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Functional Roles Assigned to the Periplasmic, Linker, and Receiver Domains of the *Agrobacterium tumefaciens* VirA Protein

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VirA and VirG activate the *Agrobacterium tumefaciens vir* regulon in response to phenolic compounds, monosaccharides, and acidity released from plant wound sites. VirA contains an amino-terminal periplasmic domain and three cytoplasmic domains: a linker, a protein kinase, and a phosphoryl receiver. We constructed internal deletions of *virA* that truncate one or more domains and tested the ability of the resulting proteins to mediate environmentally responsive *vir* gene activation in vivo. The periplasmic domain is required for sensing of monosaccharides (in agreement with earlier results), while the linker domain is required for sensing of phenolic compounds and acidity. The phosphoryl receiver domain of VirA plays an inhibitory role in signal transduction that may be modulated by phosphorylation. The carboxy terminus of the protein was also dispensable for tumorigenesis, while the periplasmic domain was required.

Protein kinases and their substrates are important in processes as diverse as cell division, organogenesis, chemotaxis, transcriptional regulation, and many other aspects of intracellular metabolism (8). Many bacterial protein kinases belong to the family of two-component regulatory proteins, each member of which contains a kinase and the substrate for that kinase, known as a response regulator (27, 39). Each of these homologous kinases autophosphorylates a specific histidine residue and transfers this phosphoryl moiety to a specific aspartate residue of the cognate response regulator. In most of these systems, the kinase is a transmembrane environmental sensor, while the response regulator is an intracellular transcriptional regulator.

The tumor-inducing (Ti) plasmid of the plant pathogen *Agrobacterium tumefaciens* encodes one of the best characterized of these systems (38, 44). During infection, approximately 25 *vir* gene products (transcribed in eight Ti plasmid-encoded operons) mediate the transfer of oncogenic DNA to the nuclei of infected plant cells (13, 31, 36). Induction of these genes is elevated by a family of related phenolic compounds and is strongly potentiated by monosaccharides and acid pH, all of which are released from plant wound sites (3, 35, 37). This induction requires the VirA transmembrane protein kinase (11, 18, 22, 23, 25) and the VirG response regulator (10, 45). VirA autophosphorylates on a histidine residue and transfers this phosphoryl group to aspartate residue 52 of VirG (10, 11). VirG protein binds to specific sites upstream of each promoter and is thought to activate transcription of these promoters in a phosphorylation-dependent fashion (6, 12, 30, 46). The stimulatory effect of monosaccharides requires periplasmic sugar-binding protein ChvE, which is thought to interact with the periplasmic domain of VirA (3, 33). Altered-function point mutations in *virA* have been described (1, 29).

The VirA protein of the octopine-type Ti plasmids is 829 amino acids long, and sequence analysis suggests that this protein contains at least four putative domains (see Fig. 1). It has a signal sequence to initiate membrane localization (amino acids 18 to 39, designated TM1), a periplasmic domain (amino acids 39 to 259; see references 24 and 44), a second hydrophobic region (amino acids 260 to 278, designated TM2), a cytoplasmic linker domain (amino acids 279 to 418), a domain homologous to the family of histidine protein kinases (amino acids 419 to 691), and a receiver domain (amino acids 712 to 829) which is homologous to the amino-terminal half of VirG and other response regulators. In contrast, VirG is 241 amino acids long and contains two domains. The amino-terminal receiver domain (amino acids 1 to 125) is the target of the VirA kinase (10), while the carboxyl-terminal domain (amino acids 126 to 241) binds to specific sites (*vir* boxes) upstream of each *vir* promoter (32).

The transmembrane topology of VirA suggests that it is an environmental sensor, a role postulated for homologous protein kinases (39), and its complex four-domain structure makes it an especially interesting sensory protein. While the function of the kinase domain is presumably to phosphorylate VirG, the roles of the other three domains are less apparent. It is plausible that these domains act to modulate kinase activity, and there is some evidence to support this hypothesis. A *virA* in-frame deletion mutation that removed part of the periplasmic domain did not abolish induction by phenolic compounds but did abolish the stimulatory effects of monosaccharides and attenuated the stimulatory effects of acid pH (4, 21, 33). It was concluded that this part of the periplasmic domain interacted with the ChvE protein and also acted as a pH sensor, although below we provide data that indicate that this domain is not required for pH sensing. It was hypothesized that the binding site for phenolic compounds is located in the transmembrane regions, although we will demonstrate that TM1 and TM2 are not required for induction by phenolic compounds.

As mentioned above, VirA contains at its C terminus a domain homologous to the receiver domain of the family of response regulators, including VirG (39; see Fig. 1). This domain of VirA contains an aspartate residue (residue 766) at the same position as the phosphorylated aspartate residues of several response regulators, suggesting that it is phosphorylated. Similar receiver domains are found in a number of homologous histidine protein kinases (2, 7, 9, 15, 20, 26,

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40). While the functions of these domains are unknown, we will provide evidence that this domain of VirA plays an inhibitory role in signal transduction.

In the current study, we tried to dissect the ChvE-VirA-VirG signal transduction system genetically by creating altered-function alleles of the *virA* gene. We describe four in-frame deletion alleles and a single-codon alteration in *virA* which encode proteins with distinctive altered functions. These studies have allowed us to reassess the contributions of each domain to the recognition of plant hosts.

MATERIALS AND METHODS

Strains, plasmids, and reagents. *Escherichia coli* JM101 and RZ1032 were obtained from E. Nester (University of Washington). Broad-host-range plasmid vector pUCD2 (5) was obtained from C. Kado (University of California, Davis). Plasmids pTZ18R and pTZ19R and the Sequenase Version 2.0 DNA Sequencing Kits were purchased from United States Biochemical Corp. (Cleveland, Ohio). Synthetic oligonucleotides used for DNA sequencing were obtained from the Cornell Nucleotide Synthesis Center. *o*-Nitrophenyl-β-D-galactopyranoside, carbenicillin, tetracycline, spectinomycin, and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma (St. Louis, Mo.). Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and were handled by using standard recombinant DNA techniques as recommended by the manufacturers. Acetosyringone (AS) was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Adenosine 5′-α-[^{35}S]thiotriphosphate was purchased from Amersham (Arlington Heights, Ill.). Tween 20, 5-bromo-4-chloro-3-indolylphosphate, *p*-Nitro Blue Tetrazolium, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G, and nitrocellulose paper were purchased from Bio-Rad Laboratories (Richmond, Calif.).

Plasmid constructions. pSW169 is a derivative of pTZ18R containing the *virA* gene from pTiA6 cloned as a *Kpn*I fragment such that transcription of P*lac* and that of *virA* proceed in the same direction (47). pSW169 was introduced into *dut ung* mutant strain RZ1032, recovered in single-stranded circular form containing uracil residues, and used to make site-directed mutations as previously described (16). Plasmid pCH101, containing a deletion of codons 11 to 283, was constructed by using synthetic oligonucleotide 5′-TA TTCACCGACGCGGCAGACCGATTGGTTAGCGCGG-3′. The resulting deletion was introduced into its parent plasmid (pSW169) by cutting both plasmids with *Eco*47III and *Bam*HI, size fractionating these fragments, and ligating the appropriate fragments in low-melting-temperature agarose (41). From this procedure, plasmid pCH203 was isolated. The DNA of the *virA* promoter and the *virA* coding sequence of pCH203 up to the *Eco*47III site were sequenced on one strand to verify the absence of additional unwanted alterations.

Plasmid pCH105, which contains a deletion of codons 712 to 829, was made by using oligonucleotide 5′-AATAA GGCACCGCGTTAGTAGAGTTGCGACGTG-3′. This deletion fuses codon 711 to the native termination codon (codon 830) and inserts an additional stop codon between them (both stop codons are underlined). Plasmid pCH231, which contains the D766N mutation, was made by using synthetic oligonucleotide 5′-GGTCATGGTCAACCAAGCG TC-3′. These mutations were introduced into a wild-type *virA* gene by cutting these plasmids and pSW169 with *Sna*BI and either *Eco*RI (for pCH105) or *Bam*HI (for pCH231), purifying the fragments from low-melting-temperature agarose, ligating them, and isolating the desired recombinants. The *Sna*BI-*Eco*RI fragments containing the Δ712-829 mutation and the *Sna*BI-*Bam*HI fragment containing the D766N mutation were sequenced to check for additional alterations. The recombinants containing the Δ712-829 and D766N mutations are designated pCH207 and pCH235, respectively.

Plasmid pCH208, which contains both the Δ11-283 and Δ712-829 mutations, was generated by cutting pCH203 and pCH207 with *Sna*BI and *Eco*RI and size fractionating and ligating the fragments as described above. Plasmid pCH242, which contains the Δ5-414 and Δ712-829 deletions, was made by treating pCH208 sequentially with *Bst*EII, nuclease S1, *Xmn*I, and T4 DNA ligase.

Plasmids pSW169 (wild-type *virA*), pTZ18R (no *virA*), pCH203 (Δ11-283), pCH207 (Δ712-829), pCH208 (Δ11-283 and Δ712-829), pCH242 (Δ5-414 and Δ712-829), and pCH235 (D766N) were linearized by digestion with *Eco*RI and ligated with pUCD2 (6) cleaved with *Eco*RI; this was followed by selection for resistance to carbenicillin and tetracycline. From these transformants, pCH114, pCH117, pCH224, pCH225, pCH222, pCH240, and pCH237 were isolated. These were introduced into *A. tumefaciens* A136(pCH116) by electroporation. This strain does not contain the Ti plasmid but does contain pCH116, an IncP plasmid containing *npt*, a P*lac-virG* fusion, and a P*virB-lacZ* fusion (unpublished data). The resulting strains were cultured and assayed for β-galactosidase as previously described (4), except that the growth medium contained 50 mM phosphate and 50 mM MES. Assays of VirA mutants were conducted at 20°C.

Western blots (immunoblots). Cultures (25 ml) of *A. tumefaciens* strains containing the wild-type or mutant proteins were grown to the mid-log phase in AB medium (18a); centrifuged; suspended in 4 ml of a buffer containing 10 mM Tris (pH 8.0), 10 mM EDTA, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride; and lysed by passage two times through a French press minicell (19,000 lb/in^2). The resulting crude cell lysate was centrifuged in a Beckman SW55Ti rotor at 12,000 rpm for 15 min. The supernatant was centrifuged in the same rotor at 50,000 rpm for 90 min. The pellet and supernatant fractions were size fractionated by using sodium dodecyl sulfate–12% polyacrylamide gels (17). Immunological staining of size-fractionated proteins was carried out as recommended by Bio-Rad Laboratories by using rabbit antiserum against a TrpE-VirA fusion protein (18, 47).

Tumorigenesis assays. Assays for tumorigenesis were conducted by using 10-cm *Kalanchoë diagremontiana* plants. The youngest full-size leaves were wounded with a wooden dowel and inoculated heavily with various *A. tumefaciens* strains grown on LB agar. Assays were conducted in quadruplicate. Plants were incubated under metal halide lights (approximately 600 microeinsteins m^{-2} s^{-1}, 16 h/day) at 50% humidity and 23°C. Photographs were taken after 3 weeks.

RESULTS

Construction and expression of in-frame deletion mutations of *virA*. To understand the functions of the putative domains of VirA, we constructed internal in-frame deletions that truncate one or more domains, as well as a single-codon alteration (D766N). A total of six *virA* alleles (shown in Fig. 1) and a negative control were introduced into Ti plasmidless *A. tumefaciens* A136 harboring plasmid pCH116. pCH116 contains a P*lac-virG* promoter fusion to permit constitutive

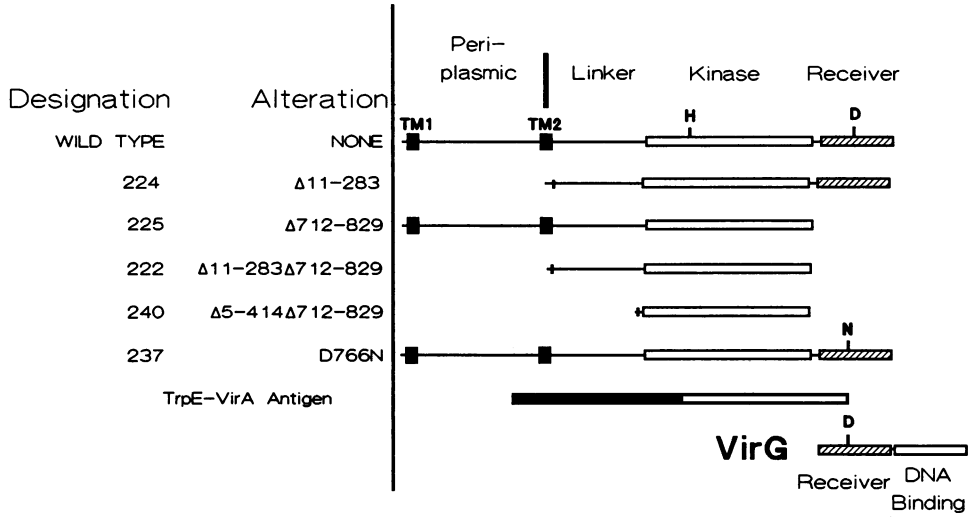

FIG. 1. Schematic diagram of the putative domain organization of the wild-type VirA and VirG proteins and of VirA variants characterized in this study. All *virA* genes utilize the native *virA* promoter. TM1 and TM2 represent hydrophobic transmembrane regions.

expression of VirG and a *virB-lacZ* gene fusion to allow measurement of a representative *vir* promoter.

To determine whether these proteins were expressed at levels similar to that of the wild type and ensure that they were truncated by the predicted amounts, proteins from these strains were visualized by immunological staining. The polyclonal antiserum used in these experiments was made against a TrpE-VirA fusion protein containing VirA sequences from amino acids 520 to 766 (Fig. 1). This antiserum detected all VirA truncated variants (Fig. 2) except the *virA240* product, which contains only the kinase domain (data not shown). Nondetection of the *virA240* product was not due to the lack of antigenic determinants (Fig. 1). This suggests that the kinase domain is highly unstable when synthesized in the absence of other domains. If this is true, then the kinase activity of that protein may be considerably higher than the levels suggested by assay of *vir* gene expression in whole cells.

The intensity of staining of the other VirA mutant proteins was approximately equivalent to that of the wild-type protein, suggesting that each of these proteins was present in approximately the same concentration, although the *virA222* product may be present in slightly smaller amounts. In each case, the antiserum stained one band which corresponded closely to the predicted molecular weight of that mutant protein. Although all of these proteins were found in the cell pellet after ultracentrifugation of crude lysates, it is premature to speculate about whether these proteins are membrane associated or soluble.

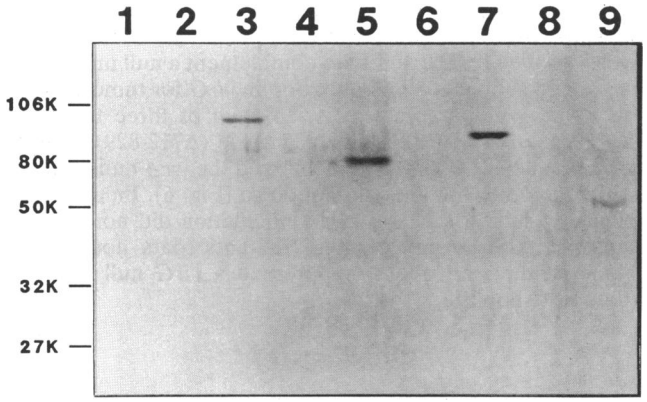

FIG. 2. Expression of the wild-type and mutant VirA proteins. *A. tumefaciens* cultures not containing *virA* (lane 1) or containing wild-type *virA* (lanes 2 and 3), *virA224* (lanes 4 and 5), *virA225* (lanes 6 and 7), or *virA222* (lanes 8 and 9) were separated into soluble (lanes 2, 4, 6, and 8) and particulate (lanes 1, 3, 5, 7, and 9) fractions and immunostained. The numbers on the left are molecular weights (10^3).

***vir* gene expression by altered-function *virA* products.** Derivatives of *A. tumefaciens* A136(pCH116) carrying the *virA* alleles described above were cultured under a variety of conditions and assayed for expression of the *virB* promoter. In all of these experiments, a control strain lacking any *virA* gene was tested in parallel, and in every assay, this strain showed less than 3 U of β-galactosidase activity (see Fig. 5B; data not shown). We first compared the wild-type VirA protein to the *virA224* product, which lacks the periplasmic domain and both transmembrane regions. Strains containing either protein were clearly stimulated by a 100 μM concentration of the phenolic compound AS (Fig. 3A), and in both cases, induction was potentiated by acid pH (Fig. 3B). This finding demonstrates that the entire periplasmic domain and both transmembrane regions are dispensable for responsiveness to these stimuli. Consistent with earlier studies (3, 33), the mutant protein was poorly responsive to a low concentration of AS and was not stimulated by monosaccharides (Fig. 3C). Consistent with results in Fig. 3A, a strain containing the *virA224* product was not detectably induced by 5 μM AS in the presence or absence of inducing sugar (Fig. 3C). In fact, glucose reproducibly caused a slight inhibitory effect, a phenomenon that remains to be investigated. The mutant protein was temperature sensitive (Fig. 3D), since it activated transcription of the reporter gene at 20°C but not at 30°C. For this reason, all assays using *virA* mutants (and the wild-type *virA* control) were conducted at 20°C.

Similar experiments were carried out by using the *virA222* product, which lacks the receiver domain. The most striking finding is that the *virA225* product was highly active even in the absence of AS. In fact, in medium optimized for *vir* gene induction, this protein was virtually as active in the absence of AS as in its presence (Fig. 4A). Stimulation of this protein

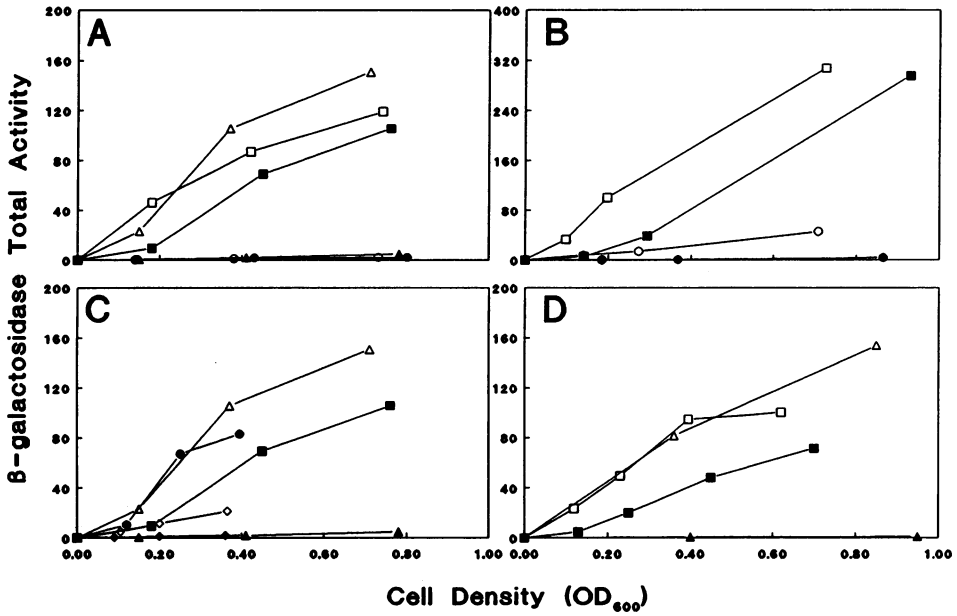

FIG. 3. Induction of the *virB* promoter by the *virA224* allele (closed symbols) or the wild-type VirA protein (open symbols). (A) Responsiveness to the phenolic inducer AS. Strains were cultured at pH 5.5 with 0.5% glycerol supplemented with 10 mM glucose and 100 μM (□, ■), 5 μM (△, ▲), or 0 μM (○, ●) AS. (B) Acid pH optimum for induction. Strains were cultured with 100 μM AS, 0.5% glycerol, and 10 mM glucose at pH 5.5 (□, ■) or 7.5 (○, ●). (C) Stimulation of induction by monosaccharides. Inductions were carried out at pH 5.5 in media containing 0.5% glycerol supplemented with 100 μM AS and 10 mM glucose (■), 100 μM AS only (●), 5 μM AS and 10 mM glucose (△, ▲), or 5 μM AS only (◇, ◆). (D) Temperature sensitivity of induction. Strains were cultured at pH 5.5 with 100 μM AS and 0.5% glucose at 30°C (△, ▲) or 20°C (□, ■).

by AS was clearly seen when either the acid pH stimulus or the monosaccharide stimulus was withheld (Fig. 4B and C). Furthermore, either AS or glucose alone efficiently stimulated this protein (compare Fig. 4A and C). This protein also retains the acid pH optimum of the wild-type protein (compare Fig. 4A and B, noting the *y* axes). These data indicate that the receiver domain inhibits the activity of the protein and suggest that the normal role of this domain is negative modulation of the kinase domain.

Two additional deletion mutants were tested. One of these (*virA222*) contains both of the deletions described above and therefore contains only the linker and kinase domains, while the second mutant (*virA240*) is even smaller and contains only the kinase domain (Fig. 1). The *virA222* allele was stimulated by AS and by acid pH. It was nonresponsive to (or even slightly inhibited by) monosaccharides in the presence of either 100 μM (Fig. 5A) or 5 μM (data not shown) AS. Expression of β-galactosidase in the absence of AS was much higher than for the wild-type protein (compare Fig. 5A and 3A), indicating that it, like *virA225*, has an elevated basal level of expression. This demonstrates that virtually half of the VirA protein is dispensable for perception of phenolic compounds and acid pH. In contrast, the strain containing the *virA240* allele activated expression of the *virB-lacZ* fusion at low constitutive levels and expression was not responsive to any of the three environmental stimuli (Fig. 5B). This constitutive expression was about fivefold higher than that seen in the absence of any *virA* gene (Fig. 5B). Since the protein encoded by *virA240* is probably unstable (see above), it is likely that these measurements understate its true kinase activity. Nevertheless, these data indicate that this protein is not responsive to any of the three environmental stimuli that activate wild-type VirA.

Alteration of aspartate residue 766. Results presented above suggest that the receiver domain of VirA plays an inhibitory role in *vir* gene expression. It seemed possible that the inhibitory role of this domain is modulated by phosphorylation of aspartate residue 766 since, as mentioned above, this residue corresponds to the site of phosphorylation of several homologous proteins. This residue was therefore altered to asparagine. In agreement with a previous report (29), this mutant was responsive to AS (Fig. 5C) but stimulated *vir* gene expression about fourfold less efficiently than did the wild-type protein, especially in conditions of limiting AS concentrations (compare Fig. 5C with 3A). In addition, the activity of this protein was still potentiated by acid pH and by monosaccharides (Fig. 5C).

Tumorigenesis by strains containing mutant alleles of *virA*. Plasmids containing the wild type or mutant alleles of *virA* were assayed for the ability to complement a null mutation in *virA* or to suppress a null mutation in *virG* for tumorigenesis on *K. diagremontiana* leaves. In each of three trials, the wild-type *virA* gene, the *virA225* allele (Δ712-829), and the *virA237* allele (D766N) complemented the *virA* null mutation while the other mutants did not do so (Fig. 6). Incubation of plants at 20°C for 2 days after inoculation did not enhance tumorigenesis by any of these mutants (data not shown). None of the *virA* alleles suppressed a *virG* null mutation (data not shown).

DISCUSSION

As described above, protein sequence analysis suggests that VirA contains four putative domains, each of which could have some degree of functional autonomy. The results presented here are consistent with this model and suggest a specific role for each domain. Our data agree with those of earlier studies (3, 33) in indicating that the periplasmic

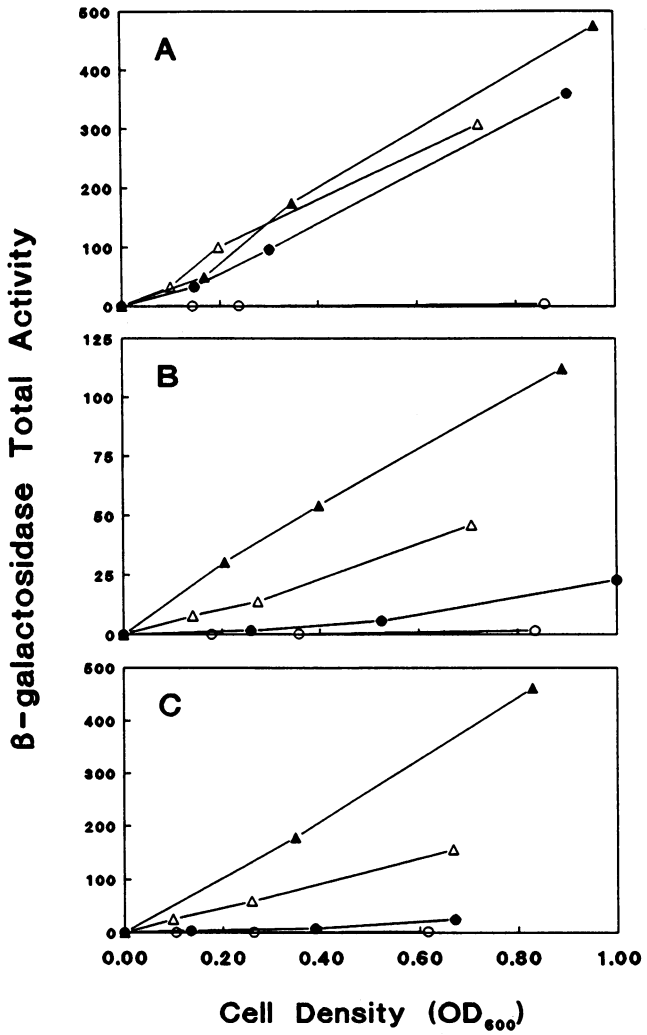

FIG. 4. Induction of the *virB* promoter by the *virA225* allele (closed symbols) or the wild-type VirA protein (open symbols). Strains were cultured with 100 μM AS (△, ▲) or no AS (○, ●) in the following media: 10 mM glucose, pH 5.5 (A), 10 mM glucose, pH 7.5 (B), no glucose, pH 5.5 (C). All media contained AB salts (18a) and 0.5% glycerol.

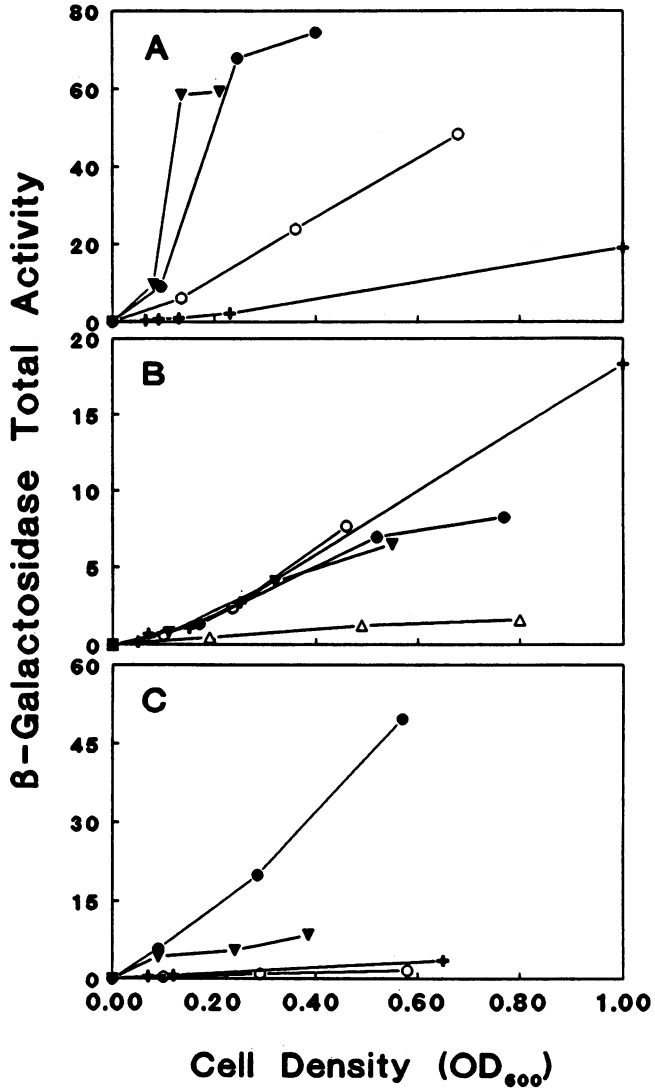

FIG. 5. Induction of the *virB* promoter by strains containing the *virA222* allele (part A), the *virA240* allele (part B), the *virA237* allele (part C), or a negative control lacking *virA* (△ in panel B). (A and B) Strains were cultured in media containing 100 μM AS and 10 mM glucose, pH 5.5 (●, △); no AS and 10 mM glucose, pH 5.5 (○); 100 μM AS and 10 mM glucose, pH 7.5 (+); or 100 μM AS and no glucose, pH 5.5 (▼). (C) Same as above, except that AS was added at 5 rather than 100 μM. All media contained AB salts (18a) and 0.5% glycerol.

domain interacts with ChvE to sense monosaccharides, and we also show that it does not play any indispensable role in the sensing of phenolic compounds or acidity. Our data further suggest that the linker domain senses phenolic compounds and pH. Our data also support an earlier proposal (29) that the receiver domain negatively modulates kinase activity, possibly in a fashion that can be neutralized by phosphorylation of aspartate residue 766.

VirA proteins lacking amino acids 11 to 283 are responsive to AS and to acidity but not to monosaccharides. This supports the hypothesis that the only known function of the periplasmic domain is to sense monosaccharides. For the following reasons, we believe that proteins lacking these amino acids do not protrude into the periplasmic space, although they could be peripherally membrane associated. In earlier studies, Tn*phoA* mutagenesis and hydropathy analysis predicted that only the region between the two transmembrane regions was periplasmically localized (22, 47). Furthermore, VirA contains a contiguous 60-kDa cytoplasmic fragment that is protease resistant in spheroplasts (47). While it is conceivable that the second transmembrane region does not lie precisely at the region designated TM2, removal of TM1 (the signal sequence) would probably nevertheless result in cytoplasmic localization of these mutant proteins. If so, both AS and acidic pH must interact (either directly or indirectly) with a cytoplasmic portion of VirA. While it is entirely possible that either stimulus may be perceived through some other protein, information about this stimulus must at some point be transferred to a cytoplasmic rather than a periplasmic domain of VirA. While not strictly necessary for this model, entry of AS into the cytoplasm is plausible, since similar compounds can serve as growth substrates (28). If VirA itself does indeed sense pH,

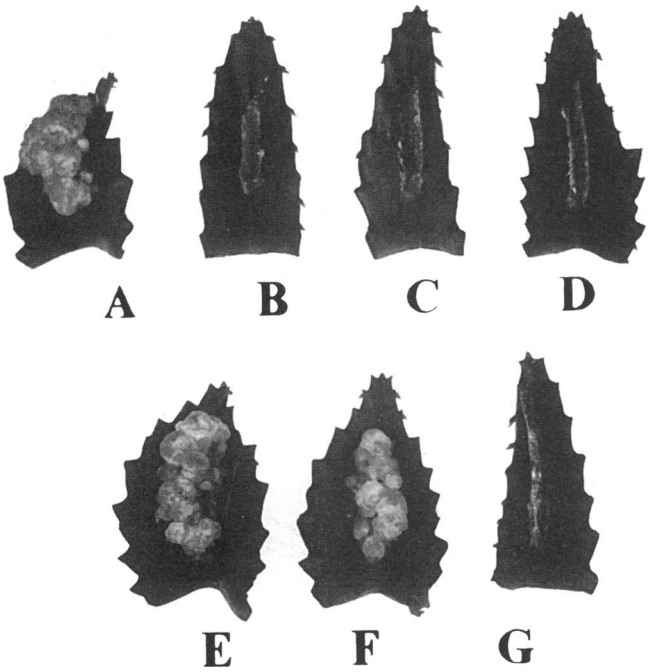

FIG. 6. Tumorigenesis by strains containing altered-function variants of VirA. *A. tumefaciens* 226MX (*virA* insertion mutant) containing plasmids that express wild-type *virA* (A), no *virA* (B), *virA224* (C), *virA222* (D), *virA225* (E), *virA237* (F), or *virA240* (G).

the protein appears to measure intracellular rather than extracellular pH. Stimulation of gene expression by acidic intracellular pH has been documented (34, 43). Melchers et al. (22) have provided data indicating that deleting part of the periplasmic domain resulted in stronger induction at neutral pH and concluded that this domain is required for pH sensing. However, in light of the present findings, it appears more probable that the allele constructed by Melchers had an elevated constitutive activity, as has been reported by others.

VirA proteins lacking the carboxyl-terminal receiver domain were responsive to all three stimuli and also directed far higher levels of transcription in the absence of AS than did the wild-type protein. This suggests that the receiver domain might in some way negatively modulate the ability of VirA to phosphorylate VirG. Since this domain is homologous to the receiver domain of VirG, one possibility is that the two receiver domains compete for access to the kinase active site. A region of approximately 20 amino acids (rich in proline and hydrophilic residues) separates the kinase domain from the receiver domain, possibly providing a flexible hinge. It is tempting to speculate that the putative negative role of this domain does not act constitutively but rather is in some way environmentally modulated. For example, VirA or another kinase might phosphorylate aspartate 766, thereby altering the inhibitory effects of this domain. Supporting this hypothesis, a D766N mutation decreases the protein's activity in the presence of AS, as was reported previously (29). It was previously reported that small truncations of the extreme carboxyl terminus strongly attenuate the activity of the protein, a phenotype opposite to that reported here (21). Such small truncations appear to strengthen the inhibitory role of this domain. VirA has certain structural similarities to at least one eukaryotic transmembrane tyrosine kinase, the epidermal growth factor receptor (42). This human proto-oncoprotein has a carboxyl-terminal domain whose removal potentiates oncogenic activity (14). This domain contains three mutually transphosphorylated tyrosine residues (19).

Since the periplasmic and receiver domains of VirA are dispensable for sensing of phenolic compounds, the binding site must lie elsewhere. There is no direct evidence that VirA contains this binding site at all. If it did, this site would have to lie either in the kinase domain itself or, more likely, in the linker domain. Consistent with this, *virA240*, which contains only the kinase domain, is nonresponsive to AS. Similarly, an allele of *virA* lacking part of the linker domain (amino acids 324 to 413) is also nonresponsive to AS (33). If AS binds to a separate protein, then that protein might contact VirA. Again, any such protein-protein contacts would have to occur in either the linker or the kinase domain.

Tumorigenesis by strains containing altered-function VirA proteins. The mutant alleles of *virA* described in this study were tested for the ability to suppress *virA* or *virG* null mutants for tumorigenesis on *K. diagremontiana* leaves. Strains containing wild-type *virA*, *virA225* (Δ712-829), or *virA237* (D766N) were proficient in tumorigenesis, while strains containing any other allele of *virA* were tumorigenesis deficient. This was somewhat surprising, since two VirA proteins lacking the periplasmic domain were responsive to 100 μM AS. However, both of these proteins were poorly responsive to 5 μM AS, and therefore one explanation for this result is that wound sites of *K. diagremontiana* do not contain sufficiently high concentrations of phenolic inducers to induce these mutants. This, in turn, suggests that monosaccharides and acidity play an even more important role in induction than previously thought.

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