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NOTES

Altered-Function Mutations of the Transcriptional Regulatory Gene *virG* of *Agrobacterium tumefaciens*

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Three point mutations were isolated in the *Agrobacterium tumefaciens virG* gene by screening for *vir* gene expression in the absence of added phenolic inducing compounds. All three mutations were localized in the predicted amino-terminal phosphoryl receiver domain of the protein. One mutant (N54D) bypasses the requirement for VirA and phenolic inducers both for transcriptional activation of all tested *vir* promoters and for plant tumorigenesis. This mutant also activates *vir* gene expression efficiently at neutral pH, indicating that the step in induction that is normally stimulated by acid pH occurs before or during VirG phosphorylation. The other two mutants (M13T and H15R) require VirA for activity but are sensitized to low levels of inducing stimuli.

Crown gall tumors of dicotyledonous plants are induced by *Agrobacterium tumefaciens* as a result of the transfer of a segment of DNA (T-DNA) from a large tumor-inducing (Ti) plasmid to wounded host cells (24). Pathogenesis requires expression of the *vir* regulon, which is transcriptionally regulated by the VirA transmembrane protein kinase (5, 7, 12, 13, 27), the VirG response regulator (4, 16, 25), and the periplasmic sugar-binding protein ChvE (1, 18). VirA autophosphorylates on a histidine residue and transfers this phosphoryl group to an aspartate residue within the amino-terminal receiver domain (amino acids 1 to 125) of VirG (4). The carboxyl-terminal domain (amino acids 126 to 241) of VirG binds to specific sites (*vir* boxes) upstream of each *vir* promoter (17). In the current study, we dissected the VirA-VirG signal transduction system genetically by creating altered-function alleles of the *virG* gene. Three point mutations in *virG*, all localized in the amino-terminal receiver domain, are described. These mutations have offered new insights into this regulatory system, including which step(s) is stimulated by acid pH and which step(s) is blocked in a heterologous host.

Efforts were made to isolate mutations in *virG* which lead to an acetosyringone (AS)-independent phenotype. Plasmid pSW207 (3), which contains an IncP replication origin, a Tc^r determinant, and a P*lac-virG* fusion, was mutagenized by using *Escherichia coli mutD5* mutant strain ES1578 (28). pSW207 DNA was purified by CsCl centrifugation and introduced into *A. tumefaciens* A136(pSW219) by electroporation (11). Plasmid pSW219 contains the *virA* gene and a *virB-lacZ* gene fusion derived from pSM243 (19), an IncW replication origin, and Kan^r, Carb^r, and Spec^r determinants. Transformants were plated on defined AB medium (8) buffered at pH 5.5 and containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Approximately 50,000 colonies in two pools were screened for elevated β-galactosidase expression in the absence of AS, and 26 potential mutants some of which may have been siblings, were obtained. Plasmid DNA was purified from them and used to transform A136(pSW219) to Tet^r by using acidic plates containing X-Gal. In each case, the resulting colonies stained darker blue than the wild type.

Plasmid DNA was isolated from these strains and used to transform *E. coli* DH5α to Tet^r. From these strains, derivatives of pSW207 containing putative mutations in *virG* were cleaved with *Eco*RI and *Pst*I and the DNA fragments containing *virG* were ligated into pTZ19R cut with *Eco*RI and *Pst*I. We ultimately isolated three different mutations that had interesting phenotypes: alteration of codon 13 from ATG to ACG, resulting in a change from methionine to threonine (M13T), alteration of codon 15 from CAT to CGT, resulting in a change from histidine to arginine (H15R), and alteration of codon 54 from AAT to GAT, resulting in a change from asparagine to aspartate (N54D). All three mutations lie within a predicted acidic pocket of the protein (21, 22), which also contains the site of phosphorylation, an aspartate residue at position 52.

To determine whether the mutations responsible for these phenotypes were contained within *virG* sequences, the three mutant alleles were subcloned as *Eco*RI-*Pst*I fragments into *Eco*RI-*Pst*I-digested pUCD2 and introduced into *A. tumefaciens* A136(pSM243) and A136(pSM243cd). pSM243 and pSM243cd have identical *virB-lacZ* fusions, and pSM243 contains the *virA* gene (20). The *virGN54D* product did not require AS for activation of transcription of *virB*, and addition of AS led to little, if any, further stimulation (Fig. 1A). The *virGN54D* mutant also did not require VirA for activity (Fig. 1B). These results strongly suggest that the *virGN54D* product does not require phosphorylation. While we have not ruled out the possibility that this protein can be activated by some other kinase, any such kinase would have to be unable to activate the wild-type protein. This mutant also efficiently activated *vir* gene transcription at pH 7.0 (see Table 1). Isolation of a mutation in *virG* that bypasses the requirement for VirA provides genetic evidence that information normally flows from VirA to VirG rather than in the other direction. One explanation for this phenotype is that the additional aspartate at residue 54 in this mutant causes a conformation which simulates the activated form of VirG. It is possible that the new aspartate residue at position 54 could

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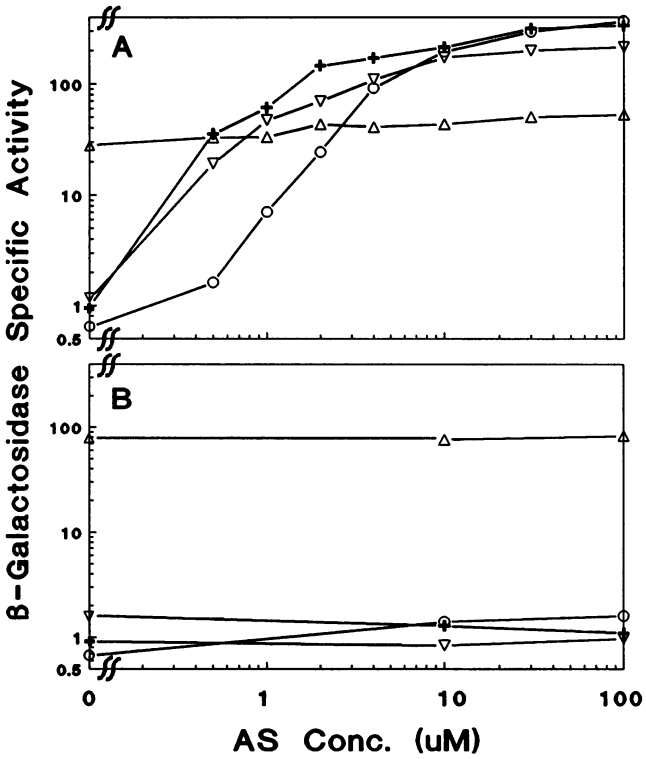

FIG. 1. Activity of altered-function VirG proteins as a function of AS concentration. *A. tumefaciens* strains containing the wild-type *virG* gene (○), the *virGN54D* allele (△), the *virGM13T* allele (+), or the *virGH15R* allele (▽). Strains also contained pSM243, which contains *virA* (A), or pSM243cd, which lacks *virA* (B). Strains were cultured for 12 h at pH 5.5 with the concentrations of AS indicated and assayed for β-galactosidase as previously described (14).

mimic the phosphoryl group that is normally bound at aspartate residue 52.

In contrast, the other two mutations, *virGM13T* and *virGH15R*, activated *virB* expression far more strongly in the presence of AS than in its absence (Fig. 1A). The *virGM13T* and *virGH15R* products activated transcription of the reporter gene about 15-fold more efficiently than the wild-type protein in broth containing low concentrations of AS (Fig. 1A). We designated this a sensitized phenotype. It was somewhat surprising that screening for AS-independent *vir* gene activation yielded this class of mutations. However, the resulting mutants showed slightly elevated transcription in the absence of AS, indicating that VirA may phosphorylate VirG at very low levels in the absence of added phenolic inducers. These mutants were strictly dependent upon VirA for activity (Fig. 1B). This finding suggests that these mutant proteins require phosphorylation by VirA but are more readily (or more stably) phosphorylated than the wild-type protein. Alternatively, the phosphorylated forms of these proteins may function more effectively than the wild type at low concentrations. These two mutants did not activate transcription efficiently at neutral pH (data not shown).

To test the activity of these mutants on other *vir-lacZ* fusions, a Kan^s derivative of pUCD2 was constructed by cutting this plasmid with *Kpn*I, digesting it with nuclease S1, and recircularizing it. The resulting plasmid, pDH99, was used to clone the wild-type *virG* gene and the N54D mutant by digesting the *virG*-containing plasmids and pDH99 with *Eco*RI and *Pst*I and ligating them. These plasmids were introduced by electroporation into *A. tumefaciens* A136(pSM243cd), A136(pSW161), A136(pSW162), A136 (pSM358cd), and A136(pSW165) to measure the activities of the *virB*, *virC*, *virD*, *virE*, and *virG* promoters, respectively. The mutant VirG protein directed higher levels of transcription than the wild-type protein for each of these promoters (Table 1). Unlike wild-type VirG, this mutant activated transcription efficiently at pH 7.0. This indicates that the normal acid pH optimum can be largely attributed to some step in signal transduction that occurs before phosphorylation. Residual (two- to fourfold) stimulation by acidic pH was observed for each promoter, indicating that acid pH could play a minor stimulatory role at some step subsequent to phosphorylation. Note that stimulation of the *virG* promoter by acid pH occurs at a VirG-independent promoter (9).

To determine whether this protein could activate *vir* gene expression in *E. coli*, these plasmids were introduced into *E. coli* MC4100. No allele of *virG* activated transcription of any *vir* promoter in this heterologous host (data not shown). The finding that the N54D mutant does not function in *E. coli* could be explained in various ways. (i) It is possible that this protein is not kinase independent, but rather that it is phosphorylated by some kinase in *A. tumefaciens* that is not found in *E. coli*. (ii) Assuming that this protein does not require phosphorylation, it may fail to make all of the correct contacts with *E. coli* RNA polymerase that are required for transcription activation. (iii) Transcription initiation could require some uncharacterized protein in addition to VirG, and this protein may not be found in *E. coli*. It is of interest

TABLE 1. *vir* gene induction by wild-type and mutant VirG proteins at neutral and acidic pHs[a]

Plasmid	Description	pH	β-Galactosidase activity[b] (Miller units)				
			virB-lacZ	*virC-lacZ*	*virD-lacZ*	*virE-lacZ*	*virG-lacZ*
pDH99	Vector	7.0	1	1.1	1.4	36.7	8.0
pDH100	*virG*$^+$	7.0	1	1.1	1.4	50.9	8.6
pDH101	*virGN54D*	7.0	20.9	7.7	7.8	460	96.7
pDH99	Vector	5.25	2.1	1.7	1.6	19.1	76.8
pDH100	*virG*$^+$	5.25	1.9	1.6	1.8	35.3	85.1
pDH101	*virGN54D*	5.25	74.1	14.4	35	801.3	392.6

[a] *A. tumefaciens* strains containing the indicated plasmids were cultured in AB medium for 12 h at the indicated pHs and assayed for β-galactosidase activity (14).

[b] Fusions between *virB*, *virC*, *virD*, *virE*, and *virG* and *lacZ* are contained in plasmids pSM243cd pSW161, pSW162, pSM358cd, and pSW165, respectively. These plasmids are described in references 20 and 26.

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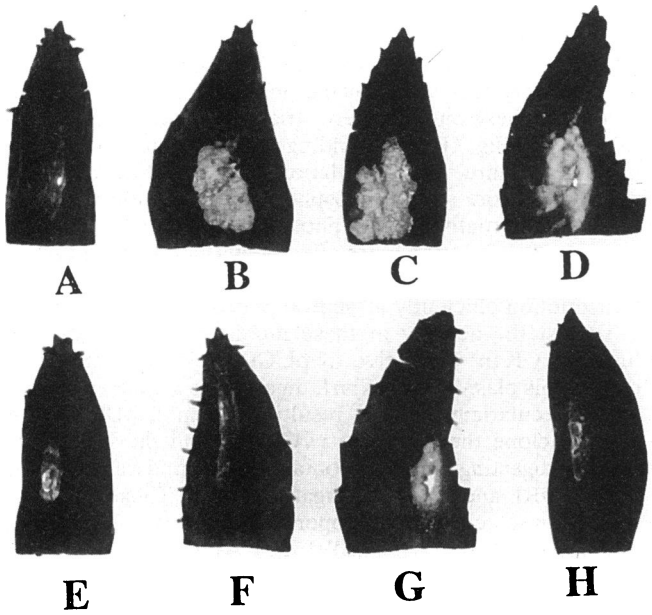

FIG. 2. Tumorigenesis by strains containing altered-function variants of VirG. *A. tumefaciens* 19MX (a *virG* insertion mutant; A, B, C, and D) or 226MX (a *virA* insertion mutant; E, F, G, and H) containing plasmids that express no *virG* (A and E), wild-type *virG* (B and F), *virGN54D* (C and G), or *virGM13H* (D and H).

that at least two other *A. tumefaciens* regulatory proteins are fully functional in *E. coli* (10, 23).

Since the N54D mutation introduces a negatively charged residue near the site of phosphorylation (D52), it seemed possible that this mutation could act by simulating the bound phosphoryl group. If this were true, then altering the leucine residue at codon 53 to an acidic residue might have the same effect. We also hypothesized that a phospho-aspartate could be simulated by a glutamate residue, owing to the larger size of glutamate than aspartate. We therefore used site-directed mutagenesis (6) to create and characterize mutations L53D and D52E. Both mutations rendered the protein nonfunctional in *vir* gene activation, even in the presence of VirA (data not shown).

Plasmids containing the wild type or mutant alleles of *virG* were assayed for the ability to complement a *virG* null mutant or to suppress a *virA* null mutant for tumorigenesis on the leaves of *Kalanchoë diagremontiana* (2). The wild-type *virG* allele, the *virGN54D* allele, and the *virGM13T* allele all complemented the *virG* insertion mutation with high efficiency (Fig. 2B to D). The N54D mutant was also able to suppress a *virA* insertion mutation (Fig. 2G). In contrast, the wild-type *virG* gene and the *virGM13T* alleles were not able to suppress a *virA* mutation (Fig. 2F and H). No *virG* allele suppressed a *virB* mutation (data not shown). The efficiency of tumorigenesis for the strain containing a *virA* mutation and *virGN54D* appears to be lower than that of strains containing wild-type *virA* and *virG* genes. The most probable explanation is that this strain expresses both the wild type and mutant VirG proteins and that these two proteins form inactive or partially active hetero-oligomers which interfere with induction by the mutant protein.

While this report was in preparation, an independent report describing a *virGN54D* mutant was published (15). That study showed that in the presence of VirA and inducing stimuli, the *virGN54D* mutant activated a target promoter fourfold more efficiently than did the wild-type protein. In contrast, we found that this mutation activated transcription only about 10 to 15% as strongly as wild type. The cause of this discrepancy is not known, but one significant difference between the two studies concerns expression of *virG*. In the present study, *virG* was expressed by using the *lac* promoter, which functions constitutively in *A. tumefaciens* (3). In contrast, Pazour et al. expressed *virG* by using its two native promoters, one of which is subject to positive autoregulation by VirG, and the other of which is induced by acidic pH (9). Use of the native *virG* promoters would therefore result in large changes in the VirG concentration over the course of an experiment, possibly complicating interpretation of the data.

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REFERENCES

1. **Cangelosi, G. A., R. G. Ankenbauer, and E. W. Nester.** 1990. Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proc. Natl. Acad. Sci. USA **87:**6708–6712.
2. **Chang, C.-H., and S. C. Winans.** 1992. Functional roles assigned to the periplasmic, linker, and receiver domains of the *Agrobacterium tumefaciens* VirA protein. J. Bacteriol. **174:** 7033–7039.
3. **Chen, C.-Y., and S. C. Winans.** 1991. Controlled expression of the transcriptional activator gene *virG* in *Agrobacterium tumefaciens* by using the *Escherichia coli lac* promoter. J. Bacteriol. **173:**1139–1144.
4. **Jin, S., R. K. Prusti, T. Roitsch, R. G. Ankenbauer, and E. W. Nester.** 1990. Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: essential role in biological activity of VirG. J. Bacteriol. **172:** 4945–4950.
5. **Jin, S., T. Roitsch, R. G. Ankenbauer, M. P. Gordon, and E. W. Nester.** 1990. The VirA protein of *Agrobacterium tumefaciens* is autophosphorylated and is essential for *vir* gene induction. J. Bacteriol. **172:**525–530.
6. **Kunkel, T. A., J. D. Roberts, and R. A. Zakoor.** 1986. Rapid and efficient site-directed mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
7. **Leroux, B., M. F. Yanofsky, S. C. Winans, J. E. Ward, S. F. Zeigler, and E. W. Nester.** 1987. Characterization of the *virA* locus of *Agrobacterium tumefaciens*: a transcriptional regulator and host range determinant. EMBO J. **6:**849–856.
8. **Lichtenstein, C., and J. Draper.** 1986. Genetic engineering of plants, p. 67–119. *In* D. M. Glover (ed.), DNA cloning: a practical approach. IRL Press, Oxford.
9. **Mantis, N. J., and S. C. Winans.** 1992. The *Agrobacterium vir* gene transcriptional activator *virG* is transcriptionally induced by acid pH and other stress stimuli. J. Bacteriol. **174:**1189–1196.
10. **Mantis, N. J., and S. C. Winans.** Unpublished data.
11. **Mattenovich, D., F. Rucker, A. da Camara Machada, M. Lamier, F. Regner, H. Steinkellner, G. Himmler, and H. Katinger.** 1989. Efficient transformation of *Agrobacterium* spp. by electroporation. Nucleic Acids Res. **17:**6747.

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12. **Melchers, L. S., D. V. Thompson, K. B. Idler, T. C. Neuteboom, R. A. deMaagd, R. A. Schilperoort, and P. J. J. Hooykaas.** 1987. Molecular characterization of the virulence gene *virA* of the *Agrobacterium tumefaciens* Ti plasmid. Plant Mol. Biol. **9:**635–645.
13. **Melchers, L. S., D. V. Thompson, K. B. Idler, R. A. Schilperoort, and P. J. J. Hooykaas.** 1986. Nucleotide sequence of the virulence gene *virG* of the *Agrobacterium tumefaciens* octopine Ti plasmid: significant homology between *virG* and the regulatory genes *ompR*, *phoB*, and *dye* of *E. coli*. Nucleic Acids Res. **14:**9933–9940.
14. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 353–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Pazour, G. J., C. N. Ta, and A. Das.** 1992. Constitutive mutations of *Agrobacterium tumefaciens* transcriptional activator *virG*. J. Bacteriol. **174:**4169–4174.
16. **Powell, B. S., G. K. Powell, R. O. Morris, P. M. Rogowsky, and C. I. Kado.** 1987. Nucleotide sequence of the *virG* locus of the *Agrobacterium tumefaciens* plasmid pTiC58. Mol. Microbiol. **1:**309–316.
17. **Roitsch, T., H. Wang, S. Jin, and E. W. Nester.** 1990. Mutational analysis of the VirG protein, a transcriptional activator of *Agrobacterium tumefaciens* virulence genes. J. Bacteriol. **172:**6054–6060.
18. **Shimoda, N., A. Toyoda-Yamamoto, J. Nagamine, S. Usami, M. Katayama, Y. Sakagami, and Y. Machida.** 1990. Control of expression of *Agrobacterium vir* genes by synergistic actions of phenolic signal molecules and monosaccharides. Proc. Natl. Acad. Sci. USA **87:**6684–6688.
19. **Stachel, S. E., and E. W. Nester.** 1986. The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. **5:**1445–1454.
20. **Stachel, S. E., and P. C. Zambryski.** 1986. *virA* and *virG* control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. Cell **46:**325–333.
21. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:**450–490.
22. **Volz, K., and P. Matsumura.** 1986. Crystal structure of *Escherichia coli* CheY refined at 1.7-Å resolution. J. Biol. Chem. **266:**15511–15519.
23. **Wang, L., J. D. Helmann, and S. C. Winans.** 1992. The *A. tumefaciens* transcriptional activator OccR causes a bend at a target promoter, which is partially relaxed by a plant tumor metabolite. Cell **69:**659–667.
24. **Winans, S. C.** 1992. Two way chemical signalling in *Agrobacterium*-plant interactions. Microbiol. Rev. **56:**12–31.
25. **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.
26. **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.
27. **Winans, S. C., R. A. Kerstetter, J. E. Ward, and E. W. Nester.** 1989. A protein required for transcriptional regulation of *Agrobacterium* virulence genes spans the cytoplasmic membrane. J. Bacteriol. **171:**1616–1622.
28. **Wu, T.-H., C. H. Clarke, and M. G. Marinas.** 1990. Specificity of *Escherichia coli* mutD and mutL mutator strains. Gene **87:**1–5.

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