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Mutational Analysis of the Input Domain of the VirA Protein of *Agrobacterium tumefaciens*

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The transmembrane sensor protein VirA activates VirG in response to high levels of acetosyringone (AS). In order to respond to low levels of AS, VirA requires the periplasmic sugar-binding protein ChvE and monosaccharides released from plant wound sites. To better understand how VirA senses these inducers, the C58 *virA* gene was randomly mutagenized, and 14 mutants defective in *vir* gene induction and containing mutations which mapped to the input domain of VirA were isolated. Six mutants had single missense mutations in three widely separated areas of the periplasmic domain. Eight mutants had mutations in or near an amphipathic helix, TM1, or TM2. Four of the mutations in the periplasmic domain, when introduced into the corresponding A6 *virA* sequence, caused a specific defect in the *vir* gene response to glucose. This suggests that most of the periplasmic domain is required for the interaction with, or response to, ChvE. Three of the mutations from outside the periplasmic domain, one from each transmembrane domain and one from the amphipathic helix, were made in A6 *virA*. These mutants were defective in the *vir* gene response to AS. These mutations did not affect the stability or topology of VirA or prevent dimerization; therefore, they may interfere with detection of AS or transmission of the signals to the kinase domain. Characterization of C58 *chvE* mutants revealed that, unlike A6 VirA, C58 VirA requires ChvE for activation of the *vir* genes.

Agrobacterium tumefaciens causes tumors on plants by transferring a segment of its DNA, encoding genes for phytohormone and opine synthesis, from the tumor-inducing plasmid (pTi) into the plant cells, where it is integrated into the genome and expressed (for recent reviews, see references 16, 17, and 52). Ti plasmids are classified according to the opines which are synthesized in the tumors they induce. pTiC58 and pTiA6 are examples of nopaline-type and octopine-type Ti plasmids, respectively. The transfer of the DNA requires a set of virulence (*vir*) genes on pTi. These genes are expressed following activation of the VirA-VirG two-component regulatory system. VirA is a dimeric transmembrane sensor protein that detects signal molecules from wounded plant cells, autophosphorylates at a histidine residue at position 474, and transfers the phosphate to VirG (22, 34). VirA is fully activated by several classes of signals from wounded plant cells: acidic pH, phenolic compounds such as acetosyringone (AS), and certain monosaccharides, including glucose, galactose, and arabinose (1, 3, 43, 45). The latter class of inducers acts synergistically with the phenolic compounds.

The VirA protein can be divided into three domains: an input domain, consisting of a periplasmic region and two transmembrane domains (TM1 and TM2); a transmitter domain; and a receiver domain (35). The periplasmic domain is required for detection of monosaccharides (3, 5, 43). Within the input domain, adjacent to the second transmembrane domain (TM2) is an amphipathic helix, an alpha helix with strong hydrophilic and hydrophobic faces (16, 40). This structure may align with the inner membrane and help anchor the protein in the membrane (41). Within the transmitter domain is the kinase domain which includes the phosphorylatable histidine and glycine-rich segments which may serve as a nucleotide binding site (23, 35). The kinase domain is critical for tumorigenesis; mutation of the His-474 residue results in an avirulent phenotype (22). The receiver domain, which has an inhibitory role, is homologous to part of the response regulator, VirG, and may act as an additional level of control. This seems to be the case in the ArcA-ArcB two-component system, in which two signals are required, one causing phosphorylation of the receiver domain within ArcB and the other resulting in the phosphorylation of the response regulator ArcA (20). VirG is a transcriptional regulator that binds to the *vir* box within the promoter regions of all the *vir* genes and activates their expression when it is phosphorylated by VirA (21, 23).

VirA detects the inducing sugars through the periplasmic glucose/galactose-binding protein ChvE (1, 3). This protein is a chromosomally encoded virulence protein that is required for the sugar enhancement of *vir* gene induction by low levels og AS ($\leq$10 μM). ChvE is also required for chemotaxis and maximum growth in sugars and tumorigenesis on some species of plants (18). The *chvE* gene is regulated by the LysR family member GbpR in response to a subset of the *vir* gene-inducing sugars (13).

The interaction between VirA and ChvE has been the subject of several recent papers (2, 3, 5, 42, 50). Shimoda et al. (42) reported that a *virA* mutant strain that no longer showed an enhancement of *vir* gene induction in response to sugars was suppressible by a mutation in *chvE*, demonstrating genetically that VirA and ChvE interact directly. In the present study, we determined what regions of VirA are important for sensing inducers by randomly mutating the C58 *virA* gene and introducing it into the isogenic C58 strain. We isolated and characterized 14 mutants with altered induction properties and, from these and other data, developed a model for the interaction of VirA with ChvE and AS.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the strains and plasmids used in this study. *Escherichia coli* DH5α was used for all routine cloning (39). C58/*virA*−, which has a deletion of the first 2,113 bp of the *virA* gene, was provided by Deanna

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TABLE 1. *Agrobacterium* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference
Strains		
C58	Nopaline-type pTiC58	51
C58/*virA*−	*virA* deletion (2.1 kb of *virA*), Ω(Sp^r Sm^r)	37a
At11054	C58 *chvE*::Tn*5*	6
At12004	C58 *chvE* Ĝm^r Δ*virA* Ŝp^r	This work
A348	C58 chromosome, octopine-type pTiA6NC	14
MX1	A348 *chvE*::Tn*5*	18
At11068	A348 *virA* deletion, Ω(Sp^r Sm^r)	5a
Plasmids		
pSM243cd	pVK102 *virB*::*lacZ* (Cb^r)	46
pTC110	pUCD2 Δ*Pvu*II-*Eco*RV (Km^r Gm^r)	6
pTC116	pTC110 *gbpR*$^+$ *chvE*$^+$ (Km^r Gm^r)	6
pDMD2	pUCD2 Δ*Sac*II-*Kpn*I (Cb^r Tc^r)	38
pJD102W	pDMD2, C58 *virA*	15a
pIB50	pVK102 *virE*::*cat virB*::*lacZ*	1
pTC182	pKT254 *aac1* (Km^r Gm^r)	7
pUCD2	IncW (Cb^r Tc^r Km^r Gm^r)	10
pSW169	pTZ18R, A6 *virA*	53
pSL4	pUC19 *mob gbpR chvE*	13
pSL45	pJD102W, 2.8-kb *Hin*dIII-*Nco*I from HA-pJD102W	This work
pSL47	pUC18, 5.6-kb *Hin*dIII-*Bam*HI *virA*	This work
pSL48	pUC18, A6 *virA*	This work
pSL50	pUCD2, A6 *virA*	This work
pPHO7	pTZ18R *phoA*	15
pSL52	pSL47, *virA-phoA* fusion	This work
pSL52ΔTM2	pSL52 with a *virA*ΔTM2 (Leu-262 to Ile-271)–*phoA* fusion	This work
pJD104	pTZ19R, C58 *virA*Δppr	15a
pSP329Gm	pSP329 derivative (Cb^s Gm^r)	37a
pSL53	pSP329Gm, C58 *virA*Δppr from pJD104	This work
pSL54	pSp329Gm *virG* (N-54→D)	This work
pSL58	pSP329Gm *Hin*dIII, pSL52 *Hin*dIII (Cb^r Gm^r)	This work
pSL59	pSP329Gm *Hin*dIII, pSL52 ΔTM2 *Hin*dIII (Cb^r Gm^r)	This work
pSL60	pSP329Gm *Hin*dIII, pUC18 *Hin*dIII (Cb^r Gm^r)	This work

[a] Cb, carbenicillin; Km, kanamycin; Gm, gentamicin; Sp, spectinomycin; Sm, streptomycin; ˆ, insertion; Δ, deletion; HA, hydroxylamine-mutagenized; ppr, periplasmic region.

Raineri. At12004 was constructed as follows. *aac1*, encoding resistance to gentamicin, was subcloned from pTC182 (7) into the *Bam*HI site within the *chvE* gene of pSL4 (13). The resulting plasmid, pSL42, was introduced into C58/*virA*−, and colonies were screened for correct marker exchange (4). Gene replacement was confirmed by Southern analysis (44).

Plasmid constructions. pJD102W was constructed as follows: a *Pst*I site was introduced into the C58 *virA* gene of pDR169 (38) by changing thymine to cytosine at position 717 in the coding sequence and guanine to cytosine at position 720 by means of PCR amplification. The mutations were confirmed by DNA sequencing using the Sequenase version 2.0 kit according to the instructions provided by the manufacturer (United States Biochemical). This plasmid was used with the intention of utilizing the *Pst*I restriction site to facilitate subsequent mapping of the hydroxylamine-induced mutations. A 5.6-kb *Hin*dIII-*Bam*HI fragment containing this *virA* gene was cloned into pDMD2, creating pJD102W (Fig. 1). A C58/*virA*− mutant was fully complemented by pJD102W for tumor formation and *vir* gene induction (15a). The two base pair changes resulted in one silent mutation and one amino acid change from Glu to Gln at codon 241; the Gln at this position matches A6 *virA*. To verify that this mutation was not responsible for the altered *vir* gene induction phenotype of the *virA* mutants, the Glu-241-to-Gln change was reversed by site-directed mutagenesis of pSL47-14, -19, and -15 using the mutagenic oligonucleotide SLD20, the selective primer SLD15, and the USE (unique-site elimination) kit from Pharmacia used essentially as described by the manufacturer. After the sequence change was verified by sequencing, the 5.6-kb *Hin*dIII-*Bam*HI fragments were cloned into pDMD2. The resulting plasmids were introduced into C58/*virA*−(pIB50), and streaked on induction medium containing 5 μM AS, 10 mM glucose, kanamycin, carbenicillin, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). All were found to be noninducible like the original mutants, demonstrating that the phenotype was not caused by the Glu-241→Gln substitution.

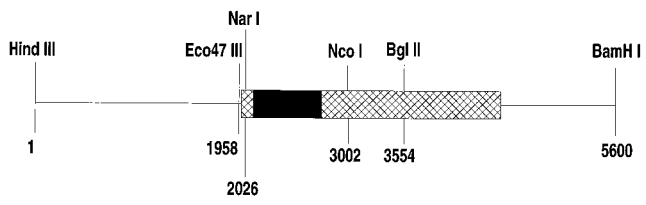

FIG. 1. Restriction map of the C58 *virA* gene. The coding sequence (cross-hatched) is from nucleotide 1983 to 4482. The sequence encoding the periplasmic domain is filled.

Two of the mutant plasmids, pSL45-10 and pSL45-14, each had two independent mutations which were separated from each other as follows. pSL45-10 and pJD102W were cut with *Nar*I and *Bam*HI. The 3.6- and 13.4-kb fragments were gel purified and switched so that the two mutations in pSL45-10 (P-7→L and A-86→V) were each isolated in separate complete *virA* genes. The two mutations in pSL45-14 were introduced separately by performing site-directed mutagenesis of pSL47, to give pSL47-14A and -14B. These plasmids were then cut with *Bam*HI and *Hin*dIII, and the two 5.6-kb fragments were ligated into pDMD2, creating pSL45-14A and pSL45-14B. pSL45-10A, -10B, -14A, and -14B were introduced into C58/*virA*−(pIB50) by triparental mating (11).

pSL48 was generated by cloning the A6 *virA*-containing *Kpn*I fragment from pSW169 (53) into pUC18. pSL48 was modified by site-directed mutagenesis using the following primers. The selection primer was SLD36; the oligonucleotides for making pSL48-2, -14, -15, -17, -19, -21, and -32 were SLD22, SLD37, SLD21, SLD17, SLD38, SLD23, and SLD39, respectively. The wild-type and mutated A6 *virA* genes were cloned into the *Kpn*I site of pUCD2, creating pSL50 and its derivatives.

Generation of *virA-phoA* fusions. pSL52 carrying a *virA-phoA* translational fusion was made as follows. pSL47, pSL47-2, -16, -21, -26, and -29 were digested with *Bgl*II, end filled with Klenow fragment, and treated with shrimp alkaline phosphatase (U.S. Biochemical). pPHO7, kindly provided by Beth Traxler (University of Washington) was cut with *Bam*HI (and *Xmn*I to facilitate purification) and end filled with Klenow fragment. The 2.6-kb *phoA*-containing fragment was ligated into pSL47 and its derivatives. pSL52 and derivatives were sequenced to

verify that the fusion was in frame. pSL52-10A and pSL52-17 resulted from site-directed mutagenesis of pSL52. The selection primer was SLD31, and the oligonucleotides SLD26 and SLD27 were used to generate pSL52-17 and pSL52-10A, respectively. pSL52ΔTM2, which has a 30-bp deletion (Leu-262 to Ile-271) of the second transmembrane domain of C58 VirA, was constructed by site-directed mutagenesis using SLD31 and SLD33.

Oligonucleotides for site-directed mutagenesis. For site-directed mutagenesis using the USE kit from Pharmacia, a selective primer and a mutagenic primer containing the desired base change were used. Selective primers were as follows. SLD15, which converted a *Kpn*I site to an *Nhe*I site, has the sequence 5′-GGA TCCCCGGCTAGCGAGCTCGAATTCG-3′. The primer SLD36, 5′-CTCTTC CTTTTTCAGGCCTATTGAAGCATTTATCAG-3′, converted the unique *Ssp*I site in pUC18 to a *Stu*I site. SLD31 (5′-CGTGAGAAGCCGAGATCTCCGGG TACCGAG-3′) converted the unique *Bam*HI site to a *Bgl*II site. Mutagenic primers were SLD20 (5′-GCCGAAAGACTGGAGCGCAAGTG-3′), SLD34 (5′-GCTCCTGCAAAATTCGCTTGC-3′), SLD35 (5′-GGAGATCAGTTACG AACTCGAC-3′), SLD22 (5′-CGAAGCCTTGGTTTATATTGGCC-3′), SLD37 (5′-GTACGCGTGCAAAATTCGCTGGCC-3′), SLD21 (5′-CATACTTGCAC GTAAAGGTCCCATTATC-3′), SLD17 (5′-GGCTACGCAAAAAAATGGAT TGGTTAGCGCGG-3′), SLD38 (5′-CTGGGAGCTCTGTGGAAGAATCTG GAAG-3′), SLD23 (5′-GCTTCAGTGGATCTTTGCCTC-3′), SLD39 (5′-CGC ATACTTGCATGTGAAGGTCCC-3′), SLD26 (5′-GCGTCGGAAAATGGCT TGGTTAAC-3′), SLD27 (5′-GAAGGTATTCACTGTCTCGGCAAG-3′), and SLD33 (5′-GAGCAGCGGGCACGGATCTTCTACATCATCTCACTGGTCT AT-3′).

Media and chemicals. All cultures of *A. tumefaciens* and *E. coli* were incubated with shaking at 28 and 37°C, respectively. *E. coli* strains were grown in Luria-Bertani broth. *Agrobacterium* strains were grown in MG/L broth (8) or ABG medium, which consists of AB minimal medium (4) with 0.5% glycerol substituted for glucose. Induction broth (IB) contains 62.5 mM phosphate buffer, AB salts (4), and 0.5% glycerol. The pH of the IB was 5.5 for all strains carrying an A6 *virA* and 5.8 for strains with C58 *virA*, so that the strains would be at their respective optimal pH for *vir* gene induction. Antiobiotics were used at the following concentrations (in micrograms per milliliter) for *E. coli*: kanamycin, 100; ampicillin, 100; gentamicin, 8; spectinomycin, 50. For *A. tumefaciens*, the concentrations were as follows: kanamycin, 100; carbenicillin, 100; gentamicin, 50; spectinomycin, 100. AS (Aldrich Chemical Company) was made up as a stock solution of 100 mg/ml in dimethylformamide.

β-Galactosidase assays for *vir* gene induction. *Agrobacterium* strains were grown overnight in 2 ml of ABG, pelleted, and resuspended in IB. One-hundred-microliter samples of the suspensions were used to inoculate 2 ml of the IB solutions. After 16 h of incubation, 1.5-ml samples of the cultures were transferred to Eppendorf tubes, pelleted, and resuspended in 600 μl of Z buffer (45). β-Galactosidase activity was assayed as described elsewhere (32, 45) with readings of optical densities at 600 and 415 nm (OD_{600} and OD_{415}) measured by a microplate reader (BIO-TECH Instruments).

The induction assays using zinnia extract employed a modified version of the protocol described by Primich-Zachwieja and Minocha (37). C58/*virA*−(pIB50, pDMD2), C58/*virA*−(pIB50, pJD102W), and At12004(pIB50, pJD102W) were grown overnight in 2 ml of ABG and washed in IB, and 100 μl was used to inoculate 2 ml of IB containing carbenicillin, 10 mM glucose, and 50 μg of kanamycin per ml. Zinnia plants (3 to 4 weeks old) were wounded by scraping the stem with a syringe needle. Six hours later, the stem tissue was harvested, weighed, and chopped thoroughly with a razor blade. Approximately equal amounts of the tissue were added to each tube of IB containing bacteria. The cultures, together with the plant tissue, were grown for 20 to 24 h. The cultures were then filtered twice through Mira Cloth (Calbiochem), and 600 μl was used to assay β-galactosidase activity as described above.

All β-galactosidase assays were performed in triplicate in at least two separate experiments.

Hydroxylamine mutagenesis and identification of mutants. Three micrograms of pJD102W DNA was mutagenized in vitro with hydroxylamine for 24 h at 37°C essentially as described elsewhere (19). The mutagen was removed by filtration as described elsewhere (1), and the mutagenized DNA (HA-pJD102W) was introduced into C58/*virA*−(pIB50) by electroporation (4). Cells were plated on induction media containing 100 μM AS, X-Gal, carbenicillin, kanamycin, and spectinomycin. After 3 days, colonies were replica plated onto induction media containing 5 μM AS and 10 mM glucose. Colonies that showed no *vir* gene induction under either condition (white colonies on both types of media) were analyzed further.

Tumor assays. *Agrobacterium* cultures were grown overnight in MG/L broth and brought to an OD_{600} equivalent of 10. Zinnia (*Zinnia elegans* cv. California Giant Mix) stems were wounded by scraping downward with a syringe needle, forming an approximately 1-cm-long wound, and 5-μl samples of the cultures were added to the wound sites. After 4 weeks, plants were scored for tumor formation.

For the dilution studies on zinnia and kalanchoë, C58/*virA*−(pJD102W), C58/*virA*−(pDMD2), and At12004(pJD102W) were grown in MG/L with carbenicillin overnight, and diluted to an OD_{600} of 1.0. The cultures were further diluted 1/1,000 and 1/10,000, and 10-μl samples were inoculated onto wound sites on kalanchoë leaves and zinnia stems.

Mapping of mutations. Plasmids (HA-pJD102W) from strains showing no *vir* gene induction either by 100 μM AS or by 5 μM AS–glucose and virulent on zinnia were isolated. Unmutated pJD102W was digested with *Hin*dIII and *Nco*I. The 2.8-kb fragment containing the 5′ third of the *virA* gene was replaced with 2.8-kb fragments containing the 5′ third of the *virA* gene was replaced with 2.8-kb fragments from the HA-pJD102W plasmids. The resulting plasmids (pSL45-plasmids) were introduced into C58/*virA*−(pIB50) by triparental mating (11), and induction was assessed by the plate assay as in the original screen. Mutations mapping to the 2.8-kb fragment were further localized to the 1-kb *Eco*47III-*Nco*I fragment.

DNA sequencing. Double-stranded-DNA sequencing was performed on the pSL45 mutant plasmid DNA by using the Sequenase kit essentially as suggested by the manufacturer. *virA* sequence was read from the *Eco*47III restriction site 24 bp upstream of the translational start site of *virA* to the *Nco*I restriction site (Fig. 1).

Western analysis. pJD104, which has the periplasmic domain of C58 VirA deleted (15a), was cut with *Bam*HI and *Hin*dIII, and the *virA*-containing band was ligated into a *Bam*HI- and *Hin*dIII-cut pSP329Gm, creating pSL53. pSL53 was introduced into all the strains to effectively induce the expression of the A6 *virA* genes without itself being detectable in the Western blot (immunoblot).

Agrobacterium cultures were grown overnight in 2 ml of MG/L and used to inoculate 50 ml of IB containing 10 mM glucose, 20 μM AS, carbenicillin, and gentamicin. After 16 h, the cells were washed with phosphate-buffered saline (PBS) and resuspended in 1 ml of PBS. Cell concentrations were adjusted to equivalent OD_{600} levels, pelleted, and resuspended in 1 ml of Laemmli buffer (25). Samples were boiled for 5 min, chilled on ice, and centrifuged for 5 min. One hundred microliters of the supernatant was loaded, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A semi-dry transfer cell Trans-Blot (Bio-Rad) was used to transfer the proteins to an Immobilon-P membrane (Millipore). The membrane was blocked with 10% milk in Tris-buffered saline (TBS), incubated with mouse antiserum against A6 VirA (28), washed with Tris-buffered saline containing 0.1% Tween, and then incubated with goat anti-mouse antibody conjugated to horseradish peroxidase. The VirA antibody complex was detected by ECL by a procedure suggested by the manufacturer (Amersham Life Science).

Dimer assay. pSL54 was constructed to facilitate expression of the mutant *virA* genes for the dimer assay. pSW167N54D (24), which encodes a constitutively active VirG protein, was cut with *Eco*RI and *Pst*I, and a 1.2-kb fragment containing the mutant *virG* gene was isolated and ligated with pSP329Gm.

The dimer assay was done essentially as described by Pan et al. (34). C58/*virA*−(pSL54) containing either pDMD2, pSL50, or pSL50-2, -17, or -21 was grown overnight in 4 ml of MG/L with carbenicillin and gentamicin. The cultures were used to inoculate 100 ml of IB containing 10 mM glucose, 0.05% yeast extract, 100 mM AS, and antibiotics. After growth for 16 h, the cells were centrifuged and the pellets were washed twice in 5 ml of PBS. The cells were then pelleted and resuspended in 1 ml of PBS, equalized to the same OD_{600}, and each sample was split and transferred into each of two Eppendorf tubes. The cells were pelleted and resuspended in 1 ml of PBS with or without freshly added Bis(sulfosuccinimidyl)suberate (BS^3, Pierce). After 60 min on ice, the cells were pelleted, resuspended in 1 ml of quenching buffer, and incubated on ice for 20 min. Sample preparation, electrophoresis, and Western blotting were done as described above.

Alkaline phosphatase assay. pSL58 and its derivatives pSL59 and pSL60 were introduced into strain C58. The cultures were grown in 2 ml of MG/L with carbenicillin overnight. Cells were washed in IB, and 100 μl was used to inoculate 2 ml of IB containing 20 μM AS and 10 mM glucose. After 16 h, the AP assay was done as described by Melchers et al. (30) with the following modifications. A 1.5-ml sample of culture was centrifuged, and the cells were resuspended in 1 ml of 10 mM Tris HCl (pH 8)–0.5 mM phenylmethylsulfonyl fluoride. The cells were centrifuged and resuspended in 1.5 ml of 1 M Tris HCl, pH 8. Five hundred microliters was reserved for reading the OD_{600}. To the remaining 100 μl, *p*-nitrophenyl phosphate (Sigma 104) was added, the reaction mixtures were incubated at 28°C for 20 min, and the reactions were stopped with 100 μl of KH_2PO_4. OD_{600} and OD_{420} values were read with a spectrophotometer (Hitachi).

RESULTS

***vir* gene induction in *chvE* mutants.** The interaction between VirA and ChvE has been studied with hybrid laboratory strains, such as A348, that contain the chromosome of the nopaline-type strain C58 and the Ti plasmid from octopine-type strains such as A6. Since the *chvE* gene is chromosomally encoded and the *virA* gene is on the Ti plasmid, we chose to carry out our studies on the wild-type C58 strain.

To determine if there are differences in *vir* gene induction involving *chvE* in the hybrid strain A348 and in the wild-type strain C58, we assayed *virB*::*lacZ* expression in the parental and two *chvE* null mutant strains. Strains A348/MX1 (18) and At11054 (6) have the same C58 chromosome with a *chvE*::Tn*5*

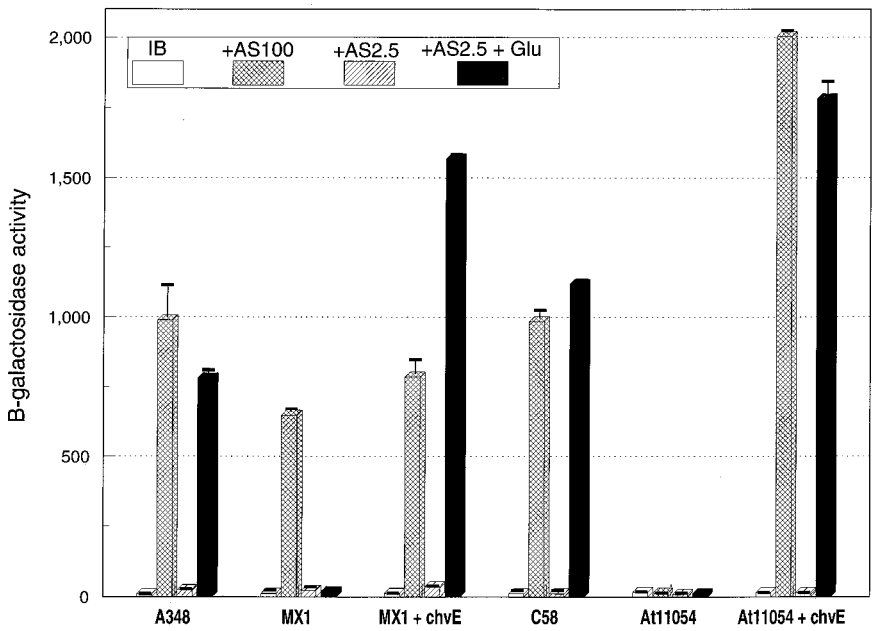

FIG. 2. *virB*::*lacZ* expression in *chvE* mutants. Strains A348(pSM243cd, pTC110), MX1(pSM243cd, pTC110), MX1(pSM243cd, pTC116), C58(pSM243cd, pTC110), At11054(pSM243cd, pTC110), and At11054(pSM243cd, pTC116) were incubated in IB with either 100 μM AS, 2.5 μM AS, or 2.5 μM AS–10 mM glucose. Standard errors are indicated.

mutant gene; they differ in that MX1 has pTiA6 and At11054 has pTiC58. Figure 2 shows that expression of the *virB*::*lacZ* gene was induced in both A348 and C58 by high levels of AS in a glycerol-containing induction medium, and glucose provided the expected synergistic effect in the presence of a low level of AS. On the other hand, the induction patterns of the *chvE* mutants were strikingly different. *vir* gene induction by high levels of AS was unaffected in the A348 *chvE* mutant (MX1), but the synergistic effect of glucose was lost, as has been previously shown (3). The synergistic effect was restored when *chvE* was added back in *trans*. However, the *chvE* mutation in C58 eliminated *vir* gene induction by AS. Addition of wild-type *chvE* restored induction by both high levels of AS alone and low levels of AS with glucose. We conclude that C58 VirA requires the ChvE protein in order to respond to even high levels of AS.

Mutagenesis of the C58 *virA* gene and screening of mutants. To determine which amino acids of the VirA protein are required for the interaction with ChvE, the entire C58 *virA* gene was randomly mutagenized with hydroxylamine. Following mutagenesis, the plasmid containing the *virA* gene was introduced into the C58/*virA* deletion strain containing a *virB*::*lacZ* fusion (pIB50) for monitoring *vir* gene induction. The transformants were replica plated onto two types of induction media, one with a high level of AS (100 μM) with no glucose and one with a low level of AS (5 μM) plus glucose. Since C58 *chvE* mutants showed no *vir* gene induction under either condition, colonies that mimicked the *chvE* mutant were selected for further study. These mutants should have mutations in *virA* affecting their ability to interact with the ChvE protein. They might also be affected in their interaction with AS or their ability to transduce the signal to VirG. These noninducible mutants were assayed for tumor-inducing ability to eliminate *virA* mutants with nonsense mutations. Those which were virulent on zinnia (like the *chvE* mutants) were chosen for further study.

Mapping and sequencing of the mutant *virA* genes. Fourteen *virA* mutants with the phenotype described above, which had mutations which mapped to the input domain of *virA* between the *Eco*47III and *Nco*I restriction sites (Fig. 1), were sequenced and characterized further.

Six mutant *virA* genes had mutations in the periplasmic region, and eight had mutations in or near the transmembrane domains. Their precise locations are identified in Fig. 3. Five of the six mutants with mutations in the periplasmic domain harbored one mutation each: pSL45-19 had an Arg-to-Trp substitution at amino acid 88, pSL45-18 had an Ala-125-to-Thr change, pSL45-1 had a Gly-131-to-Arg substitution, and pSL45-32 and pSL45-15 had an Arg-209-to-Cyt change and a Glu-210-to-Lys change, respectively. However, one of the mutant plasmids, pSL45-14, had two mutations: one led to an aspartic acid-to-asparagine substitution at codon 139; the other caused a histidine-to-tyrosine change at codon 188. Specific mutations were generated in pSL47 to make these changes individually. Only the Asp-to-Asn switch at position 139 resulted in a lack of *vir* gene induction; the other mutation (H-188→Y) gave a normal induction phenotype. Of the eight non-periplasmic-domain mutations, two were in the transmembrane domains: pSL45-2 had a serine 20-to-phenylalanine change within TM1, and pSL45-21 had a glycine 268-to-aspartic acid change within TM2. PSL45-26 had a Ser-8-to-Cyt change. pSL45-10 had two mutations, a proline 7-to-leucine change and an alanine 86-to-valine change. After the two mutations were separated, only pSL45-10A with the P-7→L change resulted in the mutant phenotype. Four of the mutant *virA* genes had mutations C terminal to the second transmembrane domain. Interestingly, two, Arg-289→Glu and Thr-284→Met, were in a recently noted amphipathic helix (40). The final two non-periplasmic-domain mutations were in pSL45-29 and pSL45-33 with Ser-333→Leu and Ala-318→Thr substitutions, respectively.

Since A6 VirA and C58 VirA seemed to interact with or respond to ChvE differently, seven of the mutations initially isolated in the C58 *virA* gene were introduced into the A6 *virA* gene by site-directed mutagenesis. These seven included four

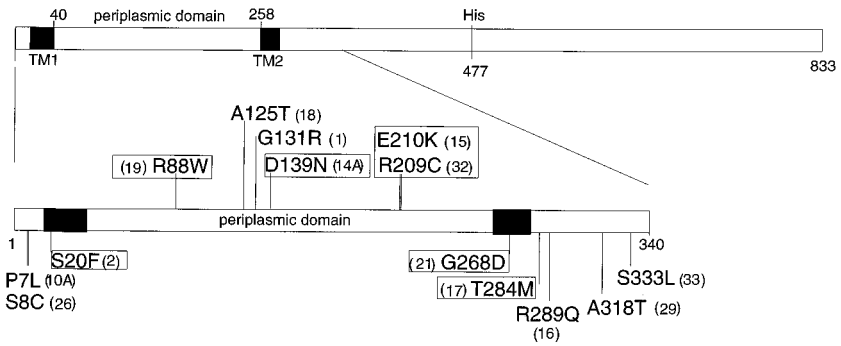

FIG. 3. Locations of the C58 VirA mutations. Mutant designations are given in parentheses, and transmembrane domains are shown as filled boxes. Mutations which were made in the corresponding A6 *virA* sequence are boxed.

mutations from the periplasmic domain, one from each of the transmembrane domains, and one from the amphipathic helix. The wild-type and mutant genes were moved into a broad-host-range vector (pUCD2) and introduced into the C58 *virA* deletion strain containing the *virB*::*lacZ* reporter plasmid.

***vir* gene induction.** Prior to comparison of *vir* gene induction levels in strains with the mutant A6 and C58 *virA* genes, a C58 *virA chvE* double mutant, At12004, was constructed in order to compare the C58 *virA* mutants with a *chvE* mutant which had the same wild-type *virA* plasmid. At12004 with the reporter plasmid pIB50 and the parental C58 *virA* gene on a plasmid (pJD102W) was assayed for induction as a control together with the C58/*virA*−(pIB50) strains carrying each of the mutant C58 *virA* genes. As shown in Tables 2 and 3, most of the *virA* mutants and the *chvE* mutant control failed to respond to either 200 μM AS or 10 μM AS with glucose. Two mutants, harboring the R-209→C and P-7→L substitutions, showed partial *vir* gene induction.

The mutations in C58 *virA* which eliminated induction by AS were compared with the corresponding A6 *virA* mutants in *vir* gene induction. As shown in Fig. 4, the strain C58/*virA*−(pIB50) containing the unmutated A6 *virA* gene (pSL50) showed *vir* gene induction by AS which was enhanced by glucose as expected. Also, the *chvE* mutant, At12004(pIB50, pSL50), behaved like its counterpart, MX1, having normal induction by AS which was not enhanced by glucose. Like this *chvE* mutant, the mutants with the A6 *virA* mutations corresponding to the C58 *virA* periplasmic-region mutations were induced by 5 μM AS like the parent but with no enhancement by glucose. This indicates that only induction by the sugar-ChvE pathway was affected in the mutants with mutations in the periplasmic domain.

Since A348 does not show significant *vir* gene induction by 5 μM AS alone, we did not expect to see induction in C58/*virA*−(pSL50, pIB50) by this small amount of AS. We considered two possible causes for this aberrant induction: (i) A6 *virA* is on a multicopy plasmid (pSL50) instead of on the Ti plasmid as in A348, and (ii) an inhibitor of A6 VirA activity is encoded on pTiA6. To distinguish between these two possibilities, we introduced pSL50 and pIB50 into the A348/*virA* mutant At11068. C58/*virA*−(pSL50, pIB50) showed significant *vir* gene induction by 5 μM AS, but A348/*virA*−(pSL50, pIB50) did not. Therefore, in A348 something prevents induction by low levels of AS. The nature of this inhibitory phenomenon is currently being investigated.

The seven mutant A6 *virA* genes were also introduced in *trans* into At11068(pIB50), and *vir* gene induction was compared with that of the parent strain (Fig. 5). The four mutants

TABLE 2. Induction of *virB*::*lacZ* expression in VirA periplasmic-domain mutants

Strain [*virA*] and medium [μM]	β-Gal (U)[a]
C58/*virA*−(pIB50, pJD102W) [wild type]	
IB	9.8 ± 0.3
+AS [200]	2,575 ± 64
+AS [10]–glucose	2,635 ± 237
C58/*virA*−(pIB50, pDMD2) [none]	
IB	12.1 ± 1.2
+AS [200]	9.8 ± 0.3
+AS [10]–glucose	9.8 ± 0.2
At12004(pIB50, pJD102W)[b] [wild type]	
IB	9.1 ± 0.9
+AS [200]	15.8 ± 1.3
+AS [10]–glucose	10.1 ± 0.1
C58/*virA*−(pIB50, pSL45-19) [R-88→W]	
IB	10.4 ± 0.7
+AS [200]	12.5 ± 0.2
+AS [10]–glucose	9.7 ± 0.2
C58/*virA*−(pIB50, pSL45-18) [A-125→T]	
IB	13.0 ± 1.2
+AS [200]	20.7 ± 2.5
+AS [10]–glucose	14.0 ± 0.5
C58/*virA*−(pIB50, pSL45-1) [G-131→R]	
IB	14.9 ± 3.4
+AS [200]	12.1 ± 1.0
+AS [10]–glucose	10.4 ± 0.7
C58/*virA*−(pIB50, pSL45-14A) [D-139→N]	
IB	10.1 ± 0.3
+AS [200]	17.5 ± 1.7
+AS [10]–glucose	11.6 ± 0.3
C58/*virA*−(pIB50, pSL45-32) [R-209→C]	
IB	13.9 ± 3.1
+AS [200]	28.9 ± 4.7
+AS [10]–glucose	513 ± 60.2
C58/*virA*−(pIB50, pSL45-15) [E-210→K]	
IB	13.3 ± 3.7
+AS [200]	25.9 ± 3.0
+AS [10]–glucose	10.2 ± 0.9

[a] β-Gal, β-galactosidase. The data are means ± standard errors of the means for three samples.
[b] chvE mutant.

TABLE 3. Induction of *virB*::*lacZ* expression in VirA non-periplasmic-domain mutants

Strain [*virA*] and medium [μM]	β-Gal (U)[a]
C58/*virA*−(pIB50, pJD102W) [none]	
IB	9.1 ± 1.3
+AS [200]	637 ± 163
+AS [10]–glucose	1,304 ± 96.4
C58/*virA*−(pIB50, pDMD2) [deletion]	
IB	5.8 ± 0.4
+AS [200]	6.2 ± 0.1
+AS [10]–glucose	6.3 ± 0.1
C58/*virA*−(pIB50, pSL45-2) [S-20→F]	
IB	13.8 ± 4.5
+AS [200]	12.9 ± 2.1
+AS [10]–glucose	38.3 ± 3.4
C58/*virA*−(pIB50, pSL45-10A) [P-7→L]	
IB	13.2 ± 3.4
+AS [200]	19.0 ± 1.3
+AS [10]–glucose	269 ± 13.7
C58/*virA*−(pIB50, pSL45-16) [R-289→Q]	
IB	13.8 ± 2.0
+AS [200]	13.6 ± 1.1
+AS [10]–glucose	42.8 ± 2.8
C58/*virA*−(pIB50, pSL45-17) [T-284→M]	
IB	6.9 ± 0.6
+AS [200]	7.0 ± 0.4
+AS [10]–glucose	6.5 ± 0.1
C58/*virA*−(pIB50, pSL45-21) [G-268→M]	
IB	12.7 ± 0.9
+AS [200]	13.2 ± 0.8
+AS [10]–glucose	23.1 ± 3.5
C58/*virA*−(pIB50, pSL45-26) [S-8→C]	
IB	21.4 ± 11.9
+AS [200]	20.6 ± 11.7
+AS [10]–glucose	28.1 ± 15.3
C58/*virA*−(pIB50, pSL45-29) [A-318→T]	
IB	9.0 ± 1.4
+AS [200]	8.4 ± 1.3
+AS [10]–glucose	11.9 ± 1.3
C58/*virA*−(pIB50, pSL45-33) [S-333→L]	
IB	9.4 ± 3.8
+AS [200]	10.3 ± 3.0
+AS [10]–glucose	54.8 ± 13.4

[a] β-Gal, β-galactosidase. The data are means ± standard errors of the means for three samples.

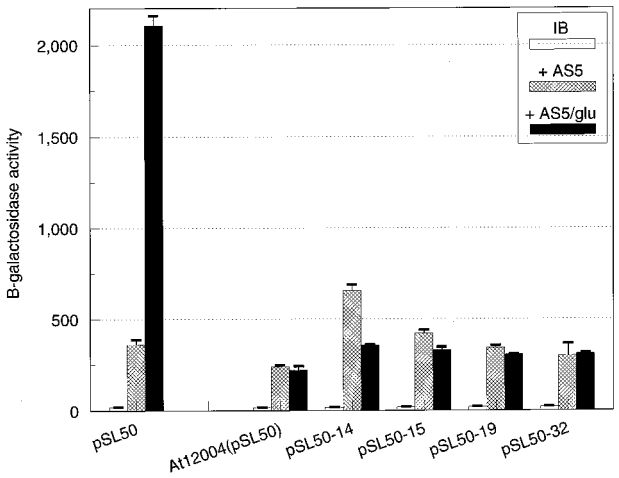

FIG. 4. Induction of *virB*::*lacZ* expression by A6 VirA in a C58 background. Strain C58/*virA*−(pIB50) containing wild-type A6 *virA* (pSL50) or an A6 *virA* mutant plasmid was incubated in IB with either 100 μM AS, 5 μM AS (AS5) or 5 μM AS–10 mM glucose.

with mutations in the periplasmic domain of VirA showed induction by high levels of AS but no induction by 2.5 μM AS in the presence of glucose. In contrast, the three non-periplasmic-domain mutants did not respond normally to AS.

VirA protein stability. To verify that a stable VirA protein was present in all of the mutants, we performed a Western analysis of the mutants. Since our VirA antiserum made against A6 VirA does not cross-react with C58 VirA, we were able to examine only the seven A6 *virA* mutants (see Materials and Methods). A functional copy of a C58 *virA* derivative (pSL53) was included in all strains as a means of expressing the A6 *virA* genes to a sufficient level to be detected by Western blot analysis. All of the mutants were found to express a stable VirA protein; although pSL50-14, -15, -19, and -21 appeared to have slightly less VirA protein (data not shown).

Virulence. Since *chvE* mutants are able to infect some but not other test plants, the *virA* mutants were tested for virulence on a variety of plants (12). The control strain C58/*virA*−(pJD102W) was virulent on kalanchoë, zinnia, tomato, datura, tobacco, and carrot. VirA is essential for virulence on all these plants, as seen by the lack of virulence of the vector control, pDMD2. The *chvE* mutant was virulent on zinnia and datura but avirulent on the other four plants. The C58 *virA* mutants had a wide range of host ranges from nearly avirulent (T-284→M) to fully virulent (R-209→C) (12).

It is surprising that the C58 *chvE* mutant was still virulent on zinnia and datura, even though there was no detectable *vir* gene induction by our in vitro assay. Since VirA is absolutely required for tumor formation, we reasoned that either (i) in zinnia, an inducer other than AS can induce the C58 *chvE* mutant, or (ii) zinnia is extra sensitive to tumor formation such that any low level of induction will lead to enough DNA transfer to result in tumors. We attempted to distinguish between these possibilities. Although the parent strain was consistently induced by an extract derived from zinnia, the *chvE* mutant was not (data not shown). Thus, there is no evidence for an inducer that functions in the absence of ChvE. To test the second possibility, dilutions of the bacteria were inoculated onto zinnia and kalanchoë (see Materials and Methods). After 4 weeks, the plants were scored for tumor formation. At high dilutions, the parent strain could induce tumors on zinnia but not on kalanchoë, suggesting that zinnia is extra sensitive to transformation (data not shown). We conclude that a level of induction too low to be measured by our reporter gene fusion is enough to induce tumors on this plant.

Dimer assay. The mutations outside the periplasmic region could interfere with *vir* gene induction in a number of ways. They might disrupt the AS binding site or prevent transmission of the activation signal to the kinase domain. Alternatively, they could prevent normal dimer formation or alter the topology of the protein. To determine if these mutants were capable of forming VirA dimers like wild-type VirA, we used BS^3, a homobifunctional cross-linker which cross-links only lysine residues that are exposed outside the cytoplasmic membrane (34). When BS^3 was incubated with intact cells containing VirA protein, cross-links were made between the seven exposed lysine residues in the periplasmic domain of VirA, resulting in several bands ranging from 205 to 222 kDa (34). We used this assay on C58/*virA*− containing pSL54, which has a mutant *virG* gene which induces the *vir* genes, including *virA*, in the absence of VirA and plant signal molecules. Into this strain, we introduced pUCD2, pSL50, or pSL50-2, -17, or -21 (see Materials and Methods). Figure 6, lanes 3 and 4, shows that addition of

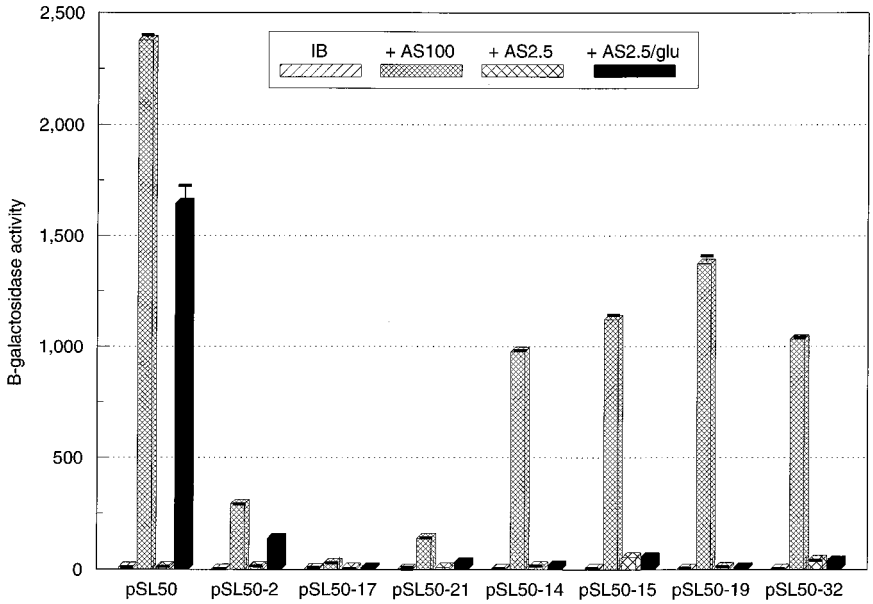

FIG. 5. Induction of *virB*::*lacZ* expression by A6 VirA in an A348 background. Strain At11068(pIB50) containing wild-type A6 *virA* (pSL50) or an A6 *virA* mutant plasmid was incubated in IB with either 100 μM AS (AS100), 2.5 μM AS, or 2.5 μM AS–10 mM glucose.

BS^3 causes the monomeric form of VirA (92 kDa) to shift to the dimeric form. The VirA Ser-20→Phe and VirA Thr-284→Met mutants show a similar shift (lanes 5 to 8); therefore, we conclude that the lack of normal *vir* gene induction in these mutants is not due to an inability to properly dimerize. The VirA Gly-268→Asp mutant, however, shows a decrease in the amount of dimer formed (lanes 9 and 10). However, it is not clear that this decrease is enough to account for the phenotype of the mutant.

Assay of membrane protein topology. It is possible that the mutations outside the periplasmic domain result in a VirA protein with an altered topology. The three N-terminal VirA mutations, P-7→L and S-8→C preceding TM1 and S-20→F within TM1, may prevent the VirA protein from inserting into the membrane. However, the P-7→L mutant (10A) is responsive to glucose (Table 3), and the S-20→F mutant (2) VirA was cross-linked with the periplasmic cross-linker. Therefore, these two mutations must not have prevented the insertion of VirA into the membrane. Seligman and Manoil (41) showed that an amphipathic helix conserved in the methyl-accepting chemoreceptor protein, Tsr, influenced the insertion of the protein into the membrane. Mutations in the amphipathic helix caused the normally cytoplasmic domain of Tsr to be exported into the periplasm. The VirA protein, like other histidine kinases and chemoreceptor proteins, has an amphipathic helix (16, 40). Since many of our mutants had mutations in the transmembrane domains and in the amphipathic helix (amino acids 278 to 288), we determined whether any of the mutations caused the C-terminal end of VirA to be exported. *virA-phoA* fusions were constructed with the parental C58 *virA* gene, the eight mutant C58 *virA* genes with mutations outside the periplasmic domain, and a positive-control *virA* gene with TM2 deleted. The fusions were made in a region of VirA that normally resides in the cytoplasm, but in the control the lack of TM2 would cause it to be periplasmic. If the mutations cause an alteration in the membrane topology of VirA, the alkaline phosphatase would be inappropriately exported to the periplasm, where it would be active. A *virD4*::Tn*phoA* fusion served as a positive control for the assay (10a). All of the constructs were introduced into C58, and alkaline phosphatase activity was measured. Figure 7 shows that, of all the *virA-phoA* constructs, only the *virA*ΔTM2-*phoA* control showed activity. Therefore, we conclude that none of the mutations outside the periplasmic domain interfere with the proper insertion of TM2 into the membrane.

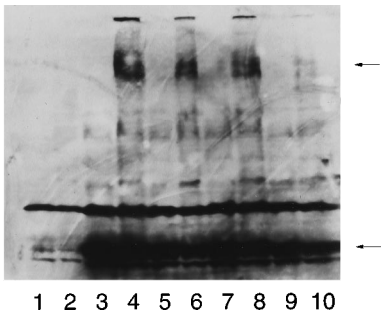

FIG. 6. Protein blot analysis of wild-type and mutant A6 VirA cross-linked with BS^3. Strain C58/*virA*−(pSL54) with pUCD2 (lanes 1 and 2), pSL50 (lanes 3 and 4), pSL50-2 (lanes 5 and 6), pSL50-17 (lanes 7 and 8), or pSL50-21 (lanes 9 and 10) was incubated with (even lanes) or without (odd lanes) BS^3. Upper arrow and lower arrow, dimer and monomer forms of VirA, respectively.

DISCUSSION

We have shown that C58 VirA requires the periplasmic glucose-galactose-binding protein ChvE in order for it to respond to AS as well as to glucose. This contrasts with numerous studies using hybrid laboratory strains containing an A6, or similar octopine-type, *virA* gene in a C58 chromosomal background in which high levels of AS induced the *vir* genes in the absence of ChvE (3, 42, 43, 50). Mutants with alterations in the periplasmic region of A6 VirA which no longer showed enhanced *vir* gene induction in response to glucose yet still responded to high levels of AS could be isolated. In contrast, of approximately 2,000 hydroxylamine-generated C58 *virA* mu-

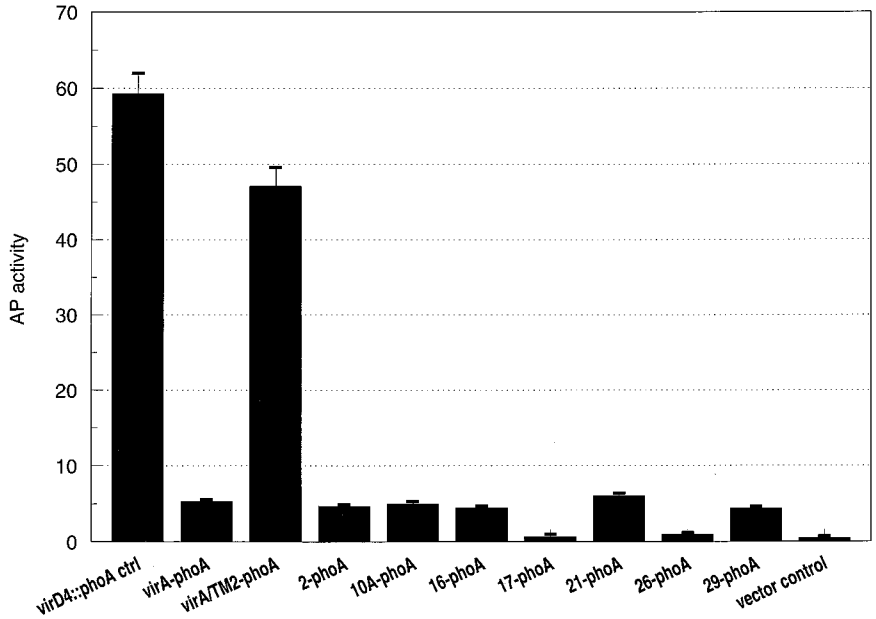

FIG. 7. Alkaline phosphatase (AP) activities of *virA-phoA* fusions. Standard errors are indicated.

tants that were screened, none were defective in the synergistic effect of glucose without also being defective in the response to high levels of AS. These data suggest that ChvE, with or without its sugar ligand, is able to interact with VirA and that this interaction is required by C58 VirA in order for AS to induce the *vir* genes. Thus, any *virA* mutant that cannot interact with ChvE also would not be induced by AS. Whether it would be possible to isolate such mutants in the wild-type strain A6 or whether it is possible only in hybrid strains is not known.

When the mutations in the periplasmic domain in C58 VirA were made in the corresponding A6 *virA* sequence, only the synergistic response by glucose on AS induction was lost. Both A6 and C58 VirA proteins require the ChvE-sugar complex in order for low concentrations of AS to induce. Heath et al. described a VirA/VirG-ChvE model for activating the *vir* genes that proposed that VirA has three states of activity: off, standby, and on (16). They suggested that monosaccharide-bound ChvE interacts with the periplasmic domain of VirA, thereby relieving this domain of a repressive or nonfunctional conformation and placing VirA into a standby conformation, poised to react to AS. In the absence of sugars, the few VirA molecules at any given time that randomly achieve the standby conformation could respond to AS only if AS was at an appropriately high concentration to saturate VirA. Our current data suggest that, unlike A6 VirA, C58 VirA is unable to achieve the standby mode without ChvE.

Random mutagenesis of the C58 *virA* gene yielded 14 mutants with altered induction properties. All of the mutations resulted in substitutions in amino acid residues which were conserved between C58 and A6. Overall, C58 VirA is 73% identical to A6 VirA in amino acid sequence (33). The mutations in the periplasmic domain are scattered from position 88 to 210 but seem to cluster in three groups. One particularly noteworthy mutant is the Glu-210→Lys mutant. Shimoda et al. introduced a mutation by site-directed mutagenesis at this location (Glu-210→Val), which resulted in a lack of sugar enhancement of AS induction. This mutant was suppressible by a mutation in *chvE* (42). Since we were able to obtain this mutant known to be defective in the VirA-ChvE interaction by random mutagenesis and screening, our method was successful. Our data are consistent with the proposal that the region between TM1 and position 229 is required for the synergistic activity of sugars on AS induction (29). We propose—on the basis of the similarity between VirA and bacterial chemoreceptors (3, 30), the known structure of the chemoreceptor Tar (31, 35), and the secondary-structure prediction program (IntelliGenetics) based on the Chou and Fasman algorithm (9)—that the periplasmic domain of VirA is a four-helix bundle (Fig. 8). We and others have found *virA* mutants with changes throughout the periplasmic domain (2, 42, 50), suggesting that the entire three-dimensional structure is important for proper signal transduction.

Although it seems clear that the mutations in the periplasmic domain of VirA are disrupting the ability of VirA to interact with or to respond to ChvE, it is unclear how the

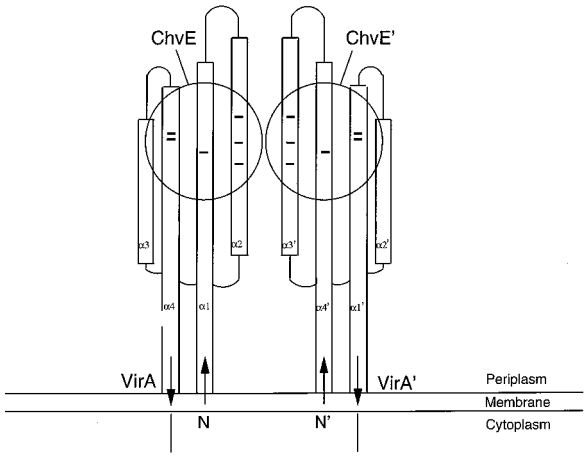

FIG. 8. Model of the interaction between ChvE and VirA. Alpha helices, numbered α1 to -4 for each monomer of VirA, are indicated. Dashes, locations of the six mutations in the periplasmic domain.

mutations outside the periplasmic domain affect induction. Since none of the mutations outside the periplasmic domain affect the stability or topology to a significant degree or prevent the dimerization of the VirA protein, these areas presumably have a role in either detecting or responding to plant signal molecules.

It is uncertain if the detection of AS by VirA is direct (27) or indirect via a phenolic binding protein (26). However, recent genetic evidence suggests that the VirA protein directly senses AS (27). Whether the interaction is direct or indirect, VirA deletion experiments suggest that the region distal to TM2 may be required for VirA to respond to AS. This response may include interacting with AS or transducing a signal. Melchers et al. interpreted their deletion data to suggest that the AS-responsive region is in or near TM2 (30). Chang and Winans, by a deletion analysis (5), localized it to a region within amino acids 324 to 413. Turk et al. deleted a nearby region, amino acids 283 to 304, also producing a loss of VirA activity (49). Several of our noninducing *virA* mutants have mutations in these areas. An amphipathic helix, an alpha helix with a strongly hydrophobic face and a strongly hydrophilic face, has been found in all chemoreceptors and a large number of the sensor histidine kinases, including the VirA protein (40). It was proposed that this helix associates with the inner membrane (41). It is possible that VirA has been adapted to detect the hydrophobic phenolic compounds or an AS-binding protein by using this amphipathic helix. Since our VirA Thr-284→Met mutant did not seem to be defective in VirA protein stability, topology, or dimerization but yet was severely affected in *vir* gene induction and virulence, the mutation may lie in the area critical for binding AS. Its location is in the area predicted by Turk et al. to be the site of AS binding.

Evidence that the transmembrane domains of chemoreceptors and histidine kinases are critical for the ability of the protein to respond to signals continues to mount. Mutations in TM1 of the sensor protein, EnvZ, cause specific signaling defects with alterations in the relative levels of kinase and phosphatase activities (47). One mutation in TM2 was shown to be suppressible by an intragenic mutation in TM1, demonstrating genetically that TM1 and TM2 may interact (48). Studies of the chemoreceptor protein Tar indicate that the transmembrane domain of each monomer within a dimer may interact to propagate a signal and that the TM1 domains of the Tar dimer are in close proximity and may act as a fulcrum for propagating structural changes (35). A previous mutational study of VirA, in which a Leu-24→Phe mutation in TM1 resulted in VirA being active in the absence of plant signals, demonstrated that the transmembrane domain of VirA is important for signaling (36). Furthermore, Turk et al. found that replacement of either TM domain of VirA with that of Tar resulted in a reduced response to AS and to the synergistic response of AS and sugars (49). Our mutations in the TM domains resulted in a lack of normal induction by high levels of AS, even in A6 VirA, suggesting that these domains are important for signaling in general.

The proposed standby position for VirA may involve an interaction of TM1 with TM2 which may cause conformational shifts that expose the amphipathic helix to AS. The amino-terminal tail may also be involved in the transmission of the signal by interacting with the region past the amphipathic helix defined by the Ala-318→Thr and Ser-333→Leu mutants.

ACKNOWLEDGMENTS

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