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Constitutive Mutations of *Agrobacterium tumefaciens* Transcriptional Activator *virG*

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The virulence (*vir*) genes of *Agrobacterium tumefaciens* Ti plasmids are positively regulated by *virG* in conjunction with *virA* and plant-derived inducing molecules. A procedure that utilizes both genetic selection and a genetic screen was developed to isolate mutations in *virG* that led to elevated levels of *vir* gene expression in the absence of *virA* and plant phenolic inducers. Mutants were isolated at a frequency of 1 in 10^7 to 10^8. Substitution mutations at two positions in the *virG* coding region were found to result in the desired phenotype. One mutant had an asparagine-to-aspartic acid substitution at residue 54, and the other contained an isoleucine-to-leucine substitution at residue 106. In both cases, the mutant phenotype required the presence of the active-site aspartic acid residue at position 52. Further analysis showed that no other substitution at residue 54 resulted in a constitutive phenotype. In contrast, several substitutions at residue 106 led to a constitutive phenotype. The possible roles of the residues at positions 54 and 106 in VirG function are discussed.

The virulence (*vir*) genes of the plant pathogen *Agrobacterium tumefaciens* catalyze the transfer of a segment of Ti plasmid-borne DNA to plant cells, leading to crown gall tumor disease (reviewed in references 26 and 43). The *vir* region encompasses about 35 kb of DNA and is composed of eight operons, *virA* to *virH* (7, 12, 17, 22, 31). Of these, *virA* and *virG*, in conjunction with plant phenolics such as acetosyringone (AS), positively control *vir* gene expression (32, 33, 42). A chromosomal gene, *chvE*, is also required for efficient induction of the *vir* genes (8). DNA sequence analysis indicated that VirA and VirG, the polypeptide products of *virA* and *virG*, respectively, are members of the bacterial two-component regulatory system family (15, 41). In two-component regulatory systems, one component, the sensor, senses an environmental stimulus and transmits a signal to the second component, the regulator, which then controls a cellular function. The signal transduction process is mediated through protein phosphorylation. In response to a stimulus, the sensor protein is autophosphorylated at a conserved histidine residue. This phosphate moiety is then transferred to a conserved aspartic acid residue of the regulator (reviewed in references 27 and 35). It is believed that in the *Agrobacterium vir* system, VirA, a transmembrane protein, functions as the sensor and VirG, a cytosolic protein, functions as the regulator. VirG is a sequence-specific DNA-binding protein that binds at conserved *vir* box sequences (11, 23) and is thought to function as a transcriptional activator when chemically modified by phosphorylated VirA.

To understand the signal transduction process, attempts were made to isolate mutations in *virA* and *virG* by using a genetic screen (25). All of the mutations identified in that study mapped to the *virA* locus, although this approach theoretically should have yielded mutations in *virG* as well. With the assumption that mutations in *virG* are rare, we used a combination of genetic selection and a genetic screen to isolate *virG* mutants. In this report, we describe the effectiveness of such a dual approach and an analysis of critical residues of the regulator component of a two-component regulatory system.

Isolation and characterization of constitutive *virG* mutants. Plasmid pGP358R (kanamycin resistant), which contains *virG* and *virB* gene fusions with the β-lactamase (*bla*) and β-galactosidase (β-gal) (*lacZ*) genes, was constructed as follows. The kanamycin resistance gene from plasmid pUC4K was first cloned as a *Pst*I fragment into plasmid pUC119 (36). An *Eco*RI site was created between the ribosome-binding site sequence (30) and the translational start codon of the β-lactamase gene (1 residue upstream of the ATG codon) by deoxyoligonucleotide-directed site-specific mutagenesis (14). The resultant plasmid was digested with *Eco*RI and filled in with T4 DNA polymerase and deoxynucleoside triphosphates. The 3.3-kb fragment containing the promoterless *bla* gene was ligated to a 330-bp *Sma*I fragment of plasmid pAD1221 (24). The *Sma*I fragment contains the promoter-regulatory region and part of the first open reading frame of the *Agrobacterium virB* locus. A clone that contained *virB* in the same orientation as the *bla* structural gene was isolated and designated pGP357. Plasmid pGP357 was cloned as an *Eco*RI fragment (the *Sma*I fragment containing *virB* sequences contains an *Eco*RI site upstream of the *virB* promoter region) into the unique *Eco*RI site of plasmid pGP220 to construct pGP358R. Plasmid pGP220 contains *virG* and a *virB-lacZ* gene fusion on wide-host-range vector pTJS75 (29). The *virB-bla* fusion was constructed such that the *bla* gene lacks its native ribosome-binding site sequence. The DNA sequence of the junction region reads dgggatccccAATTCTATG; the lowercase letters indicate *virB* sequences, and the underlined sequences indicate the translational initiation codon for the *bla* gene. It was necessary to construct this fusion because earlier experiments showed that a *virB-bla* fusion that retained the *bla* ribosome-binding site sequence can confer resistance to a high level of carbenicillin, even in the absence of an inducer (data not shown). We reasoned that the fusion in pGP358R would be translated less efficiently because the *bla* gene segment lacked its native ribosome-binding site sequence;

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therefore, increased transcription would be required to compensate for the lower translation efficiency and, consequently, for antibiotic resistance. A constitutive *virG* that is independent of *virA* and plant signal molecules is expected to induce transcription of the *virB* promoters in pGP358R, leading to formation of carbenicillin-resistant (from *virB-bla*) blue (from *virB-lacZ*) colonies on solid medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

To determine whether plasmid pGP358R can be used for selection of mutants with increased *vir* gene expression, this plasmid was introduced into *Agrobacterium* strains A136 (which lacks a Ti plasmid) and A348 (which contains octopine Ti plasmid pTiA6). Under normal growth conditions, both strains were sensitive to carbenicillin. To determine whether *vir* gene-inducing conditions confer carbenicillin resistance, 10^6 *Agrobacterium* strain A348/pGP358R bacteria were plated on AB Mes (pH 5.5) solid medium (23) with or without 100 μM AS. A filter paper disc impregnated with 300 μg of carbenicillin was placed in the center of the plate, and cells were allowed to grow at 30°C. In the absence of AS, the zone of inhibition was approximately 6 cm in diameter, while that in the presence of AS was only 0.2 to 0.6 cm (data not shown). These results indicated that this gene fusion could be used to select mutants with elevated *vir* gene expression.

Agrobacterium strain A136/pGP358R was mutagenized with nitrous acid or nitrosoguanidine (19). For nitrosoguanidine mutagenesis, cells were grown at 30°C in AB Mes (pH 5.5) liquid medium to an A_{600} of ~0.2. Nitrosoguanidine was then added at a final concentration of 0.13 μg/ml, and cell growth was continued for an additional 60 min. To stop the mutagenesis, cells were collected by centrifugation and washed with AB (pH 7.0) liquid medium. After resuspension, cells were plated on AB Mes (pH 5.5) solid medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (16 μg/ml) and carbenicillin (50 to 200 μg/ml). Nitrous acid mutagenesis was performed essentially as described by Miller (19), except that cells were not allowed to grow before plating. In both mutagenesis procedures, 75 to 80% killing of the cells was observed.

Carbenicillin-resistant cells that formed blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-containing plates in the absence of AS were picked, purified, and assayed for β-gal activity in liquid medium. Carbenicillin-resistant colonies appeared at a frequency of approximately 1 in 10^5 to 10^6 cells, of which only 1% turned blue in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. By this combination of positive selection and screening, 43 *Agrobacterium* strains with elevated levels of *virB* expression were isolated. Eight of these strains resulted from nitrosoguanidine mutagenesis, and the remainder were from nitrous acid mutagenesis. In liquid assays, the mutant strains showed 200 to 1,000 U of β-gal activity. The *virG* genes from five of these mutants were cloned into plasmid pUC119, and the complete DNA sequence of the *virG* open reading frame was determined by the dideoxy-chain termination method of Sanger et al. (28) by using a double-stranded DNA template, a series of synthetic deoxyoligonucleotide primers, and Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). All mutants were found to contain a single base change (an A-to-G change at position 432, numbered as described by Winans et al. [41]). This transition creates a new restriction endonuclease site for *Xba*I (TCTAGA) and leads to substitution of an asparagine residue at position 54 with an aspartic acid (N54D; amino acids were numbered as described by Pazour and Das [23]; this mutation has also been isolated independently by S. Winans [39]). The rest of the potential mutants were screened for this new restriction site. Of the 43 mutants, 42 contained this site. DNA sequence analysis of the other mutant (obtained by nitrosoguanidine mutagenesis) indicated that it contains an A-to-C change at position 585 which alters codon 106 from isoleucine to leucine (I106L).

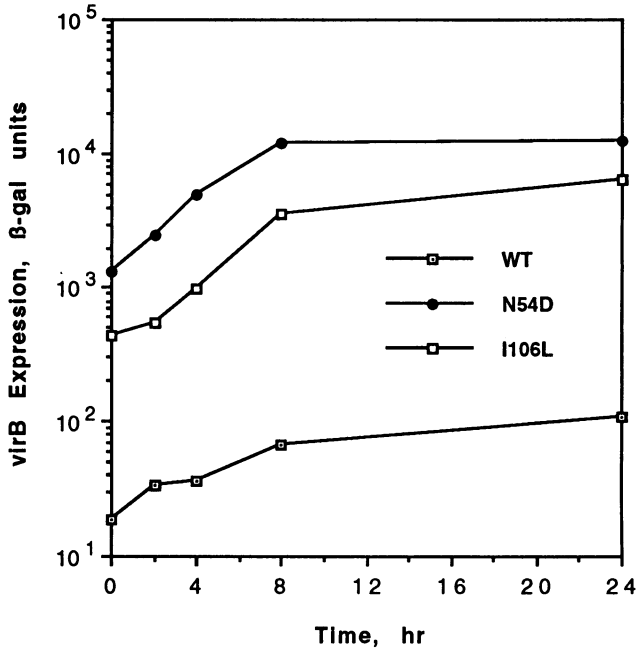

FIG. 1. Effects of *virG* and its mutations on *virB-lacZ* expression. The N54D or I106L mutation was introduced into the *virG* gene of plasmid pGP109 (23) by deoxyoligonucleotide-directed site-specific mutagenesis as described by Kunkel (14). Plasmid pGP109 or its mutant derivative was then fused to plasmid pAD1092K (5) at its respective *Eco*RI site to construct pGP229 and its derivatives. Plasmid pAD1092K contains a *virB-lacZ* gene fusion on wide-host-range plasmid vector pTJS75 (29). pGP229 and its derivatives were mobilized from *Escherichia coli* into *Agrobacterium* strain A136 by triparental mating (6). Cells were grown overnight in AB (pH 7) liquid medium, diluted 1:20 in AB Mes (pH 5.5) medium, and grown for various times as indicated. Procedures for the β-gal activity assay were as described previously (23). WT, wild-type *virG*; N54D, *virG* N54D; I106L, *virG* I106L.

To confirm that the phenotypes observed were due to the single base substitutions, these mutations were recreated by deoxyoligonucleotide-directed site-specific mutagenesis (14). *virG* and its derivatives containing the N54D or I106L mutation were cloned into plasmid pAD1092K, which contains a *virB-lacZ* reporter gene (5). After introduction of these plasmids into *Agrobacterium* strain A136, the resulting strains were assayed for β-gal activity in the absence of AS (Fig. 1). The two strains with *virG* N54D or I106L showed a significantly higher level of *virB* expression, even at time zero (time of transfer of cells grown overnight in AB [pH 7] medium into induction medium AB Mes [pH 5.5]), indicating that the constitutive phenotype of the mutants is manifested even at pH 7. In the *virG* mutant strains, *virB* expression increased for about 8 h before reaching a plateau.

In a strain harboring wild-type *virG*, the level of *virB* expression increased approximately fivefold during the 24-h incubation period. The maximum level of *virB* expression in the *virG* N54D mutant strain in the absence of the inducer

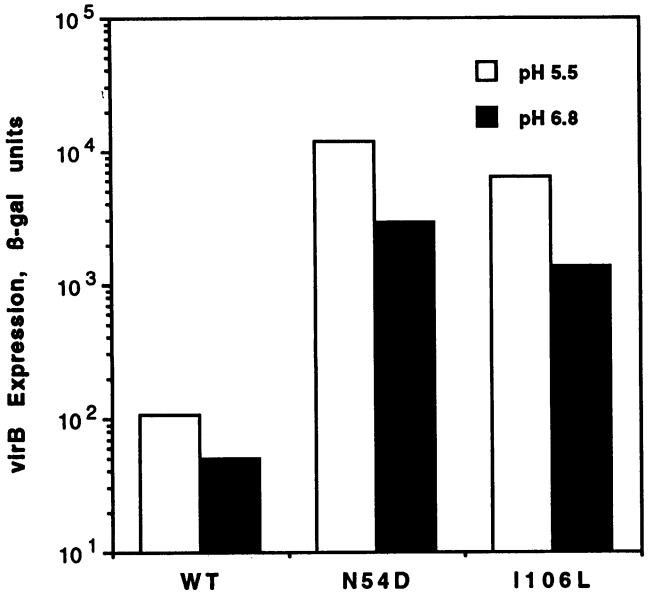

FIG. 2. Effect of pH on the phenotype of *virG* mutants. Procedures and strains used were identical to those described in the legend to Fig. 1, except that after overnight growth in AB (pH 7), cells were diluted into AB Mes (pH 5.5 or 6.8), as indicated, and grown for 24 h before assay. WT, wild type.

AS was found to be approximately fourfold higher than the fully induced level (in the presence of *virA* and AS) in strains containing wild-type *virG*, indicating that the *vir* genes are not maximally expressed under standard induction conditions. The level of *virB* expression observed with *virG* N54D and I106L was greater than that observed with *virG* from the supervirulent Ti plasmid pTiBo542 of *Agrobacterium* strain A281. The *virG* gene of pTiBo542 is believed to be responsible for the supervirulent phenotype of *Agrobacterium* strain A281. In a recent study, Chen et al. (3) reported that *virG* from pTiBo542 causes a 1.7-fold increase in *virB* expression compared with *virG* from pTiA6. Whether the large increase in *virB* expression we observed in strains bearing the *virG* mutations leads to an increase in the amount of T-strand DNA, and consequently to increased virulence, remains to be seen.

Effect of pH on the *virG* constitutive phenotype. To study the effect of pH on *vir* gene expression, strains containing *virB-lacZ* and *virG* or *virG* mutations were grown in AB Mes (pH 5.5 or 6.8) liquid medium and assayed for β-gal activity. Both *virG* mutant strains exhibited an approximately fourfold decrease in *virB* expression at pH 6.8 (Fig. 2). The basal level of expression in the strain containing wild-type *virG* was reduced twofold at the higher pH. These results indicate that the transcriptional activator function of *virG* is modulated by an extracellular acidic environment.

An acidic environment is essential for maximal expression of the *vir* genes. It has been thought that *virA* is the primary sensor of the environment, since deletion of the periplasmic domain of *virA* largely relieves the requirement for an acidic environment (18, 25). However, the phenotype produced by *virG* N54D and I106L is affected by the pH of the growth medium, even in the absence of *virA*, indicating that there is an additional step(s) that is pH dependent. Extracellular pH is not expected to have a major effect on intracellular pH, but it is known to alter the intracellular concentration of ions such as K^+ (9). The changes in ion concentration may directly affect VirG activity. Alternatively, there may be an additional unidentified component(s) that senses extracellular pH and affects *virG* expression and/or activity. Expression of *virG* is regulated by several factors, including pH (40). The pH effect is exerted through transcriptional activation of a second promoter. It is unlikely that the pH effect observed in this study is due to transcriptional activation from the pH-inducible promoter, because these mutants should autocatalytically (by transcriptional activation of the *virA*, *virG*, AS-dependent promoter) increase their cellular concentration much more significantly than expected from the induction of the pH-sensitive promoter.

TABLE 1. Effects of *virA* and AS on *virG* mutant phenotype[a]

Strain	Relevant genotype	*virB-lacZ* expression (β-gal U)	
		No AS	With AS
A136/pGP159	*virA virG*	9	3,280
A136/pGP396	*virA virG*N54D	15,100	14,900
A136/pGP404	*virA virG*I106L	864	15,800

[a] Plasmids pGP109, pGP109N54D, and pGP109I106L were cloned into pGP119 as an *Eco*RI fragment to construct pGP159, pGP396, and pGP404, respectively. After mobilization of the plasmids into *Agrobacterium* strain A136, the resulting strains were assayed for β-gal activity as described in the legend to Fig. 1. Where indicated, AS was added at a final concentration of 100 μM.

Effects of VirA and AS on *virG* mutants. To study the effect of VirA and AS on the *virG* mutants, plasmids were constructed which contained *virA*, the reporter *virB-lacZ* gene fusion, and *virG* or its mutant derivatives. After introduction of these plasmids into *Agrobacterium* strain A136, the resulting strains were assayed for β-gal activity (Table 1). Both mutations led to a large increase in *virB-lacZ* expression in the absence of the inducer (AS). The N54D mutation led to a 1,756-fold increase in *virB-lacZ* expression, while the I106L mutation caused a 96-fold increase. In the presence of AS, no significant change in *virB* expression was observed in strains bearing the *virG* N54D mutation; however, an additional 18-fold increase in *virB* expression was observed in the *virG* I106L mutant strain.

Other substitutions at position 54 or 106. To determine the effects of other substitution at either the N54 or the I106 position, additional substitutions were created by site-specific mutagenesis. Several substitutions at the N54 position (N→E, F, I, L, P, R, S, W, or Y) abolished *virG* activity, while others (N→G, H, K, M, or T) attenuated *virG* activity (Fig. 3). However, no substitution other than aspartic acid led to a constitutive phenotype. In contrast, no substitution at the I106 position abolished *virG* activity and several substitutions (I106→F, L, N, P, or Y) led to a constitutive phenotype.

Effect of *virA* on *virG* I106 substitutions. We investigated whether the phenotype of the *virG*I106 substitutions identified in studies described in the preceding section is independent of *virA*. Plasmids containing *virB-lacZ* and *virG* mutations were constructed and introduced into *Agrobacterium* strain A136, and the strains were assayed for β-gal activity (Fig. 4, open bars). All of the mutants exhibited a constitutive phenotype in the absence of *virA* and AS, indicating that the mutants function independently of VirA. The level of *virB* expression in strains bearing the *virG* I106F, I106N, or I106Y mutation did not differ significantly in the presence or absence of *virA* (Fig. 4). In contrast, *virB* expression in

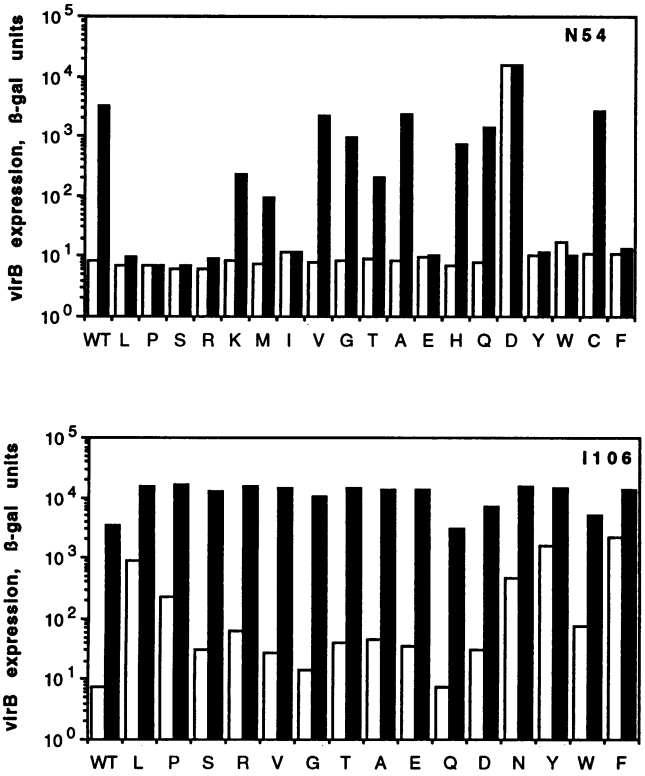

FIG. 3. Effects of amino acid substitutions at positions 54 and 106 of *virG* on *virB-lacZ* expression. Substitutions were introduced at the desired position by deoxyoligonucleotide-directed site-specific mutagenesis of pGP109 DNA as described previously (25). The mutagenic primer contained a random assortment of bases at codon position 54 or 106. The identities of the mutations were determined by DNA sequence analysis (28). The mutant pGP109 derivatives were fused to pGP119, introduced into *Agrobacterium* strain A136, and assayed for β-gal activity as described in the legend to Fig. 1. Amino acid substitutions are indicated by the single-letter code. Open bars, no AS; dark bars, with AS; WT, wild type.

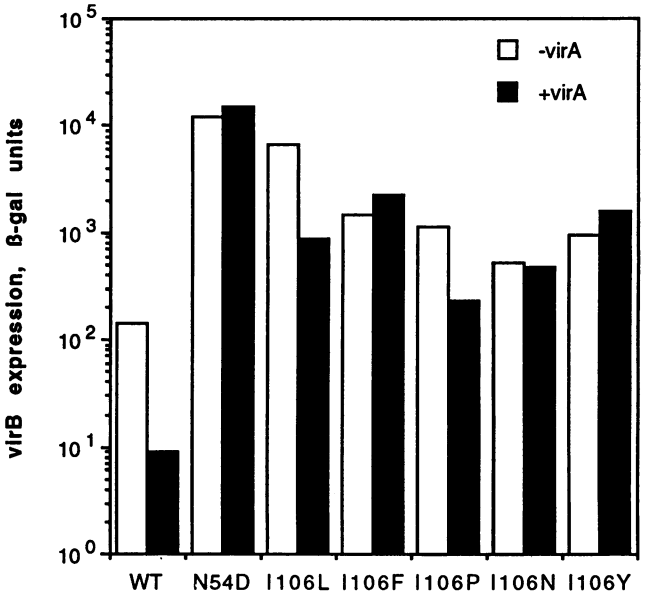

FIG. 4. Effect of *virA* on *virB-lacZ* expression. The effect of *virA* on *virB* expression was monitored in different *virG* backgrounds. *virG* and its mutant derivatives were cloned into plasmid pAD1092K (*virA*) or pGP119 (*virA*$^+$) and introduced into *Agrobacterium* strain A136, and the resulting strains were assayed for β-gal activity as described in the legend to Fig. 1. WT, wild type.

strains bearing wild-type *virG*, *virG* I106L, and *virG* I106P was higher in the absence of *virA*. The reason for this difference is not apparent and is under investigation.

Effect of alteration of the active-site aspartic acid residue. In two-component regulatory systems, an aspartic acid residue (D52 for VirG) of the regulator is phosphorylated by the corresponding sensor protein (35). The phosphorylated regulator is believed to be the active form that controls cellular functions. Phosphorylation presumably causes a structural change which activates the protein. The mutants isolated in this study can function by (i) stabilizing the aspartyl phosphate, (ii) locking the protein in an active configuration to mimic the effect of phosphorylation, or (iii) changing a site(s) distal to the site of phosphorylation, e.g., a site necessary for interaction with RNA polymerase. To distinguish between these possibilities, we sought to determine whether the N54D and I106L mutations in *virG* act independently of the aspartic acid residue at the active site. We constructed double mutants containing *virG* D52E and N54D or I106L and analyzed the effects of these mutations on *virB* expression. Substitution of the aspartic acid residue at position 52 with glutamic acid abolished VirG activity (Table 2). The loss of activity in *virG* D52E may be due to (i) the inability of this residue to be phosphorylated or (ii) structural alteration associated with this mutation. Introduction of either the N54D or the I106L mutation into *virG* D52E did not restore its transcriptional activator function, indicating that the aspartic acid residue at position 52 is required for manifestation of the constitutive phenotype of the mutants. These results indicate that the third possibility for how these mutants act is unlikely.

Activating mutations have been isolated in several two-component system regulators, including *cheY*, *degU*, *glnG*, and *ompR* (2, 4, 20, 38). Examples of mutations which act by each of the first two possibilities mentioned above have been found. *cheY* D13K, like phosphorylated CheY, causes a tumble phenotype, but this protein is not readily phosphorylated in vitro, making it likely that this mutation allows the protein to function independently of phosphorylation. A mutation in *ompR*, *ompR3*, acts by stabilizing the aspartyl phosphate (1). It seems more likely that the mutants described in the present study act by mimicking the phosphorylated state of the protein rather than by stabilizing the aspartyl phosphate, because these mutants are *virA* independent. Therefore, any phosphorylation of VirG would have to be through cross talk (21). The latter mechanism would also

TABLE 2. Effect of mutation of the active-site aspartic acid residue on VirG activity[a]

Strain	*virG* mutation	*virB-lacZ* expression (β-gal U)	
		No AS	With AS
A136/pGP411	D52E	7.6	6.9
A136/pGP408	D52E N54D	6.5	7.5
A136/pGP409	D52E I106L	19.7	27.6

[a] The desired mutations in *virG* were introduced by site-specific mutagenesis. Other procedures were as described in the legend to Fig. 1.

require that the aspartyl phosphate of wild-type VirG be labile. In vitro, the half-life of VirG aspartyl phosphate is rather long (>1 h; reference 10); this is within the range of stability expected from studies of model compounds such as acetyl phosphate (13). However, it remains to be seen whether the half-life of VirG aspartyl phosphate is as long in vivo.

The crystal structure of a VirG homolog, CheY, has been determined (34). Since the N-terminal half of VirG is homologous to CheY, it can be assumed that VirG is structurally similar to CheY. On this basis, both N54D and I106L lie very close to active-site aspartic acid D52. The conserved lysine residue at position 109 in CheY (102 in VirG) appears to be important for intramolecular changes that accompany activation. Lukat et al. (16) demonstrated the requirement of this residue for CheY function and proposed that it either forms a salt bridge or makes a hydrogen bond to the active-site aspartic acid (D54 of CheY). Those researchers also proposed that phosphorylation of the aspartic acid would disrupt the original interaction but could create a new one between the phosphate group and lysine 109. Analysis of CheY structure at a higher resolution show that the ε-amino group of lysine 109 is bound to one of the carboxyl oxygens of the active-site aspartic acid (37). This lysine is additionally hydrogen bonded to aspartic acid 12 (Asp-9 in VirG) and to an oxygen atom on a sulfate ion. This sulfate ion is also bound to the side chain of asparagine 59 (Asn-54 of VirG, the site of the N54D mutation). Phosphorylation of the active-site aspartic acid residue would lead to local changes and rearrangement in this bonding network, leading to a conformational change. The N54D substitution in *virG* would clearly disrupt many of these interactions. The other substitutions at residue 106 are very close to the conserved lysine residue (at position 102) and may affect its orientation. The wide range of amino acid substitutions (asparagine, leucine, phenylalanine, proline, and tyrosine) that confer a constitutive phenotype argues against a specific interaction mediated by these substitutions. Instead, it appears more likely that an interaction(s) is disrupted by these substitutions.

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REFERENCES

1. **Aiba, H., F. Nakasai, S. Mizushima, and T. Mizuno.** 1989. Evidence for the physiological importance of the phosphotransfer between the two regulatory components, EnvZ and OmpR, in osmoregulation in *Escherichia coli*. J. Biol. Chem. **264:**14090–14094.
2. **Bourret, R. B., J. F. Hess, and M. I. Simon.** 1990. Conserved aspartic acid residues and phosphorylation in signal transduction by the chemotaxis protein CheY. Proc. Natl. Acad. Sci. USA **87:**41–45.
3. **Chen, C.-Y., L. Wang, and S. C. Winans.** 1991. Characterization of the supervirulent *virG* gene of the *Agrobacterium tumefaciens* plasmid pTiBo542. Mol. Gen. Genet. **230:**302–309.
4. **Dahl, M. K., T. Msadek, F. Kunst, and G. Rapoport.** 1991. Mutational analysis of the *Bacillus subtilis* DegU regulator and its phosphorylation by the DegS protein kinase. J. Bacteriol. **173:**2539–2547.
5. **Das, A., and G. Pazour.** 1989. Delineation of the regulatory region sequences of *Agrobacterium tumefaciens virB* operon. Nucleic Acids Res. **17:**4541–4550.
6. **Ditta, G., S. Stanfield, D. Corbin, and D. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
7. **Garfinkel, D. J., and E. W. Nester.** 1980. *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J. Bacteriol. **144:**732–743.
8. **Huang, M.-L. W., G. A. Cangelosi, W. Halperin, and E. W. Nester.** 1990. A chromosomal *Agrobacterium tumefaciens* gene required for effective plant signal transduction. J. Bacteriol. **172:**1814–1822.
9. **Ingraham, J.** 1887. Effect of temperature, pH, water activity, and pressure on growth, p. 1543–1554. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
10. **Jin, S., R. Prusti, T. Roitsch, R. Ankenbauer, and E. W. Nester.** 1990. Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: essential role in the biological activity of VirG. J. Bacteriol. **172:**4945–4950.
11. **Jin, S., T. Roitsch, P. Christie, and E. W. Nester.** 1990. The regulatory VirG protein specifically binds to a *cis*-acting regulatory sequence involved in transcriptional activation of *Agrobacterium tumefaciens* virulence genes. J. Bacteriol. **172:**531–537.
12. **Kanemoto, R. H., A. T. Powell, D. E. Akiyoshi, D. A. Regier, R. A. Kerstetter, E. W. Nester, M. C. Hawes, and M. P. Gordon.** 1989. Nucleotide sequence and analysis of the plant-inducible locus *pinF* from *Agrobacterium tumefaciens*. J. Bacteriol. **171:**2506–2512.
13. **Koshland, D. E.** 1952. Effect of catalysts on the hydrolysis of acetyl phosphate. Nucleophilic displacement mechanisms in enzymatic reactions. J. Am. Chem. Soc. **74:**2286–2292.
14. **Kunkel, T.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
15. **Leroux, B., M. Yanofsky, W. Winans, J. Ward, S. Zeigler, and E. Nester.** 1987. Characterization of the *vir*A locus of *Agrobacterium tumefaciens*: a transcriptional regulatory and host range determinant. EMBO J. **6:**849–856.
16. **Lukat, G. S., B. H. Lee, J. M. Mottonen, A. M. Stock, and J. B. Stock.** 1991. Roles of the highly conserved aspartate and lysine residues in the response regulator of bacterial chemotaxis. J. Biol. Chem. **266:**8348–8354.
17. **Melchers, L. S., M. J. Maroney, A. den Dulk-Ras, D. Thompson, H. van Vuuren, R. A. Schilperoort, and P. J. J. Hooykaas.** 1989. Octopine and nopaline strains of *Agrobacterium tumefaciens* differ in virulence: molecular characterization of the *virF* locus. Plant Mol. Biol. **14:**249–259.
18. **Melchers, L. S., T. J. G. Regensburg-Tuink, R. B. Bourret, N. J. A. Sedee, R. A. Schilperoort, and P. J. J. Hooykaas.** 1989. Membrane topology and functional analysis of the sensory protein virA of *Agrobacterium tumefaciens*. EMBO J. **8:**1919–1925.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Nara, F., S.-I. Matsuyama, T. Mizuno, and S. Mizushima.** 1986. Molecular analysis of mutant *ompR* genes exhibiting different phenotype as to osmoregulation of the *ompF* and *ompC* genes of *Escherichia coli*. Mol. Gen. Genet. **202:**194–199.
21. **Ninfa, A. J., E. G. Ninfa, A. Lupas, A. Stock, B. Magasanik, and J. Stock.** 1988. Crosstalk between bacterial chemotaxis signal transduction proteins and the regulators of transcription of the Ntr regulon: evidence that nitrogen assimilation and chemotaxis are controlled by a common phosphotransfer mechanism. Proc. Natl. Acad. Sci. USA **85:**5492–5496.
22. **Ooms, G., P. M. Klapwijk, J. A. Poulis, and R. A. Schilperoort.** 1980. Characterization of Tn*904* insertions in octopine Ti plasmid mutants of *Agrobacterium tumefaciens*. J. Bacteriol. **144:**82–91.
23. **Pazour, G. J., and A. Das.** 1990. VirG, an *Agrobacterium tumefaciens* transcriptional activator, initiates translation at a

UUG codon and is a sequence-specific DNA-binding protein. J. Bacteriol. **172:**1241–1249.
24. **Pazour, G. J., and A. Das.** 1990. Characterization of the VirG binding site of *Agrobacterium tumefaciens*. Nucleic Acids Res. **18:**6909–6913.
25. **Pazour, G. J., C. Ta, and A. Das.** 1991. Mutants of *Agrobacterium tumefaciens* with elevated *vir* gene expression. Proc. Natl. Acad. Sci. USA **88:**6941–6945.
26. **Ream, W.** 1989. *Agrobacterium tumefaciens* and interkingdom genetic exchange. Annu. Rev. Phytopathol. **27:**583–618.
27. **Ronson, C. W., B. T. Nixon, and F. M. Ausubel.** 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell **49:**579–581.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Schmidhauser, T., and D. Helinski.** 1985. Regions of broad-host-range plasmid RK2 involved in replication and stable maintenance in nine species of gram-negative bacteria. J. Bacteriol. **164:**446–455.
30. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
31. **Stachel, S., and E. Nester.** 1986. The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. **5:**1445–1454.
32. **Stachel, S., and P. Zambryski.** 1986. *virA* and *virG* control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. Cell **46:**325–333.
33. **Stachel, S. E., E. Messens, M. Van Montagu, and P. Zambryski.** 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature (London) **318:**624–629.
34. **Stock, A., J. Mottonen, J. Stock, and C. Schutt.** 1989. Three-dimensional structure of CheY, the response regulator of bacterial chemotaxis. Nature (London) **337:**745–749.
35. **Stock, J., A. Ninfa, and A. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:**450–490.
36. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.
37. **Volz, K., and P. Matsumura.** 1991. Crystal structure of *Escherichia coli* CheY refined at 1.7-Å resolution. J. Biol. Chem. **266:**15511–15519.
38. **Weglenski, P., A. J. Ninfa, S. Ueno-Nishio, and B. Magasanik.** 1989. Mutations in the *glnG* gene of *Escherichia coli* that result in increased activity of nitrogen regulator I. J. Bacteriol. **171:** 4479–4485.
39. **Winans, S.** Personal communication.
40. **Winans, S. C.** 1990. Transcriptional induction of an *Agrobacterium* regulatory gene at tandem promoters by plant-released phenolic compounds, phosphate starvation, and acidic growth media. J. Bacteriol. **172:**2433–2438.
41. **Winans, S. C., P. R. Ebert, S. E. Stachel, M. P. Gordon, and E. W. Nester.** 1986. A gene essential for *Agrobacterium* virulence is homologous to a family of positive regulatory loci. Proc. Natl. Acad. Sci. USA **83:**8278–8282.
42. **Winans, S. C., R. A. Kerstetter, and E. W. Nester.** 1988. Transcriptional regulation of the *virA* and *virG* genes of *Agrobacterium tumefaciens*. J. Bacteriol. **170:**4047–4054.
43. **Zambryski, P.** 1988. Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. Annu. Rev. Genet. **22:**1–30.