Mutants of Agrobacterium VirA That Activate vir Gene Expression in the Absence of the Inducer Acetosyringone*

(Received for publication, August 17, 1993, and in revised form, October 11, 1993)

B. Gail McLean, Elizabeth A. Greene, and Patricia C. Zambryski
From the Department of Plant Biology, University of California, Berkeley, California 94720

In the presence of inducer molecules produced by wounded plants, the VirA/VirG two-component positive regulatory system of Agrobacterium tumefaciens initiates transcription of virulence genes required for crown gall tumor formation. Exactly how this system enables the bacterium to respond to an environmental signal is not known, but phosphorylation of VirA and VirG plays a role. To analyze further the function of VirA, we chemically mutagenized the virA gene. Two mutants that activate vir transcription without the plant inducer acetosyringone were found; these mutants alter VirA function by distinct mechanisms. One mutant functions entirely independently of acetosyringone, whereas the activity of the second mutant is enhanced by acetosyringone. Both mutants function best at acid pH, but respond differently to specific monosaccharides that stimulate induction by wild-type VirA. Both mutant phenotypes are dominant over wild-type VirA, and both need the conserved histidine at the autophosphorylation site for strong inducer-independent vir transcription.

Protein kinases regulate many diverse biological processes such as cell division, chemotaxis, and differentiation. Protein phosphorylation appears to be a signaling mechanism conserved between prokaryotes and eukaryotes. In prokaryotes, the best characterized protein kinases belong to a family of regulatory systems, termed two-component regulatory systems, in which typically two proteins transform an environmental signal into a metabolic response (1-3). When responding to an environmental cue, the sensor component functions as a protein kinase by autophosphorylating a conserved histidine residue and then transferring a phosphoryl group to an invariant aspartate residue on the second component, the response regulator protein. The response regulator component then activates transcription of other genes to allow the organism to adapt to environmental change.

The VirA/VirG two-component regulatory system on the tumor-inducing plasmid of Agrobacterium tumefaciens enables this soil bacterium to cause tumors in dicotyledonous plants (4-6). When wounded, plant cells release specific phenolic signals that induce this soil bacterium to cause tumors in dicotyledonous plants. Exactly how this system works was supported by United States Department of Agriculture Grant 90-37622-5291 (to P. C. Z.) and National Institutes of Health Postdoctoral Grant F-32-GM14124 (to B. G. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We address the effects of various media and environmental conditions on the mutant phenotype and find that the two mutations activate VirA in very different ways.

MATERIALS AND METHODS

Plasmids—The plasmid used for mutagenesis of virA (pMutA) contained the virA and virG genes, an R1 origin of replication for propagation in Escherichia coli, and a ColEl origin of replication for propagation in Agrobacterium, a ColEl origin of replication for propagation in E. coli. pMutA was constructed in three steps. First, the 2.8-kbp PstI/KpnI fragment of the vector pTZ18U (United States Biochemical Corp.) was ligated to the 8.1-kbp KpnI/XbaI fragment containing the R1 origin of replication from pJB11 (30). Next, a 1.3-kbp NheI/BstXI fragment

* This work was supported by United States Department of Agriculture Grant 90-37622-5291 (to P. C. Z.) and National Institutes of Health Postdoctoral Grant F-32-GM14124 (to B. G. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: 111 Koshland Hall, Dept. of Plant Biology, University of California, Berkeley, CA 94720. Tel.: 510-643-9204; Fax: 510-642-4995.

1 The abbreviations used are: AS, acetosyringone; kb, kilobase pair; MES, 4-morpholineethanesulfonic acid; BstRI, 2-bis(2-hydroxyethyl)amine-2-(hydroxymethyl)-propane-1,3-diol.
Agrobacterium A136, a strain lacking a tumor-inducing plasmid, to Agrobacterium; this step, however, decreased the number of transformants and was used only once.

The reporter plasmids used to assay vir transcription, pBlacZ and pABlacZ, each contain a virB::lacZ translational fusion. pBlacZ is derived from pSM405cd, which contains a virB::lacZ translational fusion in the carbenicillin- and kanamycin-resistant wide host range plasmid pVCK102 (4, 31); pABlacZ is derived from pSM402, which contains virA, virG, and a virB::lacZ translational fusion also in pVCK102 (4, 31). Digestion with BamH1 removed a fragment containing carbenicillin resistance from pSM405cd and both carbenicillin and virG from pSM402; religation formed the reporter plasmids pBlacZ and pABlacZ, respectively. The reporter plasmids then are maintained by kanamycin resistance, whereas pMutA is maintained by carbenicillin resistance. In addition to the virB::lacZ translational fusion, pABlacZ also retains wild-type virA. Each reporter plasmid was electroproporated into Agrobacterium A136, a strain lacking a tumor-inducing plasmid, to form either strain A136(pBlacZ) or A136(pABlacZ). Electroporation stocks of each reporter strain were prepared and stored at -70°C until use as recipients of chemically mutagenized DNA.

Mutagenesis—A modification of the random chemical mutagenesis method (32) was used to mutagenize virG. Approximately 2-5 μg of pMutA single-strand DNA was mutagenized with dimethyl sulfate or formic acid. Then, single-strand linear DNA complementary to all of the plasmid except the DNA targeted for mutagenesis (virA) was annealed to the mutagenized single-strand DNA. In some experiments, the entire virG coding sequence was left single-stranded; since DNA polymerase has no template to direct repair, the enzyme replaces a chemically mutagenized base nonspecifically. In other experiments, DNA representing either the 5'- or 3'-half of virA was annealed to the treated DNA to direct mutations to the single-strand half of virA. The partially double-strand plasmids were then electroproporated into Agrobacterium strain A136(pBlacZ) either to determine the extent of mutagenesis or to isolate virA mutations. The cells were plated on AB/MES pH 5.5 medium with no AS. After 3 days at 28°C, colonies from dimethyl sulfate-treated DNA, 10% were white, suggesting that 10% of the population had mutation(s) that inactivated virA, whereas 20% of the population had a mutation(s) that inactivated VirA. To identify mutations that allow constitutive expression of the virB::lacZ fusion, A136(pBlacZ+pMutA) cells were plated on AB/MES pH 5.5 medium with AS. After 3 days at 28°C, blue colonies were purified; pMutA plasmid DNA was isolated from these colonies, transformed into and isolated out of E. coli, and then electroporated back into Agrobacterium. The ability of the VirAmutant mutants to activate vir transcription was quantified in liquid cultures by measuring β-galactosidase activity of the virB::lacZ reporter fusion in the absence and presence of AS. For initial characterization, cells were induced in AB/MES pH 5.5 minimal medium containing 0.4% glucose (Fig. 2). In the absence of AS, the level of virB expression activated by the VirAmutant mutants is 900 (G471E) to 1500-fold (G665D) higher than that seen with uninduced wild-type VirA. In the presence of AS, wild-type and VirAmutant mutants increase expression to the same level. Thus, AS does not seem to hyper-induce vir transcription by either VirAmutant mutant.

Interestingly, AS affects expression by G471E, but not G665D. Since our standard assay reflects β-galactosidase ac-
cumulation over 14 h, we cannot detect differences in the kinetics of AS induction. Consequently, cells were grown with or without AS, and aliquots were taken every 2 h to assay for \(\beta\)-galactosidase activity. As shown in Fig. 3, AS has no effect on \(\text{vir}\) transcription by G665D. Compared with G665D, \(\text{vir}\) transcription by G471E seems to experience a lag phase, after which G471E cultures with AS initially show a sharper increase in expression than cultures without AS. Following this initial increase, the presence of AS in G471E cultures results in consistently higher levels of \(\text{vir}\) transcription. Increased copy number of \(\text{vir}\) (5–10 copies/cell) does not prevent the G471E lag phase, indicating that the phenotype of G471E is not a reflection of limiting VirG protein (data not shown). Although G665D and G471E promote similar rates of \(\text{vir}\) expression, G665D is fully active without AS and appears unresponsive to AS, whereas G471E is partially active and still responsive to AS. Thus, these results (Figs. 2 and 3) suggest that the mutations in G471E and G665D modify \(\text{VirA}\) in significantly different ways.

**Effects of Monosaccharides on Mutant Phenotype**—In addition to plant phenolics, \(\text{vir}\) gene transcription is affected by environmental stimuli such as specific monosaccharides and acid pH (6–11, 15). Several laboratories have shown that in the presence of low levels of phenolics (1–10 \(\mu\)M), monosaccharides such as sucrose and glycerol do not; at high concentrations of AS (100 \(\mu\)M), monosaccharides play no role in \(\text{vir}\) induction (9–11). This effect of specific monosaccharides on \(\text{vir}\) induction is mediated by \(\text{VirA}\) and ChvE, a periplasmic sugar-binding protein (9, 33).

To examine the effects of monosaccharides on \(\text{vir}\) gene induction, the \(\text{VirA}\) mutants were assayed in minimal medium containing either glucose or glycerol as the carbon source (Fig. 4A). Just as shown in Fig. 2, in the presence of glucose, G471E activates \(\text{virB}\) expression without AS, whereas addition of AS increases expression. Interestingly, when 0.5% glycerol is substituted for 0.4% glucose, G471E almost completely reverts back to a wild-type phenotype. With glycerol, G471E resembles wild-type \(\text{VirA}\) both in the absence of AS and in the presence of high levels of AS (100 \(\mu\)M); at low concentrations of AS (1 \(\mu\)M), however, \(\text{virB}\) expression is 3-fold higher with G471E than with wild-type \(\text{VirA}\). The second mutant (G665D) responds differently. With glucose, G665D increases expression of \(\text{VirB}\) without AS 1500-fold higher than wild-type \(\text{VirA}\), and addition of AS does not significantly increase \(\text{vir}\) induction. With glycerol and no AS, G665D increases \(\text{virB}\) expression 12-fold over wild-type \(\text{VirA}\); with low AS concentrations, 12–15-fold over wild-type \(\text{VirA}\); and with high AS concentrations, 3-fold over wild-type \(\text{VirA}\). Both mutants then best activate \(\text{vir}\) transcription in medium containing glucose. Substitution of glycerol for glucose represses the constitutive phenotype of G471E as well as decreases the maximal induction by wild-type \(\text{VirA}\), G471E, and, to a lesser extent, G665D.

To test whether the different carbon sources specifically influence \(\text{vir}\) induction versus general cell growth rates, cells were grown without or with 1 \(\mu\)M AS in minimal medium containing 0.5% glycerol and either 10 \(\mu\)M sucrose or glucose as metabolic sources (Fig. 4B). With sucrose, both the activity and the response to AS of each mutant resemble those seen with glycerol alone. Thus, in the absence of AS, G471E requires monosaccharides for constitutive expression of the \(\text{virB}\) fusion, and at low AS concentrations, it induces \(\text{virB}\) to a greater extent than wild-type \(\text{VirA}\), indicating that the mutant is still more active than wild-type \(\text{VirA}\). Unlike G471E, G665D does not require specific monosaccharides for constitutive expression of \(\text{virB}\), although monosaccharides enhance expression by increasing the maximum level of induction. These distinct responses to monosaccharides further illustrate how the mutants differ.

**Effects of pH on Mutant Phenotype**—Another environmental factor known to influence \(\text{vir}\) induction is pH (8, 12). If the pH of the growth medium exceeds pH 6.0, little induction of \(\text{vir}\) gene transcription occurs. Using deletion mutants, Chang and Winans (27) suggested that the \(\text{VirA}\) cytoplasmic linker domain plays an undefined yet required role in sensing both acid environment and phenolic compounds such as AS. The point mutations identified in our mutants do not map to this domain. Still, since the \(\text{VirA}\) mutants function without needing to sense AS, they also may not need an acid pH. Thus, to determine the effects of the mutations on pH sensitivity, minimal media were buffered to either pH 5.5 or 7.0 with BisTris (Fig. 5). At pH 7.0, both mutants expressed \(\text{virB}\) 2–2.5-fold higher than wild-type \(\text{VirA}\) in the absence of AS. In the presence of AS, wild-type \(\text{VirA}\) did not induce \(\text{virB}\) expression at pH 7.0; yet, relative to wild-type \(\text{VirA}\), the \(\text{VirA}\) mutants increased expression 2–3-fold with G665D, inducing \(\text{vir}\) transcription more strongly than G471E. Nevertheless, compared with expression at pH 5.6, overall expression at pH 7.0 was dramatically lower. Thus, both \(\text{VirA}\) mutants function best in an acid environment.

**Effects of Wild-type \text{VirA} on Mutant Phenotype**—To determine if the presence of wild-type \(\text{VirA}\) altered the phenotype of the \(\text{VirA}\) mutants, the mutants were expressed in A136(pABlacZ) containing both the \(\text{virB}::\text{lacZ}\) fusion and wild-type \(\text{virA}\) (Fig. 6). \(\beta\)-Galactosidase assays show that wild-type \(\text{VirA}\) has no significant effect on either \(\text{VirA}\) phenotype. These results differ from those of Pazour et al. (29), who found that the phenotype of their \(\text{VirA}\) inducer-independent mutants was codominant with wild-type \(\text{VirA}\); the presence of a tumor-inducing plasmid encoding wild-type \(\text{VirA}\) suppressed the constitutive mutant phenotype in \(\beta\)-galactosidase assays. Based on these data, they hypothesized that \(\text{VirA}\) functions as a dimer. Pazour et al. also found that disruption of \(\text{virA}\) on the tumor-inducing plasmid only partially restored the \(\text{VirA}\) mutant phenotype, suggesting that other factors on or encoded by the tumor-inducing plasmid affect this phenotype (29). In our assays, the plasmid encoding wild-type \(\text{virA}\) did not have any major effect on tumor-inducing plasmid genes other than a \(\text{virB}::\text{lacZ}\) translational fusion.

**Modification of Phosphorylation Site in Mutants**—Like all characterized sensors of two-component systems, \(\text{VirA}\) auto-phosphorylates a conserved histidine (\(\text{VirA}\) residue 474) (17, 18). The constitutive phenotype of the \(\text{VirA}\) mutants may then result from changes in autophosphorylation at histidine 474 or in phosphotransfer to \(\text{VirG}\) or possibly by phosphorylation at other sites in \(\text{VirA}\). In \text{in vitro} assays using the cytoplasmic
Agrobacterium VirA Mutants Activate vir Gene Expression

**FIG. 3.** Time course for induction of virB::lacZ by VirAs" mutants with and without inducer. Strains were grown in AB/MES pH 5.5 medium containing 1 x AB salts, 50 mM MES, 2.5 mM phosphate, 0.4% glucose, 50 μg/ml kanamycin, and 100 μg/ml carbenicillin with or without 100 μM AS as inducer. Aliquots were removed every 2 h and assayed for β-galactosidase activity. □, no AS; □, 100 μM AS.

**FIG. 4.** Effects of sugars on induction of virB::lacZ by VirAs" mutants. Strains were grown in AB/MES pH 5.5 medium containing 1 x AB salts, 50 mM MES, 2.5 mM phosphate, 50 μg/ml kanamycin, and 100 μg/ml carbenicillin. A, either 0.5% glycerol or 0.4% glucose was used as carbon source; B, both media contained 0.5% glycerol and were supplemented with either 10 mM glucose or 10 mM sucrose. Shaded bars, no AS; white striped bars, 1 μM AS; black striped bars, 100 μM AS.

A portion of VirA did not demonstrate changes in VirA autophosphorylation rates between wild-type and mutant VirA, and attempts to assay phosphorylation differences between wild-type and mutant VirA contained in Agrobacterium inner membrane extracts were not successful (data not shown). To determine if the VirAs" mutants G471E and G665D allow phosphorylation at amino acids other than the conserved histidine, histidine 474 was changed to glutamine, which cannot be phosphorylated and produces biologically inactive VirA (17). β-Galactosidase assays show that histidine 474 is required by both G471E and G665D for significant levels of expression of virB (data not shown). However, both with and without AS, G665D with the mutated histidine 474 slightly induces expression, 2-fold over wild-type VirA, suggesting that an alternative phosphorylation site may be inefficiently used in the mutant.

Use of Low Copy Number Plasmid for Isolation and Characterization of VirG Mutants—A strategy similar to that used for the isolation of VirAs" mutants was applied to the isolation of VirG" mutants. The virG coding region, contained on the pMutG plasmid, was chemically mutagenized; no VirG" mutants were found after screening 120,000 colonies, 80,000 A136(pBlacZ+pMutG) and 40,000 A136(pABlacZ+pMutG). This negative result prompted us to compare our strategy with that used previously to isolate VirG" mutants (34-36). For this comparison, a previously identified VirG" mutant, N54D, was constructed using oligonucleotide-directed mutagenesis to replace asparagine at codon 54 with aspartic acid; when present on a multicopy plasmid, the N54D mutant causes a VirG" phenotype, i.e. induction of vir gene transcription in the absence of VirA and AS (38-40). Potentially, the low copy number (1-2 copies/cell) of pMutG, the plasmid encoding virG, prevented isolation of VirG" mutants in our system, and indeed, pMutG containing the N54D mutant did not exhibit a VirG" phenotype (Fig. 7). In addition to the standard assay using a virB::lacZ reporter, the lack of the VirG" phenotype in N54D at low copy number was confirmed by a more sensitive antibiotic challenge using a virB::neomycin phosphotransferase reporter plasmid. Thus, N54D in the low copy system appears inactive (Fig. 7); that this mutant is active in the multicopy system may imply that artificially high levels of N54D are needed to activate vir transcription.

**DISCUSSION**

In this study, we isolated two VirA mutants that activate vir
transcription in the absence of the plant phenolic inducer AS. Mutant G665D, identified in three isolates, causes vir transcription in the absence of AS at the same high levels as wild-type VirA in the presence of AS. Mutant G471E causes vir transcription in the absence of AS to ~50% of maximal induction and is further induced to high levels in the presence of AS. Thus, these two mutants appear to activate VirA differently.

**Effects of Mutations on VirA**—Mutant G471E maps 3 residues from the phosphorylation site at histidine 474 and replaces glycine at position 471 with glutamic acid. Without AS, G471E requires monosaccharides for strong constitutive vir transcription; when isolated and assayed by Ankenbauer et al. (28), however, this mutant did not require monosaccharides to promote vir transcription. Differences in monosaccharide, culture medium, and method of analysis may account for these contrasting results. For example, Ankenbauer et al. compared the response of wild-type and mutant VirA in 2.5 μM AS to varying concentrations of β-glucuronide after 24 h; we compared the response of wild-type and mutant VirA in 1 μM to one concentration of glucose (10 mm) after 14 h (28). As reported previously, G471E functions best in an acid environment and induces little vir transcription at pH 7.0 (28). The second mutation replaces glycine at position 665 with aspartic acid. Unlike G471E, this mutant (G665D) does not require monosaccharides. Like G471E, G665D functions best at acid pH, but some activation of vir transcription is observed at pH 7.0.

The mutations in the VirA™ mutants may cause a conformational change that mimics the action of AS. Hess et al. (37) postulated that AS may be protonated when bound by its receptor, possibly VirA, resulting in the now-activated phenol protonating a basic site on the receptor to induce a conformational change. This conformation then activates the receptor. Alternatively, if AS does not bind VirA as has been suggested, the AS-binding protein itself may interact with VirA and induce a conformational change (38). Introduction of the negative charges of glutamic acid at position 471 or of aspartic acid at position 665 in the VirA™ mutants may resemble the AS-induced conformation of VirA. Addition of AS to G471E may induce a more appropriate activated conformation and further induce vir transcription. Interestingly, AS does not have a significant effect on G665D. Although amino acid 665 is not in the region presumed to interact with VirA and induce a conformational change (38). The mutations in the VirA™ mutants may cause a conformational change that mimics the action of AS. Hess et al. (37) postulated that AS may be protonated when bound by its receptor, possibly VirA, resulting in the now-activated phenol protonating a basic site on the receptor to induce a conformational change. This conformation then activates the receptor. Alternatively, if AS does not bind VirA as has been suggested, the AS-binding protein itself may interact with VirA and induce a conformational change (38). Introduction of the negative charges of glutamic acid at position 471 or of aspartic acid at position 665 in the VirA™ mutants may resemble the AS-induced conformation of VirA. Addition of AS to G471E may induce a more appropriate activated conformation and further induce vir transcription. Interestingly, AS does not have a significant effect on G665D. Although amino acid 665 is not in the region presumed to interact with VirA, the conformation induced by this negatively charged amino acid may reproduce exactly the AS-induced conformational change.

**Potential Effects of Mutations on Phosphorylation**—*In vitro* phosphorylation assays did not detect obvious differences between wild-type VirA and either VirA™ mutant. We then used a genetic approach to show the importance of the phosphorylation site at histidine 474 for constitutive vir transcription by the mutants. In the absence of histidine 474, both G471E and wild-type VirA cannot activate vir transcription. However, mutant G665D appears to activate vir transcription at very low levels; this minimal expression may result from weak autophosphorylation activity or from phosphorylation by another kinase in the bacterium. Thus, both VirA™ mutants still require histidine 474 for constitutive activation of vir expression, suggesting that activation of VirA involves both conformational changes and phosphotransfer.

In agrobacteria containing both wild-type VirA and VirA™, the mutant phenotype dominates. Whereas this dominance may simply result from a much higher rate of autophosphorylation or phosphotransfer by VirA™ to VirG, phosphotransfer between mutant and wild-type VirA molecules also may occur. This mechanism may be analogous to transphosphorylation between shortened CheA and kinase-deficient CheA variants that allows the variants to phosphorylate CheY, a receiver response component (39). Potentially, then, transphosphorylation of wild-type VirA by the VirA™ mutants may activate both species of VirA to maintain the AS-independent phenotype. Our results differ from those of Pazour et al. (29), who found that wild-type VirA suppressed the phenotype of their VirA™ mutants. They proposed that VirA functions as a dimer and that
components on the tumor-inducing plasmid further influence VirA activity. Our data neither support nor contradict the hypothesis that active VirA functions as a dimer. For instance, if VirA is a dimer, the dominance of our mutant phenotypes suggests that the mutants can transactivate wild-type VirA; alternatively, lack of inhibition of the mutant phenotype by wild-type VirA is consistent with the protein functioning as a monomer.

Effects of Copy Number on Mutagenesis—As noted, the characteristics of the VirA<sup>mut</sup> mutants identified in this study sometimes differ from those of VirA<sup>mut</sup> mutants isolated by other laboratories. Unlike the multicycoplasms used by other laboratories for mutagenesis, the copy number of the plasmid encoding our VirA<sup>mut</sup> mutants mimics the copy number of the tumor-inducing plasmid. How the VirA<sup>mut</sup> mutants function in our system should then mirror how the mutants would function encoded on a tumor-inducing plasmid. When this mutagenesis strategy was applied to VirG on the same copy number plasmid, no mutations were found that allowed VirG to function without VirA and AS, although such mutations have been found using a multicopy plasmid approach (38–40). Characterization of a VirG mutation previously identified on a multicopy plasmid further illustrates the differences between approaches. When this VirG mutant (N54D) is encoded on a multicopy plasmid, it functions in the absence of VirA and AS (34–36). When expressed at 1–2 copies/cell, however, this mutant does not function even when a more sensitive antibiotic selection is substituted for the β-galactosidase screen (data not shown). This result demonstrates an important contrast between our mutagenesis approach and those used previously and confirms the importance of copy number for transcriptional activation by the VirA/VirG system.

Location of Mutations Near Conserved Sensor Domains—The VirA mutants in G471E and G665D map near two of the five short blocks of common sequence found variably spaced among sensor proteins (1, 2). Of the five common motifs, the most variable is a 9-amino acid block (block H (VirA amino acids 472–480)) that includes the histidine autophosphorylation site and may function as separate nucleotide binding components, blocks G1 (VirA amino acids 627–635) and G2 (VirA amino acids 655–660), resemble glycine-rich regions of a soror proteins (2). That a mutation at this site has been produced by three laboratories using three mutagenesis methods strongly implies that this site is important for VirA function (28, 29). Exclusive of the conserved histidine, mutations at residues in block H in other sensor components show that the local structure around the histidine influences phosphorylation/dephosphorylation activities (40, 41). In the same manner, the mutation at glycine 471, just outside of block H, may activate VirA by altering the local structure around histidine 474. Two other conserved domains in the sensor components, blocks G1 (VirA amino acids 627–635) and G2 (VirA amino acids 655–660), resemble glycine-rich regions of a nucleotide-binding site and may function as separate nucleotide-binding domains (1, 2). In sensor proteins such as EnvZ, mutations in these two conserved motifs destroy autophosphorylase activity (42, 43). Mutant G665D maps just outside of block G2, and an inducer-independent mutant isolated by Pazour et al. (29) maps in the middle of block G2 at amino acid 658. Parkinson and Kofoid (1) suggested that this region could be a functional analog of the cGMP-binding site of the cGMP-dependent protein kinases; this binding site helps modulate protein conformation to activate the kinase. Identification of two mutations in this region suggests that it too plays an important role in VirA activation of vir gene transcription.

As mentioned, AS affects G471E and G665D differently, suggesting that the two mutations activate VirA by two distinct mechanisms. To activate wild-type VirA, AS is proposed to somehow cause a conformational change in VirA (29, 37). This activated conformation may bind nucleotides at the proposed binding sites in blocks G1 and G2, initiating phosphotransfer to VirG and derepressing potential inhibition by the VirA receiver domain. Rather than being directly involved in phosphotransfer, the nucleotide bound at block G2 may simply modulate VirA conformation to activate transphosphorylation either to VirG and/or within VirA to derepress potential autoinhibition. Since its mutation maps near block G2, the conformation of G665D may directly mimic AS-induced nucleotide binding at block G2; consequently, addition of AS does not influence the activity of G665D. However, with its mutation mapping near the autophosphorylation site, the conformation of G471E somehow transfers phosphate to VirG, but lacks the potential transphosphorylation and/or derepression necessary to fully activate VirA. Addition of AS to G471E may then induce nucleotide binding at block G2, leading to complete activation of VirA.

The mapping of both mutants (G471E and G665D) to two regions conserved among sensors demonstrates the importance of these regions for the interaction between VirA and VirG. The identification of mutations at or near these amino acids by other laboratories re-enforces that these two domains are crucial for VirA function. Notably, the response of the two VirA<sup>mut</sup> mutants (G471E and G665D) to the inducer AS differs significantly. Consequently, the mutants isolated from this study can be used to analyze two major aspects of vir expression. First, by separating environmental sensing from transcriptional activation, the mutants focus attention on interactions of the sensor VirA with both its own domains and its response regulator (VirG). Second, based on their very different responses to the inducer AS, the mutants provide tools for analyzing the specific role of AS in VirA activation, a complex and not well understood process that gives Agrobacterium its ability to exploit wounded plant cells.

Acknowledgments—We thank Vitaly Citovsky for critical reading of this manuscript and Kyunghee Shin and David Lynn for help with Agrobacterium inner membrane preparations.

REFERENCES

1. Parkinson, J. S., and Kofoid, E. C. (1992) Annu. Rev. Genet. 26, 71–112
2. Stock, J. B., Ninfa, A. J., and Stock, A. M. (1989) Microbiol. Rev. 53, 450–490
3. Albright, L. M., Huila, E., and Aunubel, F. M. (1989) Annu. Rev. Genet. 23, 311–336
4. Stachel, S. E., and Zambryski, P. C. (1986) Cell 46, 325–333
5. Leroux, B., Yanofsky, M. F., Winans, S. C., Ward, J. E., Ziegler, S. F., and Nester, E. W. (1987) EMBO J. 6, 849–856
6. Winans, S. C., Elbert, P. R., Stachel, S. E., Gordon, M. P., and Nester, E. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8278–8282
7. Stachel, S. E., Messens, E., Van Montagu, M., and Zambryski, P. (1985) Nature 318, 624–629
8. Stachel, S. E., Nester, E. W., and Zambryski, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 579–583
9. Cangelosi, G. A., Ankenbauer, R. G., and Nester, E. W. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6708–6712
10. Ankenbauer, R. G., and Nester, E. W. (1990) J. Bacteriol. 172, 6442–6446
11. Shimoda, N., Toyoda-Yamamoto, A., Nagamine, J., Usami, S., Katayama, M., Sakagami, Y., and Machida, Y. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6684–6688
12. Winans, S. C., Kerstetter, R. A., and Nester, E. W. (1990) J. Bacteriol. 170, 4047–4054
13. Melchers, L. S., Regenburg, T. T., Bourret, R. B., Sedee, N. J., Schilperoort, R. A., and Hoekkaas, P. J. (1989) EMBO J. 8, 1919–1925
14. Zambryski, P. C. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 465–490
15. Winans, S. C. (1992) Microbiol. Rev. 56, 12–31
16. Birns, A., and Thomasaw, M. (1988) Annu. Rev. Microbiol. 42, 575–606
17. Jin, S., Roitsch, T., Ankenbauer, R. G., Gordon, M. P., and Nester, E. W. (1990) J. Bacteriol. 172, 525–530
18. Roitsch, T., Morel, P., Powell, B., and Kado, C. I. (1990) J. Bacteriol. 172, 1142–1144
19. Jin, S. G., Prusti, R. K., Roitsch, T., Ankenbauer, R. G., and Nester, E. W. (1990) J. Bacteriol. 172, 4945–4950
20. Pazour, G. J., and Das, A. (1990) J. Bacteriol. 172, 1241–1249
21. Pazour, G. J., and Das, A. (1990) Nucleic Acids Res. 18, 6900–6913
22. Powell, B. S., and Kado, C. I. (1990) Mol. Microbiol. 4, 2159–2166
Agrobacterium VirA Mutants Activate vir Gene Expression

24. Aoyama, T., and Oka, A. (1990) FEBS Lett. 263, 1-4
25. Roitsch, T., Wang, H., Jin, S. G., and Nester, E. W. (1996) J. Bacteriol. 172, 6054-6060
26. Winans, S. C., Kerstetter, R. A., Ward, J. E., and Nester, E. W. (1989) J. Bacteriol. 171, 1616-1622
27. Chang, C. H., and Winans, S. C. (1992) J. Bacteriol. 174, 7033-7039
28. Azkanzauer, R. G., Best, E. A., Palanca, C. A., and Nester, E. W. (1991) Mol. Plant Microbe Interact. 4, 400-406
29. Pazour, G. J., Ts, C. N., and Das, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6941-6945
30. Jouanin, L., Vilaine, F., d'Enfert, C., and Casse-Delbart, F. (1985) Mol. Gen. Genet. 201, 370-374
31. Knauf, V. C., and Nester, E. W. (1982) Plasmid 8, 45-54
32. Myers, R. M., Lerman, L. S., and Maniatis, T. (1985) Science 229, 242-247
33. Huang, M. L., Cangelosi, G. A., Halperin, W., and Nester, E. W. (1990) J. Bacteriol. 172, 1814-1822
34. Pazour, G. J., Ts, C. N., and Das, A. (1992) J. Bacteriol. 174, 4169-4174
35. Han, D. C., Chen, C. Y., Chen, Y. F., and Winans, S. C. (1992) J. Bacteriol. 174, 7040-7043
36. Jin, S., Song, Y., Pan, S. Q., and Nester, E. W. (1993) Mol. Microbiol. 7, 555-562
37. Hess, K. M., Dudley, M. W., Lynn, D. G., Joerger, R. D., and Binna, A. N. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7854-7858
38. Lee, K., Dudley, M. W., Hess, K. M., Lynn, D. G., Joerger, R. D., and Binna, A. N. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8666-8670
39. Wolfe, A. J., and Stewart, R. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 10, 1518-1522
40. Russe, F. D., and Silhavy, T. J. (1991) J. Mol. Biol. 222, 567-580
41. Aiba, H., Nakazai, F., Mizushima, S., and Mizuno, T. (1989) J. Biol. Chem. 264, 14090-14094
42. Yang, Y., and Inouye, M. (1993) J. Mol. Biol. 231, 333-342
43. Kanamaru, K., Aiba, H., Mizushima, T., and Mizuno, T. (1989) J. Biol. Chem. 264, 21635-21637