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Chemotaxis to Plant Phenolic Inducers of Virulence Genes Is Constitutively Expressed in the Absence of the Ti Plasmid in *Agrobacterium tumefaciens*

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The virulence (*vir*) genes are required in the early stages of plant tumor formation and are located together on the tumor-inducing (Ti) plasmid in *Agrobacterium tumefaciens*. Five of the *vir* genes are expressed inducibly in response to the following monocyclic phenolic compounds: acetosyringone, catechol, gallate, β-resorcylate, protocatechuate, *p*-hydroxybenzoate, and vanillin. Of these compounds, only the latter five served as chemoattractants and only the latter three served as growth substrates for *A. tumefaciens* A348. Strain A136, isogenic except for lack of the Ti plasmid, demonstrated chemotactic behavior and nutritional capabilities similar to those of strain A348. The chemotactic response to the *vir* gene inducers was expressed constitutively.

The diversity of plant phenolic compounds has been proposed to afford a rich source of developmental signals or chemoattractants in the establishment of specific symbiotic interactions between microbes and plants (15). In support of this hypothesis, complex plant phenolic flavones or flavanones have recently been found to induce expression of nodulation genes in the symbiotic bacteria *Rhizobium meliloti* (16), *Rhizobium trifolii* (17), and *Rhizobium leguminosarum* (24).

In addition to the role of complex phenolic compounds as inducers of nodulation in rhizobia, monocyclic phenolic compounds have been shown to trigger expression of virulence in the soil phytopathogen *Agrobacterium tumefaciens*. These bacteria enter plants at a wound site and cause tumors to form on a wide range of dicotyledonous plants (11, 23). Tumorigenesis is ultimately accomplished through the insertion of a 25-kilobase segment of bacterial DNA, the so-called T-DNA, into the plant genome (3, 10, 12, 22). The transfer of T-DNA into the plant genome is thought to be mediated by loci within a 35-kilobase stretch of DNA located, like the T-DNA, on a large (>200-kilobase) tumor-inducing (Ti) plasmid in the bacterium (5, 6, 9, 11). The loci for virulence comprise seven complementation groups or *vir* transcription units (20). Unlike the T-DNA, the seven *vir* loci are not themselves incorporated into the plant cell nucleus. A study of the regulation of transcriptional fusions between *vir* promoters and the *Escherichia coli lacZ* gene revealed that acetosyringone, a phenolic compound exuded by metabolically active, wounded tobacco cells, serves as an effective inducer of *vir* genes (19). In addition, a mixture of several phenolic compounds at low concentrations can induce the expression of five of the *vir* loci to levels comparable to those obtained with acetosyringone; each phenolic compound alone has a fraction of the effect (2). One of the *vir* genes is expressed constitutively (21). Also expressed constitutively are two chromosomal virulence loci controlling attachment of the bacterium to the plant cell (4). Members of the family *Rhizobiaceae*, which includes *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* spp., are flagellated bacteria. At least one species of this group, *Bradyrhizobium japonicum*, responds behaviorally to diverse phenolic compounds by positive chemotaxis, i.e., by sensing a chemical and moving up a concentration gradient (15). Our study was undertaken to determine whether any of the phenolic compounds which induce expression of the *vir* genes also serve as chemoattractants or nutrients for *A. tumefaciens*. In addition, we investigated whether genetic loci for the catabolism or sensing of phenolic inducers or both are encoded by genes on the Ti plasmid.

Bacterial strains, conditions of growth, and chemotaxis assays. *A. tumefaciens* A136 is a derivative of C-58 cured of its Ti plasmid; it contains a single cryptic plasmid, pAtC58 (18). *A. tumefaciens* A348 is isogenic with strain A136 except that it carries the octopine Ti plasmid, pTiA6 (7). Both strains were enriched for uniformly motile cells as described previously (15) and were maintained on Luria broth plates. Cells were prepared for chemotaxis assays by procedures used previously with *R. trifolii* (15). Cells of both strains were frequently tested for their differential ability to utilize octopine, indicating the presence or absence of the Ti plasmid. In addition, strain A348 caused tumors to form when inoculated on *Kalanchoe daigremontiana*. Cells were prepared for auxanography as described previously for *Agrobacterium* (13). Auxanography results were confirmed by viable cell counts of cells grown in liquid medium containing a 10^{-4} M carbon source.

A capillary assay by the method of Adler (1) was used at 30°C to measure chemotaxis. Methods for performing and analyzing the capillary assay have been described in detail (15). Cells which entered a capillary tube were plated onto Luria broth plates, and colonies were counted after 2 days at 30°C. Concentrations of attractant from 10^{-6} to 10^{-1} M were tested; acetosyringone (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was tested at 10^{-8} to 10^{-6} M as well. The standard deviation for the attraction of cells into a capillary tube containing 0.1% yeast extract for assays performed on 22 different days was 20%. The number of cells entering a capillary tube containing buffer with no attractant ranged from 800 to 3,400.

Nutritional response to aromatic compounds. *A. tumefaciens* A348 and A136 were similar in their ability to utilize carbon sources tested on gradient plates. Growth was posi-

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TABLE 1. Chemotactic responses of *A. tumefaciens* A348

Compound	Doubling time (h)[a]	Threshold concn (M)	Peak concn (M)	Peak response[b]
Catechol	NG	10^{-6}	10^{-2}–10^{-1}	53,500
Gallate	NG	3×10^{-7}	10^{-3}	38,200
p-Hydroxybenzoate	2.5	5×10^{-6}	10^{-3}–10^{-2}	55,500
Protocatechuate	ND	10^{-6}	10^{-2}–10^{-1}	34,000
Quinate	2.5	10^{-6}	10^{-2}	90,400
β-Resorcylate	NG	5×10^{-7}	10^{-3}–10^{-2}	47,000
Shikimate	5.0	10^{-6}	10^{-2}–10^{-1}	92,400
Succinate	2.0	10^{-6}	10^{-2}	95,000

[a] NG, No growth; ND, not determined.

[b] Reported as the number of cells that entered a capillary tube containing the peak concentration of attractant minus the number of cells entering a capillary tube containing no attractant. Cells were harvested at an optical density of 0.36 to 0.44 at 600 nm and were suspended at a density of 2×10^8 cells ml^{-1}.

tive with quinate, shikimate, protocatechuate, vanillin, and *p*-hydroxybenzoate. Of these substrates, only the latter two compounds were toxic, as revealed by an arc of growth away from the origin of substrate application to the plate. Compounds which failed to support growth included acetosyringone, catechol, gallate, and β-resorcylate. The results with gradient plates were confirmed by incubating cells at low inoculum densities with carbon sources at a concentration of 10^{-4} M.

Chemotactic response to aromatic compounds. A number of the aromatic compounds that served as inducers of *vir* genes were also chemoattractants, regardless of whether they were metabolizable (Table 1). Absence of the Ti plasmid had no effect on the attraction of cells to any of the compounds; virtually identical results were obtained with strains A136 and A348. The nutrients *p*-hydroxybenzoate and protocatechuate were chemoattractants, as were the nonmetabolizable compounds catechol, gallate, and β-resorcylate. In addition to responding positively to five compounds which induce the expression of *vir* genes, both *Agrobacterium* strains were attracted to shikimate, quinate, and succinate (Table 1), which are common growth substrates for members of the *Rhizobiaceae* (13). The threshold concentrations for these compounds were around 10^{-6} M, with the peak concentrations being 10^{-3} to 10^{-1} M; most of the concentration-response curves had broad peaks.

Two compounds which served as inducers of the *vir* genes, vanillin and acetosyringone, share some structural resemblance in that both of them contain a 3′-methoxy and a 4′-hydroxyl group. Neither of these compounds elicited a chemotactic response. Vanillin was toxic even at 2 mM, and therefore cells were grown at the expense of quinate in the presence of 10^{-4} or 10^{-3} M vanillin. Cells of strain A348, grown under standard conditions for these assays with or without 10^{-4} M acetosyringone and rinsed twice to remove residual acetosyringone, failed to exhibit any response to this compound above background. Acetosyringone was tested in 5-fold increments in concentrations from 10^{-8} to 10^{-6} M or in 10-fold increments from 10^{-6} to 10^{-2} M. Similar negative results for acetosyringone were obtained when A348 cells were grown on Luria broth, rinsed twice, and suspended in the chemotaxis buffer of Adler (1). Others have reported that acetosyringone was a positive chemoattractant for *A. tumefaciens* C58C at 10^{-7} M under the second set of conditions described above and that the positive response required the presence of the Ti plasmid, pTiBS3 (C. H. Shaw, personal communication).

In all cases of positive chemotaxis, the responses of cells were dependent on a concentration gradient; positive responses were not observed when each chemoattractant was present in the pool with the cells and in the capillary tube. Attractants were added to cell suspensions immediately before incubation at 30°C and were tested at 10^{-4}, 10^{-3}, or 10^{-2} M, depending on the compound. The possibility remained that *A. tumefaciens* might have been responding to the sodium ions used in neutralizing the acids. Sodium hydroxide at 10^{-6} to 10^{-3} M did not stimulate cell motility or chemotaxis.

Regulation of chemotaxis towards phenolic and hydroaromatic compounds in *A. tumefaciens*. Chemotaxis to all of the positive chemoattractants examined in this work was expressed constitutively in *A. tumefaciens* A348 and A136. Cells grown at the expense of succinate showed levels of attraction to shikimate, quinate, catechol, β-resorcylate, gallate, protocatechuate, and *p*-hydroxybenzoate equal to those observed under inducing conditions of growth. In testing the response to shikimate, quinate, and *p*-hydroxybenzoate, cells were grown at the expense of the homologous substrate; in testing protocatechuate, cells were grown at the expense of quinate. In the case of catechol, gallate, and β-resorcylate, cells were grown at the expense of quinate in the presence of a 10^{-4} or 10^{-3} M concentration of the chemoattractant being tested.

Another member of the family *Rhizobiaceae*, *B. japonicum* I-110, exhibited a constitutive chemotactic response to β-ketoadipate (15) which appears to be related to constitutive synthesis of enzymes giving rise to and degrading this catabolite (14). Therefore, we checked *A. tumefaciens* A348 to see whether, unlike the *Agrobacterium* strains previously examined (14), this strain produced enzymes of protocatechuate catabolism constitutively. Like the other *Agrobacterium* strains, however, strain A348 possessed inducible enzymes for the β-ketoadipate pathway. Thus, the constitutive chemotactic response to phenolic compounds was expressed independently of the inducible synthesis of enzymes for catabolism of some of the compounds.

Properties and selective value of chemoreceptors in *A. tumefaciens*. The finding that *A. tumefaciens* has a positive chemotactic response to seven aromatic and hydroaromatic compounds raises the question of whether the identified compounds are attractants or whether they are converted to a metabolite or metabolites that interact with one or more chemoreceptors. It seems unlikely that all seven of the aromatic and hydroaromatic compounds share a common, constitutively expressed chemoreceptor. The structures of quinate, catechol, and *p*-hydroxybenzoate are dissimilar, and catechol, gallate, and β-resorcylate cannot be metabolized to a compound in common with metabolites of the other four attractants. It appears more likely that there are a number of distinct chemoreceptors for these molecules. Thus, *A. tumefaciens* has committed a portion of its genome and biosynthetic apparatus to continuously produce chemoreceptors or methyl-accepting chemotactic proteins or both.

We have shown that there is no relationship between the ability of the phenolic compounds which induce the *vir* genes to be metabolized and their recognition as chemoattractants. In addition, not all of the compounds that induce the *vir* genes appear to serve as chemoattractants. Our failure to observe chemotaxis to acetosyringone, in the face of evidence to the contrary (C. H. Shaw, personal communication), is difficult to explain. The phenotype may be strain dependent and may not be essential to virulence. It is possible that vanillin and acetosyringone are not encountered widely in association with plants by *A. tumefaciens*.

The presumptive chromosomal location of the genes which code for chemoreceptors associated with *vir* gene inducers indicates that *A. tumefaciens* strains not harboring the Ti plasmid will be attracted to sites in planta where they may acquire the Ti plasmid from virulent strains.

Generally, the unregulated synthesis of proteins is associated with functions that are used frequently or are essential for survival of the bacterium. Phenolic acid catabolism proceeds by pathways that are peripheral and seemingly expendable, at least to survival in the laboratory. In most bacteria, peripheral catabolic pathways are strongly regulated by induction and repression, and the expression of enzymes for the catabolism of protocatechuate in *Agrobacterium* spp. follows this pattern. The bradyrhizobia are an exception in expressing enzymes of the β-ketoadipate pathway constitutively (14) and in possessing an unregulated chemotactic system which responds strongly to β-ketoadipate (15). The constitutive chemotactic systems for phenolic compounds and β-ketoadipate in *Agrobacterium* and *Bradyrhizobium* spp., respectively, indicate that the compounds are significant signals for these organisms in the natural environment.

In coliform bacteria, constitutively synthesized chemotactic systems recognize nitrogenous compounds (8). The coliforms appear to reserve a relatively unregulated commitment of resources to compounds which provide both carbon and nitrogen. The fact that *A. tumefaciens* possesses constitutively synthesized chemoreceptors for nonnitrogenous compounds suggests that certain phenolic compounds may signal the proximity of a nitrogen-rich niche to the phytopathogen.

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LITERATURE CITED

1. **Adler, J.** 1973. A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. J. Gen. Microbiol. **74**:77–91.
2. **Bolton, G. W., E. W. Nester, and M. P. Gordon.** 1986. Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. Science **232**:983–985.
3. **Chilton, M.-D., M. H. Drummond, D. J. Merlo, D. Sciaky, A. L. Montoya, M. P. Gordon, and E. W. Nester.** 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell **11**:263–271.
4. **Douglas, C. J., R. J. Staneloni, R. A. Rubin, and E. W. Nester.** 1985. Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. J. Bacteriol. **161**: 850–860.
5. **Garfinkel, D. J., and E. W. Nester.** 1980. *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J. Bacteriol. **144**:732–743.
6. **Klee, H. J., M. P. Gordon, and E. W. Nester.** 1982. Complementation analysis of *Agrobacterium tumefaciens* Ti plasmid mutations affecting oncogenicity. J. Bacteriol. **150**:327–331.
7. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8**:45–54.
8. **Koshland, D. E., Jr.** 1980. Bacterial chemotaxis as a model behavioral system, p. 20–22. Raven Press, New York.
9. **Koukolikova-Nicola, Z., R. D. Shillito, B. Hohn, K. Wang, M. Van Montagu, and P. Zambryski.** 1985. Involvement of circular intermediates in the transfer of T-DNA from *Agrobacterium tumefaciens* to plant cells. Nature (London) **313**:191–196.
10. **Leemers, M., M. DeBeuckeleer, M. Holsters, P. Zambryski, A. Depicker, J. P. Hernalsteens, M. Van Montagu, and J. Schell.** 1980. Internal organization, boundaries and integration of Ti-plasmid DNA in nopaline crown gall tumours. J. Mol. Biol. **144**: 353–376.
11. **Nester, E. W., M. P. Gordon, R. M. Amasino, and M. F. Yanofsky.** 1984. Crown gall: a molecular and physiological analysis. Annu. Rev. Plant Physiol. **35**:387–413.
12. **Ooms, G., A. Bakker, L. Molendijk, G. J. Wullems, M. P. Gordon, E. W. Nester, and R. A. Schilperoort.** 1982. T-DNA organization in homogeneous and heterogeneous octopine-type crown gall tissues of *Nicotiana tabacum*. Cell **30**:589–597.
13. **Parke, D., and L. N. Ornston.** 1984. Nutritional diversity of Rhizobiaceae revealed by auxanography. J. Gen. Microbiol. **130**:1743–1750.
14. **Parke, D., and L. N. Ornston.** 1986. Enzymes of the β-ketoadipate pathway are inducible in *Rhizobium* and *Agrobacterium* spp. and constitutive in *Bradyrhizobium* spp. J. Bacteriol. **165**:288–292.
15. **Parke, D., M. Rivelli, and L. N. Ornston.** 1985. Chemotaxis to aromatic and hydroaromatic acids: comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolii*. J. Bacteriol. **163**: 417–422.
16. **Peters, N. K., J. W. Frost, and S. R. Long.** 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233**:977–980.
17. **Redmond, J. W., M. Batley, M. A. Djordjevic, R. W. Innes, P. L. Kuempel, and B. G. Rolfe.** 1986. Flavones induce expression of nodulation genes in *Rhizobium*. Nature (London) **323**: 632–635.
18. **Sciaky, D., A. L. Montoya, and M.-D. Chilton.** 1977. Fingerprints of *Agrobacterium* Ti-plasmids. Plasmid **1**:238–253.
19. **Stachel, S. E., E. Messens, M. VanMontagu, and P. Zambryski.** 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature (London) **318**:624–629.
20. **Stachel, S. E., and E. W. Nester.** 1986. The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. **5**:1445–1454.
21. **Stachel, S. E., E. W. Nester, and P. C. Zambryski.** 1986. A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. Proc. Natl. Acad. Sci. USA **83**:379–383.
22. **Thomashow, M., R. Nutter, A. L. Montoya, M. P. Gordon, and E. W. Nester.** 1980. Integration and organization of Ti plasmid sequences in crown gall tumors. Cell **19**:729–739.
23. **Turgeon, R.** 1981. Suppression of, and recovery from, the neoplastic state. Int. Rev. Cytol. **13**(Suppl.):59–81.
24. **Zaat, S. A. J., C. A. Wijffelman, H. P. Spaink, A. A. N. Van Brussel, R. J. H. Okker, and B. J. J. Lugtenberg.** 1987. Induction of the *nodA* promoter of *Rhizobium leguminosarum* Sym plasmid pRL1JI by plant flavanones and flavones. J. Bacteriol. **169**:198–204.