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Control of expression of *Agrobacterium vir* genes by synergistic actions of phenolic signal molecules and monosaccharides

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ABSTRACT **Most virulence (*vir*) genes of *Agrobacterium tumefaciens* that are required for the formation of crown gall tumors are expressed in response to such plant signal molecules as acetosyringone and lignin precursors. The phenolic signals are transduced through a receptor VirA protein in the inner membrane of the bacterial cell. The expression of these genes triggers the transfer of a specific DNA segment, called transferred DNA (T-DNA), from the Ti plasmid to plant cells, and its integration into their nuclear DNA. We show here that a group of aldoses (L-arabinose, D-xylose, D-lyxose, D-glucose, D-mannose, D-idose, D-galactose, and D-talose) can markedly enhance acetosyringone-dependent expression of *vir* genes when the concentration of acetosyringone is limited (10 μM) but does not enhance the expression of noninducible genes. Likewise, a 2-deoxy-D-glucose, a nonmetabolized sugar, is also effective. When a deletion was introduced into the *virA* gene in the region encoding the periplasmic portion of the VirA protein, enhancement by glucose disappeared, but *vir* expression was induced by acetosyringone in this mutant. These results suggest that these sugars directly enhance a signaling process initiated by phenolic inducers that results in an increase in expression of the *vir* genes.**

Agrobacterium tumefaciens harboring the Ti plasmid generate crown gall tumors on a wide variety of dicotyledonous plants (1, 2). Upon infection of plants, transferred DNA (T-DNA), a stretch of the Ti plasmid is transferred by unknown mechanisms to plant cells and integrated into plant nuclear DNA (1, 2). T-DNA transfer and processing require products of *vir* genes (*vir*A, -B, -G, -C, -D, and -E), which are located outside of the T-DNA (1). The expression of *vir*B, -C, -D, and -E is positively regulated at the transcriptional level by plant signal molecules (3, 4). The regulatory genes *vir*A and *vir*G are expressed constitutively, the expression of *vir*G increasing in the presence of the plant inducers (5). The expression of *vir*A has been reported by some investigators (6, 7), but not by others (5), to be induced by signal molecules. The plant signal is thought to be transduced into agrobacterial cells through functions of the VirA and VirG proteins, which show similarities to the two-component regulatory system that is conserved in a variety of prokaryotes (8). VirA protein is thought to serve as a sensor or receptor to detect the signal of the inducers (9, 10). It has been proposed that this protein spans the cytoplasmic membrane of *Agrobacterium*, contains approximately 270 amino acids that are flanked by two hydrophobic transmembrane domains, and protrudes into the periplasmic space (11, 12). The signal detected by the VirA protein must be transduced to the VirG protein to activate the latter protein. Activated VirG is thought to act as a positive regulator for the transcription of other *vir* genes. It has been reported that VirA has autophosphorylation activity and presumably activates VirG by phosphorylating it (13, 14) and that VirG is a DNA binding protein, consistent with its assigned role as an inducer of other *vir* genes (15–17).

vir gene induction by signal molecules is greatly affected by incubation conditions of *Agrobacterium*. High levels of induction are obtained at a pH of <6.0, but practically no induction is observed at higher pH values, even in the presence of a high concentration of inducers (5). The induction process is also temperature sensitive; maximum induction was obtained around 20°C (18). Inorganic phosphate starvation of *Agrobacterium* was shown to stimulate *vir* induction (6). Deleting most of the periplasmic domain of the VirA protein did not alter the extent of *vir* gene expression by plant inducers but made the induction process less pH sensitive and thermosensitive (11). Therefore, the periplasmic domain (and/or its adjacent regions) is thought to be responsible for the pH dependence and temperature sensitivity of *vir* gene induction (11).

Plant signal molecules in tobacco have been identified as phenolics, acetosyringone (AS), and hydroxyl-AS, which are exuded from wounded or actively growing cells (19). Components of lignin or its precursors also act as signal molecules (19, 20). Monocotyledonous plants such as wheat and oats also have been shown to contain *vir* gene-inducing factors (21). Inducing-factor activity was detected only in extracts from homogenates of these plants (21) but not in exudates of their seedlings (22). In the course of our efforts to purify the monocotyledonous inducing factors and to determine their molecular structures, we noticed that some substances extracted from homogenates of wheat and tobacco seedlings markedly enhanced *vir* induction when added to partially purified monocotyledonous factors or low concentrations of AS. Circumstantial evidence indicated that the enhancing substances might be monosaccharides or their derivatives. In the present study, we examine whether or not commercially available monosaccharides and disaccharides enhance *vir* gene induction by AS. We demonstrate that only a group of aldoses, such as D-glucose, have the ability to enhance *vir* expression strongly.

MATERIALS AND METHODS

Bacteria and Plasmids. *A. tumefaciens* C58C1Cm harboring pTiB6S3tra^c has been described (23). *A. tumefaciens* A348mx226 and A348mx358 harbor pTiA6 with insertions of transposon Tn*3*-HoHo1 in the *vir*A and *vir*E, respectively (3). Strain C58C1Cm (pTiB6S3tra^c) was used for all the induction experiments in the present study unless otherwise mentioned. *Escherichia coli* JM109 (24) was used as the cloning host. pCM110Sa, pCM110PK, and pCM110PA, which can replicate in agrobacterial cells, have been described (25).

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Abbreviation: AS, acetosyringone.

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Chemicals and Enzymes. D-Lyxose, 2-deoxy-D-glucose, D-allose, D-altrose, L-mannose, D-gulose, D-idose, D-talose, and D-sorbitol were purchased from Sigma. D-Xylose, L-arabinose, D-glucose, and D-mannitol were obtained from Wako Pure Chemical, Osaka. D-Galactose, D-mannose, and D-arabinose were obtained from Katayama Chemical, (Osaka). D-Ribose was purchased from Tokyo Kasei Kogyo. To avoid degradation of sugars by autoclaving, solutions of these sugars were sterilized by filtration. Enzymes used for plasmid constructions were purchased from Toyobo Biochemicals (Kyoto), Takara Biochemicals (Osaka), and New England Biolabs.

Plasmid Construction. pCM110PB was constructed by inserting the *Rsa* I–*Alu* I fragment (323 base pairs) containing the *virB* promoter [nucleotide positions −275 to +48 (26)] into the multiple cloning site that is located upstream of the promoterless *lacZ* gene in pCM110Sa (25). pCM110PC was constructed similarly by inserting the *Sma* I–*Ban* III fragment (141 base pairs) containing the *virC* promoter [nucleotide positions −135 to +6 (27)] into the cloning site of pCM110Sa. To generate pCM110PD, the *Sac* I–*Sma* I fragment (457 base pairs) containing the *vir*D promoter region [nucleotide positions −450 to +7 (28)] was inserted into the cloning site of pCM110Sa. These plasmids were introduced into cells of *Agrobacterium* C58C1Cm ($pTiB_6S_3tra^c$).

To generate pHK17PB, the *vir*B promoter-linked *lacZ* gene of pCM110PB was introduced into pHK17 (29). pCM110KA was constructed by inserting the *Kpn* I fragment [nucleotide positions 2–4663 (10)] containing the *vir*A gene into the cloning site of pCM110Sa. To generate pCM110KA178, pCM110KA114, and pCM110KA91, the *Pst* I fragment (see Fig. 2), the *Pma*CI–*Eco*47III fragment, and the *Eco*47III–*Bst*EII fragment in the *vir*A coding region were removed from pCM110KVA, respectively, and remaining DNA regions were joined by ligation after treatments with enzymes suitable for in-frame joining. We confirmed by nucleotide sequencing that the sequences after removal of these fragments in mutant *vir*A genes were joined in-frame. pHK17PB, pCM110KA, and its derivatives were introduced into *Agrobacterium* A348mx226 (*virA*$^-$) (3) by electroporation (30). Details of the procedure will be provided on request.

Incubation of *Agrobacterium* and Assay of β-Galactosidase Activity. Cells of *A. tumefaciens* that carry Ti plasmid and pCM110Sa derivatives containing *vir–lacZ* fusions were grown at 26°C in L broth containing carbenicillin (100 μg/ml). The bacterial cells at logarithmic phase were washed with 10 mM $MgSO_4$ and then resuspended at 2×10^8 cells per ml in MSPS medium [Murashige and Skoog medium supplemented with 62.5 mM sodium phosphate and 3% (wt/vol) sucrose (pH 5.25)] that contained 10 μM AS, carbenicillin (100 μg/ml), and the various concentrations of sugars to be examined. The cells were incubated at 26°C with shaking for 18 hr, a time sufficient for maximum induction. β-Galactosidase activity was assayed by the method of Usami *et al.* (21).

RESULTS

To determine the level of inducible expression of *vir* genes, we used *Agrobacterium* C58C1Cm (pCM110PD) containing a *vir*D–*lacZ* gene (21) in which synthesis of β-galactosidase is directed by the inducible *vir*D promoter. *Agrobacterium* cells were incubated in MSPS medium containing 3% sucrose (88 mM) as a carbon source and 10 μM AS plus various concentrations of D-glucose. The activity of β-galactosidase increased markedly in the presence of 10 μM AS and D-glucose at concentrations higher than 1 mM, whereas there was no activity when glucose was added to medium without AS (Fig. 1*A*). Results of similar experiments with other inducible *vir* genes (*vir*B, -C, and -E) are given in Table 1. As

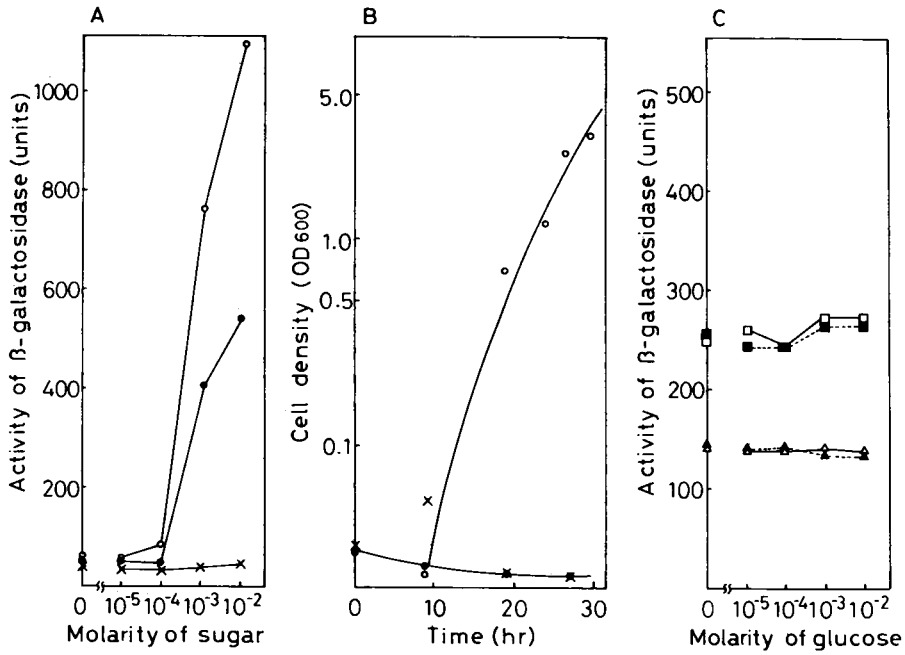

FIG. 1. (*A*) Effects of glucose and 2-deoxy-D-glucose on expression of the *vir*D gene. *A. tumefaciens* C58C1Cm cells carrying $pTiB_6S_3tra^c$ and pCM110D (*vir*D–*lacZ*) were incubated in MSPS medium containing 10 μM AS and the indicated concentrations of D-glucose (○) or 2-deoxy-D-glucose (●). The cells were also incubated in parallel in MSPS medium that contained only glucose (×). β-Galactosidase activity was assayed by the method of Usami *et al.* (21). The values given are averages of three experiments. (*B*) Utilization of glucose but not 2-deoxy-D-glucose by *Agrobacterium*. Cells grown in L broth were washed with M9 minimal medium and then divided into three suspensions. Glucose (○) or 2-deoxy-D-glucose (●) was added at the concentration of 0.2% or no sugar was added (×), and the cultures were incubated at 26°C. (*C*) Effects of glucose and 10 μM AS on the expression of *vir*A–*lacZ* and kanamycin-resistance–*lacZ* fusion genes. Agrobacterial cells that harbor $pTiB_6S_3tra^c$ and pCM110PK with the promoter of the kanamycin-resistance gene (△, ▲) (25) or pCM110PA with the promoter of the *vir*A gene (□, ■) (25) upstream of the *lacZ* coding sequence were incubated in the presence of D-glucose with 10 μM AS (△, □) or alone (▲, ■). β-Galactosidase activity was measured as described in *A*.

Table 1. Effects of glucose on gene expression directed by inducible *vir* gene promoters

Plasmid promoter	Activity of β-galactosidase, units			Activity increase, fold
	− Glc	1 mM Glc	10 mM Glc	
pCM110PB *vir*B	28 ± 12	750 ± 190	990 ± 300	35
pCM110PC *vir*C	88 ± 33	630 ± 18	880 ± 95	10
pCM110PD *vir*D	81 ± 32	780 ± 180	1100 ± 240	14
pTiA6* *vir*E	150 ± 82	2300 ± 320	2200 ± 130	15

AS at 10 μM was used except that 1 μM AS was used for induction of *vir*B since the detectable induction was observed by this concentration of AS. β-Galactosidase units (21) represent the average of three independent experimental determinations (mean ± SEM). To calculate the fold increases, units obtained at 10 mM glucose were divided by those obtained with AS alone. Glc, glucose.
*Promoterless *lacZ* [Tn*3*-HoHo1 (3)] was present in the *vir*E gene of pTiA6 in the A348mx358 strain.

shown, the addition of 10 or 1 mM glucose produced a >10-fold increase in β-galactosidase activity. The use of 10 mM glucose plus 10 μM AS produced approximately the same level of β-galactosidase as 200 μM AS alone. When glucose was added in the presence of 200 μM AS, β-galactosidase activity was enhanced just 2- to 3-fold. Thus, the lower the concentration of AS, the greater the extent of enhancement by glucose.

We also investigated effects of 2-deoxy-D-glucose, a nonmetabolized sugar. As shown in Fig. 1*A*, a marked increase of β-galactosidase activity was observed, although a higher concentration was required for this sugar than for glucose. *Agrobacterium* did not use this sugar analogue for growth (Fig. 1*B*), an indication that hexose metabolism is not necessary in agrobacterial cells for enhancement of β-galactosidase activity.

We next examined the level of activity directed by a noninducible promoter using the plasmids pCM110PK and pCM110PA (25), which have the promoters of kanamycin-resistance (*nptII*) and *vir*A genes, respectively, upstream of the *lacZ* coding sequence. Although the *vir*A gene promoter has been reported to be induced by AS (6, 7), expression directed by this promoter in our construct, pCM110PA, was barely induced as reported for pSM plasmids (5), although we employed suboptimal AS concentrations. The β-galactosidase activities expressed by the *nptII* gene and the *virA* promoters were nearly unaffected by glucose, whether or not AS was present (Fig. 1*C*). Thus, glucose specifically enhanced the amount of β-galactosidase synthesized by the *lacZ* genes driven by the AS-inducible promoters. This specific enhancement by glucose was also observed in other *Agrobacterium* strains such as C58 harboring pTiC58 and A6 harboring pTiA6 (data not shown). The pH of the culture medium did not change during the incubation.

The results obtained in the preceding section suggest that the enhancement shown by glucose is not due to improvement of bacterial growth but rather an acceleration of certain processes in *vir* gene induction. To test this hypothesis, pCM110KA containing the *vir*A gene was constructed, and

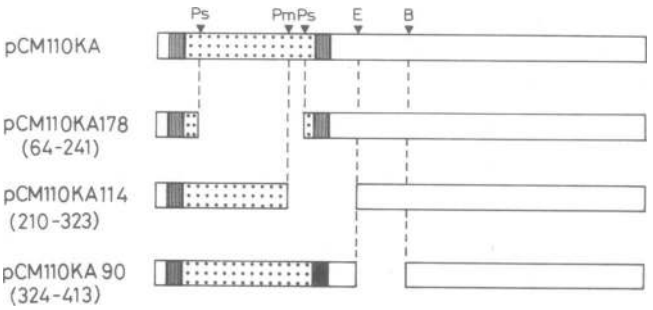

FIG. 2. Schematic representation of structures of mutant *vir*A genes. The pCM110 derivative plasmids are listed. The numbers 178, 114, and 90 in plasmid names refer to the number of amino acids deleted. Numbers in parentheses represent start and end points of amino acids deleted. Striped and dotted regions, putative transmembrane domains and periplasmic domain, respectively (11, 12); open box; cytoplasmic domain (11, 12). Ps, *Pst* I cleavage sites [nucleotide positions 1562 and 2096 (10)]; Pm, *Pma*CI cleavage site (nucleotide position 2001); E, *Eco*47III cleavage site (nucleotide position 2343); B, *Bst*EII cleavage site (nucleotide position 2615).

internal deletions were introduced into its coding region (Fig. 2). Each plasmid was introduced into *Agrobacterium* A348mx226 with a Tn*3*-HoHo1 insertion in the *vir*A gene on its Ti plasmid (3). To measure activity of the *vir*B promoter in the presence of mutant *vir*A genes, pHK17PB carrying the *vir*B–*lacZ* fusion gene was also introduced into these *Agrobacterium* cells. As shown in Table 2, activity of β-galactosidase was induced by AS in the periplasmic mutant (pCM110KA178) at a 10-fold higher level than in the wild type (pCM110KA) without glucose, but it was no longer enhanced by glucose. When regions covering the second transmembrane domain (pCM110KA114) or the cytoplasmic domain (pCM110KA91) were deleted (see Fig. 2), *vir* induction by AS itself was completely abolished. Therefore, the periplasmic region of the VirA protein seems to play an important role in the enhancement induced by glucose, although it is not clear whether glucose can directly interact with this protein. It is likely that glucose somehow amplifies a signal generated by AS through the VirA protein. The two other regions examined above are strictly required, as reported (11).

The level of *vir*B expression in A348mx226 cells carrying pCM110KA was lower than that in C58C1Cm cells having the *vir*A gene in the Ti plasmid. Since *A. tumefaciens* A348mx226 used in this experiment was a merodiploid strain containing both mutant *vir*A in pTiA6 and wild-type *vir*A in pCM110KA, this strain can produce both the wild-type VirA protein and the mutant VirA protein from pTiA6 [the N-terminal half of the VirA protein is still intact (3)]. If the number of the membrane sites where the VirA protein can be anchored is limited, the wild-type and mutant VirA proteins may compete for such sites. The lower level of *vir*B expression may be due to a diminished amount of the functional wild-type VirA protein anchored in the membrane of the A348mx226 cells having pCM110KA.

Table 2. Effects of glucose on *vir*B gene expression in the *vir*A mutant

Plasmids	Activity of β-galactosidase, units		
	− Glc	1 mM Glc	10 mM Glc
pTiA6 (virA$^-$), pHK17PB	1 ± 1	ND	ND
pTiA6 (virA$^-$), pHK17PB, pCM110KA	49 ± 6	110 ± 11	440 ± 38
pTiA6 (virA$^-$), pHK17PB, pCM110KA178	300 ± 38	280 ± 54	230 ± 31
pTiA6 (virA$^-$), pHK17PB, pCM110KA114	4 ± 3	4 ± 3	ND
pTiA6 (virA$^-$), pHK17PB, pCM110KA91	ND	ND	2 ± 2

AS at 10 μM was used. Data are expressed as mean ± SEM of three independent experimental determinations. The *vir*B promoter on pHK17PB was used. ND, not detected; Glc, glucose.

Table 3. Effects of sugars on gene expression directed by the *vir*D promoter

Sugar added	Activity of β-galactosidase, units	
	1 mM	10 mM
None	81 ± 32	81 ± 32
D-Ribose	50 ± 10	53 ± 18
D-Arabinose	48 ± 13	37 ± 3
L-Arabinose	320 ± 40	1220 ± 220
D-Xylose	660 ± 30	900 ± 40
D-Lyxose	131 ± 26	360 ± 40
D-Allose	37 ± 16	47 ± 13
D-Altrose	38 ± 14	62 ± 20
D-Glucose	780 ± 170	1100 ± 240
D-Mannose	230 ± 20	700 ± 150
L-Mannose	23 ± 6	21 ± 8
D-Gulose	56 ± 9	82 ± 8
D-Idose	330 ± 25	740 ± 45
D-Galactose	700 ± 230	1070 ± 260
D-Talose	330 ± 93	930 ± 70
D-Sorbitol	84 ± 4	46 ± 3
D-Mannitol	81 ± 32	88 ± 11

Experiment was carried out as described for Fig. 1*A* except that sugars indicated here were used. Data are mean ± SEM from three to five independent experiments. AS at 10 μM was used.

We systematically tested aldoses and other saccharides for enhancement of AS induction. As shown in Table 3, L-arabinose, D-xylose, D-lyxose, D-glucose, D-mannose, D-idose, D-galactose, and D-talose were effective. Among them, L-arabinose, D-xylose, D-glucose, and D-galactose were slightly more active than the other effective sugars. D-Ribose, D-arabinose, D-allose, D-altrose, D-gulose, L-mannose, and two reduced monosaccharides (D-sorbitol and D-mannitol) showed no enhancement of *vir* gene expression. Ketoses (D-fructose, D-ribulose, and D-xylulose) and disaccharides such as sucrose and lactose were not effective (data not shown). Note that the effective sugars except for D-idose share C-3 stereochemical structure. Aldoses that had no effect share a different C-3 stereochemical structure. In addition, L-arabinose and D-mannose are active sugars, whereas their respective stereoisomers, D-arabinose, and L-mannose, are not active at all. These findings suggest importance of stereostructures of aldoses for enhancement of *vir* gene induction and make it unlikely that contaminants in our reagents could be responsible for the effects observed.

DISCUSSION

The results presented here clearly demonstrate that a group of aldoses such as D-glucose markedly enhance the expression level of *vir* genes in the presence of limiting AS (Fig. 1). 2-Deoxy-D-glucose, a sugar not metabolized by *Agrobacterium*, is also effective. This indicates that enhancement by the monosaccharides is not due to alteration of carbohydrate metabolism that might somehow improve the physiological state of bacteria. In this connection, we noted that D-ribose, D-arabinose, and L-mannose can support bacterial growth but did not enhance *vir* gene induction. These results and the result of the experiment with the mutant *vir*A constructs support the hypothesis that effective monosaccharides directly amplify signaling by plant inducers through the VirA protein.

All the effective aldohexoses and aldopentoses except for D-idose have identical C-3 stereochemical structure. Noneffective aldoses have a C-3 stereochemical structure different from that of the effective sugars. A typical example is the inactive sugar D-allose: it differs from D-glucose only in the hydroxyl configuration at C-3. These results indicate that the C-3 stereochemical structure of aldopentose and aldohexose appears to be important for enhancement. C-2 stereochemical structure also seems to influence activity of aldoses, because activities of L-arabinose, D-xylose, D-glucose, and D-galactose were somewhat higher than those of D-lyxose, D-mannose, and D-talose: the former sugars share C-2 and C-3 stereochemical structure and the latter ones have a different hydroxyl configuration at C-2. This was clearly observed in aldopentoses, D-xylose and D-lyxose (Table 3).

The result obtained with D-idose cannot be simply explained by importance of C-3 stereochemical structure, since it has a C-3 stereochemical structure different from that of the other active aldoses. This sugar, however, can exist in two different chair conformations and both may be present at equilibrium unlike other active sugars, which can preferentially form one of alternative conformations. One of the D-idose conformations may have activity to enhance *vir* gene induction.

The finding that the stereostructures of aldoses are important for enhancement of *vir* gene induction predicts the presence of one or more proteins in *Agrobacterium* that specifically recognize the effective sugars. Experiments with the periplasmic mutant *vir*A gene indicate that the periplasmic loop of VirA protein is important for the enhancement induced by the monosaccharides. The active sugars might interact directly or through unknown mediator proteins with VirA proteins, inducing conformational alterations of this protein that could result in an increase in the extent of signaling by phenolic inducers.

Our results demonstrate that the sugar effects studied here are striking only in limited concentrations of AS. One explanation may be that there is a plateau of the extent of *vir* expression that does not increase even if higher concentrations of AS or sugars are added. Further kinetic studies of *vir* gene induction by AS and sugars, however, are required for elucidating molecular mechanisms behind this phenomenon; examination of more direct effects of AS, such as phosphorylation of VirA protein, are probably necessary.

The observation that the level of *vir* gene induction was 6-fold higher in the *vir*A periplasmic deletion mutant than in the wild type in the absence of glucose suggests that the periplasmic region of VirA protein somehow modulates the activity of this protein as a receptor for signal molecules, although it seems to be nonessential for signaling itself. The region of the VirA protein that confers pH sensitivity was reported to be linked to the periplasmic region close to the second transmembrane domain, with which AS probably interacts (11). To investigate the region(s) of the VirA protein that interacts with the effective sugars, we have constructed *vir*A mutants having amino acid substitutions in various regions of *vir*A.

It is interesting to note that most of the effective sugars listed in Table 3 are known precursors of the major components of the cell wall polysaccharides of higher plants (31). D-Galacturonic acid, which is also a component of the plant cell wall, was more effective in stimulating *vir* gene induction than D-galactose (unpublished data). Although it is not clear whether exudates from wound sites in plants contain a sufficient amount of the effective sugars to enhance *vir* gene expression, glucose has been reported to be present at a concentration of 10 mM in wound exudates from some plants (32, 33). Because the active sugars are effective even at concentrations lower than 1 mM (Fig. 1), they may, together with other extracellular conditions (e.g., pH and temperature), determine the level of *vir* gene induction by phenolic inducers when *Agrobacterium* invades plants.

Although AS is known to be a strong inducer, it is yet to be established whether it is present in all *Agrobacterium*-susceptible plants. In contrast, a wide variety of lignin

precursors or lignin components that are generally present in higher plants also function as *vir* gene inducers, although their abilities as signal molecules differ (19, 20). We have tested effective sugars added in combination with weak phenolic inducers, such as syringaldehyde, syringic acid, acetovanillone (19), and ferulate (20) which has been recently identified as one of *vir* inducing factors from wheat (37), and observed the high level of *vir* gene expression induced by AS (data not shown). Thus, even weak phenolic inducers could in synergy with effective monosaccharides induce a sufficient level of *vir* gene activity to initiate transfer of T-DNA to plant cells.

The expression of *vir* genes has been reported to increase severalfold under hypertonic conditions (34), suggesting that the enhancement we observed might be due to an increase of osmotic pressure by adding monosaccharides. However, this is unlikely, since enhancement took place even at low concentrations of the effective sugars (1 mM). Opines have been shown to stimulate *vir* induction severalfold (35). Perhaps a similar mechanism is involved in the enhancements induced by opines and monosaccharides.

Our findings should contribute to the improved introduction of foreign genes into plant cells by using the Ti plasmid vector. The addition of AS to a culture of *Agrobacterium* has been shown to improve the efficiency of transformation of *Arabidopsis* (36), which has limited contents of diffusible phenolic inducers (22). Likewise, the highly effective monosaccharides described here may prove to be useful for improving the frequency of plant transformation.

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