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The VirA Protein of *Agrobacterium tumefaciens* Is Autophosphorylated and Is Essential for *vir* Gene Regulation

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The *virA* and *virG* gene products are required for the regulation of the *vir* regulon on the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens*. VirA is a membrane-associated protein which is homologous to the sensor molecules of other two-component regulatory systems. We overproduced truncated VirA proteins in *Escherichia coli* by deleting different lengths of the 5′-coding region of the *virA* gene and placing these genes under *lacZ* control. These proteins were purified from polyacrylamide gels and renatured. The renatured proteins became radiolabeled when they were incubated with [γ-^{32}P]ATP but not with [γ-^{32}P]GTP or [α-^{32}P]ATP, which suggests an ATP γ-phosphate-specific autophosphorylation. The smallest VirA protein, which retained only the C-terminal half of the protein, gave the strongest autophosphorylation signal, which demonstrates that the C-terminal domain has the autophosphorylation site. The phosphorylated amino acid was identified as phosphohistidine, and a highly conserved histidine was found in all of the VirA homologs. When this histidine was changed to glutamine, which cannot be phosphorylated, the resulting VirA protein lost both its ability to autophosphorylate and its biological function as a *vir* gene regulator. Results of this study indicate that VirA autophosphorylation is required for the induction of the *vir* regulon and subsequent tumor induction on plants by *A. tumefaciens*.

Crown gall tumors of plants are induced by *Agrobacterium tumefaciens* by a process of natural genetic engineering. The bacterium contains a large tumor-inducing plasmid (Ti plasmid) bearing two sets of genes that are vital for tumorigenesis. One set is termed *vir* (virulence) genes and the other is termed the T-DNA or transferred DNA (for reviews, see references 18 and 37). The *vir* genes, consisting of seven genetically identified operons, *virA*, *-B*, *-C*, *-D*, *-E*, *-G*, and *-H* (13, 29), are responsible for most steps in the transfer of T-DNA from the bacterium into the plant cell. These genes are only expressed in the presence of plant exudates containing low-molecular-weight phenolic compounds such as acetosyringone (28).

Two of the *vir* genes, *virA* and *virG*, are required for the expression of all *vir* genes, since mutations in either locus eliminate the expression of all other *vir* genes (34). Protein homolgy searches have shown that the VirA and VirG proteins are homologous to a large number of two-component regulatory systems, including EnvZ-OmpR, NtrB-NtrC, and CheA-CheY (15, 16, 34). The first protein of each pair (sensor) detects a particular environmental signal and transfers this information to a second component (activator), which, in turn, activates a series of genes (regulon) whose gene products respond to the environment (34). In the homologous systems which have been studied in most detail, the sensor molecule (NtrB, CheA) is autophosphorylated, and this high-energy phosphate is then transferred to the activator molecule (7, 19). In line with this model, it was proposed that in the *Agrobacterium* system the VirA protein senses the presence of the phenolic plant signal and the cytoplasmic portion transmits this signal to VirG through phosphorylation. The phosphorylated VirG protein would then specifically activate the transcription of all *vir* genes (including *virG* itself) (34). In this report we demonstrate the autophosphorylation of the VirA protein and its physiological role in *vir* gene induction of *A. tumefaciens*.

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MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or New England BioLabs, Inc. (Beverly, Mass.), and were used according to the recommendations of the suppliers. The Klenow fragment of DNA polymerase I and T4 ligase were obtained from Bethesda Research Laboratories; [γ-^{32}P]ATP, [α-^{32}P]ATP, and [γ-^{32}P]GTP were supplied by Dupont, NEN Research Products (Boston, Mass.). A oligonucleotide-directed mutagenesis kit was obtained from Amersham Corp. (Arlington Heights, Ill.), and the Immobilon membrane was obtained from Millipore Corp. (Bedford, Mass.).

Strains and plasmids. *A. tumefaciens* A136 is a plasmidless derivative of strain C58. A348 is a derivative of A136 containing the octopine-catabolizing plasmid pTiA6NC (25). A1030 is a Tn*5* insertional *virA* mutant of A348 (4). Plasmid vectors pTZ18R and pTZ19R were purchased from U.S. Biochemical Corp. (Cleveland, Ohio). Plasmid pRS0401 contains a histidine (H) to glutamine (Q) codon change at position 474 of the *virA* gene [designated *virA*(H/Q)] and was produced by site-directed mutagenesis on pSW169, which is a *virA* clone in pTZ18R (36). Plasmid pSG673 was constructed by isolating a 3.3-kilobase (kb) *Pvu*I-*Kpn*I fragment of the *virA* gene and ligating it to a *Sma*I-cut pTZ19R vector. Plasmid pSG680 was constructed by isolating a 2.5-kb *Hae*II-*Eco*RI fragment from pSG673, blunt ending it with DNA polymerase I, and ligating it to a *Sma*I-cut pTZ18R vector. Plasmid pSG681 was constructed by isolating a 2.2-kb *Bst*EII-*Eco*RI fragment from pSG673, blunt ending it with the Klenow fragment of DNA polymerase I, and ligating the resulting fragment to *Sma*I-cut pTZ19R. Plasmids pSG673, pSG680, and pSG681 were constructed such that the *virA* open reading frame was in the same reading

frame as the *lacZ'* gene of the pTZ plasmids. Since the *virA* genes were in frame with the LacZ' proteins of the pTZ plasmids, pSG673, pSG680, and pSG681 produced LacZ-VirA fusion proteins that were under the control of the *lacZ* promoter. Plasmid pRS0925 was constructed as described above for pSG681, except that the 2.2-kb *Bst*EII-*Sma*I fragment was from pRS0401. The 4.4-kb *Kpn*I fragment encoding the wild-type *virA* or the mutated *virA*(H/Q) gene was cloned into the *Kpn*I site of the pUCD2 vector (1), resulting in pTB108 and pRS0824, respectively.

Purification and renaturation of overproduced insoluble proteins. An overnight culture of *Escherichia coli* cells containing the *lacZ::virA* fusion construct was diluted 1:100 into fresh LB medium and grown at 37°C until the optical density at 600 nm reached 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 1 mM, and the cells were incubated for 3 h. The cells were collected by centrifugation, washed once with buffer A (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4], 50 mM KCl, 10% glycerol), and then suspended in 0.02 times their original volume in buffer A. They were disrupted by two passages through a Fench pressure cell at 20,000 lb/in^2. Insoluble proteins were collected by centrifuging the solution for 5 to 10 min at 10,000 × *g* in a microcentrifuge. The pellet was washed once with buffer A, and the protein was gel purified as described by Hager and Burgess (5). The purified protein was resuspended in buffer D (50 mM Tris hydrochloride [pH 8.0], 1 mM EDTA; 5 mM dithiothreitol, 6 M guanidine hydrochloride) to a final concentration of 0.05 mg/ml. This solution was dialyzed against 200 volumes of renaturation buffer R (20 mM HEPES [pH 7.4], 50 mM KCl, 5 mM dithiothreitol, 50% glycerol) for 18 h at 4°C without stirring. Dialysis was continued for an additional 18 h in fresh renaturation buffer with stirring. The renatured protein was stored at −20°C.

Phosphorylation protocol. Ten-microliter portions of the reaction buffer (20 mM HEPES [pH 7.4], 50 mM KCl, 7.5 mM $MgCl_2$) containing 5 μCi of [γ-^{32}P]ATP (made from 3,000 Ci/mmol) were delivered into 0.5-ml Eppendorf tubes, and the phosphorylation reactions were started by adding 5 μl of protein (0.5 μg). The mixtures were incubated at room temperature for 5 min, and the reactions were terminated by the addition of 5 μl of 4× loading buffer (14). The reaction mixtures were subjected to electrophoresis on sodium dodecyl sulfate (SDS)–12% polyacrylamide gels, and the proteins were transferred onto either nitrocellulose or Immobilon membranes. The membrane blots were washed in solution W (50 mM Tris hydrochloride [pH 7.4], 6 M guanidine hydrochloride) for 5 min to remove any nonspecifically bound radioactive materials and were then rinsed with water and air dried. The blots were wrapped with Saran Wrap and exposed to XAR film at −70°C by using an intensifying screen.

Determination of the phosphorylated amino acid. ^{32}P-phosphorylated VirA fusion protein from pSG681 (VirA681) was electrophoresed and blotted onto an Immobilon membrane as described above. To determine the class of amino acid which was phosphorylated in the VirA protein, three different Immobilon blots were treated under the following separate conditions: (i) 16% trichloroacetic acid at 90°C for 45 min, (ii) 1 M KOH at 55°C for 2 h, and (iii) water at room temperature for 1 h (control). All blots were rinsed several times with water, allowed to air dry, and then exposed to XAR film at −70°C. Similarly, ^{32}P-phosphorylated VirA681 was electrophoresed into SDS-polyacrylamide gels. Three separate gels were treated under the three conditions described above. Following the treatments, the gels were neutralized by incubating them in 0.5 M Tris hydrochloride (pH 8.0) for 30 min, rinsed with H_2O, and dried onto Whatman 3MM filter paper (Whatman, Inc., Clifton, N.J.). The dried gels were wrapped with Saran Wrap and exposed to XAR film at −70°C.

In order to determine whether phosphohistidine was present in phosphorylated VirA protein, 10 μg of VirA681 was phosphorylated with 100 μCi of [γ-^{32}P]ATP. The phosphorylated VirA681 protein was separated from [γ-^{32}P]ATP by denaturing it with 6 M guanidine hydrochloride and passing it over a Sephadex G-25 column equilibrated with H_2O. Protein elution was monitored at 280 nm. The ^{32}P-phosphorylated VirA681 sample was lyophilized and hydrolyzed with 3 M KOH as described previously (27). Following neturalization and removal of the precipitated potassium perchlorate, the solution was lyophilized and the residue was dissolved in 50 μl of H_2O. This solution was mixed with both histidine and phosphohistidine, which served as internal standards. Authentic phosphohistidine was prepared as described previously (9, 10, 26). The samples were spotted onto a thin-layer chromatography plate (Silica Gel G; Fisher Scientific Co., Pittsburgh, Pa.), and chromatograms were developed with chloroform–methanol–17% (wt/wt) ammonia (2:2:1; vol/vol) (6). Following chromatography, the plate was dried, wrapped with Saran Wrap, and exposed to XAR film at −70°C. After the chromatogram was exposed to X-ray film, it was treated with glacial acetic acid and the Pauly reagent as described previously (10) to visualize histidine and its derivatives.

Oligonucleotide-directed mutagenesis. The *virA* gene in pSW169 was mutagenized by the method of Taylor et al. (30, 31). Components were purchased as a kit from Amersham Corp. and used according to their recommendations. A 36-mer oligonucleotide, 5'-GCCGCCGGAATAGCCCAGG AATTTAATAACATTTTG-3', which was made on a DNA synthesizer (8600; BioSearch), was used to direct the codon change of CAT, which codes for histidine (position 474) in *virA*, to CAG (see underscored codons in the 36mer above), which codes for glutamine. At the same time, the neighboring codon GCA was changed to GCC (see underscored codons in the 36mer above), which did not change the amino acid for which it codes (alanine) but created a new restriction site (*Bst*NI). The *Bst*NI restriction enzyme was used in the initial screening for the mutagenized plasmid. To verify these changes, this region was sequenced by the chain-termination method (24).

Plant inoculation and *vir* gene induction. Virulence assays were performed on *Kalanchoe* leaves and *Nicotiana glauca* leaf disks as described by White and Nester (33) and Horsch et al. (8), respectively. *vir* genes were induced by acetosyringone as described by Winans et al. (35) and the immunoblotting assay with anti-VirE2 was performed as described previously (20).

RESULTS

VirA overproduction. VirA is a membrane-spanning protein which has an N-terminal hydrophobic signal sequence and a hydrophobic stop-transfer sequence (15). Attempts to overproduce a native-sized VirA protein were unsuccessful, presumably because the overproduced protein in the membrane is lethal for the bacteria (36). The support of this hypothesis, when we deleted the signal sequence and put it under the control of the *lacZ* promoter, a truncated VirA protein was overproduced with little effect on bacterial growth. Three different plasmid constructs, pSG673,

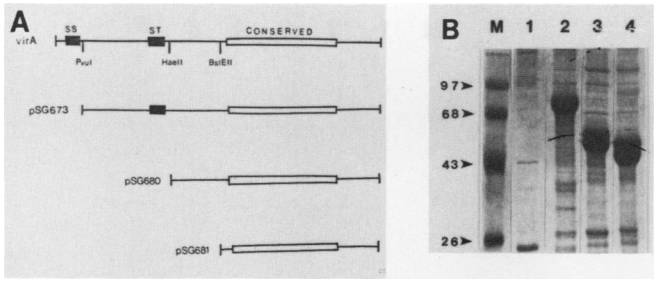

FIG. 1. Construction of three truncated VirA-overproducing plasmids. (A) Comparison of the wild-type *virA* structural gene with the truncated *virA* genes of the three constructs. Abbreviations: SS, signal sequence; ST, stop transfer sequence. The open box marked conserved represents the sequence which was highly conserved among the sensor proteins of the two-component regulatory systems. (B) Coomassie brilliant blue staining of isopropyl-β-D-thiogalactopyranoside-induced insoluble protein pellets from *E. coli* harboring plasmids. Lane 1, pTZ19R; lane 2, pSG673; lane 3, pSG680; lane 4, pSG681; M, molecular size standards (indicated to the left of the gel, in kilodaltons).

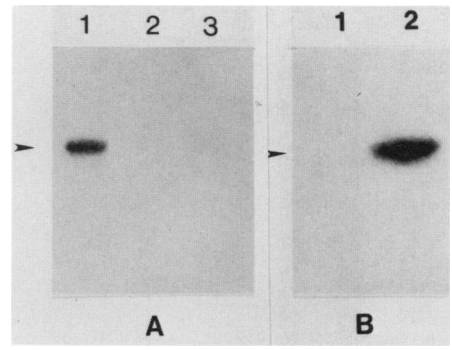

FIG. 3. (A) Autoradiogram showing nucleotide specificity of the VirA autophosphorylation. VirA681 (0.5 μg) was incubated with 5 μCi of ^{32}P-labeled nucleotide in each reaction. Lane 1, Incubation with [γ-^{32}P]ATP; lane 2, incubation with [γ-^{32}P]GTP; lane 3, incubation with [α-^{32}P]ATP. (B) Autophosphorylation of the VirA protein from pSG681 and pRS0925 by using [γ-^{32}P]ATP. Both proteins were purified at the same time, and 0.5 μg of protein was used in each reaction. Lane 1, VirA from pRS0925; lane 2, VirA from pSG681. The arrowheads indicate the VirA protein bands.

pSG680, and pSG681 (Fig. 1A), yielded the LacZ-VirA fusion proteins VirA673, VirA680, and VirA681, respectively. In the VirA673 protein, the 54 amino acids from the N-terminal end were deleted; this included the signal sequence. In the VirA680 protein, 324 amino acids from the N-terminal end were deleted. This included the two hydrophobic sequences and the periplasmic domain. Therefore, Vir680 retained only the cytoplasmic domain of the VirA protein. Protein VirA681 contained the C-terminal region of the protein, which is the most conserved region in all the VirA homologs (15). Proteins VirA673, VirA680, and VirA681 had 18, 11, and 18 N-terminal amino acids from LacZ′ of the pTZ vectors, respectively. These overproduced fusion proteins were all insoluble in *E. coli* cells (Fig. 1B).

Truncated VirA proteins are autophosphorylated. All three truncated VirA proteins were gel purified and renatured (Fig. 2A). These proteins were assayed for autophosphorylation by incubating them with [γ-^{32}P]ATP for 5 min in the presence of 5 mM $MgCl_2$. Each of the three proteins became radiolabeled with various intensities of the radioactive signals. The smallest construct (VirA681) gave the strongest signal of all constructs (Fig. 2B). Radiolabeling was obtained only when the truncated VirA protein was incubated with [γ-^{32}P]ATP but not with [γ-^{32}P]GTP or [α-^{32}P]ATP (Fig. 3A),

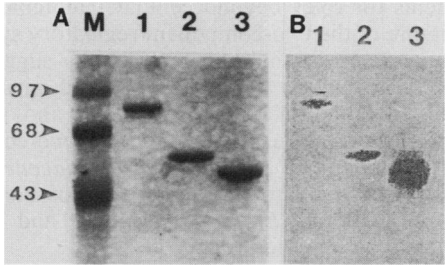

FIG. 2. (A) Coomassie brilliant blue staining of the purified VirA proteins (2 μg each). Lane 1, VirA673; lane 2, VirA680; lane 3, VirA681; M, molecular size standards (indicated to the left of the gel, in kilodaltons). (B) Autoradiogram showing autophosphorylation of the three truncated VirA proteins. Proteins (0.5 μg) and [γ-^{32}P]ATP (5 μCi) were used in each reaction. Lane 1, VirA673; lane 2, VirA680; lane 3, VirA681.

suggesting an ATP γ-phosphate-specific autophosphorylation. Inclusion of the acetosyringone in the reaction mixtures did not affect the autophosphorylation of the three truncated VirA proteins (data not shown).

The phosphorylation site in VirA is histidine. In order to characterize the phosphorylated amino acid of the VirA protein, we tested the stability of the phosphate bond under alkaline and acidic conditions. The phosphorylated VirA protein was run on an SDS–12% polyacrylamide gel and transferred to an Immobilon membrane. Both SDS-polyacrylamide gels and the blots were treated under alkaline and acidic conditions as described in the Materials and Methods. The autoradiograms demonstrated that the phosphate bond was stable to base and labile to acid treatment (data not shown). This suggests an amidophosphate bond, possibly phosphohistidine (2, 3, 12).

The phosphorylated amino acid of VirA was further identified. The ^{32}P-phosphorylated VirA681 was hydrolyzed as described in the Materials and Methods, and the hydrolysate was subjected to thin-layer chromatography with histidine and phosphohistidine internal standards. Radioactivity was found to comigrate with free phosphate at the origin and the phosphohistidine standard at an R_f of 0.24 (Fig. 4). By using this chromatography system, phosphohistidine was separated into two spots, as shown in Fig. 4. The preparation of phosphohistidine by the reaction of histidine with potassium hydrogen phosphoramidate has been shown to yield both 1-phosphohistidine and 3-phosphohistidine (6, 10). According to those previous reports (6, 10) and the time course of phosphohistidine formation, we believe that the spots designated P His1 (phosphohistidine form 1) and P His2 (phosphohistidine form 2) in Fig. 4 correspond to 1-phosphohistidine and 3-phosphohistidine, respectively.

Histidine phosphorylation has also been observed in two VirA-homologous proteins, NtrB (19) and CheA (6). In the case of the CheA protein, the histidine at position 48 is phosphorylated (6), and this is the only histidine residue that occurred in a sequence of amino acids which was highly conserved among all VirA homologs. This conservation strongly suggested that VirA is also phosphorylated at this histidine residue (amino acid 474 in VirA).

Effect of altering the conserved histidine in the VirA protein. To gain further evidence that the histidine residue at

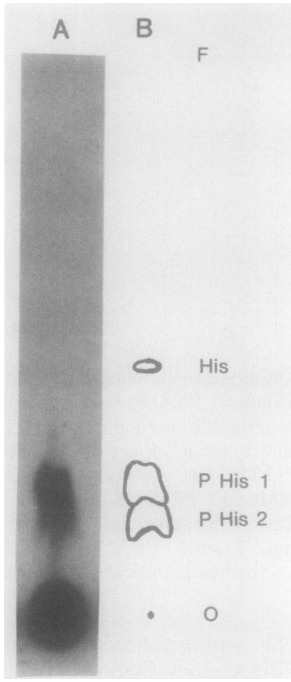

FIG. 4. Demonstration of phosphohistidine in phosphorylated VirA. ^{32}P-phosphorylated VirA681 was purified, hydrolyzed, and prepared for chromatography as described in the text. The hydrolyzed sample was applied to a silica gel chromatogram and developed in $CHCl_3$–methanol–17% (wt/wt) ammonia (2:2:1; vol/vol). Histidine and phosphohistidine were added to the hydrolyzed sample to serve as internal standards. (A) Autoradiogram of the thin-layer chromatogram following development. (B) Graphic representation of spots generated following treatment of the thin-layer chromatogram with glacial acetic acid and the Pauly reagent to visualize histidine and its derivatives. Abbreviations: F, solvent front; O, origin; His, histidine; P His 1, phosphohistidine form 1; P His 2, phosphohistidine form 2.

position 474 was the site of phosphorylation, we studied the biological consequences of changing it to another amino acid by site-directed mutagenesis. Glutamine was chosen as a conserved substitute for histidine. Furthermore, glutamine cannot be phosphorylated. An oligonucleotide, 5′-GCCGCC GGAATAGCCCAGGAATTTAATAACATTTTG-3′, was used to direct the change. This oligonucleotide not only directed the change of histidine to glutamine but also created a new restriction site of *Bst*NI, which we used to screen for the correct changes (see Materials and Methods). This region of the resulting mutant (pRS0401) was sequenced to confirm that only the predicted changes occurred (data not shown).

Using this mutated *virA*(H/Q) gene, we constructed a VirA-overproducing plasmid, pRS0925, which corresponded to pSG681. This pRS0925-encoded VirA protein, designated VirA681(H/Q), failed to autophosphorylate (Fig. 3B), demonstrating that the histidine residue at amino acid 474 is essential for VirA autophosphorylation.

Autophosphorylation is biologically important. The VirA protein was required for *vir* gene induction, and therefore for the virulence of *A. tumefaciens*. In order to determine whether His-474 is essential for the biological activity of VirA, the acetosyringone-mediated induction of *vir* genes and tumorigenic potential of the strains containing mutated *virA*(H/Q) were assayed. The broad-host-range plasmids pTB108 and pRS0824, containing the wild-type *virA* and the

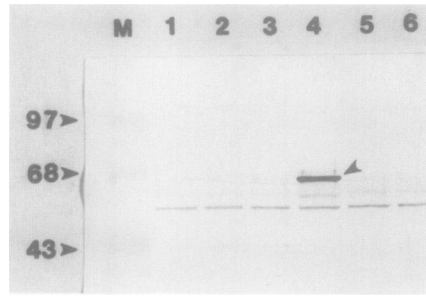

FIG. 5. *virE* gene induction assay. A total of 10 μg of induced (lanes 2, 4, and 6) and uninduced (lanes 1, 3, and 5) *Agrobacterium* cell extracts was run on a SDS–12% polyacrylamide gel and immunoblotted with antibody against VirE2 protein. Lanes 1 and 2, A1030; lanes 3 and 4, A1030(pTB108); lanes 5 and 6, A1030 (pRS0824). The arrowhead indicates the VirE2 protein band.

mutated *virA*(H/Q) genes, respectively, were mobilized into A1030, a *virA* mutant of *A. tumefaciens*, resulting in strains A1030(pTB108) and A1030(pRS0824), respectively. The inducibility of the *virE* gene in these two strains was assayed by immunoblotting the acetosyringone-induced cell extracts with VirE2 antibody. The VirE2 protein was induced in A1030(pTB108) but not in A1030(pRS0824) (Fig. 5), while the mutant *virA* gene was expressed, as was the wild-type gene (data not shown). This suggests that the mutated VirA(H/Q) protein no longer functions as a *vir* gene regulator. This result was supported by testing the virulence of these strains on plants. A1030(pRS0824) did not induce tumors on either *Kalanchoe* leaves (Fig. 6) or *N. glauca* leaf disks (data not shown), while the control strain A1030(pTB108) induced tumors on both plants. Thus, the induction of *Agrobacterium vir* genes and the subsequent tumor-inducing activity are correlated with the autophosphorylation of the VirA protein.

DISCUSSION

The VirA and VirG proteins are members of a family of two-component regulatory systems (23). The best studied of these systems, CheA-CheY, NtrB-NtrC, and EnvZ-OmpR, are *E. coli* regulatory proteins that are involved in the physiological maintenance of the cell. Reports on other regulatory systems such as VirA-VirG (15, 34), DctB-DctD of *Rhizobium leguminosarum* (22), the *ctxAB* operon of *Vibrio cholerae*, the *ptx* operon of *Bordetella pertussis*, and the virulence loci of *Shigella* species (17) demonstrate that there is a wide-ranging adaptation of two-component regulatory systems for roles beyond cellular maintenance. The essential nature of the two-component regulatory systems in these pathogenic and symbiotic interactions suggests that these signal transduction mechanisms played an important role in the evolution of the host-parasite and symbiont relationship. Furthermore, the presence of two-component systems in members of the family *Rhizobiaceae* demonstrates that such regulatory circuits are not confined to members of the family *Enterobacteriaceae* and are very likely prevalent among the procaryotes.

Here we have reported on the autophosphorylation of the VirA sensor protein. This result is consistent with autophosphorylation of other sensor molecules, CheA, NtrB, and EnvZ (7, 11, 19). It has been proposed that the phosphorylated sensor molecule then transfers the phosphate group to an activator protein which promotes transcription. The transfer of phosphate from sensor to activator has been

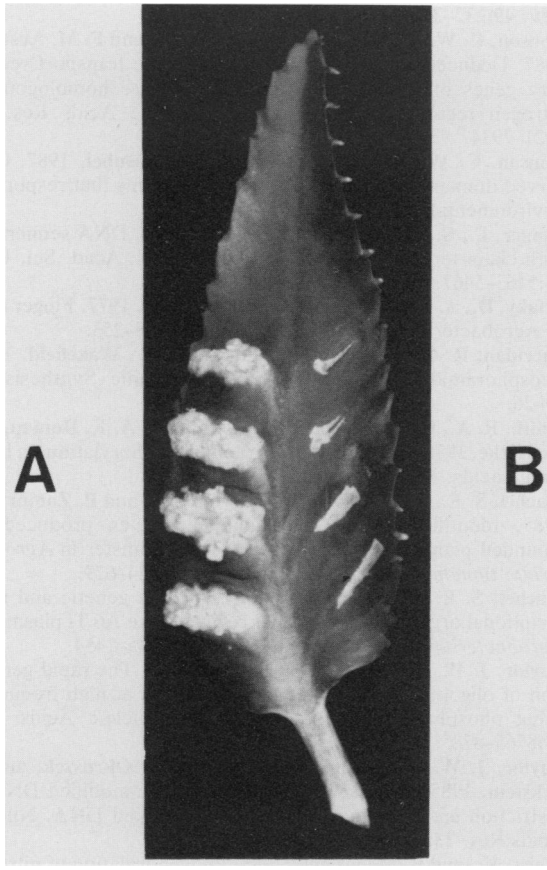

FIG. 6. Virulence test of the strains A1030(pTB108) and A1030(pRS0824) on a *Kalanchoe* leaf. Side A was inoculated with A1030(pTB108); side B was inoculated with A1030(pRS0824).

demonstrated in the CheA-CheY (6), NtrB-NtrC (32), and EnvZ-OmpR (11; T. Silhavy, personal communication) systems but has not yet been shown in the case of VirA-VirG. However, in view of studies on other two-component systems, phosphate transfer to VirG is likely.

In the two-component systems of CheA-CheY and NtrB-NtrC, the sensor molecules are cytoplasmic proteins, while in the VirA-VirG system, VirA is a membrane-spanning protein anchored to the membrane by two hydrophobic sequences. It has been previously reported (36) that overproduction of the native VirA protein was not possible due to the toxicity associated with directing too much protein to the membrane. To overcome this problem, we deleted the signal sequence of the VirA protein which prevented membrane localization and resulted in the formation of insoluble inclusion bodies. The three different truncated VirA proteins, VirA673, VirA680, and VirA681, gave different levels of autophosphorylation, with the smallest, VirA681, yielding the highest level. This result demonstrates that the C-terminal conserved region of VirA is sufficient for autophosphorylation. One interpretation for the lower levels of autophosphorylation obtained with VirA673 and VirA680 is that the N-terminal amino acids attached to the VirA conserved region have a negative regulatory effect. However, since none of the truncated VirA proteins were in their membrane-bound native state, it is difficult to assess whether there is any physiologic significance to the observed autophosphorylation levels. It is probable that the truncated VirA proteins fold incorrectly compared with the native form of VirA. It remains to be determined whether plant-derived molecules, such as acetosyringone, signal for the autophosphorylation of VirA or for the transfer of phosphate from a preexisting autophosphorylated VirA protein to VirG or for some other undefined function. It would be of interest to determine whether these truncated VirA proteins are able to promote *vir* gene expression independent of acetosyringone in *A. tumefaciens*.

Of the 10 histidine residues in VirA681, only 1, which was located in a highly conserved block of amino acids, was found in all of the VirA homologs. This same histidine of the CheA protein has been shown to be phosphorylated (6). Glutamine was chosen to replace His-474 because of its similar size and structural similarities in the position of the nitrogen atom, which minimizes spatial effects on the structure of the molecule. In addition, glutamine cannot be phosphorylated, which makes it an appropriate substitute for the phosphate-receiving site. The replacement of this histidine by glutamine eliminated the ability of the VirA protein to autophosphorylate and strongly suggests that His-474 is the phosphorylation site of the VirA protein, although it is not absolutely certain that the amino acid change had no effect on some other aspect of the autophosphorlyation mechanism. Since *A. tumefaciens* mutants with the histidine to glutamine replacement [VirA(H/Q)] could not induce the *vir* genes or form tumors on plants, a strong correlation exists between VirA autophosphorylation and its physiological role in pathogenicity.

Histidine phosphorylation of proteins has been observed in both procaryotic and eucaryotic organisms, in which it mainly serves as an intermediate in phosphoryl group transfer (21). As additional information becomes available on the function of various domains of the VirA and VirG molecules, it should be possible to modify a variety of biological activities by altering relevant amino acids.

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