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Ti Plasmid-Specified Chemotaxis of *Agrobacterium tumefaciens* C58C[1] toward *vir*-Inducing Phenolic Compounds and Soluble Factors from Monocotyledonous and Dicotyledonous Plants

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Twelve phenolic compounds with related structures were analyzed for their ability to act as chemoattractants for *Agrobacterium tumefaciens* C58C[1] and as inducers of the Ti plasmid virulence operons. The results divided the phenolic compounds into three groups: compounds that act as strong *vir* inducers and are chemoattractants for *A. tumefaciens* C58C[1] harboring the nopaline Ti plasmid pDUB1003Δ31, but not the isogenic cured strain; compounds that are at best weak *vir* inducers and are weak chemoattractants for Ti plasmid-harboring and cured *A. tumefaciens* C58C[1]; and compounds that are *vir* noninducers and are also nonattractants. A strong correlation between *vir*-inducing ability and Ti plasmid requirement for chemotaxis is thus established. In addition, chemical structure rules for *vir* induction and chemotaxis are outlined. Positive chemotaxis toward root and shoot homogenates from monocotyledonous and dicotyledonous plants was observed. At low extract concentrations, chemotaxis was enhanced by the presence of Ti plasmid. The chemoattractants do not derive from intact cell walls. Lack of attraction is not responsible for the apparent block to monocot transformation by *A. tumefaciens*.

Plant wounding is a prerequisite for crown gall tumor induction by Ti-plasmid-harboring *Agrobacterium tumefaciens*. The Ti plasmid virulence operons, induced by wound exudates such as acetosyringone (24, 32, 33), mediate T-DNA transfer to the plant cell, where neoplastic overgrowth results from its expression (19, 20, 23).

A. tumefaciens C58C[1] possesses a highly sensitive chemotaxis system, which responds to a range of sugars and amino acids (19a). For example, the chemotactic peak for sucrose occurs at 10^{-6} M. Thus, release of these compounds from plant roots and chemotaxis of *A. tumefaciens* toward them can account for the prevalence of the bacterium in the rhizosphere (15, 16).

The majority of chemotactic responses in *A. tumefaciens* appear to be chromosomally encoded (3, 19a, 25). However, chemotaxis toward acetosyringone, one of the major plant phenolic inducers, requires the presence of a Ti plasmid and occurs with a threshold sensitivity of $<10^{-8}$ M (3), some 1,000-fold below the maximal *vir*-inducing concentration (32). *Agrobacterium* strains harboring either octopine or nopaline Ti plasmids respond at this concentration (29), indicating that chemotaxis is important in guiding virulent *A. tumefaciens* toward a susceptible plant (30). The Ti plasmid genes involved in this specific chemotactic response are *virA* and *virG* (29). *virA* and *virG* are also involved in mediating *vir* induction in response to acetosyringone (18, 21, 34, 37). This suggests a multifunctional role for *virA* and *virG*: at low concentrations of acetosyringone they trigger chemotaxis, whereas at high concentrations *vir* induction is effected.

To confirm and extend our previous observations, we embarked upon a survey of the *vir*-inducing and chemotactic properties of a variety of related phenolic compounds found in plant exudates. We present evidence indicative of a strong correlation between the ability of a phenolic compound to be a *vir* inducer and its requirement for a Ti plasmid function in chemotaxis. To correlate these observations with the in planta situation, we attempted to demonstrate attraction of *A. tumefaciens* toward natural plant extracts. We report chemotaxis of *A. tumefaciens* toward soluble factors from monocotyledonous and dicotyledonous plants which, at low attractant concentrations, is enhanced by the presence of Ti plasmids. This suggests that *A. tumefaciens* recognizes, and is attracted to, both monocots and dicots, and that this step is not the basis of the apparent block to monocot transformation.

MATERIALS AND METHODS

Bacterial strains and growth media. Chemotaxis assays were performed with the highly motile (19a) strain *A. tumefaciens* C58C[1] (36), either cured of its Ti plasmid or harboring the nopaline Ti plasmid pDUB1003Δ31 (31), a Km^r derivative of the *tra*(Con) occ^+ pTiC58 isolate pGV3105 (13), or the octopine Ti plasmid pTiB6S3 (9). All strains were periodically enriched for motile bacteria by passage through swarm plates (19a). Bacterial cultures were grown in either L broth or minimal A medium supplemented with glucose (22, 29), centrifuged, washed, and suspended in chemotaxis medium (1, 3).

Induction assays. Phenolic compounds were assayed for induction of the virulence region by using *A. tumefaciens* A348(pSM30) streaked onto plates containing MS plus 0.1 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) · ml^{-1}, 1% Bacto-Agar (Difco Laboratories), and 50 μM phenolic compound (pH 5.7 with potassium phosphate buffer [5, 17, 32]). The plates were incubated at 28°C for 3 days and compared with a control plate lacking the phenolic compound.

Shoot homogenates. Equal weights of plant aerial parts were homogenized in chemotaxis medium (CM) (1) in a Waring blender, and the homogenates were centrifuged at 9,000 × *g* for 10 min, filter sterilized, and adjusted to the same total protein concentration.

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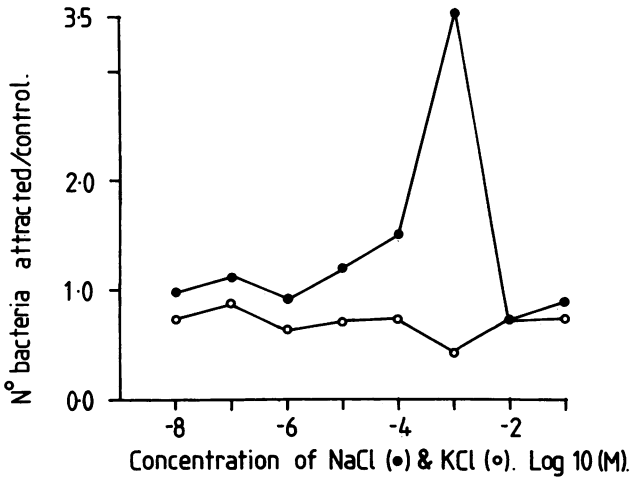


FIG. 1. Dose-response curve of motility of *A. tumefaciens* C58C^1 toward sodium chloride (●) and potassium chloride (○).

Protoplast isolation. After the lower epidermis was removed from surface-sterilized tobacco SR1 leaves, they were cut in half and incubated in the dark for 16 h in CPW10 (26); half of each leaf was digested with 0.4% cellulase plus 0.1% macerozyme, and the other half was not. The nondigested leaf half was then homogenized as described above. Large material was removed from the digested half, and the protoplasts were sedimented at 50 × *g* for 10 min and suspended in 10 ml of CPW20 plus CPW10. After centrifugation for 10 min at 50 × *g*, the protoplast interface was removed and washed twice in 10 ml of CPW10. After microscopic enumeration, half the protoplasts were sonicated in CM, and the other half was regenerated in T_0 (6) for 48 h, until cell walls were visible by Calcofluor (Sigma) staining and then sonicated in CM.

Chemotaxis assays. Both the capillary assay (1) and the modified blind-well chamber assay (2, 29) were used. Incubation times were 90 min at room temperature for the blind-well assay and 60 min for the capillary assay. All phenolic compounds were neutralized with potassium hydroxide, filter sterilized before use, and assayed in the concentration range 10^{-2} to 10^{-8} M in triplicate for capillary assays and in quadruplicate for blind-well assays. Bacteria attracted into capillaries were plated out onto selective media (3). Bacteria swimming into the upper chamber of the blind-well apparatus in response to an attractant were counted after dilution in Isoton (Coulter Electronics, Inc.) by using a model TAII/PCA Coulter Counter. The numbers of bacteria attracted were divided by control values derived from assays with chemotaxis medium, and the results were plotted.

Microscopy. After washing and resuspension in chemotaxis medium, the motility of each strain was checked by using a Nikon Optiphot microscope with phase-contrast optics.

RESULTS

Chemotaxis toward sodium chloride. It was important to ensure constant assay pH, since fluctuations from neutrality could result in spurious chemotactic responses evoked by H^+ ions. This could mask an attraction or produce a false-positive, particularly for compounds with chemotactic optima at high concentrations. Thus, it was crucial to neutralize phenolic acid solutions with NaOH or KOH. Chemotaxis toward NaCl was assayed by using both *A. tumefaciens* C58C^1 and C58C^1(pDUB1003Δ31) to eliminate the possibility that Na^+ ions were chemoattractants (Fig. 1). However, a response was observed with both strains at 10^{-3} M, indicating chemotaxis toward either Na^+ or Cl^- ions. To determine which was responsible, we assessed chemotaxis toward KCl. Chemotaxis medium consists of 10^{-2} M KPO_4 and 10^{-4} M dipotassium EDTA. Thus, any effects at 10^{-3} M for K^+ ions would be saturated. Therefore, a response with KCl would be indicative of attraction toward Cl^- ions only. No response was observed toward KCl (Fig. 1). Thus, the response observed with NaCl was a result of attraction to, or motility enhancement by, Na^+ ions. As a result, for all experiments, phenolic compounds were neutralized with KOH.

***vir* induction and chemotaxis toward aromatic compounds.** A total of 12 phenolic compounds were compared for their abilities to induce the *virB-lacZ* fusion plasmid pSM30 (32) and as attractants for *A. tumefaciens* C58C^1 and *A. tumefaciens* C58C^1(pDUB1003Δ31). Chemotaxis was generally assessed by using the blind-well chemotaxis assay. However, for vanillin and acetosyringone, both the capillary and blind-well assays were used.

The results obtained divide the phenolic compounds into three main categories (Table 1). The first category consists of compounds that are strong to moderate inducers of the virulence region and chemoattractants for Ti plasmid-harboring strains only. These include acetosyringone (chemotactic optimum, 10^{-7} M [3, 29]), sinapinic acid (optimum, 10^{-7} [Fig. 2a]), syringic acid (optimum, 10^{-7} M [Fig. 2b]), ferulic acid (optimum, 10^{-4} M [Fig. 3a]), vanillin (optimum, 10^{-4} M [Fig. 3b]), and 3,4-dihydroxybenzoic acid (optimum, 10^{-2} M [data not shown]). This category may represent two subcategories differentiated by the sensitivity of the chemotactic response. Thus, acetosyringone, sinapinic acid, and syringic acid, which evoke extremely sensitive responses, would belong in one subcategory, whereas vanillin, ferulic acid, and 3,4-dihydroxybenzoic acid, which evoke responses several orders of magnitude less sensitive, would be members of a second subcategory.

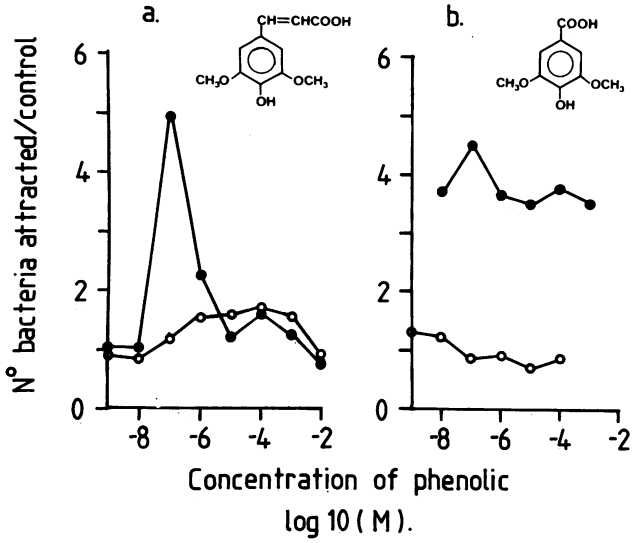
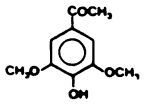
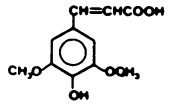
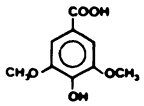
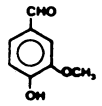
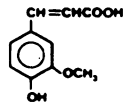
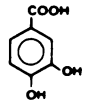
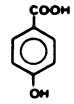
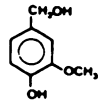
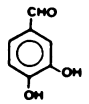
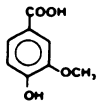
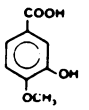


FIG. 2. Dose-response curve of motility of *A. tumefaciens* C58C^1(pDUB1003Δ31) (●) and *A. tumefaciens* C58C^1 (○) toward (a) sinapinic acid and (b) syringic acid.

TABLE 1. Correlation of *vir*-inducing ability and chemoattractant properties of phenolic compounds

Plant Phenolic	Structure	Vir-inducer	Chemotaxis	Peak Conc^{n}	Ti plasmid required
Acetosyringone		+[a]	+	10^{-7}	+
Sinapinic acid		+[a]	+	10^{-7}	+
Syringic acid		+[a]	+	10^{-7}	+
Vanillin		+[b]	+	10^{-4}	+
Ferulic acid		+[c]	+	10^{-4}	+
3,4 dihydroxy - benzoic acid		+[b]	+	10^{-2}	+
Catechol		±[b]	+	10^{-2}	-
p-hydroxy benzoic acid		±[b]	+	10^{-3}	-
Vanillyl alcohol		-[c]	+	10^{-2}	-
3,4 dihydroxy - benzaldehyde		-[c]	+	10^{-2}	-
Vanillic acid		-[b]	-	NC[d]	NC
Isovanillic acid		-[c]	-	NC	NC

[a] From reference 32.
[b] From reference 5.
[c] See text.
[d] NC, Nonchemotactic.

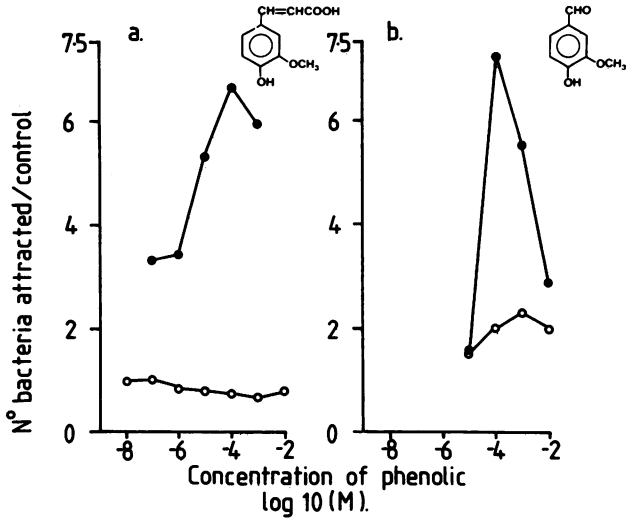


FIG. 3. Dose-response curve of motility of *A. tumefaciens* C58C^1(pDUB1003Δ31) (●) and *A. tumefaciens* C58C^1 (○) towards (a) ferulic acid and (b) vanillin.

Within the second major category are weak *vir* inducers (or noninducers) which are weak chemoattractants for both cured and Ti plasmid-harboring strains. These include *p*-hydroxybenzoic acid (optimum, 10^{-3} M), catechol, vanillyl alcohol (3), and 3,4-dihydroxybenzaldehyde (optima, 10^{-2} M) (data not shown). Catechol and *p*-hydroxybenzoic acid were recently reported to be *vir* noninducers (20), contrary to a previous report (5).

Within the third category are compounds that can neither induce the virulence region nor act as chemoattractants. These include vanillic acid and isovanillic acid.

The results indicate that the Ti plasmid plays an important role in chemotaxis toward plant phenolic inducers of the virulence region and suggest the existence of two separate phenolic receptor systems in *A. tumefaciens*.

Chemotaxis toward shoot homogenates. Undiluted shoot homogenates from a dicot, *Kalanchoë* sp., and a monocot,

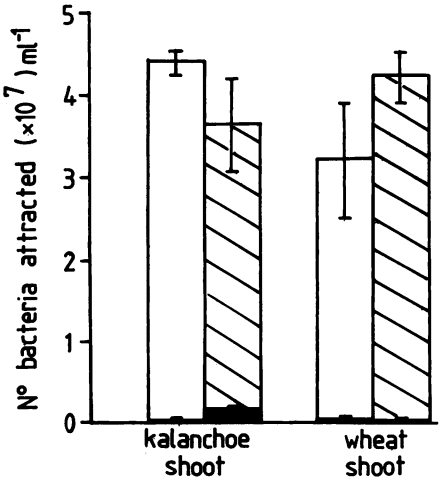


FIG. 4. Chemotaxis of *A. tumefaciens* C58C^1 (□) and C58C^1(pDUB1003Δ31) (▨) toward *Kalanchoë* and wheat shoot extracts. Control values obtained with CM in the capillary are also shown (■). Values presented are the mean of duplicate assays performed on four separate occasions.

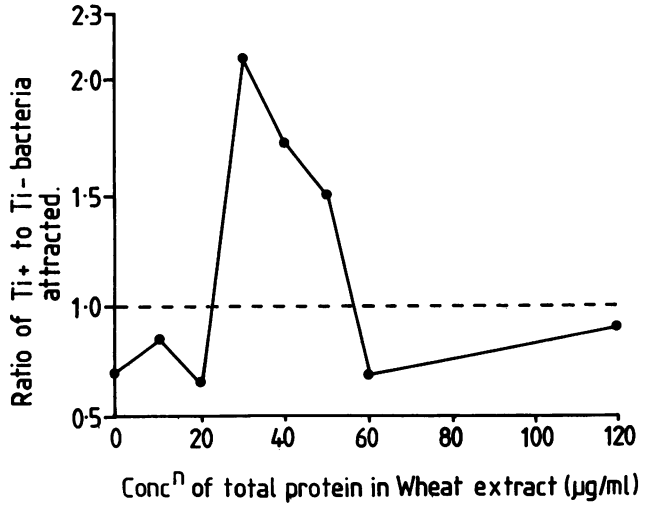


FIG. 5. Chemotaxis toward diluted wheat shoot extract by a mixed population of *A. tumefaciens*. Dilutions of wheat shoot extract were tested as chemoattractants for a 1:1 mixture of *A. tumefaciens* C58C^1 and C58C^1(pDUB1003Δ31) by the capillary assay. The number of each strain entering the capillary was assessed by plating onto agar containing rifampin (selective for C58C^1) or kanamycin [selective for C58C^1(pDUB1003Δ31)]. The ratio of Ti-containing to Ti-lacking bacteria attracted was calculated prior to plotting. Values presented are the mean of duplicate assays performed on six separate occasions.

wheat, elicited a chemotactic response from both Ti plasmid-harboring and cured *A. tumefaciens* C58C^1 (Fig. 4). Similar results were observed with root extracts (data not shown). However, when the wheat extract was diluted through the range 120 to 10 µg of total protein · ml^{-1} and assayed as a chemoattractant for mixed (1:1) populations of cured and Ti plasmid-harboring strains, a distinct enhancement of chemotaxis was conferred by the Ti plasmid (Fig. 5). Twofold dilution of the extract resulted in an equivalent attraction of both strains. However, increasing the dilution factor resulted in an increasing attraction of the Ti plasmid-containing strain relative to the cured strain. A maximum enhancement of >2:1 (Ti plasmid-harboring relative to cured strain) was achieved at 30 µg · ml^{-1}. At dilutions lower than 20 µg · ml^{-1}, significant chemotaxis was not detected. A similar curve was obtained with the dicot extract (data not shown). On the basis of equal protein concentration, the monocot and dicot shoot extracts are of equivalent potency as chemoattractants for *A. tumefaciens* C58C^1. Moreover, the response to plant extracts is considerably more intense than to individual chemicals (3, 19a), suggestive of synergy. Furthermore, possession of a Ti plasmid enhances chemotaxis toward low concentrations of monocot extracts.

Chemotaxis toward protoplast homogenates. To assess whether cell wall components are involved in chemotaxis of *A. tumefaciens* towards natural extracts, we prepared homogenates from freshly isolated leaf protoplasts, regenerated protoplasts, and leaves of tobacco plants. The homogenates were diluted to the same total protein concentration and tested as chemoattractants in capillary assays for *A. tumefaciens* C58C^1(pDUB1003Δ31). Equivalent chemotaxis was observed toward the three extracts (Fig. 6). This indicates that native cell wall components are not required for chemotaxis of *A. tumefaciens* C58C^1 toward plant extracts.

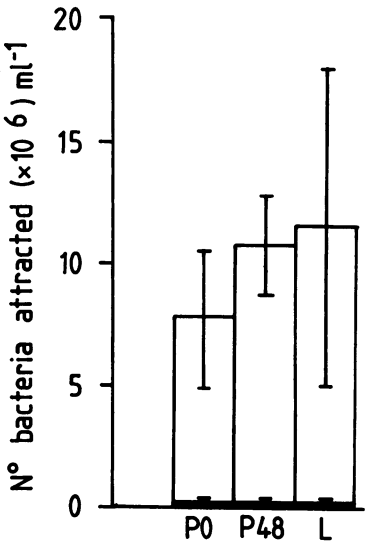


FIG. 6. Chemotaxis of *A. tumefaciens* C58C[1] toward homogenates of freshly isolated (P0) and regenerating (P48) protoplasts and leaves (L) of tobacco. Control values obtained with CM as the attractant are shown for comparison (■). Values presented are the mean of duplicate assays performed on six separate occasions.

DISCUSSION

In this report we present evidence indicating a strong correlation between the *vir*-inducing ability of a phenolic compound and its requirement for Ti plasmid functions for chemotaxis. In conjunction with previous reports on chemotaxis of *A. tumefaciens* C58C[1] toward acetosyringone (3), sugars, and amino acids (19a), this confirms the existence of a highly sensitive chemotaxis system in *A. tumefaciens* C58C[1]. The data indicate that *A. tumefaciens* can express two separate receptor systems for chemotaxis toward phenolic compounds: a highly sensitive, Ti plasmid-encoded system specific for *vir*-inducing phenolic compounds; and a less sensitive, chromosomally encoded system. *virA* and *virG* are the Ti plasmid functions required for both chemotaxis and *vir* induction (29, 34) mediated by acetosyringone and related phenolic compounds. The identity of the chromosomal receptor is obscure.

The spectrum of compounds assayed here allows certain operational rules to be established regarding the degree of substitution of the benzene ring necessary for optimum chemotaxis and *vir* induction. Bolton et al. (5) established the key role of the 4′ hydroxyl group in *vir* induction. All of the compounds used here except isovanillic acid have a 4′ hydroxyl substituent, but they fit into three or possibly four distinct categories, as defined by the chemotactic or *vir*-inducing response that they evoke. For optimum Ti plasmid-determined chemotaxis and *vir* induction, the presence of a 4′-hydroxyl group, 3′ and 5′ O-methyl groups, and a polar side chain at the 1′ position appear to be important. Loss of O-methyl groups results in a loss of sensitivity, but not specificity, unless other substitutions are changed. Chemotactic responses determined by the Ti plasmid are, in general, much more sensitive than those encoded elsewhere.

Parke et al. (25) reported recently that Na^+ ions did not stimulate cell motility or chemotaxis in *A. tumefaciens* A136 and A348 and were able to neutralize all phenolic compounds in NaOH. The contrary response observed here for *A. tumefaciens* C58C[1] may well be a variant-specific trait; it may also be due to the poor motility of *A. tumefaciens* A136 (19a). They obtained similar results to ours with catechol and *p*-hydroxybenzoic acid when using *A. tumefaciens* A136, but observed broad peaks in each case (25). Using *A. tumefaciens* C58C[1], we observed very defined peaks in most cases. They reported in the same paper (25) that they were unable to demonstrate chemotaxis toward acetosyringone or vanillin with *A. tumefaciens* A348. We found that *A. tumefaciens* A348 chemotaxis toward acetosyringone was only weakly evident, even after selection of motile populations by five passages through swarm plates (C. H. Shaw and A. M. Ashby, unpublished observations). Moreover, on microscopic examination, *A. tumefaciens* A136 and A348 were shown to be very poor swimmers in comparison with *A. tumefaciens* C58C[1] (19a). These facts may explain the discrepancy between our data and those of Parke et al. (25). Furthermore, in our laboratory, using two independent assay techniques, we have confirmed that both the octopine Ti plasmid pTiB6S3 and the nopaline Ti plasmid pDUB1003Δ31 confer on strain C58C[1] the property of chemotaxis toward 10^{-7} M acetosyringone (3, 29) and 10^{-4} M vanillin (see above). We have also demonstrated that *virA* and *virG* are the Ti plasmid functions responsible for the specific chemotactic response toward acetosyringone (29). In view of the continual accumulation of evidence supporting the specific chemotaxis of Ti plasmid-harboring *A. tumefaciens* C58C[1] toward plant phenolic inducers of the virulence region (3, 29), *A. tumefaciens* C58C[1] may be regarded as a suitable model system with which to elucidate the primary events that occur in the rhizosphere prior to virulence induction and T-DNA transfer to the plant cell.

Chemotaxis of *A. tumefaciens* C58C[1] toward plant extracts appears to be a response to diffusible factors in the homogenates. The data suggest that native cell wall components are not required for the attraction, but they do not rule out the possibility that cell wall precursors are involved. In this regard, it is interesting that acetosyringone and other *vir*-inducing phenolic compounds are thought to be either degradation products or biosynthetic precursors of lignin (32). The Ti plasmid-determined chemotaxis toward low concentrations of phenolic compounds correlates well with the Ti plasmid-mediated enhancement of chemotaxis toward monocot factors observed here. The role of chemotaxis in the *Agrobacterium*-plant interaction must be in attracting and guiding the bacterium toward the plant. The initial attraction will involve spatial separation of the two organisms, thus implying that the recognition would occur at low attractant concentrations, as demonstrated here.

A limited range of monocots appear to be susceptible to *Agrobacterium* infection (8, 10, 11, 12, 14, 27). Recent reports have attempted to correlate the paucity of *vir* inducers in monocots with their apparent nonsusceptibility (24, 35), although the evidence is conflicting. Our results indicate that monocots and dicots possess equally potent chemoattractants for *A. tumefaciens* C58C[1]. Moreover, there appears to be Ti plasmid-mediated enhancement of attraction to monocots. Since Ti plasmid functions are involved in chemotaxis toward *vir* inducers (3, 29), this strongly suggests that recognition of, and attraction to, susceptible plants is not the step blocked in monocot transformation.

The results presented here, together with previous work (3, 19a, 29, 30), allows us to build up a detailed scenario of the behavior of *Agrobacterium* in the rhizosphere. Attraction to sugars, etc., in plant exudates may explain the prevalence of agrobacteria in the rhizosphere. Bacteria are capable of moving great distances in soil; for example,

Azospirillum brasilense Cd, a rhizosphere bacterium, can move up to 160 cm in response to the presence of plants (4). Virulent *A. tumefaciens* cells will be attracted to wounded plant cells by release of *vir*-inducing phenolic compounds. Chemotaxis toward phenolic *vir* inducers (3, 29) occurs at concentrations well below those producing *vir* induction (32). Thus, the bacteria are guided to wound sites, where the higher inducer concentrations effect *vir* induction. In this report we demonstrate that the observed attraction to individual chemicals can be reproduced by natural extracts. The results correlate well with early reports of attraction of *A. tumefaciens* to plant roots (28) and of *Pseudomonas lachrymans* chemotaxis toward extracts from susceptible and resistant plants (7). Since nonphytopathogenic forms of *A. tumefaciens* predominate in the rhizosphere (15), the Ti plasmid-mediated determination and enhancement of chemotaxis toward *vir* inducers and plant extracts would provide virulent agrobacteria with a competitive advantage in this stressful habitat.

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LITERATURE CITED

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