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Genetics

Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein

(crown gall/signal transduction/ChvE/VirA/galactose-binding proteins)

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ABSTRACT **Phenolic plant metabolites such as acetosyringone induce transcription of the virulence (*vir*) genes of *Agrobacterium tumefaciens* through the transmembrane VirA protein. We report here that certain sugars induce the *vir* genes synergistically with phenolic inducers by way of a distinct regulatory pathway that includes VirA and a chromosomally encoded virulence protein, ChvE. Sequence comparison showed that ChvE is a periplasmic galactose-binding protein corresponding to the GBP1 protein isolated from *Agrobacterium radiobacter*. Like homologous sugar-binding proteins in *Escherichia coli*, ChvE was required for chemotaxis toward galactose and several other sugars. These sugars strongly induced *vir* gene expression in wild-type cells when acetosyringone was absent or present in low concentrations. Mutations in *chvE* abolished *vir* gene induction by sugars and resulted in a limited host range for infection but did not affect *vir* gene induction by acetosyringone. A mutant lacking the periplasmic domain of VirA exhibited the same regulatory phenotype and limited host range as *chvE* mutants. These data show that the *vir* genes are regulated by two separate classes of plant-derived inducers by way of distinct regulatory pathways that can be separated by mutation. Induction by sugars is essential for infection of some but not all plant hosts.**

Ti plasmids in *Agrobacterium tumefaciens* strains carry >20 virulence (*vir*) genes that code for most of the proteins involved in crown gall infection of wound sites on dicotyledonous plants. The biology of crown gall tumorigenesis, which involves transfer and integration of a piece of bacterial DNA into the host plant genome, has been reviewed recently (1, 2). The *vir* genes are induced by phenolic plant metabolites such as acetosyringone. Two Ti plasmid gene products, the transmembrane VirA protein and the cytoplasmic VirG protein, carry out this induction as a typical bacterial two-component sensory transduction system (3, 4). VirA is the putative environmental sensor for acetosyringone, although the site for this recognition has not been identified. The N-terminal periplasmic domain of VirA does not contain this site, since it is not required for *vir* gene induction by acetosyringone (5). The C-terminal cytoplasmic domain of VirA has autophosphorylating activity and also can phosphorylate VirG (6, 7). VirG is a DNA-binding protein that recognizes *vir* gene regulatory sequences (8, 9).

A. tumefaciens chromosomal genes participate in some of the early steps in crown gall tumorigenesis (1, 2). Several of these genes are involved in attachment of the bacteria to plant cells, and at least three affect *vir* gene regulation. Mutations in the chromosomal *ros* locus result in a pleiotropic phenotype that includes constitutive expression of two of the *vir* operons, *virC* and *virD*, but do not affect virulence (10). The *chvD* locus is required for virulence and for induction of *virG* in response to environmental stress (11). The *chvE* locus is required for virulence on four of seven plants that were examined and for wild-type levels of *vir* gene induction (12).

Sequence analysis of *chvE* showed that it codes for a protein of at least 31.5 kDa that is homologous to the periplasmic ribose-binding protein and galactose/glucose-binding protein (GBP) of *Escherichia coli* (12). Upon binding their respective sugars, these proteins in *E. coli* interact with separate sites in the periplasmic domain of the methyl-accepting transmembrane signal protein Trg, resulting in chemotaxis toward the sugars. The sugar-bound proteins also interact with separate membrane-bound uptake complexes, resulting in transport of the sugars into the cell (reviewed in refs. 13 and 14).

In this paper, we show that ChvE is a GBP that mediates chemotaxis, uptake, and *vir* gene induction in response to galactose, glucose, and several other sugars. We show that *vir* gene induction by sugars occurs through ChvE and the periplasmic domain of VirA, neither of which is required for *vir* gene induction by acetosyringone.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Wild-type *A. tumefaciens* strain A723 (C58 chromosome, pTiB6806) and its derivatives A1059 (*chvE*::Tn*5*), A1068 (*chvE*::Tn*5*), and A1030 (*virA*::Tn*5*) have been described (12, 15). Wild-type strain A348 (C58 chromosome, pTiA6NC) (16) and its derivatives A348-MX1 (*chvE*::Tn*5*) (12) and A348-MX226 (*virA*::Tn*3*-HoHo1) (17) were substituted for A723 and its derivatives when indicated. Plasmids used in this work are listed in Table 1. Plasmids were introduced into *A. tumefaciens* strains by triparental mating (20).

Media and Chemicals. *A. tumefaciens* strains were maintained on MG/L medium (12), supplemented when indicated with kanamycin (100 μg/ml), carbenicillin (100 μg/ml), and tetracycline (6 μg/ml). Induction broth (11) was modified by substituting 0.5% (54 mM) glycerol for 2% glucose and buffering to pH 5.5 with 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes). The extra buffering capacity assured uniform pH in media supplemented with various inducing sugars and other carbon sources at 10 mM. All components of induction media, including added sugars and other compounds, were sterilized by filtration. All chemicals were obtained from Sigma, except acetosyringone (Aldrich), Mes (Research Organics), D-(+)-glucose (EM Science), and sucrose (Enzyme Grade, United States Biochemical).

Chemotaxis Assays. Sterile 1-cm filter paper disks soaked in filter-sterilized 15% solutions of the indicated carbon sources were placed on the surface of swarm agar plates (induction broth lacking a carbon source, solidified with 0.3% agar).

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Abbreviation: GBP, galactose/glucose-binding protein.

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Table 1. Plasmids

Plasmid	Relevant features	Ref.
pSM243cd	*virB*::*lacZ*, Cb^r, Km^r, IncP replicon	3
pSM358cd	*virE*::*lacZ*, Cb^r, Km^r, IncP replicon	3
pSW169	pTZ18R (Cb^r, United States Biochemical) with *virA*	18
pGP159	*virA*, *virG*, *virB*::*lacZ*, Cb^r, Tc^r, IncP + pUC replicons	19
pIB400	*virA*Δ63/240, Cb^r, pUC replicon	
PIB410	*virB*::*lacZ*, Tc^r, IncP replicon	
pIB415	*virA*Δ63/240, *virB*::*lacZ*, Cb^r, Tc^r, IncP + pUC replicon	
pIB422	*virA*, *virB*::*lacZ*, Cb^r, Tc^r, IncP + pUC replicon	

Cb, carbenicillin; Km, kanamycin; Tc, tetracycline; ^{r}, resistance.

Bacteria grown overnight on MG/L medium supplemented with the appropriate antibiotics were pelleted and resuspended to 1/40 original volume in induction broth lacking a carbon source. Two microliters of each bacterial suspension was placed in the center of each plate, 4 cm from the filter paper disk, and plates were incubated at room temperature for 2 days. Positive chemotactic responses were clearly evident as sharply defined arcs of bacteria pointing toward the carbon source.

***vir* Gene Induction.** *A. tumefaciens* cells containing pSM-243cd (*virB*::*lacZ*) (3), pSM358cd (*virE*::*lacZ*) (3), or one of the constructs described below were grown overnight on MG/L medium supplemented with the appropriate antibiotics, pelleted, and resuspended to the original volume in induction broth. Fifty microliters of this suspension was inoculated into screw-cap tubes containing 3 ml of induction broth alone or supplemented with the indicated concentrations of acetosyringone. Sugars and other compounds were added to 10 mM as indicated. Tubes were shaken for 24 hr at 28°C, and β-galactosidase activity was measured as described (21).

Plasmid Construction. A region of *virA* encoding ≈81% of the periplasmic domain was deleted with the construction of plasmid pIB400, as follows. Plasmid pSW169 (18) was digested with *Pst* I to yield four fragments. A 4.4-kilobase (kb) fragment carrying pTZ18R, the *virA* promoter region, and the first 61 codons of *virA* and a 2.1-kb fragment carrying codons 241–829 of *virA* and downstream sequences were purified by agarose gel electrophoresis. They were ligated together to result in plasmid pIB400, which encodes a VirA protein with a translational fusion of Leu-62 to Gln-241, and the deletion of the intervening 178 amino acids. This construct was designated *virA*Δ63/240.

Plasmid pIB415 carrying *virA*Δ63/240 and a *virB*::*lacZ* fusion was constructed as follows. The regions of plasmid pGP159 (19) encoding *virA*, *virG*, and the pUC origin of replication were removed by digestion with *Kpn* I and religation in dilute solution. The resulting plasmid, pIB410, carries *virB*::*lacZ*. pIB400 and pIB410 were cut at their unique *Kpn* I sites and ligated together to yield plasmid pIB415. Plasmid pIB422 carrying the wild-type *virA* and a *virB*::*lacZ* fusion was constructed by cutting plasmids pIB410 and pSW169 at their unique *Eco*RI sites and ligating them together.

RESULTS

ChvE is Homologous to a Periplasmic GBP Isolated from *Agrobacterium radiobacter*. Our initial sequence analysis (12) demonstrated that *chvE* codes for a protein of over 31.5 kDa which is homologous to certain periplasmic sugar binding proteins of *E. coli*. Recently, an N-terminal amino acid

Table 2. Effects of sugars on chemotaxis and induction of *virB* by 2.5 μM acetosyringone

Attractant or addition	Chemotaxis*			β-Galactosidase,† units		
	A723	1059	A1068	A723	A1059	A1068
Glycerol	+	+	+	14	10	5
D-(+)-Galactose	+	D	D	1857	7	6
D-(+)-Glucose	+	D	D	2051	10	11
L-(+)-Arabinose	+	D	D	2079	10	13
D-(+)-Fucose	+	D	D	1519	10	13
D-(+)-Xylose	+	D	D	2247	9	11
Cellobiose	+	+	+	1081	6	8
2-Deoxy-D-glucose	–	–	–	2741	10	17
6-Deoxy-D-glucose	–	–	–	2456	8	11
Citric acid	+	+	+	7	3	9
L-Glutamic acid	+	+	+	9	7	10
α-Lactose	+	+	+	23	8	5
Maltose	+	+	+	10	8	14
α-L-Rhamnose	+	+	+	6	10	6
D-(–)-Ribose	+	+	+	76	4	7
Sorbitol	+	+	+	11	4	3
Sucrose	+	+	+	12	6	13
D-(–)-Arabinose	–	–	–	42	3	4
Lactobionic acid	–	–	–	2	2	2

*Chemotaxis was tested in triplicate on swarm agar plates as described in the text. D, delayed by 2 days or more.

†Induction of *virB*::*lacZ* in the indicated strains containing pSM243cd was determined as described in the text. All cultures contained 54 mM glycerol, 2.5 μM acetosyringone, and the indicated additions to 10 mM. Parallel cultures lacking acetosyringone had <1.5 unit of β-galactosidase activity in all cases. Similar results were obtained in duplicate experiments with these strains and with strains containing pSM358cd (*virE*::*lacZ*).

sequence was obtained from an internal proteolytic fragment of a 36.5-kDa periplasmic GBP (GBP1) isolated from *A. radiobacter* (22). Twenty-one of the 22 amino acids in the partial GBP1 sequence (AGYTTDLQYADDDIPNQLS-IEN) matched amino acids 56–78 (AGYKTDLQYADDDIP-NQLSLIEN) of ChvE (12). Therefore, ChvE of *A. tumefaciens* corresponds to GBP1 of *A. rabiobacter*.

ChvE is Involved in Chemotaxis. Since the homologs of ChvE in *E. coli* are sugar receptors involved in chemotaxis, we used a swarm agar assay to determine whether ChvE functions in chemotaxis toward specific sugars in *A. tumefaciens*. Cells of wild-type *A. tumefaciens* strain A723 were chemotactic toward a variety of sugars and other compounds in this assay (Table 2). *ChvE* mutants A1059 and A1068 resembled the wild-type cells except that chemotaxis toward D-galactose, D-glucose, L-arabinose, D-fucose, and D-xylose was delayed by several days. This phenotype is expected if ChvE functions as a specific periplasmic receptor protein for chemotaxis, as do its homologs in *E. coli*. Growth of the mutant bacteria on solid medium containing D-glucose, D-galactose, or D-fucose as sole carbon source was also slower than that of wild-type cells, whereas growth on other carbon sources was indistinguishable in mutant and wild-type bacteria.

Stimulation of *vir* Gene Expression by Sugars Recognized by ChvE. In previous experiments measuring the *vir* gene induction defect of *chvE* mutants (12), bacteria were incubated in the presence of acetosyringone and glucose. Since ChvE functions as a sugar receptor for chemotaxis, we tested whether a defect in sugar recognition (as opposed to acetosyringone recognition) limits *vir* gene induction in *chvE* mutants. In the presence of glycerol, which did not appear to interact with ChvE in chemotaxis or growth experiments, a *virB*::*lacZ* fusion was induced 5- to 10-fold by a low concentration (2.5 μM) of acetosyringone in wild-type and *chvE* mutant cells. The addition of certain sugars caused an additional 60- to 200-fold induction in wild-type cells but not in *chvE* mutant cells (Table 2). Significantly, most of these inducing sugars were the same sugars that exhibited reduced chemoattractant activity for *chvE* mutants. The nonmetabolizable glucose analogs 2-deoxy-D-glucose and 6-deoxy-D-glucose, and the glucose-containing disaccharide cellobiose also stimulated *vir* gene induction. Other sugars and carbohydrates did not have this effect.

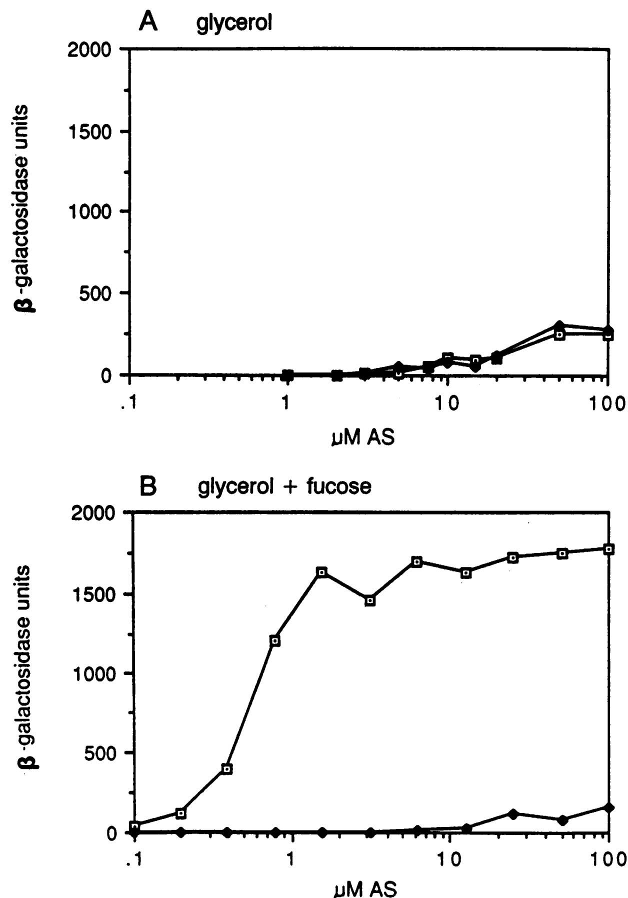

FIG. 1. Effects of acetosyringone and D-fucose on *virB* induction. Cells containing pSM243cd (*virB*::*lacZ*) were incubated with the indicated concentrations of acetosyringone (AS), and *virB* expression was determined. Cultures lacking acetosyringone had <1.5 unit of β-galactosidase activity. Open symbols, wild-type strain A723; closed symbols, *chvE* mutant A1059. (*A*) Induction broth. (*B*) Induction broth supplemented with 10 mM D-(+)-fucose.

In the absence of an inducing sugar, expression of *virB* in wild-type and mutant bacteria increased equally with increasing acetosyringone concentrations, with both strains exhibiting a 100-fold induction by 100 μm acetosyringone (Fig. 1*A*). Addition of an inducing sugar (D-fucose) resulted in additional *virB* expression in wild-type but not in *chvE* mutant cells (Fig. 1*B*). This effect was most pronounced at low acetosyringone concentrations. Therefore, ChvE is required for the regulatory response to inducing sugars but not to acetosyringone.

Requirement for ChvE and VirA for Acetosyringone-Independent Induction of *virE* by Sugars. Some of the inducing sugars stimulated *virB* expression in the absence of acetosyringone after extended (48 hr) incubation (data not shown). When induction of a *virE*::*lacZ* fusion was examined, this was observed in the standard 24-hr incubation period in wild-type strain A723 (Table 3). *VirA* mutant A1030 as well as *chvE* mutants A1059 and A1068 did not exhibit this induction. Therefore, VirA as well as ChvE are required for induction of *virE* by the inducing sugars. Maltose, which did not appear to be recognized by ChvE in chemotaxis experiments, did not induce *virE*.

Requirement for the Periplasmic Domain of VirA for *vir* Gene Induction by Sugars. The VirA protein structurally resembles the Trg protein, which interacts with the sugar/GBP complex in *E. coli* to transmit the signal for chemotaxis (14). VirA (5, 18) and Trg (23) each have an N-terminal transmembrane domain (TM1), a periplasmic domain, a second transmembrane domain (TM2), and a C-terminal cytoplasmic domain that interacts with other proteins in their respective regulatory cascades. VirA was not homologous to Trg in a global sequence comparison, but a strongly homologous 15-amino acid overlap was detected in their periplasmic domains (Fig. 2). This region of Trg is the site of the *trg*-8 mutation, which decreases the chemotactic response to sugar recognition by the galactose- and ribose-binding proteins (25). The conserved region is situated near TM2 in VirA, but it is closer to TM1 in Trg.

Table 3. Acetosyringone-independent induction of *virE* by sugars

	β-Galactosidase, units			
Addition	A723 (wt)	A1059 (*chvE*)	A1068 (*chvE*)	A1030 (*virA*)
None	193	151	133	175
L-(+)-Arabinose	9886	162	152	138
D-(+)-Fucose	2872	150	155	117
Maltose	160	167	131	162

All cultures contained 54 mM glycerol, and the indicated compounds were added to 10 mM. Induction of *virE*::*lacZ* in strains containing pSM358cd was determined as described in the text. wt, Wild-type strain. Similar results were obtained in duplicate experiments with these strains and with wild-type strain A348, *chvE* mutant A348::MX1, and *chvA* mutant A348::MX226.

We examined the effects of deleting the periplasmic domain of VirA on *vir* gene induction by sugars. A 178-codon *Pst* I fragment encoding most of the periplasmic domain (including the 15-amino acid conserved region) was removed from *virA* as described in *Materials and Methods*. This construct, designated *virA*Δ63/240, was introduced into the *virA* mutant A1030 on plasmid pIB415. Unexpectedly, induction of *virB::lacZ* by 5 μM acetosyringone was 30-fold higher in these cells than in isogenic cells carrying the wild-type *virA* gene. However, addition of L-arabinose enhanced induction only in cells carrying the wild-type VirA (Table 4). Therefore, the periplasmic domain of VirA is required for the response to L-arabinose.

Role for ChvE and the Periplasmic Domain of VirA in Host Range. We previously reported that *chvE* mutants had a reduced host range (12). If the periplasmic domain of VirA is part of the same regulatory pathway as ChvE, mutants lacking this domain should have the same host range defects as *chvE* mutants. This proved to be the case. Mutants carrying *virA*Δ63/240 and mutants in *chvE* both formed tumors on *Nicotiana glauca* stems and sunflower stems but not on *Kalanchoe* leaves. Wild-type *A. tumefaciens* formed tumors on all of these plants, whereas *virA* mutant A1030 was avirulent on all of them.

DISCUSSION

The inducing sugars and the inducing phenolics are separate classes of signal molecules that regulate the *vir* genes in *A. tumefaciens*. The regulatory pathway for *vir* gene induction by sugars can be distinguished mutationally from the pathway for induction by phenolics. The unique components of the pathway for induction by sugars include the periplasmic sugar-binding protein ChvE and the periplasmic domain of the transmembrane signal protein VirA.

Comparison of the predicted amino acid sequence of ChvE (12) and the sequenced fragment of the periplasmic GBP (GBP1) from *A. radiobacter* (22) indicated that these two proteins are the same. An immunologically identical protein was also isolated from *A. tumefaciens* (22). Like the GBP of *E. coli*, ChvE (or a protein affected by polar mutations in *chvE*) was required for chemotaxis toward a specific set of sugars. These sugars stimulated *vir* gene induction in wild-type but not *chvE* mutant cells. Five of the inducing sugars (D-galactose, D-glucose, D-xylose, D-fucose, and 6-deoxy-D-glucose) bound strongly to GBP1 in competition experiments (26). Binding of 2-deoxy-D-glucose to GBP1 was slightly above background levels. L-Arabinose was not tested for binding to GBP1, but it induces the synthesis of GBP in *E. coli*, presumably by virtue of structural analogy to galactose (27). Cellobiose, a disaccharide consisting of β-1,4-linked glucose, also induced the *vir* genes in wild-type but not in *chvE* mutant cells. ChvE was not required for chemotaxis toward cellobiose, which suggests that another cellobiose receptor exists that is involved in chemotaxis but not in *vir* gene induction.

The mutants grew more slowly than wild-type cells on the inducing sugars as sole carbon sources, presumably because

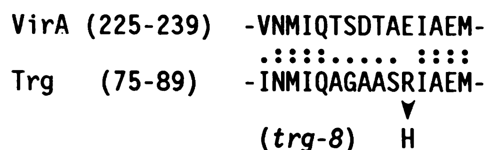

FIG. 2. Homologous regions of the periplasmic domains of VirA (24) and Trg (25). The positions of the homologous regions within each amino acid sequence and the site of the *trg*-8 mutation are indicated. :, Amino acid identity; ., conserved substitution.

Table 4. Effects of deleting the periplasmic domain on VirA on induction of *virB* by acetosyringone and L-arabinose

Acetosyringone, μM	β-Galactosidase, units			
	No addition		L-Arabinose added	
	virA⁺	*virA*Δ63/240	*virA*⁺	*virA*Δ63/240
0	2	5	5	5
0.625	2	3	402	9
1.25	3	13	450	33
5.0	13	449	243	516

All cultures contained 54 mM glycerol. L-(+)-Arabinose was added to 10 mM as indicated. Induction of *virB::lacZ* in strains A1030(pIB422) (*virA*⁺) and A1030(pIB415) (*virA*Δ63/240) was determined as described in the text. Similar results were obtained in a duplicate experiment. β-Galactosidase activities at 2.5 or 10 μM acetosyringone were similar to those observed at 5 μM.

of a defect in GBP-dependent sugar transport. Therefore, we included in our induction medium 54 mM glycerol, a carbon source that does not differentially affect chemotaxis, growth, or *vir* gene induction in mutant and wild-type cells. Mutant cells grown in induction broth with 54 mM glycerol and 10 mM D-fucose did not differ from wild-type cells in growth rate or cell yield (data not shown). Thus, the effects of mutations in *chvE* on *vir* gene induction were probably not due to defects in growth on these sugars. This is supported by the inducing activity of 2-deoxy-D-glucose and 6-deoxy-D-glucose, which do not support the growth of *A. tumefaciens*.

Previous observations (28) indicated that a carbon source is necessary for maximum levels of *vir* gene induction. Our observations demonstrate that this is a true regulatory (as opposed to nutritional) effect and that it is specific to sugars that are recognized by ChvE. This specificity has not been observed previously, probably because laboratory media for *vir* gene induction typically include high concentrations of glucose or autoclaved sucrose (which may contain significant amounts of glucose) as carbon sources and high concentrations of acetosyringone. We used such media previously to find that *chvE* mutants were defective in *vir* gene induction (12).

In *E. coli*, the sugar/GBP complex interacts with periplasmic sites on the transmembrane protein Trg to stimulate chemotaxis (14). By analogy, ligand-bound ChvE may interact with periplasmic sites on VirA to stimulate *vir* gene induction. We have made four observations that support this model. (*i*) Transposon insertions in *virA* abolished acetosyringone-independent induction of *virE* by sugars. (*ii*) A region in the periplasmic domain of VirA was strongly homologous to a periplasmic region of Trg involved in signal transduction. (*iii*) Deletion of the periplasmic domain of VirA abolished *vir* gene induction by L-arabinose but not by acetosyringone. (*iv*) Strains carrying such a deletion had the same host range for virulence as *chvE* mutants.

Melchers *et al.* (5) previously observed that the periplasmic domain of VirA was not required for *vir* gene induction by 100 μM acetosyringone. We observed that in the absence of an inducing sugar, 5 μM acetosyringone induced *virB* much more strongly in cells lacking the periplasmic domain of VirA than in cells containing a wild-type VirA. We cannot presently explain this enhanced activity. The deletion may cause conformational changes in VirA that mimic transient changes caused by positive signal recognition. L-Arabinose strongly stimulated *virB* induction in wild-type cells but did not affect *virB* induction in the mutant.

The data support a model for *vir* gene induction in which the sugar/ChvE complex interacts with periplasmic sites on VirA to stimulate induction, whereas acetosyringone appears to interact with VirA in the membrane or cytoplasm (5). The signal pathway for chemotaxis by *A. tumefaciens* toward the

inducing sugars is not known, but by analogy to *E. coli* it probably includes an interaction between ChvE and a Trg-like protein. It does not include VirA, since *virA* mutants are not defective in chemotaxis toward galactose or glucose (data not shown). ChvE probably also participates in uptake of these sugars by interacting with a membrane-bound uptake complex, as does the GBP of *E. coli* (13).

Induction of the *vir* genes by sugars appears to work synergistically with induction by acetosyringone. Since *chvE* mutants are avirulent on some but not all plants, *vir* gene induction by sugars may influence host range. The plant tissues that permit tumorigenesis by *chvE* mutants may contain enough phenolic inducers to overcome the lack of induction by sugars. Alternatively, these tissues may require lower levels of *vir* gene expression for infection. The inducing sugars may be present in susceptible wounded plant tissue as degradation products of plant cell wall polysaccharides such as cellulose, galactans, arabinogalactans, and xyloglucans (29). It is interesting that cellobiose, a breakdown product of cellulose, is active in *vir* gene induction. Determination of the inducing activity of higher-molecular weight β-1,4-linked glucans awaits further investigation. Such oligosaccharides may be more significant than monosaccharides to induction of the *vir* genes in plant wounds. It would also be interesting to determine whether periplasmic binding proteins function in recognition of phenolic inducers, opines (30), or glycosidic *vir* gene inducers such as coniferin (31).

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