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Sugar-Mediated Induction of *Agrobacterium tumefaciens* Virulence Genes: Structural Specificity and Activities of Monosaccharides

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The virulence genes of *Agrobacterium tumefaciens* are induced by specific plant phenolic metabolites and sugars (G. A. Cangelosi, R. G. Ankenbauer, and E. W. Nester, Proc. Natl. Acad. Sci. USA, in press). In this report, monosaccharides, derivatives, and analogs which induce the *vir* regulon have been identified and the structural requirements for monosaccharide-mediated induction have been determined. Pyranose sugars with equatorial hydroxyls at C-1, C-2, and C-3 displayed strong *vir* gene-inducing activity; the C-4 hydroxyl could be epimeric and a wide variety of substitutions at C-5 were permissible. The acidic monosaccharide derivatives D-galacturonic acid and D-glucuronic acid were the strongest inducers among the monosaccharides tested. Eight of the 11 inducing compounds are known plant metabolites, and 7 are monomers of major plant cell wall polysaccharides. A role for monosaccharides and plant phenolic compounds as wound-specific plant metabolites which signal the ChvE/VirA/VirG regulatory system is proposed.

The *vir* regulon of *Agrobacterium tumefaciens* is transcriptionally regulated by the *virA* and *virG* gene products (25, 26). The VirA-VirG protein pair (15, 32) are members of a large family of bacterial two-component regulatory systems (27). The VirA and VirG proteins have been identified respectively as a histidine protein kinase (11, 13) and response regulator (12, 14, 18) by biochemical and genetic methods.

Previous work has demonstrated that the VirA and VirG proteins activate transcription of the *vir* regulon in the presence of wound-induced plant phenolic metabolites such as acetosyringone (24). Recently, another level of signal transduction involved in activation of the *vir* regulon has been discovered. A number of sugars act synergistically with acetosyringone to afford high levels of *vir* gene expression. This sugar-mediated induction requires the ChvE protein (10), a periplasmic glucose- and galactose-binding protein. Upon binding its sugar ligand, the ChvE-sugar complex appears to interact with the periplasmic domain of the VirA protein to signal for *vir* gene induction (G. A. Cangelosi, R. G. Ankenbauer, and E. W. Nester, Proc. Natl. Acad. Sci. USA, in press). A glucose-galactose-binding protein from *Agrobacterium radiobacter*, GBP1, corresponds to the ChvE protein of *A. tumefaciens* (Cangelosi et al., in press), and its sugar-binding ability has been confirmed by a number of biochemical and physical methods (3, 4). This new class of *vir* gene-inducing compounds has been analyzed, and the monosaccharide structural requirements and specificity for *vir* gene induction were determined.

MATERIALS AND METHODS

Bacteria and plasmids. *A. tumefaciens* A723 (C58 chromosome, pTiB6806) (6) was used throughout this study. The IncP plasmid pSM243cd (32) carries a *virB*::*lacZ* fusion and specifies resistance to carbenicillin and kanamycin.

Media and chemicals. *A. tumefaciens* was maintained on MG/L medium and supplemented with carbenicillin (100 μg/ml) and kanamycin (50 μg/ml). Induction broth consisted of 0.5% glycerol, 50 mM MES [2-(*N*-morpholino) ethanesulfonic acid, pH 5.5], 1× AB salts (33), and 0.5 mM NaH_2PO_4. Acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone; Aldrich Chemical Co., Milwaukee, Wis.) was dissolved in dimethyl sulfoxide and added to induction broth at a concentration of 2.5 μM. The monosaccharides, derivatives, and analogs used in this study were obtained from Sigma Chemical Co. (St. Louis, Mo.) with the following exceptions: 2-deoxy-D-glucose, 1,4-anhydro-L-threitol, and *trans*-1,2-cyclohexanediol were from Aldrich Chemical Co.; D-(+)-glucose was from EM Science, Cherry Hill, N.J.; isopropylthio-β-D-galactoside was from Research Organics Inc., Cleveland, Ohio; and sorbitol was from Eastman-Kodak, Rochester, N.Y. The monosaccharides, derivatives, and analogs were dissolved in water and filter sterilized except for 1,4-anhydro-L-threitol, *trans*-1,2-cyclohexanediol, and salicin, which were dissolved in dimethyl sulfoxide. All solutions were stored at 4°C. The monosaccharides, derivatives, and analogs were added to induction broth at the concentrations indicated in the text.

***vir* gene induction assays.** Twenty-four-hour cultures of A723(pSM243cd) in MG/L medium with carbenicillin and kanamycin were centrifuged, and the bacterial pellets were suspended in an equal volume of water. The cells were diluted 1:50 into 3 ml of induction broth (with indicated supplements), and the cultures were incubated with shaking at 28°C for 24 h. *vir* gene induction was determined as a function of β-galactosidase activity in the cultures. β-Galactosidase activity was determined as described previously (Cangelosi et al., in press).

RESULTS

Identification of monosaccharides, derivatives, and analogs which induce *vir* genes. Previous studies in this laboratory demonstrated that arabinose, fucose, galactose, glucose, and xylose strongly induced *vir* genes in concerted action with acetosyringone. In order to determine the structural requirements for monosaccharide-mediated induction of the *vir* genes, 28 monosaccharides, derivatives, and analogs were tested for their ability to induce a *virB*::*lacZ* fusion. Each of the compounds was added to induction broth at a concentration of 5 or 10 mM, and *vir* gene induction was measured as a function of β-galactosidase activity. A natural division

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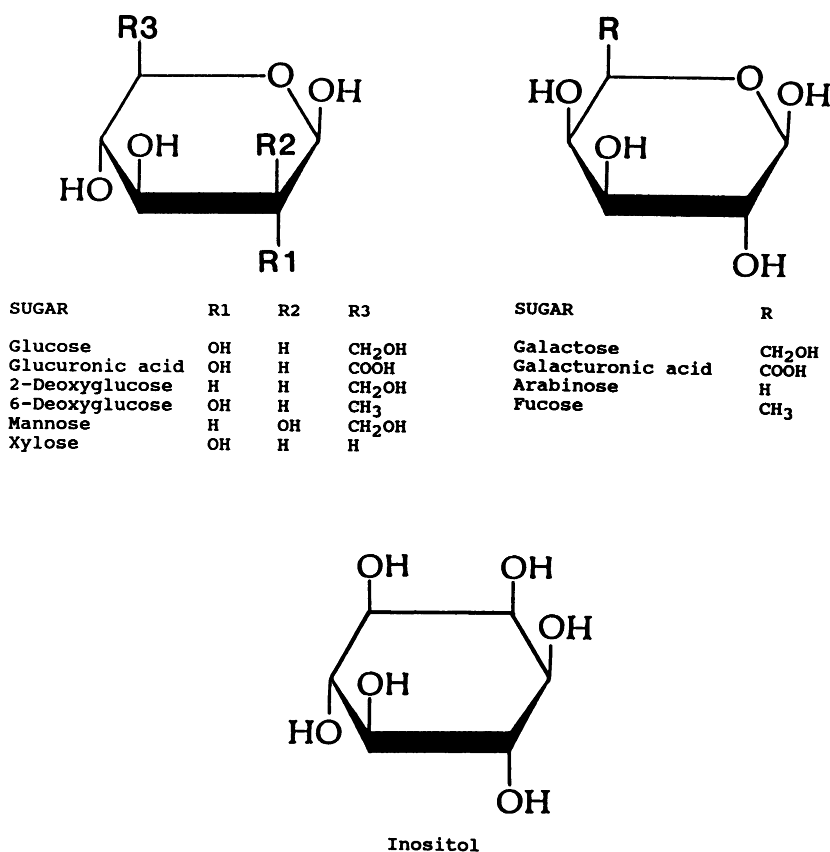

FIG. 1. Structures of inducing monosaccharides, derivatives, and analogs in Haworth projection. These compounds elicited high levels of *vir* gene induction (>600 U of β-galactosidase activity from a *virB*::*lacZ* fusion) in the presence of 2.5 μM acetosyringone. *A. tumefaciens* A723(pSM243cd) was used in the induction assays.

among the *vir* gene-inducing activities of the 28 compounds was observed. Eleven of the compounds [L-(+)-arabinose, 2-deoxy-D-glucose, 6-deoxy-D-glucose, D-(+)-fucose, D-(+)-galactose, D-galacturonic acid, D-(+)-glucose, D-glucuronic acid, inositol (*myo*-inositol), D-(+)-mannose, and D-(+)-xylose] were found to yield high *vir* gene induction (β-galactosidase activity, >600 U), while the remaining 17 compounds [D-allose, D-altrose, 1,4-anhydro-L-threitol, D-(−)-arabinose, *trans*-1,2-cyclohexanediol, β-D-(−)-fructose, L-(−)-fucose, D-(+)-glucosamine, isopropylthio-β-D-galactoside, methyl-β-D-galactopyranoside, methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside, 3-*o*-methylglucose, L-(−)-rhamnose, D-(−)-ribose, salicin, and sorbitol] had little or no *vir* gene-inducing activity (β-galactosidase activity, <80 U). In addition to the compounds identified previously (Cangelosi et al., in press), D-galacturonic acid, D-glucuronic acid, inositol, and D-(+)-mannose were also found to induce *vir* genes.

Structural requirements for monosaccharide-mediated induction. The structures of the monosaccharides, derivatives, and analogs which induced the *vir* genes are shown in Haworth projection in Fig. 1. The common structural features of these 11 compounds include a six-membered pyranose ring (with the exception of inositol), an equatorial hydroxyl at C-3, an open anomeric C-1 hydroxyl, equatorial or axial hydroxyls at C-2 and C-4, and a variety of permissible equatorial substitutions off the C-5 carbon (including hydroxyl, methyl, hydroxymethyl, and carboxyl groups). The compounds which failed to induce the *vir* genes indicate the following structural requirements for induction. (i) The anomeric C-1 hydroxyl cannot be blocked in glycosidic form (isopropylthio-β-D-galactoside, methyl-β-D-galactopyranoside, methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside, and salicin), nor can it be absent (*trans*-1,2-cyclohexanediol). (ii) The sugar must be able to exist in a six-membered pyranose ring, since five-membered furanose structures were inactive [1,4-anhydro-L-threitol, β-D-(−)-fructose, and D-(−)-ribose], as were sugar analogs which cannot close to form pyranose rings (sorbitol). (iii) The C-3 hydroxyl cannot be axial (D-allose and D-altrose), nor can it be blocked by methylation (3-*o*-methylglucose). (iv) Optical isomers of inducing sugars were inactive [D-(−)-arabinose, L-(−)-fucose, and L-(−)-rhamnose, which is 6-deoxy-L-mannose], as expected, since the C-3 hydroxyls in such compounds are axial. (v) Positively charged amino group substitutions for hydroxyls were inactive, at least at C-2 [D-(+)-glucosamine], despite the varied substitutions permissible for *vir* gene induction at this position [equatorial hydroxyl, axial hydroxyl, and no hydroxyl; D-(+)-glucose, D-(+)-mannose, and 2-deoxy-D-glucose, respectively].

Effect of concentration on monosaccharide-mediated induction. Although the inducing sugars were found to have a number of common features, it was presumed that the different ring substitutions would affect the affinity of the ChvE-ligand interaction and therefore *vir* gene induction. To test this idea, each of the 11 inducing sugars was tested for *vir* gene induction at 10 mM, 1 mM, 100 μM, and 10 μM. The levels of *vir* gene induction afforded by the different concentrations of monosaccharides are presented in Table 1. At a concentration of 10 mM, all of the compounds except inositol yielded very high levels of *vir* gene induction. Only

TABLE 1. Induction of *virB*::*lacZ* fusion at different concentrations of inducing sugars[a]

Substrate	Mean β-galactosidase activity (U) ± SD			
	10 mM	1 mM	100 μM	10 μM
L-(+)-Arabinose	2,649 ± 21	695 ± 131	24 ± 1	10 ± 1
2-Deoxy-D-glucose	1,232 ± 19	291 ± 72	9 ± 1	3 ± 1
6-Deoxy-D-glucose	ND[b]	1,605 ± 341	404 ± 145	3 ± 1
D-(+)-Fucose	2,102 ± 118	485 ± 57	24 ± 4	3 ± 1
D-(+)-Galactose	1,133 ± 73	875 ± 120	36 ± 6	3 ± 1
D-Galacturonic acid	—[c]	1,916 ± 150	2,059 ± 278	253 ± 159
D-(+)-Glucose	2,666 ± 168	1,899 ± 181	32 ± 3	3 ± 1
D-Glucuronic acid	2,532 ± 142	2,393 ± 45	1,865 ± 284	20 ± 17
Inositol	621 ± 180	26 ± 6	5 ± 1	7 ± 2
D-(+)-Mannose	2,592 ± 82	278 ± 77	5 ± 2	3 ± 1
D-(+)-Xylose	2,313 ± 208	2,254 ± 141	164 ± 32	3 ± 1
No addition	6 ± 2	ND	ND	ND

[a] A723(pSM243cd) was grown in induction broth for 24 h, and β-galactosidase activity (nanomoles of *o*-nitrophenol per minute per milligram of protein) was determined as described in Materials and Methods. Results are presented as the mean of three independent assays followed by the standard deviation. All substrates were added to induction broth which contained 0.5% glycerol and 2.5 μM acetosyringone.

[b] ND, Not determined.

[c] Inhibitory to cell growth; not determined.

at lower concentrations could the differences between the monosaccharides be detected. The monosaccharides are listed here in order of descending *vir* gene-inducing activity: D-galacturonic acid > D-glucuronic acid > 6-deoxy-D-glucose > D-(+)-xylose > D-(+)-glucose > D-(+)-galactose > L-(+)-arabinose > D-(+)-fucose > D-(+)-mannose > 2-deoxy-D-glucose > inositol. D-Galacturonic acid and D-glucuronic acid were by far the strongest inducers of the 11. The strongest inducer, D-galacturonic acid, was inhibitory to bacterial growth at a concentration of 10 mM.

Determination of the relative *vir* gene-inducing activities of the sugars allowed refinement of the optimal structural requirements for *vir* gene induction by monosaccharides. Inositol, the weakest of the inducing compounds, does not have a pyranose ring but does have a cyclohexane ring; furthermore, it has an axial hydroxyl at C-6, a feature that the other compounds cannot possess because an oxygen atom occupies that position. It is possible that the C-6 axial hydroxyl of inositol sterically hinders its interaction with ChvE or that a ring oxygen is optimal for ligand binding by ChvE. Equatorial hydroxyls at C-2 are strongly favored, as demonstrated by the low inducing activities of D-(+)-mannose and 2-deoxy-D-glucose. The eight sugars which exhibited the highest *vir* gene-inducing activity differed only in the hydroxyl configuration about C-4 and the substitution at C-5. The optimal structure for an inducing sugar is shown in the three-dimensional "chair" configuration in Fig. 2. This structure demonstrates that all of the hydroxyls were in the sterically favored equatorial positions with the exception of the C-4 hydroxyl, which could be epimeric.

DISCUSSION

The relative strengths of the different monosaccharides in *vir* gene induction correspond with the biochemical data on the affinity of sugar binding by the GBP1 protein of *Agrobacterium radiobacter* (3, 4). In in vitro competition assays, Cornish et al. (3) determined that GBP1 bound the following monosaccharides (in decreasing order of affinity): D-galactose > D-glucose > D-fucose > 6-deoxy-D-glucose > D-xylose > 2-deoxy-D-glucose. The methylated derivatives methyl-α-D-glucopyranoside and 3-*o*-methylglucose do not bind to GBP1. This order was similar to the results obtained for *vir* gene induction except that 6-deoxy-D-glucose and D-(+)-xylose were found to be more active in the in vivo assays. Methylated monosaccharides incapable of binding to GBP1 were also unable to elicit *vir* gene induction. It would appear that the structural specificity required by GBP1 for monosaccharide binding correlates with the ability of these monosaccharides to induce the *vir* genes.

The ChvE protein is strongly homologous to the galactose-glucose-binding protein (GBP) of *Escherichia coli* (10). Although both ChvE and the *E. coli* GBP bind galactose and glucose with high affinity, there appear to be substantial differences in the spectrum of monosaccharides bound by the proteins. The *E. coli* GBP is unable to bind L-(+)-arabinose, D-(+)-mannose, and D-(+)-xylose (2, 9), monosaccharides which interact strongly with ChvE. Additionally, ChvE does not recognize methyl-β-D-galactopyranoside, a compound known to be bound by the *E. coli* GBP (9, 31).

The differences in monosaccharide specificity between ChvE and the *E. coli* GBP should not be surprising in view of the evolutionary distance between *A. tumefaciens* and *E. coli* in the purple subdivision of the eubacteria (34). Furthermore, unlike *E. coli*, *A. tumefaciens*, as a member of the *Rhizobiaceae*, has evolved in close relationship with plants. The wider range of monosaccharides bound by ChvE may in

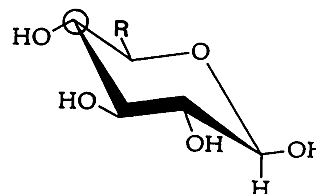

FIG. 2. Optimal monosaccharide structure for *vir* gene induction in three-dimensional chair configuration. This structure was determined by structural analysis of inducing monosaccharides, derivatives, and analogs and their respective *vir* gene-inducing abilities. The R group can be -COOH, $-CH_3$, $-CH_2OH$, or -H. The C-4 carbon is circled to show that it can be epimeric, with the hydroxyl being in either the equatorial (as shown) or axial position. The C-1 carbon is shown as the β anomer with the hydroxyl in the equatorial position, since the β configuration is the predominant form of these monosaccharides in solution; however, the absolute structural requirement at the anomeric carbon in *vir* gene induction was not determined because of rapid equilibration between α and β anomers.

fact reflect the natural history of the *Agrobacterium*-plant relationship. Eight of the 11 inducing compounds are known plant metabolites, and 7 are monomers of plant cell wall polysaccharides, including cellulose, xylans, mannans, and pectin (20). Of the nine monosaccharides and derivatives known to compose the basic primary walls of plants [L-(+)-arabinose, L-(−)-fucose, D-(+)-galactose, D-galacturonic acid, D-(+)-glucose, D-glucuronic acid, D-(+)-mannose, L-(−)-rhamnose, and D-(+)-xylose] (5, 30), only L-(−)-fucose and L-(−)-rhamnose did not yield *vir* gene induction. The lack of response to L-(−)-fucose and L-(−)-rhamnose was expected, since both are optical isomers of inducing sugars. Inositol, although not a monomer of cell wall polysaccharides, is also found in plants and serves as a biosynthetic precursor to a number of the inducing monosaccharides (5).

How can the phenomenon of monosaccharide-mediated induction of *A. tumefaciens vir* genes be placed into the context of natural tumorigenic interactions between plant and bacterium? We currently envision the following scheme. Plant cell walls contain a variety of glycosidases (7, 30), which are presumably involved in plant defense mechanisms and expansion of the plant cell wall as necessary for cell growth (23). Upon wounding of the plant and disruption of the cell wall structures, free sugars and oligosaccharides are generated by both mechanical means and the enzymatic activity of these cell wall glycosidases. These plant cell wall fragments elicit the production of phytoalexins and other secondary plant metabolites (16). Acetosyringone and related phenolic inducers of the *vir* genes are likely produced in such a response. In fact, it has been shown that treatment of *Nicotiana tabacum* suspension cultures with the cell wall-degrading enzyme cellulase elicits high levels of acetosyringone production (29). The mechanism of the production of *vir* regulon-inducing plant phenolics is unknown but may involve de novo synthesis or hydrolysis of phenolic glycosides by glycosidases of either plant (7) or microbial (17) origin.

Upon localization of *A. tumefaciens* to the wound site, possibly mediated by ChvE-mediated chemotaxis towards these cell wall monomers (8; Cangelosi et al., in press), the bacteria can attach to the plant cells and the *vir* regulon is then induced by plant phenolic compounds and sugars. Production of the phytohormone auxin by *A. tumefaciens* (22) may also be involved in this process, since auxin promotes hydrolysis of cell walls (28) to mono- and oligosaccharides and the acidification of plant cell walls (19). It is significant that the presence of inducing sugars and a low pH are both required for high levels of *vir* gene induction (1, 21, 33; Cangelosi et al., in press). Cell wall-hydrolyzing enzymes of microbial origin may also be involved in the production of monosaccharides; however, the production of such enzymes by biovar I strains of *A. tumefaciens* has not been established (P. Allenza, O. Carmi, L. Wu, and E. W. Nester, unpublished observations). However, enzymes produced by epiphytic microorganisms other than *Agrobacterium* species might play an important role in this regard.

Thus, the *A. tumefaciens* ChvE/VirA/VirG sensory transduction system is capable of detecting two separate classes of wound-induced plant metabolites (i.e., plant phenolics and inducing sugars), and the bacterium may play direct or indirect roles in the generation of these metabolites.

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