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Mutants of the *Agrobacterium tumefaciens virA* Gene Exhibiting Acetosyringone-Independent Expression of the *vir* Regulon

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Hydroxylamine-induced mutations in the *virA* gene of *Agrobacterium tumefaciens* that do not require the plant phenolic-inducing compound acetosyringone for *vir* regulon induction were isolated. The isolation was based on the activation of both *virB*::*lacZ* and *virE*::*cat* fusions by mutant *virA* loci in the absence of acetosyringone. Three of these *virA*(Ais) (*a*cetosyringone-*i*ndependent *s*ignaling) mutants were characterized. All three mutants expressed a *virB*::*lacZ* fusion at high levels in the absence of acetosyringone. One *virA*(Ais) mutant, *virA112*, exhibited *vir* gene expression in the absence of inducing monosaccharides and acidic growth conditions, both of which are normally required for *vir* gene induction. The phenotype of the *virA112* mutant resulted from a glycine to glutamic acid change near His-474, the site of VirA autophosphorylation.

The gram-negative phytopathogen *Agrobacterium tumefaciens* (Smith and Townsend) Conn is the etiologic agent of crown gall, a neoplastic disease of plants. Crown gall tumor formation is a complex process that requires the products of the *Agrobacterium vir* (virulence) regulon for transfer of bacterial DNA into plant cells (Binns and Thomashow 1988; Zambryski 1989).

The *vir* regulon is under transcriptional control of the *virA* and *virG* gene products, VirA and VirG, respectively (Stachel and Nester 1986; Stachel and Zambryski 1986). The VirA/VirG pair (Leroux *et al.* 1987; Winans *et al.* 1986) are members of a family of prokaryotic two-component regulatory systems that serve to transcriptionally activate subject operons in response to specific environmental stimuli (for reviews see Albright *et al.* 1989; Bourret *et al.* 1989; Gross *et al.* 1989; Stock *et al.* 1989). The VirA/VirG regulatory system activates *vir* regulon transcription in response to specific plant phenolic metabolites (e.g., acetosyringone) (Stachel *et al.* 1985), plant-derived monosaccharides and derivatives (Ankenbauer and Nester 1990; Cangelosi *et al.* 1990; Shimoda *et al.* 1990), and acidic conditions (Stachel *et al.* 1986; Winans *et al.* 1988).

Whereas the VirG protein serves as a transcriptional activator, the VirA protein has the crucial role in sensing the inducing stimuli. VirA is a transmembrane protein with a short N-terminal periplasmic domain and a large C-terminal cytoplasmic region (Melchers *et al.* 1989; Winans *et al.* 1989). The VirA protein autophosphorylates at histidine-474 within the cytoplasmic domain (Huang *et al.* 1990b; Jin *et al.* 1990b) and then transfers this phosphate group directly to VirG (Jin *et al.* 1990a), a DNA-binding protein that subsequently activates transcription (Jin *et al.* 1990c; Pazour and Das 1990). The periplasmic domain of VirA is required for sensing inducing monosaccharides via the ChvE protein (Cangelosi *et al.* 1990; Huang *et al.* 1990a) but not for the detection of acetosyringone (Cangelosi *et al.* 1990; Melchers *et al.* 1989; Shimoda *et al.* 1990).

In this report, we describe the isolation and characterization of *virA* mutants that express *vir* genes at high levels in the absence of acetosyringone, herein designated *virA*(Ais) for *virA*(*a*cetosyringone-*i*ndependent *s*ignaling). One of the *virA*(Ais) mutants, *virA112*, was relatively insensitive to acetosyringone and contained a single amino acid change in the conserved domain of VirA at a glycine residue near the site of VirA autophosphorylation.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1. Plasmids were introduced into *A. tumefaciens* by high-voltage electroporation (Cangelosi *et al.*, in press) or triparental matings (Ditta *et al.* 1980) using pRK2073 as a helper plasmid.

Media and chemicals. *A. tumefaciens* strains were grown in MG/L or AB media (Lichtenstein and Draper 1985). *E. coli* strains were grown in Luria-Bertani medium (LB) (Maniatis *et al.* 1982). Standard induction broth (SIB) consisted of 2% glucose, 0.5 mM NaH_2PO_4, 50 mM MES (2-[*N*-morpholino]ethanesulfonic acid) (pH 5.5), and AB salts (Lichtenstein and Draper 1985). Glycerol induction broth (GIB) was identical to standard induction broth except for the substitution of 0.5% glycerol for 2% glucose. PIPES-SIB was identical to SIB except for the substitution of 50 mM PIPES (piperazine-*N,N'-bis*[2-ethanesulfonic acid]) (pH 7.0) for 50 mM MES. PIPES was substituted for MES to provide higher buffering capacity near neutrality to test the effect of pH on induction. Aceto-

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Nucleotide and/or amino acid sequence data are to to submitted to GenBank, EMBL, and DDBJ as accession no. JO3703.

syringone (Aldrich Chemical Co., Milwaukee, WI) was prepared in dimethyl sulfoxide as a 1 M stock solution and added to media at the appropriate concentration. Inducing sugars were prepared in distilled water, filter sterilized, and added to media at concentrations indicated in the text. Antibiotics were used at the following concentrations in μg/ml for both *A. tumefaciens* and *E. coli*: kanamycin, 50; carbenicillin, 100; chloramphenicol, 100. X-Gal was added to media to yield a final concentration of 40 μg/ml and media were solidified with 1.5% agar.

Hydroxylamine mutagenesis and identification of acetosyringone-independent signaling mutants. Plasmid DNA was mutagenized *in vitro* with the chemical mutagen hydroxylamine for 2 hr at 68° C essentially as described by Humphreys and co-workers (1976). Following mutagenesis, hydroxylamine was removed by centrifugation of the DNA-mutagen mixture through a Centricon-30 microconcentrator (Amicon, Beverly, MA). Approximately 0.5 μg *in vitro* mutagenized plasmid DNA was electroporated into *A. tumefaciens* A114 (pIB50). Following electroporation, 1 ml of MG/L medium was added to cells and cultures were incubated for 2 hr at 28° C with shaking. Cells were washed, resuspended in distilled H_2O, and plated on SIB plates supplemented with chloramphenicol to select for *virE*::*cat* expression, and X-Gal to screen for *virB*::*lacZ* expression.

Plasmid constructions. Restriction enzyme analysis and molecular cloning were done using standard procedures (Maniatis *et al.* 1982). *E. coli* strains DH5α and C2110 (*polA*) served as transformation recipients.

Plasmid pIB50 was constructed as follows: the 2.8-kb *Sal*I fragment of pSW110 containing *virE*::*cat* (a pUCD2 derivative provided by S. C. Winans, Cornell University, Ithaca, NY) was ligated to *Sal*I digested pSM243cd (Winans *et al.* 1986) to yield pIB20. pIB20 was digested with *Bam*HI and recircularized by ligation to delete a 3.2-kb fragment bearing the *bla* gene of pSM243cd. The resulting plasmid, pIB50, encodes resistance to kanamycin, and contains two *vir* gene fusions, *virE*::*cat* and *virB*::*lacZ*.

Plasmid pIB100 was constructed by ligating a 1.6-kb *Pvu*II fragment of pSW164 containing *virG* to the blunt-ended *Bam*HI site of pTB108 (Winans *et al.* 1988). pEB103 was derived from pIB100 by deleting the 4.4-kb *Kpn*I fragment bearing *virA*, which regenerated the kanamycin resistance marker of the parent plasmid pUCD2 (Close *et al.* 1984).

To assay for acetosyringone-independent *vir* gene expression by *virA* hybrids constructed on pUC derivatives, it was necessary to introduce them into *A. tumefaciens* on broad host range vectors. The pUC derivatives were ligated into the unique *Eco*RI site of pIB410 (Cangelosi *et al.* 1990), an IncP replicon encoding a *virB*::*lacZ* fusion and then mobilized into *A. tumefaciens* by triparental matings.

***vir* induction assays.** *vir* gene induction was assayed using three separate induction media, SIB for assaying induction elicited by acetosyringone, GIB for detecting induction mediated by specific inducing sugars, and PIPES-SIB for detecting induction at neutral pH. The procedure for *vir* gene induction assays and calculations for β-galactosidase activity have been described previously (Ankenbauer *et al.* 1990; Cangelosi *et al.* 1990).

DNA sequencing. Double-stranded DNA sequencing was carried out using Sequenase Version 2.0 (U.S. Biochemical Corp., Cleveland, OH) following the methods provided by the manufacturer.

Table 1. Bacterial strains and plasmids used in this study

Strains	Relevant characteristics	Source or reference
Agrobacterium tumefaciens		
A114	C58 chromosome, no Ti plasmid	Watson *et al.* 1975
A1030	pTiB6806 *virA*::Tn*5*	Garfinkel and Nester 1980
Escherichia coli		
DH5α	*lacZΔM15 endA1 recA1 hasR17 supE44 thi-1 gyr96 Δ(lacZYA-argF) U169*	Bethesda Research Laboratories[b]
C2110	*polA rha his* Nal[r]	Prince and Barlam 1985
Plasmids		
pSM243cd	*virB*::*lacZ* Cb[r] Km[r] IncP	Winans *et al.* 1986
pSW110	*virE*::*cat* Km[r] IncW	S. C. Winans
pSW164	*virG* Cm[r] pUC derivative	S. C. Winans
pSW169	*virA* Cb[r] pUC derivative	Winans *et al.* 1988
pTB108	*virA* Cb[r] Tc[r] IncW	Winans *et al.* 1988
pIB50	*virB*::*lacZ virE*::*cat* Km[r] IncP	This study
pIB100	*virA virG* Cb[r] IncW	This study
pIB410	*virB*::*lacZ* Tc[r] IncP	Cangelosi *et al.* 1990
pEB103	*virG* Cb[r] Km[r] IncW	This study

[a] Nal, nalidixic acid; Cb, carbenicillin; Km, kanamycin; Cm, chloramphenicol; Tc, tetracycline; and [r], resistant.
[b] Gaithersburg, MD.

RESULTS

Isolation of acetosyringone-independent signaling mutants. The strategy that was used to isolate mutants of *virA* and *virG* which express the *vir* genes in the absence of acetosyringone (*virA*[Ais] and *virG*[Ais], respectively) involved physically separating the *virA*/*virG* sensor/regulator pair from a pair of reporter genes that allow the monitoring of *vir* gene expression. To this end, two types of compatible plasmids were constructed. One type of plasmid contained either *virA* and *virG* (pIB100) or *virG* alone (pEB103). These plasmids were constructed using IncW replicons and served as mutagenesis targets. The other type of plasmid (pIB50) was an IncP replicon and contained two *vir* genes fused to genes whose expression could be monitored easily (*virB*::*lacZ* and *virE*::*cat*). Plasmid pIB50 served as a differential screening and selective tool. *Agrobacterium* strains containing pIB100 (or pEB103) and pIB50 produce white, chloramphenicol-sensitive colonies on acetosyringone-free SIB medium supplemented with the chromogenic substrate X-Gal. We sought to isolate *virA*(Ais) and *virG*(Ais) mutants by mutagenizing plasmids pIB100 and pEB103 *in vitro* with hydroxylamine and then electroporating the plasmids into the chloramphenicol-sensitive *A. tumefaciens* strain A114 (Garfinkel and Nester 1980; Watson *et al.* 1975) containing pIB50. By mutagenizing pIB100 and pEB103 *in vitro* rather than *in vivo*, constitutive mutations in the reporter fusions could be prevented. Transformants were plated on acetosyringone-

free induction medium containing chloramphenicol to select for *virE*::*cat* expression, and X-Gal to screen for *virB*::*lacZ* expression. Mutants that were able to form blue, chloramphenicol-resistant colonies were obtained at a frequency of approximately 0.1% when the mutagenesis target was pIB100. No acetosyringone-independent signaling mutants were obtained when pEB103 was used as the mutagenesis target.

Identification of acetosyringone-independent signaling loci. Six mutants derived from pIB100 that demonstrated acetosyringone-independent signaling were analyzed to determine the locus responsible for the phenotype. By transferring the 4.4-kb *Kpn*I *virA* fragment of each mutant into pEB103, electroporating the resulting plasmids into A114 (pIB50), and screening for *vir* expression in the absence of acetosyringone, the presence of *virA*(Ais) mutants could be detected. Similarly, by deleting the *virA* genes of the mutant plasmids, *virG*(Ais) mutants could be detected. This procedure identified pEB112, pEB129, pEB137, pEB145, and pEB146 as encoding *virA*(Ais) mutants. The mutation in plasmid pIB100-32 was found not to map in *virA* and, surprisingly, the mutation did not map in *virG* either.

Phenotypes of *virA*(Ais) mutants. The *virA*(Ais) mutants were selected for their ability to express the *vir* genes in the absence of acetosyringone. It was expected that there would be differences in their respective levels of *vir* regulon expression in the absence and presence of acetosyringone. The three *virA*(Ais) loci carried by pEB112, pEB129, and pEB137, *virA112, virA129,* and *virA137*, respectively, were isolated independently (pEB145 and pEB146 were mutagenesis siblings of pEB137) and were further analyzed to determine their respective levels of acetosyringone-independent *vir* expression. In SIB lacking acetosyringone, all three mutants expressed the vir genes at levels ranging from 80- to 550-fold greater than that of a wild-type strain (Table 2). One mutant, virA129, displayed an additional 11-fold induction of *vir* gene expression when grown in standard induction broth supplemented with acetosyringone. The other two mutants, *virA112* and *virA137*, were relatively insensitive to the addition of acetosyringone with increases of 1.5- and 3-fold, respectively. Because acidic conditions (pH <6.0) are required for *vir* gene induction (Stachel *et al.* 1986; Winans *et al.* 1988), the ability of the *virA*(Ais) mutants to express *vir* genes was determined at pH 7.0 (Table 2). Only *virA112* yielded measurable *vir* gene expression at neutrality.

Because *virA112* exhibited the strongest acetosyringone-independent *vir* expression of the three *virA*(Ais) loci examined and also functioned at pH 7.0, it was analyzed further. The response of *virA112* to increasing concentrations of acetosyringone was compared with that of wild-type *virA* (Fig. 1). At saturation levels of acetosyringone a *virB*::*lacZ* fusion was induced 750-fold by wild-type *virA*, whereas *virA112* induced *vir* genes only 1.6-fold. The final

Table 2. Effects of acetosyringone and pH on *vir* gene induction by *virA*(Ais) mutants

Strain	β-Galactosidase activity[a]			
	SIB[b]	SIB + AS	PIPES-SIB	PIPES-SIB + AS
A114 (pIB50)	3.12	2.77	2.50	3.07
A114 (pIB50, pIB100)	3.36	2,840	2.46	4.36
A114 (pIB50, pEB112)	1,810	2,570	37.1	48.9
A114 (pIB50, pEB129)	264	2,830	3.04	4.93
A114 (pIB50, pEB137)	781	2,170	2.73	3.81

[a] Units of β-galactosidase activity (nanomoles of *o*-nitrophenol/min/mg protein) were determined as described in the text.
[b] Media composition and induction assay protocol are listed in the text. Abbreviations: SIB, standard induction broth; AS, 200 μM acetosyringone; PIPES, piperazine-*N,N'-bis*[2-ethanesulfonic acid].

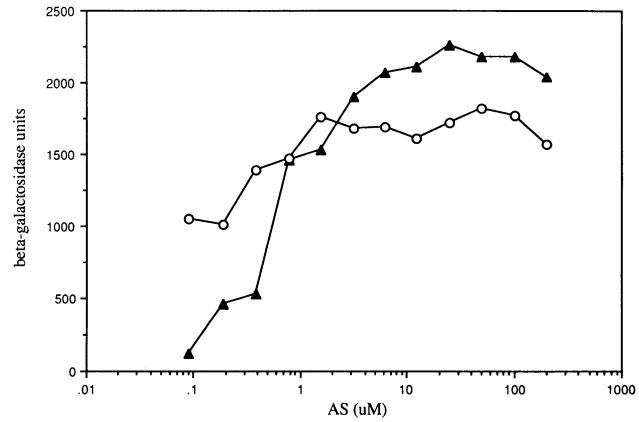

Fig. 1. Effect of acetosyringone on *vir* gene induction by *virA* and *virA112*. *Agrobacterium tumefaciens* strain A114 (pIB50) carrying pIB100 (*virA*) (▲) or pEB112 (*virA112*) (○) were inoculated into standard induction broth supplemented with indicated concentrations of acetosyringone. *vir* gene induction was determined by measuring β-galactosidase activity expressed from a *virB*::*lacZ* fusion. Assays were done at 24 hr after inoculation and data points are the means of two independent assays. β-Galactosidase acitivity was determined as described in the text. A114 (pIB50) carrying pEB112 (*virA112*) exhibited 1,135 units of β-galactosidase activity in the absence of acetosyringone.

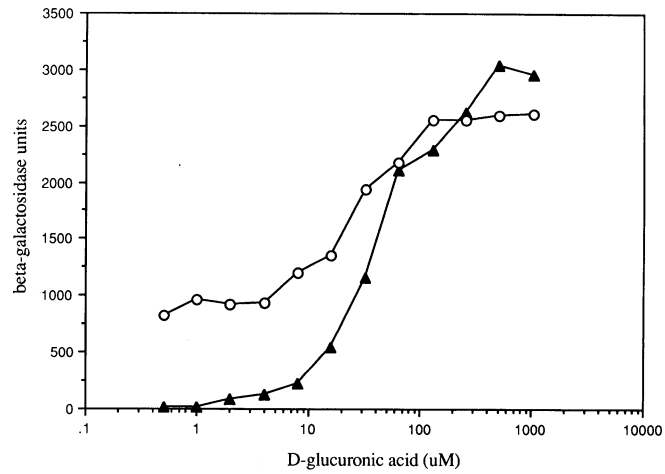

Fig. 2. Effect of D-glucuronic acid on *vir* gene induction by *virA* and *virA112*. *Agrobacterium tumefaciens* strain A114 (pIB50) carrying pIB100 (*virA*) (▲) or pEB112 (*virA112*) (○) were inoculated into glycerol induction broth supplemented with 2.5 μM acetosyringone and indicated concentrations of D-glucuronic acid. *vir* gene induction was determined by measuring β-galactosidase activity expressed from a *virB*::*lacZ* fusion. Assays were done at 24 hr after inoculation and data points are the means of two independent assays. β-Galactosidase acitivity was determined as described in the text. A114 (pIB50) carrying pEB112 (*virA112*) exhibited 864 units of β-galactosidase activity in the absence of D-glucuronic acid.

level of *vir* induction by *virA112* was lower than that of wild-type *virA*, as observed previously. The response of *virA112* and the wild-type *virA* to increasing concentrations of D-glucuronic acid was also compared (Fig. 2). In results similar to those with acetosyringone, wild-type *virA* induced more strongly than *virA112*. Wild-type *virA* induced a *virB*::*lacZ* fusion 180-fold at saturation while *virA112* induced only threefold.

A time course experiment comparing the *vir* gene induction by wild-type *virA* and *virA112* over 24 hr (Fig. 3) demonstrated that the *virA*(Ais) mutant *virA112* did not yield faster induction kinetics as might be expected. Although wild-type *virA* gave higher levels of *vir* gene expression, the times at which the final levels were achieved were similar. The only difference indicating the nature of the *virA*(Ais) mutant is that *virA112* shows approximately 100 units of β-galactosidase activity at time zero, whereas the *vir* gene expression by wild-type *virA* was negligible.

In all the induction assays, the acetosyringone-independent level of *vir* expression by *virA112* was approximately 50–65% of the *vir* gene expression elicited by wild-type *virA* in the presence of high concentrations of acetosyringone. Thus, the *virA112* locus yielded *vir* expression in the absence of acetosyringone, acidic conditions, and inducing monosaccharides, conditions all required for the induction of *vir* genes by the wild-type *virA* locus.

Mapping and identification of the *virA112* mutation. The specific nucleotide change yielding the *virA*(Ais) phenotype in plasmid pEB112 was determined. Because hydroxylamine mutagenesis may have induced multiple mutations in virA112, we initially mapped the region of the *virA112* gene that conferred the *virA*(Ais) phenotype by constructing hybrids with the wild-type *virA* gene. In this way, only those base changes in the specific region conferring the acetosyringone-independent signaling phenotype need be considered. The 4.4-kb *Kpn*I fragment bearing the *virA*(Ais) mutation of pEB112 was introduced into pBluescript KS(+) to yield pEB115. Specific fragments of the mutant *virA* gene were substituted for the corresponding fragments in the wild-type *virA* gene in pSW169, yielding the *virA* hybrids in Figure 4. The ability of a periplasmic deletion mutant of VirA to induce *vir* genes in response to acetosyringone was exploited because many of the hybrid constructions would have been extremely difficult with the full-length *virA* gene. Because pUC derived plasmids cannot replicate in *A. tumefaciens*, it was necessary to clone the *virA* hybrids into a broad-host-range vector to determine the acetosyringone-

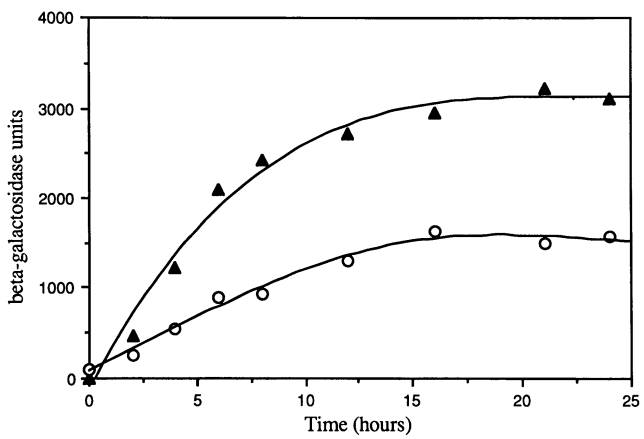

Fig. 3. Time course of *vir* gene induction by *virA* and *virA112*. *A. tumefaciens* strain A114 (pIB50) carrying pIB100 (*virA*) (▲) was inoculated into standard induction broth (SIB) supplemented with 2.5 μM acetosyringone and A114 (pIB50,pEB112) (*virA112*) (○) was inoculated into SIB containing no acetosyringone. *vir* gene induction was determined by measuring β-galactosidase activity expressed from a *virB*::*lacZ* fusion. Assays were done at the indicated time points and data points are the means of three independent assays. β-Galactosidase acitivity was determined as described in the text. Curves were generated by fourth-order polynomial approximations.

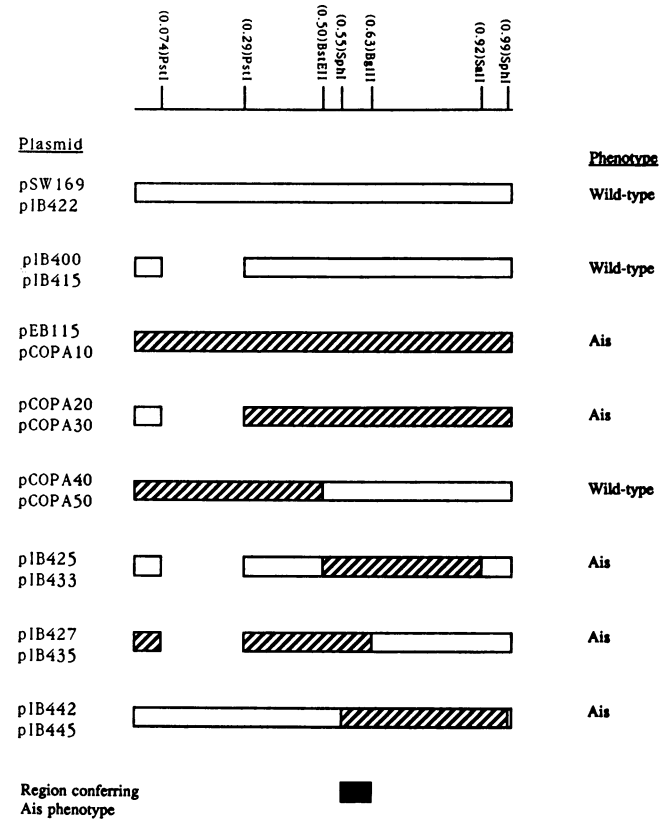

Fig. 4. Mapping of the *virA*(Ais) mutation in *virA112*. The top line represents the *virA* coding region with relevant restriction enzyme sites indicated. The fractions next to the sites indicate the distance from the *virA* start codon with the full-length gene being 1.00. Hybrids between wild-type *virA* and *virA112* are shown as bar figures with wild-type *virA* sequences represented as open bars and *virA112* sequences as hatched bars. The plasmid pairs listed in the left column are, on top, the pUC derivatives on which the hybrids were constructed and, at bottom, the broad host range derivatives bearing the *virA* hybrids that were transferred into *Agrobacterium tumefaciens* strain A1030 (e.g., pSW169 is a pUC derivative and pIB422 is a broad host range IncP derivative). The column on the right, headed phenotype, indicates the vir expression phenotype of the hybrids in *A. tumefaciens* strain A1030 cultured in SIB with no acetosyringone; Ais represents acetosyringone-independent signaling. Phenotypes were determined by measuring β-galactosidase activities expressed from a *virB*::*lacZ* fusion. Assays were done 24 hr after inoculation. Hybrids exhibiting >200 units of β-galactosidase activity were considered *virA*(Ais) and those with <2 units were considered wild type. All wild-type hybrids gave high levels of induction in the presence of acetosyringone. The filled-in box at the bottom of the figure is the region of *virA112* conferring the *virA*(Ais) phenotype on the hybrids as determined by the overlap of the hatched bars in the *virA*(Ais) hybrids.

independent signaling phenotype of these *virA* hybrids in *A. tumefaciens*. Each of the pUC derivatives carrying the different *virA* hybrids were ligated to pIB410 (Cangelosi *et al.* 1990), an IncP plasmid carrying a *virB*::*lacZ* fusion, at the plasmids' unique *Eco*RI sites. These plasmid fusions were introduced and maintained in *E. coli* C2110 (*polA*) because pUC/IncP replicon fusions are unstable in the *polA*$^+$ strain, *E. coli* DH5α. The broad host range constructs were mobilized into *A. tumefaciens* A1030 (*virA*::Tn*5*) via triparental matings. The *vir* expression phenotypes of the resulting strains were determined as a function of *virB*::*lacZ* expression in SIB without acetosyringone (Fig. 4).

By comparing the overlaps of *virA112* fragments present in each of the *virA*(Ais) hybrids, the mutation conferring the *virA*(Ais) phenotype in *virA112* was localized to an *Sph*I-*Bgl*II fragment corresponding to nucleotides 1377–1558 of the *virA* coding sequence. Nucleotides 1162–1558 of *virA112* were sequenced and the only nucleotide change found in this 403 base pair stretch was a G to A transition at nucleotide 1412. This G to A transition resulted in the replacement of a GGA codon with GAA (Fig. 5), which substitutes a glutamic acid residue for glycine-471 of the VirA protein.

DISCUSSION

Sensory protein mutants similar to *virA*(Ais) mutants described here have been isolated in other two-component regulatory systems (Albright *et al.* 1989). These stimulus-independent signaling mutants have similar phenotypes in that they activate transcription of subject operons in the absence of the stimulus specific for that two-component regulatory system. In the case of *virA112, vir* transcription is promoted in the absence of both acetosyringone and inducing monosaccharides, plant metabolites necessary for high levels of *vir* expression. The location of the *virA112* mutation in the VirA cytoplasmic domain correlates with the acetosyringone- and monosaccharide-independent phenotypes. Because detection of monosaccharides occurs in the periplasm and the detection of acetosyringone presumably occurs in the membrane (Melchers *et al.* 1989), it is unlikely that mutations in the cytoplasmic domain would yield phenotypes representing solely acetosyringone or monosaccharide independence to the exclusion of the other stimulus. Stimulus-independent signaling mutations in a variety of sensor proteins do not appear to be clustered in any specific region of the proteins (Albright *et al.* 1989). Thus, mutations in a wide variety of locations would likely yield *virA*(Ais) mutants.

It is interesting that the glycine to glutamic acid mutation is only three residues away from histidine-474, the active site of VirA, which transfers a phosphate group to aspartic acid-52 of the VirG protein. The G to A transition observed at codon 471 in *virA112* reflects the GC to AT mutational specificity of hydroxylamine. This glycine residue is conserved among a number of the sensor proteins, VirA, LHR VirA, NtrB, EnvZ, DctB, and SpoIIJ (Stock *et al.* 1989). Comparison of the predicted secondary structures of the wild-type VirA and VirA112 proteins by the Chou-Fasman algorithm (Chou and Fasman 1978) indicates that the glycine to glutamic acid mutation in VirA112 replaces a turn in the wild-type VirA protein with an α-helix. The unique characteristics of glycine in proteins and the presence of tandem glycines at sites 470–471 in VirA may indicate the presence of a reverse turn or hinge between two structural domains of the VirA protein at this point (Pakula and Sauer 1989). The glycine to glutamic acid change may directly mimic the active state configuration of the VirA protein in contrast to merely an allosteric change in protein configuration elicited by stimuli detection.

A. tumefaciens strains carrying *virA112* responded very weakly to acetosyringone and inducing monosaccharides; the various experiments indicated a range of 1.4- to 3-fold induction. This result indicates that the signaling mechanism in *virA112* is strongly dissociated from external stimuli, as might be expected from the cytoplasmic location of the lesion. Although *vir* expression by *virA112* was no longer dependent on external stimuli, the level of expression observed at neutral pH was much lower than that at pH 5.5. This observation supports the hypothesis that *vir* induction requires both the detection of plant signal molecules by VirA and the transcriptional induction of *virG* by acidic conditions (Winans *et al.* 1988; Winans 1990). Even though the signaling mechanism of *virA112* is dissociated from extracellular stimuli (acetosyringone, inducing monosaccharides, and, to a certain extent, low pH), the lack of high level *virG* transcription at pH 7.0 prevents the high acetosyringone-independent level of *vir* expression normally observed with *virA112*. Recent reports have indicated that VirA also plays an important role in the acidic pH optimum for *vir* regulon induction (Chen and Winans 1991; Melchers *et al.* 1989). Although it is likely that acidic conditions are sensed through VirA, the data presented here with the *virA112* allele indicates that transcriptional induction of *virG* by acidic conditions is absolutely required for efficient *vir* regulon induction.

The inability to isolate *virG*(Ais) mutants, reflected by the results using pEB103, was unusual in view of the isolation of similar stimulus-independent signaling mutants of other regulator proteins (Albright *et al.* 1989). Perhaps the absolute GC to AT specificity of hydroxylamine contributed to the difficulty. Other mutagens with broader mutagenic spectra (e.g., UV light or nitrous acid) may prove useful in the isolation of such mutants. Alternatively, it must be considered that *virG*(Ais) mutants may be unstable or at a selective disadvantage. In this regard, it has been

virA

5' CTT GCC GGC GGA ATA GCA CAT GAA TTT 3'

L A G G I A H E F

virA112

5' CTT GCC GGC G<u>A</u>A ATA GCA CAT GAA TTT 3'

L A G <u>E</u> I A H E F

Fig. 5. Nucleotide mutation and resulting amino acid change in *virA112*. The top lines of the indicated genes are the sequences of nucleotides 1402–1428 (numbered as in Leroux *et al.* 1987) and the lower lines are the translated amino acid sequences from residues 468–476. The mutant base and amino acid in *virA112* are underlined.

observed that *A. tumefaciens* cultures demonstrate very long generation times in the presence of acetosyringone and glucose. Fast-growing variants in these cultures have been determined to carry mutations and deletions in the *virG* or *virA* loci (C. Fortin, P. Dion, and E. W. Nester, unpublished observations).

Another unusual result was obtained with the mutant plasmid pIB100-32 in which the acetosyringone-independent signaling phenotype was not associated individually with either the *virA* or *virG* loci on pIB100-32. This plasmid, which does not carry either *virA*(Ais) or *virG*(Ais) mutations, likely requires the presence of its specific *virA* and *virG* loci to yield acetosyringone-independent *vir* expression.

Some laboratories have reported enhanced transformation of recalcitrant plant species by employing preinduction of the *A. tumefaciens* transformation vector by acetosyringone (Mathews *et al.* 1990; Sheikholeslam and Weeks 1987). Because the basis of the inability to transform a number of plants via *Agrobacterium* vectors is unknown, it would be useful to consider the employment of *virA*(Ais) mutants if inducing plant metabolites are limiting in certain plants. Alternatively, the use of *virG* constructs in which transcription is independent of acidic conditions may also be useful. The use of *virA*(Ais) mutants in the transformation of such plants is currently being analyzed in this laboratory.

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