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Research Note

Regulation of *Agrobacterium tumefaciens* Virulence Gene Expression: Isolation of a Mutation that Restores *vir*GD52E Function

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Expression of *Agrobacterium tumefaciens* virulence (*vir*) genes is controlled by *vir*A, *vir*G, and a plant inducer. Isolation of two constitutive mutants of the transcriptional activator *vir*G, *vir*GN54D, and *vir*GI106L, that support *vir* gene expression in a *vir*A independent manner has previously been reported. Characterization of *vir*GN54D by several groups showed considerable variation in its ability to activate *vir* gene transcription. In this study we demonstrate that these differences can be accounted for by plasmid copy number. We report the isolation of a third constitutive mutation, *vir*GI77V, that partially restores transcription activation function of a nonfunctional *vir*G mutant, *vir*GD52E. The second regulator, VirA, in its extreme C-terminus, contains a domain that is homologous to the N-terminal domain of VirG. Deletion of this domain of VirA leads to a fully constitutive phenotype.

Expression of the *Agrobacterium tumefaciens* virulence (*vir*) genes is controlled by *vir*A, *vir*G and a plant inducer(s) such as acetosyringone (Stachel et al. 1985; Stachel and Zambryski 1986). Certain monosaccharides sensitize the bacterium to respond to a low level of inducer (Cangelosi et al. 1990; Shimoda et al. 1990). The two regulatory genes are members of the two-component regulatory system found in many bacteria and some eukaryotes. One component, VirA or its homolog, functions as an environmental sensor and the second one, VirG or its homolog, functions as a regulator that controls cellular functions, usually transcription activation (reviewed in Parkinson and Kofoid 1992). The activity of the regulator is controlled by protein phosphorylation. The sensor, VirA, contains at least four domains: periplasmic, linker, kinase, and a C-terminal response regulator homologous domain (Chang and Winans 1992). The last domain is present only in a small subset of VirA homologs. The periplasmic domain is responsible for sugar-mediated sensitization of the bacteria to a low concentration of the inducer and the kinase domain is the site of autophosphorylation (Banta et al. 1994; Cangelosi et al. 1990; Shimoda et al. 1990; Jin et al. 1990b; Huang et al. 1990). The role of the extreme C-terminal domain is not known but it is believed to function as an autoinhibitory domain (Pazour et al. 1991; Chang and Winans 1992).

VirG consists of at least two domains: the N-terminal signal receiving domain and the C-terminal DNA binding domain (Winans et al. 1986; Powell and Kado 1990). The N-terminal domain contains a conserved aspartic acid at position 52 that is phosphorylated by phospho-VirA (Jin et al. 1990a). We previously reported the isolation of two constitutive *vir*G mutations, *vir*GN54D and *vir*GI106L, that function independent of both VirA and the plant inducer acetosyringone (Pazour et al. 1992). Characterization of *vir*GN54D by different laboratories showed that this mutation confers a constitutive phenotype on *vir*G, but the level of constitutive expression varied widely in the different studies (Pazour et al. 1992; Han et al. 1992; Jin et al. 1992; Schreen-Groot et al. 1994). In one study, this mutation failed to exhibit a constitutive phenotype when expressed in 1 or 2 copies per cell (McLean et al. 1994). In our own study, while the original isolate of *vir*GN54D supported a modest level of the reporter *vir*B–*lac*Z expression, a very high level of reporter gene expression was observed when the N54D mutation was introduced into *vir*G by site-specific mutagenesis (Pazour et al. 1992).

In a recent study we described the isolation and characterization of two spontaneous deletion mutations in the wide-host-range cloning vector pTJS75 that led to an increase in plasmid copy number in *A. tumefaciens* (Schmidhauser and Helinski 1985; Das and Xie 1995). The two mutants, Δ1 and Δ2, have a 56- and 505-bp deletion within the origin of vegetative replication of pTJS75, and have a 3- and 7-fold higher copy number, respectively. To investigate whether the previously reported differences in *vir* gene activation by *vir*GN54D are due to a copy number effect, we studied the effect of plasmid copy number on *vir* gene expression. The two copy number mutations were introduced into plasmids pGP229, pGP229N54D, and pGP229I106L by molecular cloning (Sambrook et al. 1989). Plasmid pGP229 contains wild-type *vir*G and a *vir*B-*lac*Z reporter gene on the wide-host-range vector pTJS75 (Pazour et al. 1992). Introduction of the Δ1

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and Δ2 mutations led to a 9- to 20-fold increase in the basal level of *vir*B-*lac*Z expression (Fig. 1). When the same mutations were introduced into pGP229N54D and pGP229I106L, the effect of plasmid copy number on *vir* gene expression was rather obvious. Both mutants show a modest constitutive level of *vir*B expression (a 50- to 100-fold increase over basal level) in a wild-type pTJS75 background (open bars). This is consistent with the reports of Han et al. (1992) who observed a low level of constitutive expression of *vir*GN54D. Introduction of the two copy number mutants into these plasmids led to an additional 10- to 25-fold increase in *vir*B expression. As noted previously (Pazour et al. 1992), the *vir*GN54D mutation consistently exhibited a higher level of *vir*B expression than *vir*GI106L did.

VirA contains a 117-amino acid residue segment in its extreme C-terminus that is homologous to the N-terminal signal receiving domain of VirG. To study the effect of this domain on *vir* gene expression we used site-specific mutagenesis (Kunkel 1985) to construct a deletion derivative of VirA, VirAΔ, that lacks residues 712 to 828. VirA or VirAΔ was cloned into pGP229 and its derivatives and the effect on *vir* gene expression was monitored by measuring *vir*B–*lac*Z expression (Fig. 2). As expected, in the presence of VirA expression of *vir*B requires the inducer acetosyringone (compare columns 1 and 2). However, when VirA was substituted with VirAΔ, a high level of *vir*B expression was observed in the absence of AS and addition of AS had little or no effect (columns 3, 4). These results indicate that the deletion of the extreme C-terminal domain of *vir*A leads to a fully constitutive phenotype and that this domain functions as a negative regulator of *vir* gene expression. Similar results were previously reported by Chang and Winans (1992).

Analysis of similar strains containing *vir*GI106L provides additional support for this conclusion. In the presence of VirA, VirGI106L supported a modest level of constitutive expression (column 9). When AS was added a further increase in *vir*B expression was observed (column 10). In the presence of VirAΔ, VirGI106L exhibited a fully constitutive phenotype and *vir*B expression was not affected by the addition of AS (columns 11,12). In contrast, when *vir*G was substituted with *vir*GN54D, VirAΔ had little or no effect on *vir*B expression (columns 5 to 8). These results are not surprising because *vir*GN54D is not affected by *vir*A (Pazour et al. 1992; Han et al. 1992; Jin et al. 1993).

The effect of copy number and *vir*AΔ on *vir*B expression was studied by using strains that harbored either wild-type pTJS75 plasmid or its copy number mutant, Δ1 or Δ2. In strains containing wild-type VirG, the presence of VirAΔ led to a high level of *vir*B expression in the absence of AS and *vir*B expression was essentially insensitive to AS (Fig. 3, WT). The two copy number variants showed an increased

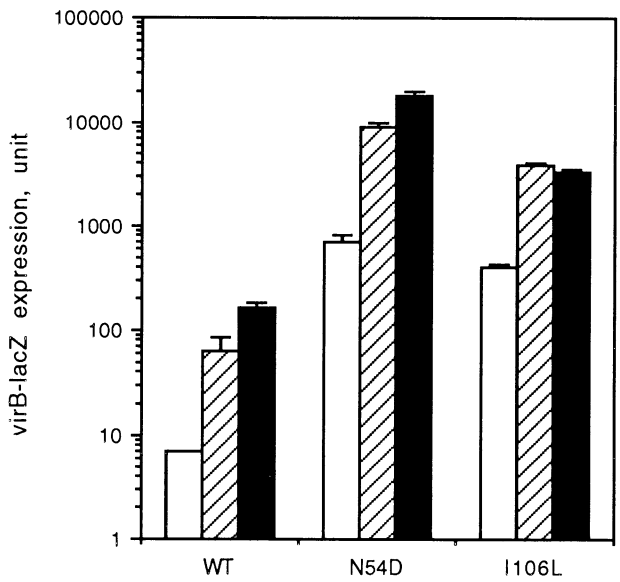

Fig. 1. Effect of plasmid copy number on *vir*B expression. *Agrobacterium tumefaciens* A136 harboring plasmid pGP229 (WT), pGP229N54D (N54D), or pGP229I106L (I106L) was used for *vir*B–*lac*Z expression assays as described previously (Pazour et al. 1992). Open bar, wild type pTJS75 vector; hatched bar, pTJS75Δ1; closed bar, pTJS75Δ2.

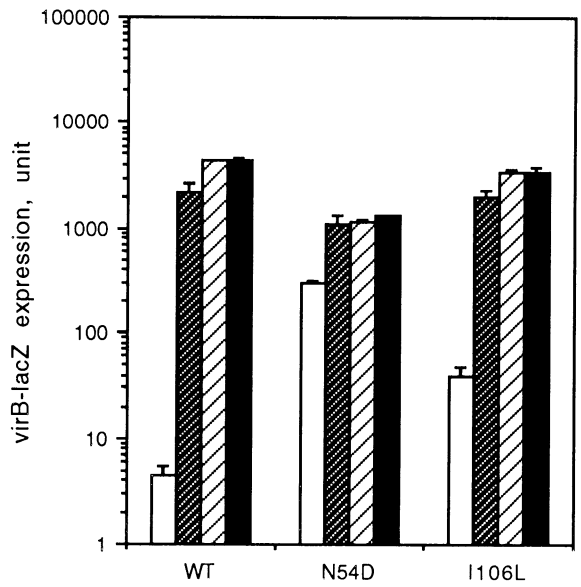

Fig. 2. Effect of deletion of C-terminal response regulator homologous domain of VirA on *vir*B expression. Assays were performed as described. Assay plasmids contained wild-type pTJS75, *vir*B–*lac*Z, *vir*G (WT) or *vir*GN54D (N54D) or *vir*GI106L (I106L) and *vir*A (bars 1 and 2) or virAΔ (bars 3 and 4). Bars 1,3—minus acetosyringone; bars 2,4—plus 100 μM acetosyringone.

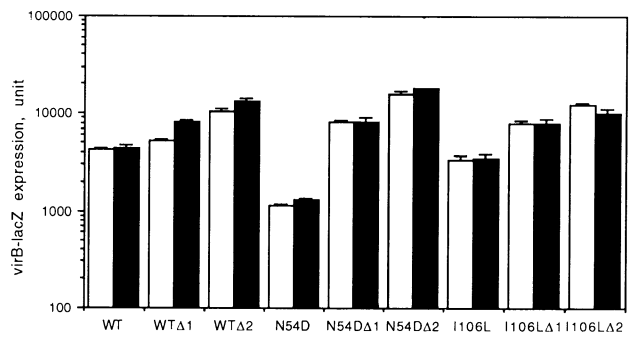

Fig. 3. Effect of plasmid copy number and deletion of C-terminal response regulator homologous domain of VirA on *vir*B expression. Assays were performed as described in the absence (open bars) or presence (dark bars) of 100 μM acetosyringone. All plasmids harbored virAΔ, *vir*B-*lac*Z, and *vir*G or its mutants. Where indicated, the deletion derivatives of pTJS75, Δ1 and Δ2, were used.

level of *vir*B expression in the absence of AS (WTΔ1, WTΔ2—open bars). Addition of AS showed a marginal increase (filled bars). Essentially similar results were obtained when *vir*G was substituted with *vir*GI106L. Of the three *vir*G derivatives, the *vir*GN54D showed the lowest level of expression when pTJS75 was used as a vector. Since this mutation is not affected by *vir*A the level of expression in *vir*GN54D is not modulated by *vir*AΔ.

Noteworthy in these results is the level of *vir*B expression in the strain harboring a high copy plasmid containing wild-type VirG and VirAΔ (lane WTΔ2). In the absence of acetosyringone this strain exhibited a very high level of *vir*B expression indicating that a fully constitutive phenotype results from the deletion of the C-terminal response regulator homologous domain of VirA. Since unmodified VirG is virtually nonfunctional as a transcriptional activator these results suggest that in the absence of acetosyringone VirAΔ can activate VirG. Therefore, the C-terminal response regulator domain of VirA must function in the prevention of inducer independent phosphorylation probably by blocking access to the active site. A likely mode of action of the inducer is to cause a conformational change that will destroy intramolecular interaction between the active site and the VirA C-terminal domain. This loss of interaction will lead to VirA autophosphorylation and subsequent phosphotransfer to VirG. The conformational change induced by the inducer can result from its binding to VirA or is mediated by other proteins as suggested by Lee et al. (1992).

Transcription activation of *vir* genes requires phosphorylation of the aspartic acid 52 of VirG. Alteration of this aspartic acid to glutamic acid (VirGD52E) completely abolishes its transcription activation function (Pazour et al. 1992). To understand the mechanism of transcription activation we sought to isolate second-site mutations that restore *vir*GD52E function. By nitrous acid and nitrosoguanidine mutagenesis we isolated five mutants that partially restored the transcription activation function of *vir*GD52E. These mutants led to a ~10- to 18-fold increase in *vir*B expression. To ensure that the mutation(s) lies in *vir*GD52E, the mutant *vir*G genes were cloned into an unmutagenized *vir*B-*lac*Z reporter plasmid and assayed for *vir*B expression. In all cases the mutation(s) mapped to the *vir*GD52E gene. DNA sequence analysis of each of the mutant genes showed that all mutants had a single base substitution, an A --> G change at nucleotide 501, that resulted in a change of isoleucine at position 77 to valine (I77V). Analysis of one of these mutants is presented in Table 1. To confirm that this single substitution led to the constitutive phenotype we introduced this alteration by site-specific mutagenesis and found that I77V confers a partial constitutive phenotype to *vir*GD52E (line 4). To study the effect of the I77V mutation without the complication of the D52E substitution, we introduced the I77V mutation into the wild-type *vir*G by site-specific mutagenesis. Analysis of the mutant showed that *vir*GI77V has a constitutive phenotype (line 6). When this mutant was cloned into a high copy plasmid a large increase in *vir*B–*lac*Z expression was observed (line 7).

The *vir*GI77V mutation is responsive to both *vir*A and acetosyringone (Table 2) indicating that the I77V mutation does not negatively affect the site of phosphorylation. In contrast the *vir*GD52E, I77V double mutant is not responsive to *vir*A and acetosyringone. This is expected because the VirGD52E cannot be phosphorylated by VirA. These results demonstrate that the I77V mutation confers a constitutive phenotype to both *vir*G and *vir*GD52E, indicating that this mutation acts independent of the aspartic acid at position 52. In a recent study Scheeren–Groot et al. (1994) reported the isolation of the *vir*GI77V mutation. Our results differ from that study in that we observed a low level of constitutive expression with this mutation. The reason for the apparent anomaly is not known.

Expression of the virulence genes require VirA, VirG, and a plant inducer. Under normal conditions a plant inducer is an absolute requirement for *vir* gene activation (Stachel and Zambryski 1986). Results presented in this study demonstrate that this requirement cannot be bypassed by a substantial increase in the copy number (70 to 100 copies per cell) of *vir*G (Fig. 1). In addition to the control by the inducer, this system also contains a negative regulatory mechanism possibly to prevent inappropriate activation of the *vir* genes. This role is carried out by the C-terminal response regulator homologous domain of VirA. This domain most likely blocks the VirA active site preventing autophosphorylation and/or preventing the access of VirG. In the presence of an inducer a conformational change weakens the intramolecular interaction, allowing phosphorylation of VirA. Once phosphorylated the C-terminal domain of VirA may be a poor competitor for the interaction between the VirA active site and the N-terminal domain of VirG.

A third factor that affects *vir* gene expression is the level of the regulatory proteins. Data presented in this study demonstrate that an increase in the copy number of the constitutive *vir*G mutants or that of both regulatory genes leads to a substantial increase in *vir*B expression. That this effect of copy number leads to an increase in the expression of other *vir* genes as well is supported by the observations that the level of T-strand DNA synthesis varies with plasmid copy number (N. R. Madamanchi and A. Das, unpublished observations). Similar effects of *vir*G copy number on plant transformation have been previously noted (Zyprian and Kado 1990; Liu et al. 1992).

Table 1. A second-site mutation restores activity of VirGD52E mutation

Strain	*vir*G phenotype	*vir*B-*lac*Z expression, β-Gal units
SG6	D52E	13 ± 1
Mutant NA3	D52E I77V	180 ± 7
Cloned NA3	D52E I77V	141 ± 12
SG13[a]	D52E I77V	312 ± 74
GP229	WT	8 ± 1
SG14	I77V	196 ± 33
AD1396[b]	I77V	2,882 ± 100

[a] I77V mutation was reconstructed by site-specific mutagenesis.
[b] High copy derivative of SG14. Assays were performed in the absence of acetosyringone.

Table 2. Effect of *vir*A on *vir*GI77V mutation

		*vir*B-*lac*Z expression, β-Gal units[a]	
Strain	Relevant phenotype	–AS	+AS
GP159	*vir*A, *vir*G	6 ± 1	1,742 ± 200
SG23	*vir*A, *vir*GI77V	407 ± 21	2,355 ± 57
SG22	*vir*A, *vir*GD52EI77V	407 ± 18	370 ± 11

[a] Data (± standard error) shown is an average of two experiments.

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