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SPECIFICITY OF SIGNAL COMPOUNDS DETECTED BY *AGROBACTERIUM TUMEFACIENS**

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Abstract—*Agrobacterium tumefaciens* is known to respond to the presence of certain phenolic compounds by expressing genes prerequisite for transformation of plant cells. One report has described the isolation and identification of virulence-inducing acetosyringone and hydroxyacetosyringone from a host plant. Since these compounds have never previously been reported from plant tissues and are not likely of widespread occurrence, it seems unlikely that these are the only signal compounds for this wide host range pathogen. We report here the structure-activity specificity of *vir*-induction, and present results which indicate that *Agrobacterium* is capable of detecting compounds which are ubiquitous or at least widespread amongst its host range. Also, the *vir*-inducing activities of the lignin precursors coniferyl and sinapyl alcohols are presented and discussed in terms of the biology of *Agrobacterium*.

INTRODUCTION

Plant–microbe interactions must all be characterized initially by a stage in which the microbe detects a susceptible host. The mechanism of detection is on a chemical level, in which the host either normally exudes or under certain conditions produces compounds which act as signals for the microbial pathogen or symbiont. Presumably, the microbe responds to these signals by expression of genes necessary in subsequent stages of the interaction. In a few cases the signal compounds involved have been identified [1–4].

The study of signal compounds in relation to the biology of *Agrobacterium tumefaciens* is potentially of great importance. This is a gram-negative soil bacterium which causes crown gall disease of a wide variety of dicotyledonous plants [5]. The bacterium causes a neoplastic growth of the plant tissue by passing T-DNA, a part of its tumour inducing plasmid (pTi), into the host plant genome [6–11]. This T-DNA includes genes which encode enzymes of auxin [12, 13] and cytokinin [14, 15] biosynthesis, and these genes are expressed in the transformed plant cell [16, 17]. A useful vector for genetic engineering in plants may be constructed by replacing certain of the normal T-DNA genes with new genes of interest. Plant cells are then infected with the bacterium containing the modified Ti-plasmid and finally plants are regenerated from the transformed cells. This system has been successful in a number of cases [18]; however, it is ultimately dependent on the ability of the bacterium to detect and successfully transform the plant tissue.

Detection of susceptible host cells and early stages of tumorogenesis are mainly controlled by a set of pTi genes known as the virulence (*vir*) genes [19, 20]. These genes are expressed upon cocultivation of the bacteria with host plant cells [21, 22]. Because of their role in the early stages of tumorogenesis, and therefore their central importance in transformation of plant genomes, research has been directed to understanding the mechanism involved in *vir* gene expression and identifying the *vir* gene products. Two of the *vir* genes (A and G) are regulatory in nature [23, 24] *vir* A is also a host range determinant and is thought to be the environmental sensor of the plant derived inducer molecules [25]. At least one more *vir* locus (*vir*C) is connected with host range [26–28], and another (the *vir* D operon) is now known to encode an endonuclease which recognizes and cleaves the left and right border sequences of T-DNA [29]. Activation of *vir* gene expression is known to result in the production of multiple single-stranded T-DNA molecules within the bacterium [30].

Bolton *et al.* [31] found that a mixture of low M_r phenolic compounds could be used to induce expression of most of the *vir* genes, but quantitative analysis of *vir* gene induction by each component of the mixture was not reported. Stachel *et al.* [1] identified two active signal compounds, acetosyringone and hydroxyacetosyringone, from plant tissues. In that report a few other related compounds were assayed at one or more concentrations for their *vir*-inducing activity. This comprised a very brief structure–activity study which yielded useful but incomplete information about the structural features required to confer activity. At the concentrations tested none of these compounds displayed the level of activity observed with acetosyringone. It was implicit in these signal compound studies that *Agrobacterium* is attracted to susceptible plant tissues by following a concentration gradient of these virulence-inducing substances, and some results which support this idea were obtained by Ashby *et al.* [32].

* This work was presented at the Phytochemical Society of North America meeting in June at Tampa, Florida.

Thus the compounds acetosyringone and hydroxyacetosyringone have come to be regarded as the chemicals which *Agrobacterium* detects in nature and which trigger the initial events within the bacterium, resulting in tumour formation. Reports are appearing in the literature concerning the use of wound exudates from host plants or of acetosyringone to induce virulence of *Agrobacterium* and thereby extend the normal host range [33] or to boost transformation efficiency [34]. However, it has yet to be shown that these acetophenones are the signal compounds produced by all susceptible hosts. In fact, it may well be that other phytochemicals are involved in the induction of virulence in *Agrobacterium*.

The present work reports the *vir*-inducing activity over a range of concentrations of a variety of plant-derived phenolic compounds with structures related to that of acetosyringone and presents concise information regarding the structural features involved in the activation of *vir* genes. Included are the activities of some cinnamic acid derivatives, chalcones, and of the lignin precursors sinapyl alcohol and coniferyl alcohol. A number of these compounds are known to be of widespread occurrence,

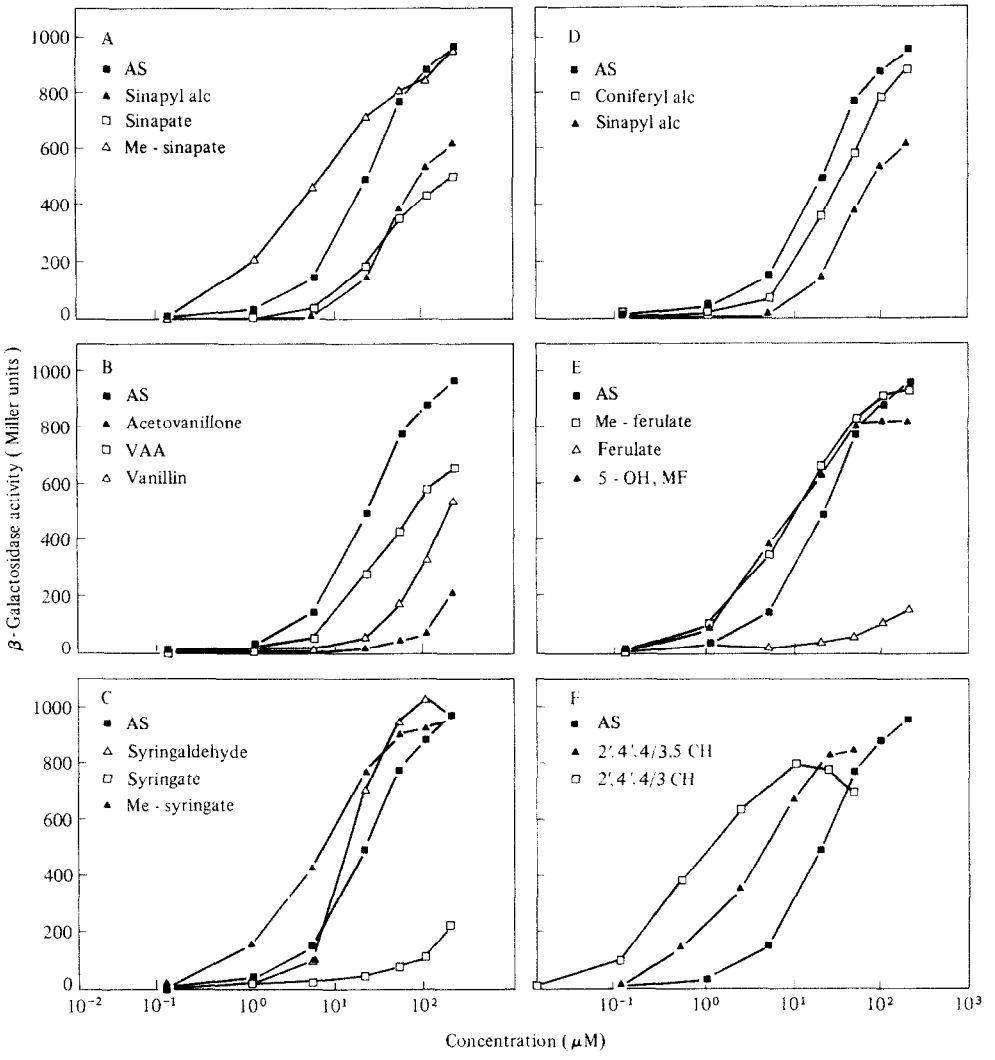


Fig. 1. The virulence inducing activity of a variety of phenolic compounds. Following incubation with a compound in aqueous solution, β-galactosidase activity in a strain of *Agrobacterium* carrying a *vir* E::*lac* Z fusion plasmid (A348/pSM358) was assayed as an indicator of *vir* gene induction. To assist in comparing the curves, the activity curve of acetosyringone is shown with each group of compounds. Shown in different panels are the activities of (A) sinapic (sinapinic) acid and related structures, (B) vanillin and related structures, (C) syringic acid and related structures, (D) the lignin precursors, (E) ferulic acid and related structures and (F) chalcones of guaiacyl and syringyl substitution. Abreviations used: AS, acetosyringone; Coniferyl alc., coniferyl alcohol; Sinapyl alc., sinapyl alcohol; Me-Sinapate, sinapic acid methyl ester; VAA, vanillalacetone; Me-syringate, syringic acid methyl ester; Me-ferulate, ferulic acid methyl ester; 5-OH,MF,5-hydroxyferulic acid methyl ester, 2',4',4/3,5 CH,2',4',4-trihydroxy-3,5-dimethoxy chalcone; 2',4',4/3 CH,2',4',4-trihydroxy-3-methoxy chalcone.

and the monolignols ubiquitous, amongst plants. The results are discussed in relation to their possible ecological and technological significance.

RESULTS AND DISCUSSION

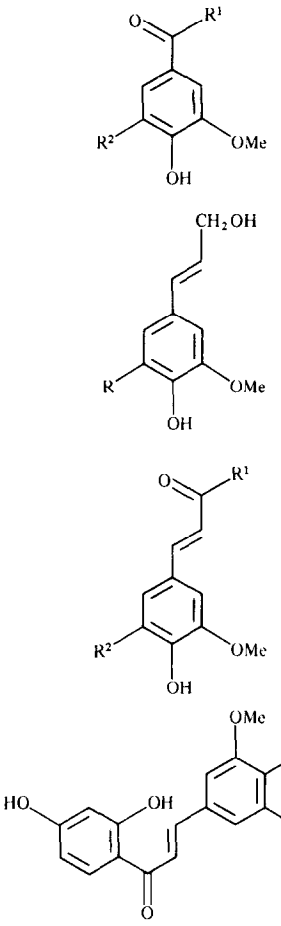
The structures of the 16 *vir*-inducing phenolic compounds examined may be assembled into four groups: (**1**) acetophenones and related structures, (**2**) monolignols, (**3**) structures related to cinnamic acid and (**4**) chalcone derivatives. Each structure contains a guaiacyl or syringyl nucleus, most possess a carbonyl group, and many are common plant-derived compounds. The dose-response curves obtained for these compounds are shown in Fig. 1. and are discussed below.

In Fig. 1D the activities of the lignin precursors sinapyl alcohol (**2b**) and coniferyl (**2a**) alcohol are shown. The activity of coniferyl alcohol approaches that of acetosyringone (**1d**) while that of sinapyl alcohol is somewhat less, for the greater part of its range approximating that of sinapic acid (**3c**)(Fig. 1A). To our knowledge this is the first report of virulence induction by compounds generally regarded as immediate precursors of lignin [35]. This important result establishes that *Agrobacterium* may be capable of detecting cells which are undergoing lignin synthesis or cell wall repair and thereby target those cells for transformation.

vir-Induction by ferulic acid (**3b**) (Fig. 1E), syringic acid (**1e**) (Fig. 1C) and acetovanillone (**1c**) (Fig. 1B) was significantly less than that of any of the other compounds tested. The low activity of acetovanillone (of guaiacyl substitution) in comparison with that of acetosyringone (of syringyl substitution) indicates that for acetophenones a syringyl nucleus is more effective at *vir*-induction. This supports the results of Stachel *et al.* [1] who, by using a *vir*B::*lac* Z strain of *Agrobacterium*, assayed the relative activities of these acetophenones at four concentrations. Vanillalacetone (**3a**) possesses a structure similar to that of acetovanillone except that its carbonyl group is separated from the guaiacyl nucleus by a C–C double bond, as is present in the cinnamic acid derivatives. Its dose-response curve (Fig. 1B) lies between that of the two acetophenones, indicating either that the double bond or simply the increased distance between the carbonyl group and the guaiacyl nucleus enhances the activity of the structure. However, the former appears to be the case because dihydroferulic acid, which is saturated at this bond, was found to be inactive.

Interestingly, the methyl esters of ferulic (**3d**), syringic (**1f**), and sinapic acids (**3f**) (Figs 1E, C, A) exhibited significantly greater activity than the corresponding free acids. The possible effects of this esterification are discussed below. The ethyl esters tested were less active again (data not shown), perhaps due to some steric hindrance at the bacterial receptor site not evident with the methyl esters.

The curves of activity induced by the chalcones (Fig. 1F) are markedly different than that of any of the other compounds tested. 2′,4′,4-Trihydroxy-3-methoxychalcone (**4a**) displayed its greatest *vir*-inducing activity at 10 μM. The maximum levels of induction by all of the other compounds (except syringaldehyde, **1b**) were obtained at the highest concentration tested, 200 μM. Unlike any of the other compounds, this chalcone was capable of low level *vir*-induction at 0.1 μM. The curve for 2′,4′,4-trihydroxy-3,5-dimethoxychalcone (**4b**) is shifted



	R^1	R^2
1a	H	H
1b	H	OMe
1c	Me	H
1d	Me	OMe
1e	OH	OMe
1f	OMe	OMe

	R
2a	H
2b	OMe

	R^1	R^2
3a	Me	H
3b	OH	H
3c	OH	OMe
3d	OMe	H
3e	OMe	OH
3f	OMe	OMe

	R
4a	H
4b	OMe

more to the right than the other chalcone, closer to that of acetosyringone and its maximum activity is observed at 50 μM. Perhaps this is as a result of the syringyl substitution of its B-ring, thereby affording a structure more similar to that of acetosyringone. Neither of these chalcones is very soluble in the aqueous medium used, but they do exhibit significantly greater activity at lower concentrations than do any of the simpler phenolics tested. A number of chalcones are known to exhibit biological activity [36] and it will be interesting to investigate whether chalcones play any part in *vir*-induction in nature.

Other compounds tested, most of which possessed guaiacyl or syringyl substitution patterns but which exhibited little or no *vir*-inducing activity, include the following: phloridzin, chrysosplenol-6-*C*-glucoside, homoeridiodictyol, tricin, 3,5,7,4′-tetrahydroxy-3′-methoxyflavonol, plicatic acid, conidendrin, substituted aurones, vanillic acid, vanilloyl methyl ketone, 5-hydroxyvanillin, dihydrodiferulic acid, 5-hydroxyferulic acid, isoferulic acid, glucoferulaldehyde, and syringic, ferulic and vanillic acid ethyl esters. Apparently, hydroxylation at the 5 position of any active compound possessing a guaiacyl nucleus decreases the compound's activity. This appears to be true even of 5-hydroxy ferulic acid methyl ester (**3e**), which retains activity similar to that of methyl ferulate, but reaches its maximum at a concentration of 50 μM (Fig. 1E). The lack of activity of 5-hydroxyferulic acid is of

interest because it has recently been found as one of a few cell wall bound cinnamic acid derivatives in monocots [37]. Whereas we have yet to demonstrate inhibition of *vir*-induction by a phenolic compound, these observations lead one to speculate about the possibility of phenolic *vir*-inhibitors, perhaps common to all monocots.

The structure of the aglycone of glucoferulaldehyde meets the putative requirements of an active signal compound (see below) and yet the glycoside itself was found to be inactive. Although only two phenolic glycosides were tested, the results indicate that, in the time allowed, *Agrobacterium vir* genes are not induced by such compounds. If the vacuolar phenolics, which must be exuded upon wounding, are a source of *vir*-inducing phytochemical precursors, then it appears that plant glucosidases must act to yield the effective compound. The activity of other glycosides such as coniferin [4-(3-hydroxy-1-propenyl)-2-methoxyphenyl-D-glucopyranoside] and isoconiferin [1-(4-hydroxy-3-methoxyphenyl)-propenyl-3-D-glucopyranoside] remain to be investigated. Coniferin is found in *Asparagus* [38], which appears to be the only monocot from which *Agrobacterium*-transformed tissue has been obtained by the usual methods [39].

In addition to the inactive compounds listed above, each of the compounds used by Bolton *et al.* [31] was assayed individually, and only vanillin (**1a**) resulted in any significant *vir*-induction (Fig. 1B). Interestingly, the remaining compounds (gallic, β-resorcylic, pyrogallic, *p*-hydroxybenzoic, and protocatechuic acids, and catechol) were essentially inactive. None of these inactive compounds possesses a guaiacyl or syringyl nucleus. It remains to be determined in what manner each of these compounds effects the activity curve of vanillin.

The results clearly indicate that two basic structural features together in general are required to confer activity upon a compound. These features are: (i) guaiacyl or (conferring enhanced activity) syringyl substitution on a benzene ring, and, with the exception of the monolignols, (ii) a carbonyl group on a substituent *para* to the hydroxy substituent on the ring. There are restrictions on the nature of the carbonyl carbon. It may be one or three carbon atoms removed from the ring. However, to confer maximal activity, in the latter case there must be a double bond between the carbonyl carbon and the ring, as is present in the chalcones and cinnamic acid derivatives. Furthermore, the carbonyl group of a free acid is less effective than that of the corresponding ester. Esterification alters the solubility of the compound. In addition, esterification blocks one oxygen of the carboxyl group from forming a partial double bond, thereby rendering the carbonyl group more reactive. In these cases, and the case of the aldehydes and chalcones, this carbonyl group forms the terminus of a long conjugated double bond system running from the hydroxyl group and through the ring. A completed C ring in the flavonoids tested virtually abolishes activity.

The structure–activity relationships reported here are somewhat different from those reported for the activation of *nod* genes in *Rhizobium* species [40]. Hydroxylated flavones or flavanones from nM to μM concentrations induce expression of *nod* genes [2–4]. Each *Rhizobium* species is highly specific for its host plant species and appears to exhibit a high degree of specificity towards its signal compound. The original strain of *Agrobacterium tumefaciens* from which the strain used in this study was derived exhibits a wide host range (WHR) and, as we have shown in this report, a comparatively much lower degree of signal compound specificity. Another potentially important piece to this biochemical puzzle is the finding that some of the very compounds which induce *vir* genes in *Agrobacterium* strongly inhibit *nod* gene activation by these flavonoids [4]. At higher concentrations most of the *vir*-inducing phenolics are bacteriostatic even against *Agrobacterium* (data not shown), and presumably they act in this way against *Rhizobium* species, or they may act more directly by competitive inhibition of *nod*-induction.

A number of the active compounds reported here are of widespread occurrence amongst dicotyledonous plants. Indeed, the lignin precursors must be ubiquitous amongst susceptible hosts. It would therefore be possible to conclude that presence of one or another of these compounds alone would determine whether a given plant is susceptible to infection by *Agrobacterium*. However, it is well known that monocots also produce such compounds, even exuding them into the rhizosphere from intact roots [41] and yet, with one exception [39], lie outside of the natural host range of any strain of *Agrobacterium*. This limitation of host range remains a significant problem in the use of this organism as a vector for genetic engineering in plants. It is hoped that a better understanding of the underlying mechanism of host range determination may be obtained through the results presented here, and perhaps also that this important barrier to Ti-plasmid mediated plant transformation may be overcome.

EXPERIMENTAL

The chemicals used in this study were commercially available, synthesized in our lab, or supplied by others (see Acknowledgements). Their purity was confirmed by TLC on silical gel or polyamide and where necessary purified by recrystallization or preparative TLC.

β-galactosidase activity was assayed as a measure of *vir*-gene induction in a strain carrying a *vir* E::*lac* Z gene fusion. The compounds tested were dissolved in DMSO and diluted in citrate–phosphate buffered pH 5.70 MS medium [42] to a final concn of 0.1% DMSO. 100 μl of bacterial cells from an overnight culture of A348/pSM358 [21] were inoculated into each 25×150 mm culture tube and subjected to continuous shaking at 200 rpm and at 28° for 8 hr to allow for induction of *vir* E::*lac* Z expression. Cell density was determined by measuring absorbance at 600 nm and 1 ml aliquots were removed for β-galactosidase assay essentially as described by Miller [43].

Each point on the activity curve of a test compound represents the average of the results of each concentration tested in triplicate. *vir*-Induction is strongly pH dependent ([22], our results, data not shown), so the buffer system was used to minimize variation due to pH shifts otherwise observed in the results. Standard deviations rarely reached 10%, the average being 4.7% ($n = 92$) for results of 100 Miller units and above. The curve shown for acetosyringone represents the average of the results of numerous assays conducted under identical conditions. Because acetosyringone has been reported as a *vir*-inducer, its curve of activity with increasing concentration is reported here as a standard with which to compare the activity of each of the other compounds assayed. Acetosyringone was used as a positive control in each experiment to ensure that the system was functioning correctly.

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