Plant Phenolic Compounds Induce Expression of the *Agrobacterium tumefaciens* Loci Needed for Virulence

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The virulence loci of *Agrobacterium tumefaciens* are a set of linked transcriptional units that play an essential role in the early stages of plant tumorigenesis. These loci are induced upon cocultivation of the bacteria with plant cells. Seven phenolic compounds that are widely distributed among the angiosperm plants—catechol, gallic acid, pyrogallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, β-resorcylic acid, and vanillin—are able to induce the expression of the virulence loci. These phenolics in combination induce each transcriptional locus of the *vir* loci. Furthermore, this induction displays similar kinetics and genetic control to that observed during cocultivation of the bacteria with plant cells.

IT HAS LONG BEEN RECOGNIZED THAT bacteria can sense and respond to their environment. For bacteria that interact with plants, recent data suggest that some of these bacteria respond to signals emanating from the plants. For example, during initiation of the symbiosis between *Rhizobium* and its leguminous plant hosts, the bacterial genes involved in the formation of the nitrogen fixing nodules that develop on the plant roots are specifically induced by root exudates (*1*). In another example, the formation of tumors on the dicotyledonous plants infected by *Agrobacterium* requires that a set of genes concerned with early stages of the infection process be expressed (*2*). These *Agrobacterium* genes are induced following contact with wounded plant cells (*3, 4*). We now report the results of studies to identify the plant signals that induce the virulence loci of *A. tumefaciens*.

Agrobacterium tumefaciens causes crown gall disease, a tumorous growth of dicotyledonous plant tissue (*5*). These soil bacteria gain access to plant tissue through a wound site. Binding of the bacteria to plant cells is most likely required for tumor formation, and the bacterial loci involved in binding map to the chromosome (*6*). Another set of genes, which is located on the tumor-inducing (Ti) plasmid that these bacteria harbor, is responsible for virulence (*vir*) and, in part, host range of *Agrobacterium* (*7*). The *vir* loci lie within a 35-kb region of the Ti plasmid (*2*), and they are induced during cocultivation of the bacteria with plant cells. These loci do not become incorporated into the plant genome. The tumor formed on the plant results after a 25-kb region of the Ti plasmid, the transferred DNA (T-DNA), becomes inserted into the genome of the plant cell (*8*). The T-DNA genes code for enzymes that catalyze the formation of the phytohormones auxin and cytokinin, and for opines, compounds characteristic of crown gall tumors (*9*). Production of the phytohormones gives rise to the tumorous growth on the plant. The opines produced are modified amino acids that provide a nutrient source for the bacteria. The mechanism of the transfer of T-DNA into the plant genome is not known, but appears to involve the *vir* loci (*2*).

It is possible to study the expression and regulation of a genetic locus of unidentified function by placing under its control a gene (reporter) that codes for a protein with easily measurable activity. This latter gene, with its regulatory signals removed, is inserted into the coding sequences of the locus under study to give a fusion protein that reports on the activity of the locus being studied (*10*). Fusion reporter genes have been constructed to investigate the developmental events leading to crown gall formation. Stachel *et al.* (*11*) constructed a Tn*3-lacZ* transposon system, Tn*3*-HoHol, that generates β-galactosidase proteins when placed under the control of either a bacterial or a plant locus. Using this system, Stachel *et al.* (*3*) demonstrated induction of the *vir* loci of *A. tumefaciens* strain A348 during cocultivation of the bacterial and plant cells, during cultivation in medium conditioned by plant cells, or with partially purified extracts of conditioned medium. They concluded that the *vir* loci located on the plasmid pTiA6NC of strain A348 were induced by one or more water-soluble plant metabolites, whose molecular weight is less than 1000.

Our approach to identifying the inducing substance (or substances) of the *A. tumefaciens vir* loci was to screen 40 commercially available chemicals, most of which are derivatives of plants (*12*). Our screening assay utilized a petri plate containing MS^- medium (*13*) incorporating the β-galactosidase substrate X-gal. The plate was spread with a lawn of A348(pSM358), which contains a *virE*–β-galactosidase fusion as a representative of the *vir* loci (Table 1), and a small amount of the compound being tested placed in the center of the plate. The data from these screening assays indicated that seven simple plant phenolic compounds induce the *virE* locus. They are: catechol, *p*-hydroxybenzoic acid, β-resorcylic acid, protocatechuic acid, pyrogallic acid, gallic acid, and vanillin. Our tests indicated that a *p*-hydroxyl group was required for all but one of the phenolics that induced *virE*. Analysis of *Nicotiana tabacum* cell extracts by high-pressure liquid chromatography and thin-layer chromatography confirmed the presence of all but one (vanillin) of these phenolics. The concentrations of these, among other phenolics detected, were comparable to the amounts used to induce the *vir* loci (Fig. 2). These seven phenolics are known to be constituents of intact and wounded plant cells and, although not found in this combination in every plant, nevertheless are widely distributed among the angiosperms (*14, 15*).

To confirm the results of the screening assays, we next determined the *vir*-inducing activity of these phenolics in liquid culture. Added singly, or in combinations of two, three, or four, *virE* induction was only three- to sixfold, considerably less than rou-

Table 1. The relevant characteristics of strains employed.

Plasmid or strain	Relevant phenotype or genotype	Reference
pVCK 219	Cosmid clone carrying PIN* or *virF*, *virA*, and a portion of *virB*	(*19*)
pVCK 242	Cosmid clone carrying *virB*, *virG*, and *virC*, and a portion of *virD*	(*19*)
pVCK 225	Cosmid clone carrying *virG*, *virC*, *virD*, *virE*, and a portion of *virB*	(*19*)
A348	pTiA6NC	(*19*)
A348(pSM202)	pVCK 219 with Tn*3*-HoHol inserted into *virA*	(*3, 11*)
A348(pSM30)	pVCK 242 with Tn*3*-HoHol inserted into *virB*	(*3, 11*)
A348(pSM379)	pVCK 225 with Tn*3*-HoHol inserted into *virC*	(*3, 11*)
A348(pSM304)	pVCK 225 with Tn*3*-HoHol inserted into *virD*	(*3, 11*)
A348(pSM358)	pVCK 225 with Tn*3*-HoHol inserted into *virE*	(*3, 11*)
A348(pSM363)	pVCK 225 with Tn*3*-HoHol inserted into *virG*	(*3, 11*)
A1030(pSM358)	A348 with Tn*5* inserted into *virA* of pTiA6NC and Tn*3*-HoHol inserted into *virE* of pVCK 225	(*3, 11*) Garfinckel and Nester (*2*)

*PIN, plant-inducible Ti-plasmid locus not required for virulence.

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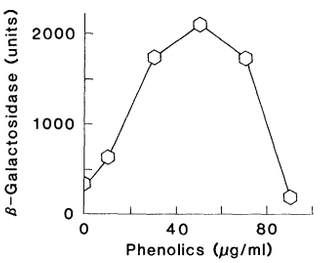

Fig. 1. Optimum concentration of phenolic compounds for the induction of *A. tumefaciens virE* locus. A348(pSM358) was assayed as described in the legend to Fig. 2 in MS* medium (*13*) with increasing concentrations of phenolic compounds (*16*).

tinely observed during cocultivation of the bacteria with plant cells. When the seven phenolics were combined, induction of *virE* was equal to or greater than that achieved during cocultivation of bacteria with plant cells (Fig. 2).

The optimum concentration of the combined phenolics for the induction of the *virE* locus of *A. tumefaciens* is presented in Fig. 1. The seven phenolics used to induce this locus are present at equal concentrations (*16*). No effort was made to optimize the concentration of each component. Concentrations of total phenolics in excess of about 70 μg/ml led to a sharp drop in the production of β-galactosidase activity because higher concentrations are bacteriostatic. This may indicate that phenolic concentrations in plant tissue above this amount will inhibit *Agrobacterium* during early stages of infection.

The *vir* loci of A348/pSM*vir* (Table 1) were induced under four different conditions (Fig. 2). The β-galactosidase activity produced by these bacterial strains was measured during their cultivation with phenolics, *N. tabacum* cell conditioned medium, or fresh medium, or by cocultivation with *N. tabacum* cells (*13*). Among the observations that can be made from this experiment is that the *virB, virC, virD, virE*, and *virG* loci were each induced more than 15-fold and the *virA* locus was induced less than twofold. This agrees with results obtained with partially purified extracts of plant cell conditioned medium (*3*).

Stachel *et al.* (*3*) reported that a functional *virA* locus is required for induction of the other *vir* loci. To further demonstrate that the phenolic compounds identified here are related to the natural inducing substance or substances, we utilized A1030, an A348 derivative that has a mutation in the *virA* locus of the pTiA6NC plasmid and a pSM358/*virE* plasmid. We found that a functional *virA* is required for induction of *virE* both by the phenolic compounds and during cocultivation with plant cells (Fig. 3). Similar data were obtained with plant cell conditioned medium. These data support the notion that the induction of the *vir* loci with these selected phenolic compounds and the inducer or inducers produced by plant cells is similar.

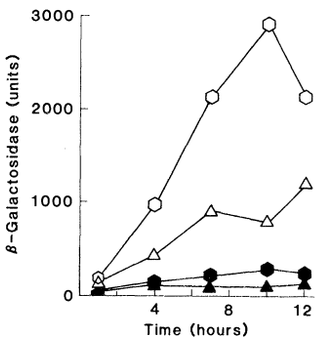

Fig. 3. Effect of a *virA* locus mutation on induction of the *virE* locus during cultivation of *A. tumefaciens* with phenolic compounds or cocultivation with *N. tabacum* cells. A348(pSM358), *virE* plus *virA*, and A1030(pSM358), *virE* minus *virA*, were assayed as described in the legend to Fig. 2; (⬡) A348(pSM358) and (⬢) A1030(pSM358) with 50 μg/ml total phenolic compounds (*16*); (△) A348(pSM358) and (▲) A1030(pSM358) during cocultivation with tobacco cells.

A previous study of the plant-derived compound showed that a metabolite with a molecular weight of less than 1000 induced the *vir* loci (*3*). We conclude from the experiments presented here that the inducer compound is most likely a simple plant phenolic of the type described above, and that induction of the *vir* loci may not be limited to a single unique phenolic structure. This is not unexpected in view of the fact that *Agrobacterium* has a wide host range and infects many dicotyledonous plants. A low molecular weight polypeptide has also been shown to induce the *vir* loci of *A. tumefaciens* (*4*).

It is known that a variety of phenolic compounds are released from intact plant cells and many more are released after a cell is wounded. Further, wounding appears to be a requirement for the initiation of many plant diseases (*15*). The availability of the Tn*3*-HoHol system should speed investigation of these interactions between host and parasite. The ability to induce the *A. tumefaciens vir* loci with commercially available compounds should greatly facilitate the analysis of the early stages of *Agrobacterium*-plant interactions (*17*).

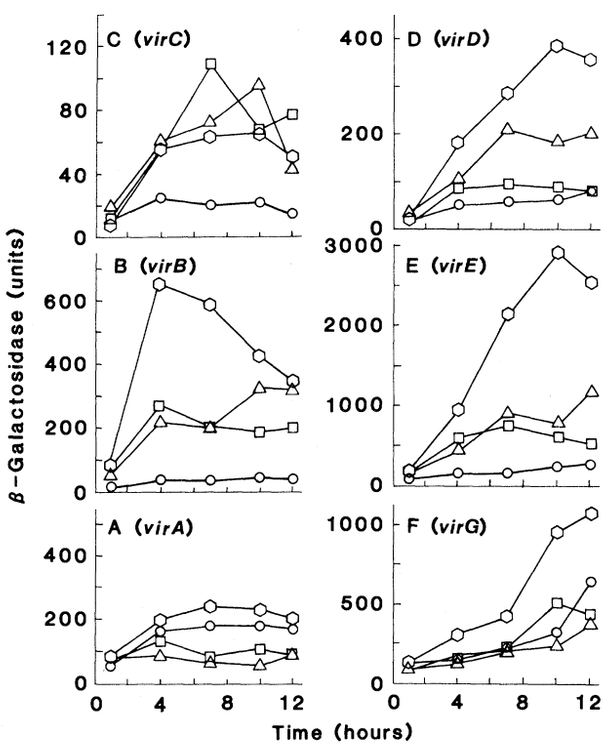

Fig. 2. Induction of *A. tumefaciens vir* loci during cultivation with phenolics, *N. tabacum* cell conditioned medium, or fresh medium, or by cocultivation with tobacco cells. A348 strains, each containing a different Tn*3*-HoHol plasmid (Table 1), were grown at 28°C on a reciprocating shaker (150 cycles per minute) in MG/L medium (*13*) to an $A_{600\ nm}$ of 1 to 2. Each bacterial strain (100 μl) was inoculated into 18 × 180 mm glass screw-top tubes containing 10 ml of the following MS* media: (⬡) to (○) with 50 μg/ml total phenolic compounds (*16*); (△) cocultivation with 3-day-old tobacco cells (*13*) (1 to 2 ml of cells per tube); (□) with medium conditioned for 3 days by tobacco cells; (○) fresh medium. The 24 tubes (four conditions per strain) were incubated at 28°C as described above. At the times indicated, 1.5-ml portions were withdrawn from each tube; 1 ml was used to determine the β-galactosidase activity (*18*) and 0.5 ml was used to measure the $A_{600\ nm}$ of the culture. The tubes containing plant cells stood for 5 minutes to allow plant cells to settle before bacteria were assayed. (A) pSM202/*virA*; (B) pSM30/*virB*; (C) pSM379/*virC*; (D) pSM304/*virD*; (E) pSM358/*virE*; and (F) pSM363/*virG*.

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12. Compounds that can induce the *virE* locus are: catechol (1,2-dihydroxybenzene), *p*-hydroxybenzoic acid (4-hydroxybenzoic acid), β-resorcylic acid (2,4-dihydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), pyrogallic acid (2,3,4-trihydroxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), and vanillin (3-methoxy, 4-hydroxybenzaldehyde). Other compounds tested are: anthrone, benzidine dihydrochloride, benzoic acid, chlorogenic acid, 2,4-dichlorophenoxyacetic acid, 2,3-dihydroxybenzoic acid, D-galacturonic acid, genetisic acid, hydroquinone, 8-hydroxyquinoline, indole, indoleacetic acid, napthalene, orcinol, *p*-aminobenzaldehyde, *p*-dimethylaminobenzaldehyde, *p*-hydroxybenzaldehyde, *p*-nitrophenol, poly-*N*-acetylglucosamine, polygalacturonic acid, pyrogallol, (−)-quinic acid, resorcinol, α-resorcylic acid, γ-resorcylic acid, salicylic acid, scopletin, (−)-shikimic acid, tannic acid, *trans*-cinnamic acid, traumatic acid, and vanillic acid.
13. Each experiment was conducted at least three times, variation in results was ±20 percent. The data presented in the figures represents results of one experiment. Experiments were carried out with the following: MS* medium (*p*H adjusted to 5.7) containing MS salts [T. Murashige, F. Skoog, *Physiol. Plant.* **15**, 473 (1962)] (4.3 g/liter, available from Gibco), thiamine (1 mg/liter), *myo*-inositol (0.1 g/liter), K_2HPO_4 (0.18 g/liter), 2,4-dichlorophenoxyacetic acid (2,4-D) (0.2 mg/liter). MS$^-$ medium is MS* (less the 2,4-D) with 0.6 percent (w/v) agar and 0.1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). MG/L medium is from J. E. Beringer [*J. Gen. Microbiol.* **84**, 188 (1974)]. *Nicotiana tobacum* cells were grown in MS* in 50-ml cultures. The tobacco cells were incubated in continuous light at 28°C on a rotary shaker (120 cycles per minute). Fresh cell cultures were started each week by using a 1-ml inoculum from the previous week's culture. Three-day-old tobacco cells were used in the cocultivation experiments. Conditioned medium was the supernatant from 3-day-old tobacco cell cultures.
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16. These seven phenolics were combined at 0.5 mg/ml in MS$^-$ medium (*13*) to make a stock solution.
17. Since this report was submitted, S. E. Stachel, E. Messens, M. Van Montagu, and P. Zambryski [*Nature (London)* **318**, 624 (1985)] have isolated several simple phenolics from conditioned medium, identified other commercially available phenolics, and have shown that they induce the *virB* locus. These phenolics are structurally similar to those described here.
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20. We thank Dr. Steve Winans, Dr. Vilas Sinkar, Jim Pringle, and Dean Regier for unpublished observations, Dr. Lisa Albright and Marty Yanofsky for discussions and help with bacterial strains, Dr. Gynheung An for suggesting cocultivation experiments, and Sharon Bradley for preparing the manuscript. We also thank Scott Stachel for an introduction to *vir* induction, and all other members of our laboratories for discussions. Supported by grant R01 GM32618 from the NIH and grant 84-CRCR-1-1438 from the USDA.

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Induction of Synaptic Potentiation in Hippocampus by Patterned Stimulation Involves Two Events

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Electrical stimulation of axons in the hippocampus with short high-frequency bursts that resemble in vivo activity patterns produces stable potentiation of postsynaptic responses when the bursts occur at intervals of 200 milliseconds but not 2 seconds. When a burst was applied to one input and a second burst applied to a different input to the same target neuron 200 milliseconds later, only the synapses activated by the second burst showed stable potentiation. This effect was observed even when the two inputs innervated completely different regions of the postsynaptic cells; but did not occur when the inputs were stimulated simultaneously or when the second burst was delayed by 2 seconds. Intracellular recordings indicated that the first burst extended the decay phase of excitatory postsynaptic potentials evoked 200 milliseconds later. These results suggest that a single burst of axonal stimulation produces a transient, spatially diffuse "priming" effect that prolongs responses to subsequent bursts, and that these altered responses trigger spatially restricted synaptic modifications. The similarity of the temporal parameters of the priming effect and the theta rhythm that dominates the hippocampal electroencephalogram (EEG) during learning episodes suggests that this priming may be involved in behaviorally induced synaptic plasticity.

EXCITATORY POSTSYNAPTIC POTENTIALS (EPSP's) in hippocampal neurons exhibit a persistent potentiation, termed long-term potentiation (LTP), following high-frequency stimulation (*1*). Similar effects are seen in other parts of the brain (*2*) and nervous system (*3*), and it has been proposed that the underlying mechanisms might be involved in memory storage (*4*). LTP is triggered by transient events in the postsynaptic neuron (*5–7*) and requires cooperative action among many synapses (*8, 9*). We recently reported that short bursts of high-frequency axonal stimulation (four pulses at 100 Hz) do not induce LTP unless repeated at intervals of less than 2 seconds; the optimal interburst interval proved to be 200 msec (*10*). These short bursts mimic a discharge pattern commonly seen in hippocampal pyramidal cells (*11*), and the optimal interburst interval corresponds to the theta EEG frequency found in exploring rats (*12*). The mechanisms through which naturally occurring activity patterns might produce synaptic modifications of the type represented by LTP are not known. One possibility is that each burst of high-frequency synaptic activity produces an effect at the activated synapses that accumulates maximally when the bursts are repeated at 200-msec intervals; this effect is then directly involved in producing LTP. Alternatively, a burst of synaptic activity might produce a diffuse "priming" effect, which is maximal 200 msec after the burst and alters the postsynaptic response to high-frequency activity at any synapses on the target neuron; these altered responses would then serve to trigger LTP. Our results support the priming mechanism and, in addition, demonstrate that primed responses directly induce LTP without the need for further stimulation-dependent processes. The findings clarify the nature and number of events that translate bursting activity into LTP and suggest that LTP is caused by a relatively simple physiological mechanism.

The experiments were conducted on slices of rat hippocampus with stimulating electrodes activating separate groups of Schaffer-commissural projections converging on a common set of CA1 pyramidal neurons. This preparation is used widely in studies of synaptic interactions in the hippocampus (*9, 13, 14*). A short burst was delivered to one set of fibers (S1) every 2 seconds, with each burst followed by an identical burst to the other set (S2) after a 200-msec delay (Fig. 1A). If LTP is caused by a specific accumulation mechanism, similar effects should have been seen on subsequent synaptic responses to each input (that is, LTP on each or no LTP on each) because each pathway received the same pattern of stimulation. If, however, induction of LTP

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