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Specificity of signal molecules in the activation of *Agrobacterium* virulence gene expression

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Summary

The activation of the *Agrobacterium* virulence system is known to be induced by certain phenolic compounds. We have tested the *vir*-inducing ability of fifty compounds, by using a *virB-lacZ* gene fusion, and analysed the relationship between structure and activity of these compounds. In this way we have identified several new *vir*-inducers: coniferylalcohol, 3,5-dimethoxy-4-hydroxybenzene, homovanillic acid, ferulic acid, 3-ethoxy-4-hydroxybenzaldehyde and guaiacol, all of which are compounds with strong or moderate activity and four compounds with weak *vir*-inducing activity. In view of the specificity of *vir*-inducers, our data extended observations of others and enabled us to define the specific structural features of a *vir*-inducer molecule. In addition we show here that induction of the octopine Ti *vir*-genes is (i) optimal at 29°C and totally abolished at 37°C, and (ii) strongly inhibited at low concentrations of sodium chloride. The implications for plant transformation are discussed.

Introduction

The soil bacterium *Agrobacterium tumefaciens* causes the crown gall disease in dicotyledonous plants. The bacterium provokes a neoplastic growth at wound sites of plants, by introducing oncogenic T-DNA, a specific part of its tumour-inducing (Ti) plasmid, into plant cells, (for recent reviews see Koukolikova-Nicola *et al.*, 1987 and Melchers and Hooykaas, 1987).

A first step in this bacterial-plant interaction involves recognition of susceptible plant cells by *Agrobacterium*, which might involve chemotaxis. The bacterium possesses a highly sensitive chemotaxis system responsive to a variety of sugars, amino acids and phenolics present in wounded plant tissue (Loake *et al.*, 1988; Ashby *et al.*, 1987). Thereafter the bacterium attaches to plant cell walls at specific receptor sites (Lippincott and Lippincott, 1969; Schilperoort, 1969). This step is mediated by several genes (*chvA, chvB, att* and *pscA* or *exoC*) located in the bacterial chromosome (Douglas *et al.*, 1985, Matthysse, 1987; Thomashow *et al.*, 1987; Cangelosi *et al.*, 1987). The Ti plasmid genes required for transfer of the T-DNA to plant cells are located in the 40kb Vir region. Genetic analysis has revealed that at least seven *vir* operons (*virA* to *virG*) are present in the Vir region of the octopine Ti plasmid (Hille *et al.*, 1982; Klee *et al.*, 1983; Hille *et al.*, 1984; Hooykaas *et al.*, 1984; Stachel and Nester, 1986). In *in vitro* cultures of *Agrobacterium*, only the regulatory genes *virA* and *virG* are significantly expressed (Stachel and Nester, 1986). However, in the presence of specific plant factors the expression of the other *vir* genes (Stachel and Nester, 1986), including *virF* (L. S. Melchers *et al.*, submitted), is induced. The activation of *vir* expression by plant factors initiates the products of single-stranded linear T-DNA molecules (T-strands) in *Agrobacterium* (Stachel *et al.*, 1987) which are probably the T-DNA intermediates that are transferred to the plant cells. Characterization of the *vir* genes in more detail has shown that the VirA protein is an inner membrane protein, which probably acts as a sensor for specific plant signal molecules (Leroux *et al.*, 1987; Melchers *et al.*, 1987). In the presence of these plant factors, VirA activates the second regulatory protein VirG (possibly via phosphorylation), which then can act as a positive regulator for the remaining *vir* genes (Winans *et al.*, 1986; Melchers *et al.*, 1986). Products determined by these other *vir* genes include the proteins VirD1 and VirD2, which are involved in the generation of T-strands (Stachel *et al.*, 1987), whereas most VirB products are thought to be membrane proteins involved in the actual T-DNA transfer process (Engström *et al.*, 1987; Thompson *et al.*, 1988).

The recognition of plant signal molecules by *Agrobacterium* is an important trigger of T-DNA transfer. Stachel and co-workers (1985) identified from wounded *Nicotiana tabacum* tissue two signal compounds, acetosyringone and α-hydroxyacetosyringone, which specifically activate expression of the Ti plasmid *vir* genes. Later, Bolton *et al.* (1986) reported that seven different phenolic compounds could induce expression of the *vir* genes. Together, these two reports gave no clear picture about the structural features which confer on a compound *vir*-inducing activity.

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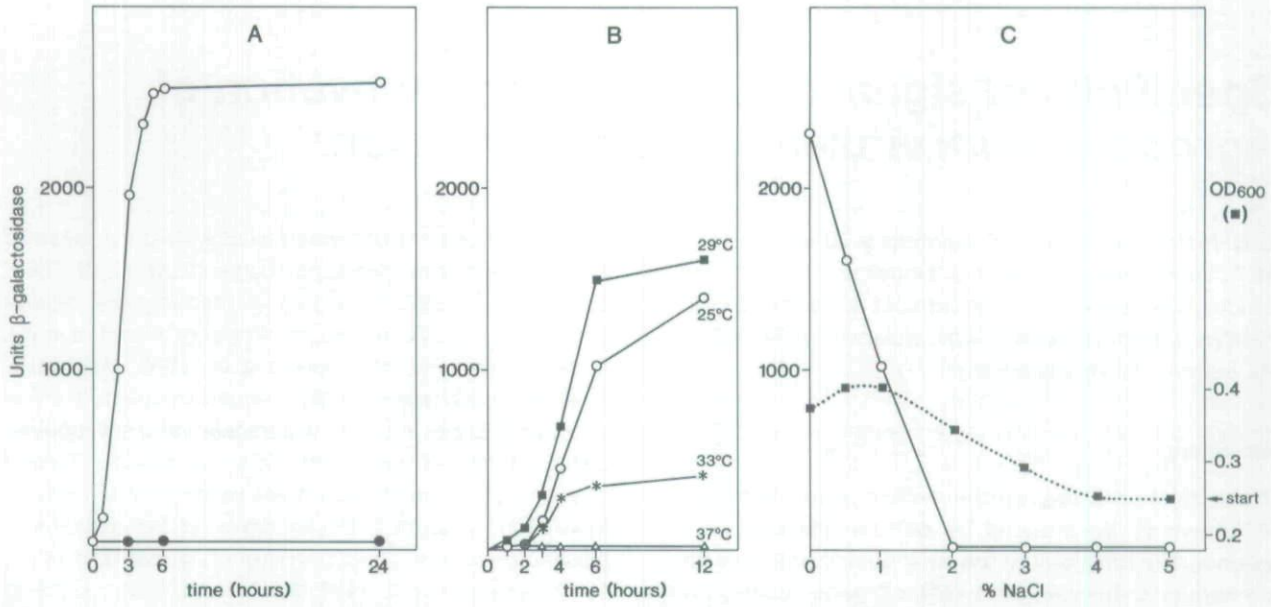


Fig. 1. Characteristics of *Agrobacterium vir* gene induction by acetosyringone. The bacteria were grown in IM and the *vir* genes were induced with 200 μM acetosyringone.

A. Kinetics of *vir* gene induction of the *Agrobacterium* strains LBA2516 (○), LBA2520 (●) and LBA2522 (●).

B. Effect of growth temperature on *vir* gene induction. Strain LBA2516 (*virB-lacZ* fusion) was grown at 25°C (○), 29°C (■), 33°C (*) and 37°C (△). The β-galactosidase activity (Miller Units) of each culture was determined during 12 hours of incubation.

C. Growth curve (■) and *vir*-induction response (○) of strain LBA2516 grown in the presence of different concentrations of NaCl.

In this paper, we report a quantitative analysis of the *vir*-inducing activity of a large number of mostly phenolic compounds. This study extends the observations of earlier studies and defines the structural features of a *vir*-inducer molecule. In addition, we define a number of parameters which influence *vir* gene expression.

Results

Induction of virulence genes

It was previously reported that low pH is a prerequisite for induction of the *Agrobacterium vir* genes (Stachel *et al.*, 1985). In order to be able to develop a medium optimized for *vir* induction, we studied the effect of a number of different parameters, i.e. pH, temperature, medium type, salt concentration, carbon source and acetosyringone concentration, and monitored induction. As an indicator for *vir* expression we used the *Agrobacterium* octopine Ti strain, LBA2516, which carries a translational fusion of *lacZY* to the *virB2* open reading frame (ORF) (see *Experimental procedures*). Negative controls were derivatives of LBA2516 with mutations either in *virA* (LBA2520) or *virG* (LBA2522). As can be seen in Fig. 1A, *vir* expression by LBA2516 reaches its optimum six hours after incubation in induction medium (IM) (see below) with 200 μM acetosyringone, while even after 24 hours of incubation no expression is found for the regulatory mutants LBA2520 and LBA2522. When we varied the pH in our original minimal medium (MM), which is buffered with 12.5 mM potassium phosphate, we found that, irrespective of the initial pH, the pH of the medium dropped rapidly to about pH 4.2 during six hours of incubation. Unexpectedly, the same turned out to be true for the media that have been described in the literature on *vir*-induction assays, and that are buffered by 12.5 mM sodium phosphate (MSSP-medium) (Stachel *et al.*, 1985; Rogowsky *et al.*, 1987) or 12.5 mM 2-(*N*-Morpholino) ethane sulphonic acid (MES) (Winans *et al.*, 1988). Therefore, in order to be able to test the effect of pH properly, we used minimal medium, supplemented with 62.5 mM potassium phosphate. This medium exhibited adequate buffering capacity during the incubation period of six hours, and in using it we found that optimal induction in LBA2516 occurred at pH 5.3 in the presence of 200 μM acetosyringone (not shown). Testing of the effect of different carbon sources (glucose, sucrose, glycerol, succinate) in this medium, at 0.25%, 1% and 3%, revealed that the highest sugar concentration (3%) provided optimal *vir* induction, and that sucrose was preferred over the other sugars for induction. The sugar composition did not significantly influence the growth of

the culture; in all cases growth was slow. Thus for induction medium (IM) used thereafter we used 62.5 mM potassium phosphate, pH 5.3 and 3% sucrose as a carbon source (see *Experimental procedures*). The need for such a high sugar content in the medium reflects a need either for a rich energy supply or for a relatively high osmolarity of the medium. In order to find out whether increasing just the osmolarity of the medium would lead to induction, we added NaCl at different concentrations to the standard IM medium. It turned out that the addition of NaCl had a strong inhibitory effect on *vir* induction (Fig. 1C), which indicates that the *vir*-induction system does not require a high osmolarity medium, but rather a rich energy supply. We therefore tested whether richer media, adjusted to pH 5.3 and containing 200 μM acetosyringone, would be more suitable for *vir* induction. Neither the use of rich media (LC, TY or YMB) nor the sole addition of yeast extract to the minimal medium IM had a positive effect on *vir* induction. In contrast to Rogowsky *et al.* (1987), who studied the nopaline Ti system, we found that yeast extract concentrations of ≥0.1% inhibit *vir* gene induction in the octopine Ti system. Therefore, we used IM medium as defined for further experiments.

The temperature dependence of *vir* induction is shown in Fig. 1B. A normal *vir*-induction response is seen for *Agrobacterium* grown at 25 °C or 29 °C in IM after exposure to 200 μM acetosyringone. Induction of *Agrobacterium vir* genes was significantly reduced at 33 °C, whereas no induction was detected at 37 °C. These data clearly show that the transcriptional activation of *vir* genes is temperature-sensitive, although the levels of growth of *Agrobacterium* in IM did not differ significantly over the whole temperature range from 25 °C to 37 °C.

Specificity of vir *inducers*

The plant phenolic compounds acetosyringone and α-hydroxyacetosyringone have been identified by Stachel *et al.* (1985) as the inducers that activate *vir* gene expression in *Agrobacterium*. In order to define the specific features of a *vir* inducer in more detail, we tested 50 mostly phenolic chemicals for their ability to activate *vir* gene expression. *Agrobacterium* strain LBA2516, which contains a *virB-lacZ* reporter gene construct, was grown in the presence of 200 μM of each compound in IM medium (see above). Activation of *vir* gene expression was measured via a β-galactosidase enzyme assay after 24 hours. The results shown in Table 1 indicate that besides acetosyringone, 15 of the 50 tested compounds induce the *vir* genes. The following compounds are strong to moderate *vir* inducers: acetosyringone (1), sinapinic acid (2), coniferylalcohol (3), syringaldehyde (4), acetovanillone (5), vanillin (6), 3,5-dimethoxy-4-hydroxybenzene (7), syringic acid (8), homovanillic acid (9), ferulic acid (10) 3-ethoxy-4-hydroxybenzaldehyde (11) and guaiacol (12). Other compounds like 3,4-dihydroxybenzaldehyde (13), vanillylalcohol (14), 4-hydroxy-3-methylacetophenone (15) and 3,4,5-trimethoxybenzaldehyde (16) are weak *vir* inducers.

The level of *vir* induction in *Agrobacterium* is dependent on the *vir*-inducer concentration. Therefore, different inducer concentrations (ranging from 5 nM to 500 μM) were tested to establish the lowest concentration of each *vir* inducer which resulted in maximal *vir* induction (see Table 1). Acetosyringone was the most active *vir* inducer (10 μM was sufficient for maximum induction). Ferulic acid is optimally active at 50 μM while other inducers require relatively high concentrations, i.e. 100–500 μM. Comparison of the *vir*-inducing ability of all the different compounds tested reveals a number of important features of a *vir*-inducer molecule. For strong *vir*-inducing activity, the para-hydroxyl group (R4-position, see Table 2) is absolutely essential, as shown previously by Stachel and co-workers (1985) and is illustrated in Table 2A. Only one *vir* inducer, 3,4,5-trimethoxybenzaldehyde (16), which is classified as a weak *vir* inducer, lacks the hydroxyl group at the R4-position and contains a methoxyl group instead. The results summarized in Table 2B show that the presence of one methoxyl group at the R3-position is essential, while strong *vir* inducers contain a methoxyl group at both the R3- and R5-positions. The compounds 3-ethoxy-4-hydroxybenzaldehyde (11), 3,4-dihydroxybenzaldehyde (13) and 4-hydroxy-3-methylacetophenone (15) have partial inducing activity, although they lack both methoxyl groups. This residual activity is probably due to the presence of an ethoxyl group, a hydroxyl group or a methyl group at the R3-position in compounds (11), (13) and (15), respectively. A large number of different functional groups can be substituted at the R1-position (see Table 2C) of an inducing compound without having a strong negative effect on the *vir*-inducing activity. For example, sinapinic acid (2) which has a relatively large group, and 3,5-dimethoxy-4-hydroxybenzene (7), which has no side group at all at the R1-position, are both strong *vir* inducers. The results obtained with the compounds shown in Table 2C indicate that not all substituents are tolerated at the R1-position, since vanillylalchohol (14) appeared to be a weak *vir* inducer only, and 4-hydroxy-3-methoxybenzoic acid (38) totally lacked inducing activity. Ferulic acid (10) possesses a structure similar to that of 4-hydroxy-3-methoxybenzoic acid (38), except that its carboxyl group (R1-position) is separated by a C-C double bond from the guaiacyl nucleus. Interestingly, the compound with the extra C-C double bond exhibited significantly greater *vir*-inducing activity. This is also the case for coniferylalcohol (3) and vanillylalcohol (14): the extra C-C double bond in coniferylalcohol leads to a much stronger

Table 1. List of compounds classified to their ability to stimulate *vir* gene expression.

Compound	*vir* inducers: Units β-gal.[a]	*vir* inducers: conc.[b] (μM)
1. acetosyringone	3400	10
2. sinapinic acid	3360	100
3. coniferyalcohol	3120	100
4. syringaldehyde	3060	500
5. acetovanillone	2652	500
6. vanillin	2580	500
7. 3,5-dimethoxy-4-hydroxybenzene	2480	200
8. syringic acid	2040	500
9. homovanillic acid	1870	100[c]
10. ferulic acid	1700	50
11. 3-ethoxy-4-hydroxybenzaldehyde	1530	500
12. guaiacol	1140	500
13. 3,4-dihydroxybenzaldehyde	815	500
14. vanillylalcohol	442	100
15. 4-hydroxy-3-methylacetophenone	400	500
16. 3,4,5-trimethoxybenzaldehyde	306	200

Non-inducers

Compound	Compound
17. Salicylic acid	34. 3,5-dimethoxyacetophenone
18. 2-aminobenzoic acid	35. 1,2-benzopyrone
19. 4-hydroxybenzoic acid	36. 1,2-dihydroxybenzene
20. tetra-hydroxybutane	37. 3,4,5-trimethoxyacetophenone
21. 2,3,4-trihydroxyacetophenone	38. 4-hydroxy-3-methoxybenzoic acid
22. 3,5-dimethylphenol	39. 4-hydroxy-3-nitrobenzaldehyde
23. 2,5-dihydroxybenzoic acid	40. 3-hydroxy-4-methoxybenzoic acid
24. 3,4-dihydroxycinnamic acid	41. 4-hydroxy-3-methoxymandelic acid
25. 2,4-dihydroxybenzoic acid	42. 4-hydroxyacetophenone
26. indole-3-acetic-acid	43. 4-hydroxybenzaldehyde
27. 3,4,5 trihydroxybenzoic acid	44. 2,3-dihydroxybenzoic acid
28. 3,5-dimethoxybenzoic acid	45. 3-hydroxy-4,5-dimethoxybenzoic acid
29. 4-hydroxycinnamic acid	46. naringenin
30. quinic acid	47. luteolin
31. 1,2,3-trihydroxybenzene	48. quercetin
32. 3,4-dihydroxybenzoic acid	49. genistein
33. 3,5-dimethoxybenzaldehyde	50. umbelliferone

a. β-galactosidase activities determined after a 24h incubation of LBA2516 with 200 μM of compound. In the absence of an inducer, the background level of expression was <50 Units.
b. Minimum concentration which gave maximum *vir* induction (range 5 nM–500 μM).
c. Compound is bacteriostatic at 500 μM.
Note: compound (38) totally inhibited cell growth at 200 μM.

vir-inducing activity relative to vanillylalcohol. Thus, a double bond at the R1-position enhances the activity of the structure.

We investigated whether inhibitors of stimulatory activity are present amongst the group of non-inducers or weak *vir* inducers. *Agrobacterium* cells were pre-exposed for one hour to a 40-fold molar excess of a potential inhibitor before the addition of 5 μM acetosyringone. None of the following compounds — 3,4-dihydroxybenzaldehyde (13), vanillylalcohol (14), 3,4,5-trimethoxybenzaldehyde (16), 4-hydroxybenzoic acid (19), 3,5-dimethylphenol (22), 3,4-dihydroxy-cinnamic acid (24), 3,5-dimethoxybenzoic acid (28), 3,5-dimethoxybenzaldehyde (33), 3,5-dimethoxyacetophenone (34), 3,4,5-trimethoxyacetophenone (37) and naringenin (46) — displayed an inhibitory effect on *vir* gene expression (data not shown). These results indicate that none of these compounds, which are structurally closely related to acetosyringone, is able to compete with acetosyringone for its binding site.

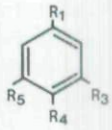
Discussion

In this study we have investigated several aspects of the stimulation of *vir* gene expression by plant signal molecules. Plant tumour induction requires no more than 18 hours (Lipetz, 1966). The minimum time required by *A. tumefaciens* to provoke tumour induction at wound sites of *Kalanchoe daigremontiana* was estimated to be 4–8 hours (Sykes and Matthyse, 1986). In agreement with these reports, we observed that maximum *vir* induction is

Table 2. Structure and *vir*-induction activity of different compounds.

A. R3=R5=OCH_3

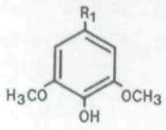
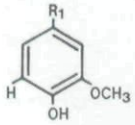
Compound	R1	R4	Induction
acetosyringone	$O=C-CH_3$	OH	++++
3,5-dimethoxyacetophenone		H	−
3,4,5-trimethoxyacetophenone		OCH_3	−
syringaldehyde	$O=C-H$	OH	++++
3,5-dimethoxybenzaldehyde		H	−
3,4,5-trimethoxybenzaldehyde		OCH_3	+
syringic acid	$O=C-OH$	OH	+++
3,5-dimethoxybenzoic acid		H	−



B. R4=OH

Compound	R1	R3	R5	Induction
acetosyringone	$O=C-CH_3$	OCH_3	OCH_3	++++
acetovanillone		OCH_3	H	+++
4-hydroxy-3-methylacetophenone		CH_3	H	+
4-hydroxyacetophenone		H	H	−
syringic acid	$O=C-OH$	OCH_3	OCH_3	+++
4-hydroxy-3-methoxybenzoic acid		OCH_3	H	−
syringaldehyde	$O=C-H$	OCH_3	OCH_3	++++
vanillin		OCH_3	H	+++
4-hydroxybenzaldehyde		H	H	−
3,4-dihydroxybenzaldehyde		OH	H	+
3-ethoxy-4-hydroxybenzaldehyde		OC_2H_5	H	++

C.

Structure	Compound	R1	Induction
	acetosyringone	$COCH_3$	++++
	sinapinic acid	$CH=CHCOOH$	++++
	syringaldehyde	CHO	++++
	syringic acid	COOH	+++
	3,5-dimethoxy-4-hydroxybenzene	H	+++
	coniferylalcohol	$CH=CHCH_2OH$	++++
	vanillin	CHO	+++
	acetovanillone	$COCH_3$	+++
	ferulic acid	$CH=CHCOOH$	++
	homovanillic acid	CH_2COOH	++
	guaiacol	H	++
	vanillylalcohol	CH_2OH	+
	4-hydroxy-3-methoxybenzoic acid	COOH	−

a. *vir*-inducing activity of the individual compounds expressed as Units of β-galactosidase activity (see Table 1) was classified as follows: −, 0–100; +, 150–1000; ++, 1000–2000; +++, 2000–3000; and ++++, 3000–4000..

reached after 5–6 hours. Our results indicate that the temperature-sensitive step in plant tumour induction reported several decades ago by Riker (1924) and Braun (1943) is likely to be partially due to the inhibition of *vir* induction at high temperatures. We found that, at 33°C, only 25% of the maximum activity was present, while at 37°C, induction of *vir* gene expression was completely abolished. In addition, our results were supported by the observations of Alte-Moerbe *et al.* (1988). Using the nopaline Ti system, they found that the production of both VirD2 and *trans*-zeatin, which is also induced by acetosyringone, is optimal at 20–28°C and is significantly reduced at higher temperatures. It is most likely that one of the regulatory proteins (VirA and VirG), or even both, determines a thermo-sensitive step in *Agrobacterium* tumour-induction.

In order to investigate the structural features of a *vir* inducer compound in more detail, we analysed a large

number of compounds which revealed several new *vir* inducers: coniferylalchohol, which is a strong *vir* inducer, 3,5-dimethoxy-4-hydroxybenzene, homovanillic acid, ferulic acid, 3-ethoxy-4-hydroxybenzaldehyde and guaiacol, all of which are moderate *vir* inducers, and 3,4-dihydroxybenzaldehyde, vanillylalcohol, 4-hydroxy-3-methylacetophenone and 3,4,5-trimethoxybenzaldehyde, which are weak *vir* inducers. Using a plate assay, Bolton and co-workers (1986) identified seven plant phenolics that induce the *virE* locus. Our results confirmed that vanillin is able to induce *vir* gene expression, but in contrast to the observations of Bolton *et al.*, we found, using a quantitative assay, that the other six compounds, 4-hydroxybenzoic acid (19), 2,4-dihydroxybenzoic acid (25), 3,4,5-trihydroxybenzoic acid (27), 1,2,3-trihydroxybenzene (31), 3,4-dihydroxybenzoic acid (32) and 1,2-dihydroxybenzene (36), are unable to activate the *Agrobacterium vir* genes. It is not likely that this discrepancy is due to the different transcriptional loci (*virE* and *virB*) used for monitoring *vir* gene expression, since it is known that both the *virB*- and the *virE* operon are transcriptionally activated via the same VirA-VirG regulatory system (Stachel and Zambryski, 1986). The six non-inducers mentioned above all lack both methoxyl groups at the R3- and R5-positions, which implicates, according to our structure-activity analysis and reports of others (Stachel *et al.* 1985), that they are unable to stimulate *vir* gene expression. Moreover, our findings regarding the important structural features of a *vir*-inducer molecule corroborate and extend the observations of Stachel *et al.* (1985). From the analysis of fifty compounds, we found that guaiacol illustrates the basic structural features required to confer *vir*-inducing activity on a compound, as follows: a benzene molecule with a hydroxyl substituent at the R4-position and one methoxyl group at the R-3 position. The presence of a methoxyl group at both the R3- and R5-positions, enhances the activity of the inducer. Furthermore, the structural feature of the substituent opposite the hydroxyl group on the benzene ring (R1-position) is a less strict requirement. Several substituents at this position, a hydrogen-, aldehyde-, acetyl- or carboxyl-group confer inducing activity on the compound, whereby the presence of a C-C double bond, at the R1-position, has a positive effect and increases the activity of the *vir*-inducer. We show here that some phenolic compounds, i.e. 3-ethoxy-4-hydroxybenzaldehyde (11), 3,4-dihydroxybenzaldehyde (13), 4-hydroxy-3-methylacetophenone (15) and 3,4,5-trimethoxybenzaldehyde (16), although they lack a hydroxyl group at the R4-position (16) or a methoxyl-group at the R3- or R5-positions (11, 13, 15), can still have partial inducing activity. To date, it is not known whether the trigger of a weak *vir* inducer received by *Agrobacterium* is sufficient to induce the complete virulence system and the transfer of T-DNA to plant cells.

Comparison of the plant-mediated gene expression in *Agrobacterium* and in *Rhizobium* shows several differences between these members of the Rhizobiaceae. First, the flavanoid activation of nodulation genes in *Rhizobium* occurs at relatively low concentrations (from 0.1 μM to 10 μM) while for activation of the *Agrobacterium* virulence genes, higher inducer concentrations (10 μM–500 μM) are required. Secondly, specific compounds which are structurally related to the flavonoid inducers are able to strongly inhibit the induction of *Rhizobium* (Firmin *et al.*, 1986; Djordjevic *et al.*, 1987). Moreover, acetosyringone and a number of analogous compounds, like acetovanillone and 4-hydroxyacetophenone, can also strongly inhibit *nod* gene activation (Firmin *et al.*, 1986). The structural similarity of inhibitory and stimulatory compounds associated with *Rhizobium nod* gene expression suggests that there is a common binding site for these molecules. Among the compounds we have tested, including analogues of acetosyringone and the flavonoid naringenin, there are no antagonists of acetosyringone induction of *Agrobacterium vir* genes. This observation suggests that, unlike *Rhizobium*, the recognition of specific inducing plant compounds by *Agrobacterium* is not inhibited by analogous compounds. Thirdly, *Rhizobium* species differ in their responsiveness to different flavonoids, which is in agreement with their high specificity for certain legume hosts (Spaink *et al.*, 1987). In contrast, *A. tumefaciens* induces tumours on a wide range of dicotyledonous plants. Therefore, it is not surprising that at least some of the *vir* inducers, like ferulic acid, syringaldehyde and coniferylalcohol (a lignin precursor), are widely distributed in plants.

The inability of monocotyledons to synthesize substances with strong *vir*-inducing activity (Usami *et al.*, 1988) is a major barrier in gene transfer to monocots. It has been shown that transformation of a monocotyledonous crop plant is possible upon pre-incubation of *Agrobacterium* with wound exudates from dicotyledons prior to infection (Schäfer *et al.*, 1987). A better understanding of the structural features of a plant signal molecule, present in wound exudates, may be useful for the genetic engineering of monocots as well as dicots.

Experimental procedures

Materials

All chemicals listed in Table 1 were purchased from Aldrich Chemical Co. or EGA-chemie and were of the highest possible grade of purity. Restriction endonucleases were purchased from either Promega or New England Biolabs and used according to the suppliers' recommendations. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was from Boehringer Mannheim Biochemicals. *o*-Nitrophenyl-β-D-galactopyranoside and carbenicillin were purchased from Sigma Chemical Co., and rifampicin was a gift from Ciba Geigy.

Table 3. List of bacterial strains and plasmids.

Strain or plasmid	Characteristics	Reference or source
E. coli		
KMBL1164	Δ(*lac-pro*) XIII, *thi, supE*	Giphart-Gassler and Van de Putte (1979)
p3478	*polA, thy* (KA208)	De Lucia and Cairns (1969)
Agrobacterium		
LBA288	*rif*^{R}, *nal*^{R} Ti-cured	Hooykaas
LBA969	LBA288 (pAL969) Km^R	Hille *et al.* (1983)
LBA2505	LBA969: ΔKpn10, Sp^R; *vir*A-mutant	Melchers *et al* (1987)
LBA2516	LBA969::*virB-lacZ*, Cb^R	This study
LBA2518	LBA969: Δ*Xho*I-fragment (position 457 to 550)[a], Sp^R; *virG* mutant	This study
LBA2520	LBA2505::*virB-lacZ*, Cb^R	This study
LBA2522	LBA2518::*virB-lacZ*, Cb^R	This study
Plasmid		
pNM482	Vector for *lac* gene fusions, Cb^R	Minton (1984)
pRAL3220	pBR322-(*Bam*HI-3), Cb^R	Melchers *et al.* (1987)
pRAL3227	pIC19R-(*Hpa*I-16) (cloned into *Sma*I-site)	This study
pRAL3260	pNM482 (*virB2-lacZ*); *virB-lacZ* fusion	This study
pAL969	Cointegrate R772::pTiB6, Km^R	Hille *et al.* (1983)
pAL2516	pAL969::*virB-lacZ*, Cb^R	This study

a. bp positions refer to the *virG* DNA sequence (Melchers *et al.*, 1986).

Bacterial strains and plasmids

All strains used in this study are listed in Table 3. Plasmid conjugal transfer was performed as described by Hooykaas *et al.* (1977), plasmid isolation was by the method of Birnboim and Doly (1979), and standard recombinant DNA procedures were according to Maniatis *et al.* (1982).

Media and antiobiotics

Escherichia coli strains were grown at 37°C in LC-medium (Hooykaas *et al.*, 1977) and *A. tumefaciens* strains were grown at 29°C in minimal medium (MM) (Hooykaas *et al.*, 1979). The induction medium (IM) routinely used for *Agrobacterium vir*-induction experiments contained minimal medium (MM) salts, 3% sucrose with 62.5 mM potassium phosphate pH 5.3. The MSSP-medium was used as described by Stachel *et al.* (1985).

Antibiotic concentrations for *Agrobacterium* strains were as follows: rifampicin, 20 μg ml^{-1}; spectinomycin, 250 μg ml^{-1}; carbenicillin, 100 μg ml^{-1}; and kanamycin, 100 μg ml^{-1}; and for *E. coli*: spectinomycin, 50 μg ml^{-1}; carbenicillin, 100 μg ml^{-1}; and kanamycin, 25 μg ml^{-1}.

Induction of agrobacteria

Bacteria were grown at 29°C in liquid minimal-medium (MM) overnight to an optical density (OD_{600}) of 0.7–0.8. Cells were pelleted by centrifugation and resuspended in 25 ml induction medium (IM) at a concentration of 5×10^8 bacteria per ml. Standard assays were done at 29°C, pH 5.3 with 0.2 mM acetosyringone (stock solution 0.2 M in DMSO) in IM. At various time intervals, two samples of 1 ml were taken from the culture. Bacteria were harvested by centrifugation and the pellet was resuspended in 1 ml of Z-buffer (Miller, 1972) and stored at 4°C. After collection of all samples, aliquots were assayed for β-galactosidase activity as described (Miller, 1972).

Construction of LBA2516

Restriction fragment *Hpa*I-16 of the octopine type Ti plasmid pTi15955 was subcloned from pRAL3220 (Melchers *et al.*, 1987) and inserted into the *Sma*I site of vector pIC19R (Marsch *et al.*, 1984) to produce the plasmid pRAL3227. A 1.3 kb *Hin*dIII fragment of pRAL3227, containing the promoter and 5′ end of the *virB* operon, was cloned into the unique *Hin*dIII site of the promoterless *lacZ* reporter construct, pNM482 (Minton, 1984). A recombinant clone was isolated (pRAL3260) in which the insertion of the 1.3 kb *Hin*dIII fragment resulted in a translational fusion of the 29th codon of the *virB2* gene with the 5th codon of the β-galactosidase gene. The *virB-lacZ* fusion construct, pRAL3260, was introduced into the Ti plasmid of *Agrobacterium* strain LBA969 via homologous recombination as described below. The stable R772::Ti cointegrate plasmid (pAL969) (Hille *et al.*, 1983) was transferred from LBA969 to the *E. coli* strain KMBL1164 (pRAL3260) via conjugation. Transconjugants were selected at 37°C in the presence of kanamycin and carbenicillin. Subsequently, one such transconjugant carrying pRAL3260 as well as pAL969 was mated with the *polA*-deficient *E. coli* strain, p3478. Propagation of plasmid pRAL3260 in the *polA*-recipient strain was only possible after its integration into plasmid pAL969 via homologous recombination. Transconjugants were selected on *E. coli* minimal medium agar plates supplied with 0.25%

thymine and the antibiotics kanamycin and carbenicillin. The cointegrate construct pAL969::pRAL3260 (named pAL2516) was transferred via conjugation to the *Agrobacterium* strain LBA288 to yield LBA2516. The recombinant Ti plasmid was checked via Southern blotting of *Agrobacterium* total DNA isolated from strain LBA2516.

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