pSW169 (50) as a template for site-directed deletion mutagenesis. This oligonucleotide fuses codon 1 of the *lacZ* α peptide to codon 712 of *virA*. The resulting plasmid, pCH251, then was digested with *Bam*HI and ligated with the 11-kb fragment of *Bam*HI-digested pCH114 or pCH225 to make plasmids pCH260 and pCH263. All of the resulting plasmids were then introduced into *A. tumefaciens* A136(pCH116) by electroporation (25) and assayed for β-galactosidase activity as described previously (6). Plasmid pCH116 is an IncP plasmid containing *npt*, a P*lac-virG* fusion, and a P*virB-lacZ* fusion (4). In addition, a pCH116 derivative containing the constitutive *virG* (*N54D*) allele was created by ligating *Eco*RI-digested pYF3a (9) with *Eco*RI-digested pRSETA vector (Invitrogen, San Diego, Calif.), creating pCH394. Plasmid pCH394 was then digested with *Bgl*II and *Xba*I and ligated with *Bgl*II-*Xba*I-digested pCH116 to make pCH395, which is identical to pCH116 except for the *virG*(*N54D*) mutation.

TABLE 2. Restoration of low basal levels of VirA kinase activity by overexpression of the receiver module

Plasmids	VirA	Receiver module	β-Galactosidase activity with the following VirG protein[a]:			
			VirG		VirG(N54D)	
			Without AS	With AS	Without AS	With AS
pCH385, pCH116	Δ712-829	None	565	1,084	342	384
pCH365, pCH116	Δ712-829	712-829	20	716	172	192
pCH355, pCH116	Δ712-829	712-829(D665N)	7	411	101	115
pCH381, pCH116	Wild type	None	5	887	373	432
pCH361, pCH116	Wild type	712-829	5	402	132	157
pCH351, pCH116	Wild type	712-829(D665N)	6	245	104	111
pCH387, pCH116	None	None	6	6	362	392
pCH367, pCH116	None	712-829	6	5	158	180
pCH357, pCH116	None	712-829(D665N)	5	5	101	109

[a] Miller units (29a).

Construction of *virA* alleles lacking codons 324 to 423. Plasmids pSW169, pCH225, and pCH126, which contain *virA*, *virA*(Δ*712-829*), and *virA*(*G665D*), respectively, were treated with *Eco*47III, *Bst*EII, Klenow fragment, and T4 DNA ligase. The resulting plasmids were linearized by digestion with *Eco*RI and ligated with *Eco*RI-digested pUCD2, resulting in plasmid pCH169, which contains *virA*(Δ*324-423*); pCH310, which contains virA(Δ*324-423*)(Δ*712-829*); and pCH136, which contains *virA*(Δ*324-423*)(*G665D*). These plasmids were introduced into *A. tumefaciens* A136(pCH116) and assayed for β-galactosidase activity at 20°C.

Isolation of altered-function alleles of *virA*. To mutagenize *virA*, the IncW-type plasmid pCH114 containing the wild-type *virA* was introduced into the *Escherichia coli mutD5* strain ES1578 (52), and the resulting strain was cultured to stationary phase in 1 liter of L broth. pCH114 DNA was purified from this culture and introduced into *A. tumefaciens* A136(pCH116) by electroporation. Transformants were plated on acidified (pH 5.25) AB medium (3a) (pH 5.25) containing the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), carbenicillin, kanamycin, and 100 μM HAP. Colonies that stained darker blue than the wild type were purified and screened on solid medium for HAP-inducible β-galactosidase production.

DNA sequencing. Plasmid DNA was purified by using SpinBind columns (FMC Bioproducts, Rockland, Maine) or QIAprep columns (QIAGEN, Chatsworth, Calif.). Synthetic oligonucleotides used for DNA sequencing were obtained from the Cornell Nucleotide Synthesis Center. Automated DNA sequencing was carried out with an ABI-373A Stretch Automated DNA Sequencer (Applied Biosystems) at the Cornell Sequencing Facility.

RESULTS

The receiver module of VirA restores low-level basal activity to VirA(Δ712-829). We wanted to know whether the elevated kinase activity of VirA(Δ712-829) could be decreased by expressing the receiver module as a separate protein. Plasmids that express the receiver module from the P*tac* promoter [P*tac-virA*(*712-829*)] along with either *virA*(Δ*712-829*) or wild-type *virA* were constructed. Similar plasmids were also made by using P*tac-virA*(*712-829*)(*D665N*). The intracellular concentrations of receiver modules were found by Western immunoblotting to be considerably greater than the concentrations of intact VirA or VirA(Δ712-829) (data not shown).

As shown previously (4), the strain expressing VirA(Δ712-829) and not expressing any receiver module strongly expressed P*virB* even in the absence of AS (Table 2, line 1) and was stimulated only about twofold by AS. In the absence of AS, expression of this promoter was decreased about 30-fold by overexpressing VirA(712-829) (line 2) and was decreased about 80-fold by overexpressing VirA(712-829)(D766N) (line 3). In contrast, P*virB* expression in the presence of AS was inhibited only slightly (approximately two- to threefold) (Table 2, lines 2 and 3).

The basal levels of P*virB* expression were also inhibited in strains that contain VirG(N54D) rather than wild-type VirG (Table 2, lines 2 and 3), despite the fact that this mutant of VirG does not require VirA for activity (9, 19, 32, 33). However, this inhibition was far more subtle than that described above [approximately twofold for VirA(Δ712-829) and fourfold for VirA(Δ712-829)(D665N)]. This inhibition could be due to inhibitory interactions between receiver modules and VirG(N54D) or could be an indirect consequence of receiver overexpression. Even when these effects are taken into account, overexpression of receiver modules still caused a 15-fold inhibition of VirA(Δ712-829) AS-independent activity.

Strains expressing wild-type VirA and wild-type VirG showed a low level of expression of P*virB* in the absence of AS and a high level of AS-induced expression (Table 2, line 4). Mild inhibition of AS-induced kinase activity was observed by overexpressing receiver modules (lines 5 and 6). However, when these data were normalized by using similar strains that express VirG(N54D) (lines 4 to 6), it appeared that receiver domains did not detectably inhibit the function of the wild-type VirA protein.

Similar experiments were performed with the P*lac* promoter rather than the P*tac* promoter used to express the receiver module. In contrast to the results described above, this did not inhibit wild-type VirA or VirA(Δ712-829) (data not shown). We conclude that inhibition by receiver modules occurs only when they are strongly overexpressed. This is not surprising, since in the wild-type VirA protein, the receiver is tethered to the kinase domain, resulting in very high apparent concentrations. Similar studies using other two-component kinases have shown that the inhibition of kinase activity by liberated receiver fragments requires high-level expression of these fragments (13, 30, 44). VirA itself is expressed at high levels compared with many regulatory proteins (49).

The null phenotype of VirA(Δ324-413) requires the receiver module. Deletion of codons 324 to 413 was previously shown to abolish detectable VirA activity (35). It was hypothesized that this inactivity was due to the loss of a binding site for phenolic compounds (or for phenolic compound-binding proteins). However, it seemed equally plausible that the inactivity of this protein could be due to denaturation of the kinase module. To distinguish between these two possibilities, we attempted to restore the activity of VirA(Δ324-413) with second-site mutations that by themselves enhance kinase activity. One of these mutations, *virA*(*G665D*), causes strong *vir* gene expression even in the absence of AS (26). This mutation and *virA*(Δ*712-829*) were therefore combined with the *virA*(Δ*324-413*) mutation, and the resulting strains were assayed for their response to different combinations of *vir* gene stimuli. These deletions are shown in Fig. 1.

Consistent with previous observations (35), the strain containing *virA*(Δ*324-413*) was not detectably induced in response

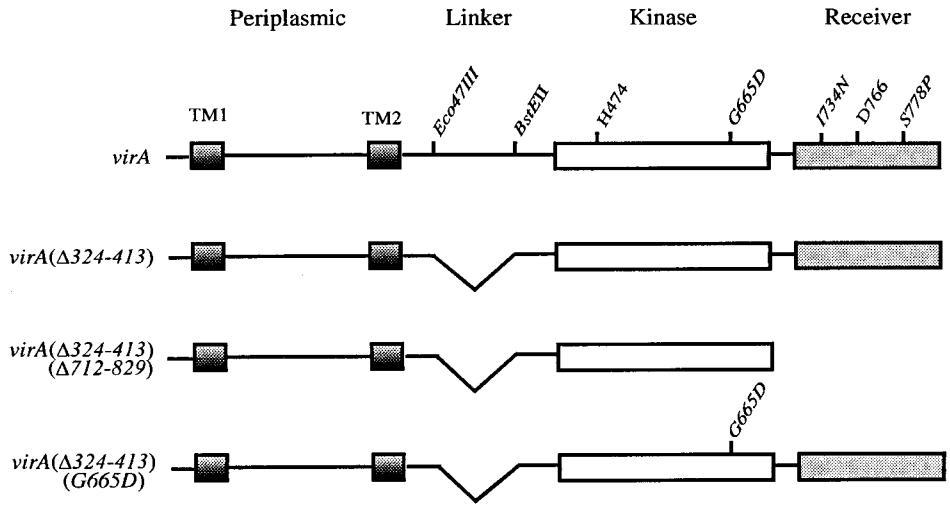

FIG. 1. Locations of *virA* point mutations and deletion mutations described in this study. *Eco*47III and *Bst*EII restriction sites were used to construct *virA*(Δ*324-413*). Mutations *virA*(*I734N*) and *virA*(*S778P*) confer responsiveness to HAP, while *virA*(*G665D*) causes constitutive kinase activity (26). H-474 is the probable site of VirA autophosphorylation, while D-766 may also be phosphorylated. TM1 and TM2 are the hydrophobic transmembrane regions.

to any combination of stimuli (Table 3, column 5). On the other hand, *virA*(Δ*324-413*)(Δ*712-829*) and *virA*(Δ*324-413*)(*G665D*) showed significantly greater activities under many conditions (columns 7 and 9). This indicates that both the *virA*(Δ*712-829*) mutation and the *virA*(*G655D*) mutation can partially restore activity to the *virA*(Δ*324-413*) mutant.

P*virB* expression was not stimulated by AS in any strain containing the *virA*(Δ*324-413*) mutation under any condition tested (Table 3, compare lines 1 and 4, lines 2 and 7, lines 3 and 6, and lines 5 and 8). This finding supports the hypothesis that the linker module is essential for sensing phenolic compounds. Similarly, no strain containing the *virA*(Δ*324-413*) allele was stimulated by monosaccharides, despite the presence of the periplasmic module (Table 3, compare lines 1 and 3, lines 2 and 5, lines 4 and 6, and lines 7 and 8). This indicates that the linker domain is essential for sensing this stimulus and suggests that any conformational change caused by ChvE may be transmitted through the linker module to the kinase module. Somewhat surprisingly, both mutants containing the *virA*(Δ*324-413*) allele were stimulated by acidity (Table 3, compare lines 1 and 2, lines 3 and 5, lines 4 and 7, and lines 6 and 8). Although we previously showed that a VirA mutant containing just the linker and kinase modules was stimulated by acidity, it appears that a protein containing just the periplasmic and kinase modules [VirA(Δ324-423)(Δ712-829)] can also respond to this stimulus.

TABLE 3. Responsiveness to three environmental stimuli by *VirA* mutants containing deletions within the linker region[a]

Stimulus			β-Galactosidase activity with the following VirA protein:					
Acid	Glucose	AS	Wild-type	Δ324-413	Δ712-829	Δ324-413, Δ712-829	G665D	Δ324-413, G665D
−	−	−	3	4	21	7	209	72
+	−	−	4	5	79	35	379	147
−	+	−	3	4	31	5	101	45
−	−	+	4	4	241	7	252	67
+	+	−	5	4	487	77	944	122
−	+	+	8	5	210	8	252	50
+	−	+	25	5	505	36	862	145
+	+	+	680	6	587	70	1,541	129

[a] *Agrobacterium* strain A136(pCH116) containing the indicated plasmid(s) was cultured in AB minimal medium supplemented with 0.5% glycerol at 20°C and assayed for β-galactosidase activity under various conditions as indicated. Acid (+), pH 5.25; acid (−), pH 7.0; glucose (+), with 10 mM glucose; glucose (−), without glucose; AS (+), with 100 μM AS; AS (−), without AS.

Isolation of altered-function alleles of *virA*. In order to understand which region of VirA is involved in the recognition of phenolic compounds, 300,000 colonies containing *virG*, a *virB-lacZ* reporter, and a mutagenized *virA* gene were screened for P*virB* activity by using medium containing HAP. Three clones that showed HAP-stimulated P*virB* expression were isolated. Sequence analysis revealed that two of these were identical and could be siblings. Therefore, only two isolates were characterized further. Both mutant genes differed from wild-type *virA* by just one nucleotide, and surprisingly, both mutations were localized in the receiver module. One mutant gene contained a T-to-A substitution at the second position of codon 734, creating an isoleucine-to-asparagine mutation [designated *virA*(*I734N*)], while the other mutation contained a T-to-C substitution at the first position of codon 778, creating a serine-to-proline substitution [designated *virA*(*S778P*)]. In addition to the two HAP-inducible *virA* alleles, we isolated many constitutive alleles. One such mutant gene has a G-to-A substitution at the second position of codon 665, creating a glycine-to-aspartic acid substitution [denoted *virA*(*G665D*)], which is identical to a *virA* mutant described previously (26). This mutation was used in the studies described above. Figure 1 shows the locations of these mutations within the VirA protein. Plasmid DNA was purified from these strains and reintroduced into the reporter strain A136(pCH116). The resulting strains showed the same phenotypes as the original mutants, indicating that the mutations that caused these phenotypes were localized on pCH114.

The HAP-inducible *virA* alleles were tested for responsiveness to AS and HAP over a range of concentrations. The strain expressing VirA(I734N) was detectably induced by HAP at concentrations as low as 10 μM, while the strain expressing wild-type VirA was not detectably induced by HAP concentrations of up to 500 μM (Fig. 2). This mutant was also stimulated by concentrations of AS too low to induce the wild-type protein. VirA(S778P) was also stimulated by HAP, especially at higher concentrations. However, unlike VirA(I734N), this pro-

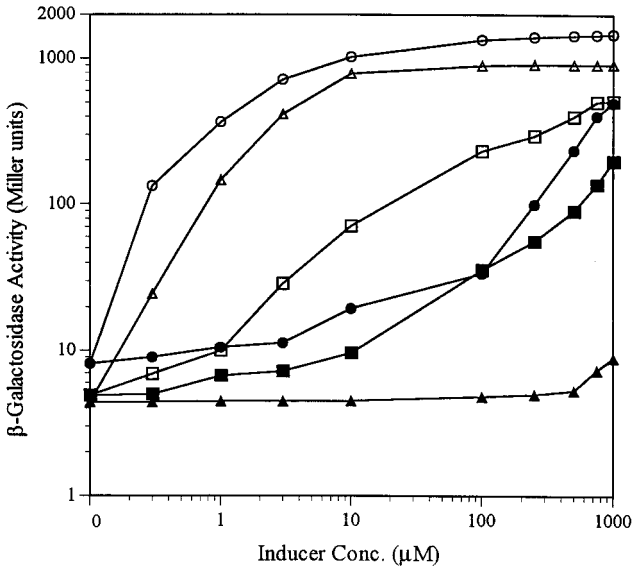

FIG. 2. *virB-lacZ* expression by mutant VirA proteins as a function of inducer concentrations. *A. tumefaciens* A136(pCH116) containing a second plasmid with the wild-type *virA* (△, ▲), the *virA*(*I734N*) (○, ●), or the *virA*(*S778P*) (□, ■) allele was cultured for 16 h in pH 5.25 medium supplemented with 10 mM glucose in the presence of AS (open symbols) or HAP (closed symbols) at the indicated concentrations (Conc.). The cultures were then assayed for β-galactosidase specificity activity.

tein was stimulated more weakly by AS than the wild-type VirA protein. In the presence of AS, the activity of both proteins was stimulated by glucose (data not shown), a phenomenon similar to that observed with the VirA(Δ712-829) mutant described previously (4).

Activation of VirA mutants by various phenolic compounds. We tested both mutants for responsiveness to 12 phenolic compounds (Table 4). The strain expressing VirA(I734N) was induced by several phenolic compounds that either did not induce or weakly induced the strain expressing wild-type VirA. We therefore describe the *virA*(*I734N*) allele as having a sensitized phenotype. On the other hand, the strain expressing VirA(S778P) showed attenuated responses to most of the inducers but increased sensitivity to guaiacol and several noninducers, such as HAP, 4-hydroxybenzoic acid, and 4-hydroxybenzaldehyde, all of which lack both methoxy groups (Table 4). While these data could be interpreted to suggest that VirA (S778P) recognizes an altered set of phenolic compounds, we propose an alternative model below.

Sensing of phenolic compounds by a VirA mutant lacking the receiver domain. Since both of these mutant proteins have mutations on their receiver modules, we tested whether a strain expressing VirA(Δ712-829) had similar properties. Such strains strongly activate *vir* genes even in the absence of phenolic compounds when the pH and sugar stimuli are provided (4). We therefore performed these experiments in the absence of glucose to eliminate this background. VirA(Δ712-829) was highly responsive to many phenolic compounds that induce the wild-type protein and was stimulated by several phenolic compounds that failed to induce the wild-type protein, including HAP and *p*-coumaric acid (Table 4). In contrast, DMA, which lacks the hydroxyl group at the R4 position, was not recognized either by VirA(Δ712-829) or by the two point mutants described above, indicating the importance of this substituent. Surprisingly, VirA(Δ712-829) was also detectably responsive to high but subinhibitory concentrations of phenol (Table 4).

DISCUSSION

A previously proposed model to describe the functions of each VirA module (4, 51) was tested in this study by several genetic approaches. One approach was to liberate the receiver module and study the interactions of this module with the kinase module. This approach has been used in several recent studies (1, 30, 44). We found that overexpressing VirA(712-829) or VirA(712-829)(D766N) strongly decreased the basal activity of VirA(Δ712-829). The fact that VirA(712-829)(D766N) strongly inhibited kinase activity indicates that inhibition does not require phosphorylation of this module. We conclude that the mechanism of inhibition does not involve competition between the receiver and VirG for phosphoryl groups. Rather, inhibition is more likely due to kinase-receiver interactions that

TABLE 4. *virB-lacZ* expression by VirA proteins in response to various phenolic compounds

Compound	R1	R3	R4	R5	β-Galactosidase activity with the following VirA protein[b]:				
					Wild type	VirA(I734N)	VirA(S778P)	Wild type[c]	VirA(Δ712-829)[c]
AS	-CO-CH_3	-OCH_3	-OH	-OCH_3	414[d]	716[d]	29[d]	195	726
Syringic acid	-COOH	-OCH_3	-OH	-OCH_3	161	255	31	39	385
Acetovanillone	-CO-CH_3	-OCH_3	-OH	-H	39[e]	104[e]	21[e]	27	703
Vanillin	-CHO	-OCH_3	-OH	-H	52	111	25	50	79
Ferulic acid	-CH═CHCOOH	-OCH_3	-OH	-H	183	252	33	87	485
Guaiacol	-H	-OCH_3	-OH	-H	26	137	70	13	213
HAP	-CO-CH_3	-H	-OH	-H	5[f]	100[f]	56[f]	6	42
4-Hydroxybenzoic acid	-COOH	-H	-OH	-H	5[f]	66[f]	15[f]	7	18
4-Hydroxybenzaldehyde	-CHO	-H	-OH	-H	5[f]	31[f]	17[f]	6	24
p-Coumaric acid	-CH═CHCOOH	-H	-OH	-H	5[f]	10[f]	5[f]	9	52
Phenol	-H	-H	-OH	-H	5	12	6	5	29
DMA	-CO-CH_3	-OCH_3	-H	-OCH_3	5	10	6	5	9
None					4	8	5	5	10

[a] *A. tumefaciens* A136(pCH116) containing the indicated VirA protein was cultured in pH 5.25 (0.5% glycerol) medium supplemented with 10 mM glucose and assayed for β-galactosidase in the presence of the phenolic compounds at 100 μM, unless otherwise indicated.
[b] Miller units (29a).
[c] These experiments were carried out in the absence of glucose.
[d] Concentration of phenolic compound, 3 μM.
[e] Concentration of phenolic compound, 20 μM.
[f] Concentration of phenolic compound, 250 μM.

mimic those of the wild-type protein. These results differ from those of a study in which the receiver module of the response regulator UhpA was overproduced (44). In that study, overproduced UhpA receivers competed against intact UhpA protein for phosphorylation. However, our results are similar to those of a study in which various modules of the *Bordetella pertussis* BvgS protein were overproduced. These modules were able to complement full-length nonfunctional BvgS proteins containing point mutations in these domains (1).

Our finding that kinase activity can be restored by intragenic suppression to a mutant lacking the linker module strongly suggests that ablation of the linker does not denature the kinase module. Rather, removal of the linker is likely to cause the loss of an activation signal that is ordinarily transmitted from it to the kinase. Furthermore, these data support models in which the linker module sends a stimulatory signal to the kinase in the presence of AS rather than sending an inhibitory signal that is overcome by AS.

Our observations that the receiver module can decrease the basal activity of the kinase module indicate that each of these modules must be able to attain an approximately wild-type conformation even when not tethered to each other. Similarly, the fact that kinase activity can be restored to a mutant lacking the linker module suggests that the linker deletion does not denature the kinase module but rather that the kinase folds correctly even in the absence of the linker. These data provide support for the functional autonomy of these domains and provide support for a modular structure of VirA that was originally proposed on the basis of sequence alignments.

It was initially surprising that both mutations that cause responsiveness to HAP were localized in the receiver domain. Since the linker and kinase domains are sufficient to sense AS (4), it seems unlikely (though not inconceivable) that these mutations directly perturb the phenolic compound binding site. It seems more likely that the receiver module interacts with some part of VirA after phenolic compound binding to modulate kinase activity. Residues I-734 and S-778 are located at conserved positions (corresponding to L-25 and L-69 of the CheY proteins of *S. typhimurium* and *E. coli*). These CheY residues are located on the hydrophobic faces of two amphipathic α helices and therefore contribute to the hydrophobic interiors of these proteins (2, 39, 41, 42).

Of the two VirA mutants that respond to HAP, one mutant [VirA(I734N)] responded more sensitively than the wild type to AS and to several other phenolic compounds. Interestingly, a mutation at an adjacent codon [VirA(A735V)] causes a constitutive phenotype (31), while VirA(I734N) showed low-level activity in the absence of phenolic compounds. The VirA (I734N) protein has properties somewhat similar to those of the VirA(Δ712-829) protein (Table 4), and we therefore propose that this point mutation may partially unfold or denature the receiver domain or in some other way make it function inefficiently. The other mutant [VirA(S778P)] appeared to be more strongly induced than the wild type by weak inducers but more weakly induced than the wild type by strong inducers. One way to interpret these data is to propose that this mutation locks the receiver into a conformation that is less inhibitory than that of wild type but which is not efficiently neutralized by phosphorylation. If so, this could be due to reduced phosphorylation or to a reduced conformational change in response to phosphorylation.

We previously showed that removal of the VirA receiver module sharply increases kinase activity in vivo (4). We now show that the same mutation also broadens the substrate specificity of VirA. We propose that under noninducing conditions, a high energy of activation prevents VirA autophosphorylation and/or phosphotransfer and that inducing stimuli lower this energy threshold. Noninducing phenolic compounds such as HAP may also lower this threshold somewhat, but not sufficiently to permit detectable expression of target genes. We further propose that this energy threshold may be lower in the VirA(Δ712-829) mutant than in the wild-type protein and that this lowered threshold might allow activation by compounds that fail to activate the wild-type protein.

As we described above, previous studies from several groups have suggested that guaiacol contains the basic structural features required for the recognition by its receptor (28, 36). Our results indicate that all of the phenolic compounds having a hydroxyl group but lacking methoxy groups, including phenol, HAP, and *p*-coumaric acid, are able to induce *virA*(*Δ712-829*). In contrast, DMA, which lacks the hydroxyl group, could not detectably activate the mutant. This is consistent with previous results and suggests that the phenol rather than guaiacol contains the essential features for detectable induction, although efficient induction appears to require other structural features, including at least one methoxy group.

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