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# NOTES

## Specific Attachment of *Agrobacterium tumefaciens* to Bamboo Cells in Suspension Cultures

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***Agrobacterium tumefaciens* was tested for its ability to attach to tissue culture cells of bamboo, a monocotyledonous plant. Phase-contrast microscopy and kinetic experiments with radiolabeled bacteria showed that attachment to bamboo cells was indistinguishable from attachment to cells of dicotyledonous plants. Bacterial mutants defective in attachment to dicotyledonous plants showed similar behavior with bamboo, and extensive washing of the bamboo cells had no effect on the number of bacteria which attached.**

The plant pathogen *Agrobacterium tumefaciens* induces crown gall tumors on a wide variety of dicotyledonous plants. Virulent agrobacteria contain tumor-inducing (Ti) plasmids (18, 19), and during tumor formation a portion of this plasmid, the T region, is transferred to plant cells where it is stably integrated and expressed as T-DNA in plant nuclear DNA (2, 9, 17, 20, 21). Recently, T-DNA genes have been shown to encode enzymes for the synthesis of auxin and cytokinin (10, 16; D. E. Akiyoshi, H. J. Klee, R. M. Amasino, E. W. Nester, and M. R. Gordon, Proc. Natl. Acad. Sci. U.S.A., in press), plant hormones which control growth. Although the attachment of *A. tumefaciens* to plant cell surfaces is among the first steps required for tumor initiation (11), the mechanism of T-DNA transfer to plant cells remains to be elucidated. Ti-plasmid derivatives have been widely studied as vectors for the introduction of desirable foreign DNA into plant cells in the hope of genetically improving crop plants (1, 15), but monocotyledonous plants are not generally susceptible to gall formation by *Agrobacterium* spp. (4). We were interested in determining at what stage tumorigenesis is blocked in monocots. As a part of these studies, we analyzed the ability of *A. tumefaciens* to attach to monocot cells. Previous reports have indicated that monocots may be resistant to gall formation by *A. tumefaciens* due to an inability of *A. tumefaciens* to attach to these plant cells (12). We demonstrate here that *A. tumefaciens* can specifically attach to certain monocot cells in a manner which is indistinguishable from its attachment to cells of susceptible dicot plants. This indicates that the surfaces of at least some monocot cells contain receptors for *A. tumefaciens* attachment and suggests that tumor initiation in these monocots is blocked at a later point. These results also suggest that agrobacterium-mediated T-DNA transfer to monocot cells may be possible.

It has been previously shown that *A. tumefaciens* attaches to suspensions of mechanically isolated *Zinnia* leaf mesophyll cells (5) and tobacco cells in suspension cultures (5, 14). The attachment we have measured to tobacco and *Zinnia* cells is determined by the C58 chromosome of virulent *A. tumefaciens* A723 and is not dependent on Ti-plasmid genes (5). We have also demonstrated that mechanically isolated *Zinnia* cells can be transformed by virulent *A. tumefaciens* A348 (C. Douglas, unpublished data), indicating that the attachment measured can lead to Ti-plasmid transfer. Strain A348 (8) is identical to the previously described wild-type strain A723 (5) except that it contains pTiA6 instead of pTiB6806 in the C58 chromosomal background. It is identical in virulence and attachment properties to strain A723 (Douglas, unpublished data). To determine whether strain A348 can attach to cells of a monocotyledonous plant in a manner similar to its attachment to cells of tobacco and *Zinnia* sp., we monitored the interaction of this strain with bamboo cells by using phase-contrast microscopy. Bamboo is a member of the grass family, whose members are not susceptible to crown gall tumor formation (4). A suspension culture of bamboo cells was obtained from T. Murashige, University of California, Riverside, Calif., and was grown under constant light in Murashige and Skoog medium supplemented with 10 mg of (2,4-dichlorophenoxy)acetic acid per liter. Plant cells in mid-log growth were centrifuged out of growth medium and resuspended in fresh medium. Bacteria were added to the plant cell suspensions at a final concentration of approximately $5 \times 10^7$ cells per ml. Two hours after the addition of *A. tumefaciens*, plant cell suspensions were filtered through Miracloth filters (Calbiochem-Behring, La Jolla, Calif.) and washed to remove unattached bacteria. After this treatment, numerous bacteria were observed to be attached to the surfaces of the monocot cells (Fig. 1). The bacteria appeared initially to attach singly to the plant cell surface and tended subsequently to form large aggregates on the cell surface (Fig. 1b), a phenomenon also observed in the attachment to dicot cells. The bacteria appeared to be firmly attached since extensive washing failed to reduce the number of bacteria observed adhering to the cell surfaces.

To further characterize the attachment to monocot cells, we analyzed the kinetics of attachment of strain A348 to bamboo cells and compared them with the kinetics of attachment to tobacco cells. The attachment of radiolabeled bacteria to plant cell suspensions was assayed as described previously (5) by filtering mixtures of plant cells and bacteria through Miracloth filters which retained plant cells but

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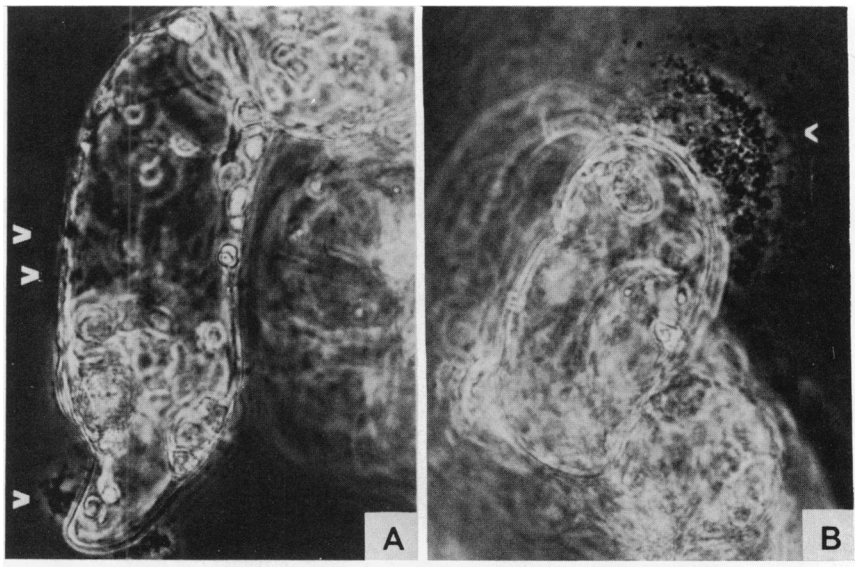


FIG. 1. Light micrographs of bamboo cells in suspension culture in the presence of virulent *A. tumefaciens* A348. (A) After 2 h of incubation, cells of strain A348 were observed adhered to the plant cell surface either singly or in small clumps (arrowheads). (B) Large aggregates of bacteria adhered to the plant cell surface after longer incubation (arrowhead).

allowed the passage of free bacteria. Bacteria were added to reach a final concentration of $2 \times 10^6$ to $5 \times 10^6$ cells per ml. After washing, the radioactivity on the filters was counted in a liquid scintillation counter, and the percentage of bacteria attached was determined. Attachment to both bamboo and tobacco cells was detectable 15 min after addition of the bacteria (Fig. 2). The kinetics of attachment to the two kinds of plant cells were very similar; attachment proceeded rapidly for about 60 min and then leveled off. In both cases, after 2 h approximately 20% of the added bacteria were attached to the plant cells. Thus, both microscopic analysis and measurement of the kinetics of attachment suggest that cells of *A. tumefaciens* have the ability to attach to monocot cells in a manner indistinguishable from their attachment to tobacco cells.

The specificity of the attachment of *A. tumefaciens* to monocot cells was investigated with avirulent *A. tumefaciens* mutants which are defective in attachment to tobacco and *Zinnia* cells (5). The mutations in such strains are due to the inactivation of genes clustered in two distinct but closely linked loci on the *Agrobacterium* chromosome (C. Douglas, W. Halperin, and E. W. Nester, submitted for publication). If the attachment to bamboo cells results from the same type of interaction that occurs between *A. tumefaciens* cells and dicot cells, such attachment-defective mutants should have impaired ability to attach to the monocot cells. We chose three representative mutants and compared their attachment to bamboo cells with that of the wild-type, virulent strain A348. Strain A348 attached to bamboo cells to the same extent as to tobacco cells after 2 h of incubation, whereas the mutant strains A1011, A1020, and A1045 showed a loss in attachment ability to bamboo cells and tobacco cells (Table 1). This suggests that the attachment observed to monocot cells is qualitatively similar to that observed to dicot cells.

Tomato cells in suspension cultures produce a cell surface substance which agglutinates *Agrobacterium* cells and can lead to the nonspecific attachment of bacteria to these cells (N. Neff and A. Binns, submitted for publication). This substance can be removed from the surface of tomato cells by washing them with plant growth medium. To determine whether a similar type of substance allowed nonspecific attachment of strain A348 to bamboo cells, we compared attachment to bamboo cells that had been washed five times in plant growth medium with attachment to cells which had been centrifuged out of growth medium and resuspended in

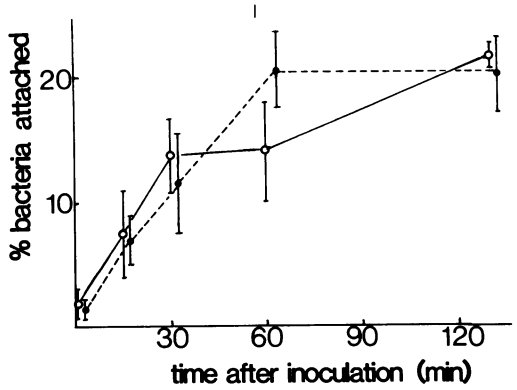


FIG. 2. Time course of attachment of *A. tumefaciens* A348 to tobacco and bamboo cells in suspension culture. Radiolabeled bacteria were added to plant cell suspensions, and the number of bacteria attached was determined by filtration through filters which allowed the passage of free bacteria but not plant cells. After washing, the number of attached bacteria was determined by liquid scintillation counting of the filters. Symbols: ○, bamboo cells; ●, tobacco cells.

TABLE 1. Attachment of *A. tumefaciens* to bamboo and tobacco cells

| Strain[a] | Bacteria attached to [b]: | |
|---|---|---|
| | Bamboo cells | Tobacco cells |
| A348 | 21 ± 2 | 24 ± 4 |
| A1011 | 7 ± 1 | 5 ± 1 |
| A1020 | 6 ± 2 | 4 ± 0.3 |
| A1045 | 8 ± 1 | 6 ± 1 |

[a] Strains A1011, A1020, and A1045 contain Tn*5* in the C58 chromosome (7). Strain A1011 contains pTiA6, and strains A1020 and A1045 contain pTiB6806. Wild-type strain A348 contain pTiA6 in the C58 chromosomal background (8). Strain A348 is virulent; all other strains are avirulent.

[b] The percentage of radiolabeled bacteria attached to the plant cells was determined after 2 h of incubation as described previously (5). The numbers represent the mean ± standard error of at least three separate experiments.

fresh medium without washing. No loss in the ability to attach to washed cells was found (data not shown), indicating that attachment to bamboo cells is not of the nonspecific type observed to unwashed tomato cells.

It is clear from the data presented that *A. tumefaciens* cells can attach to monocot tissue culture cells in suspension. Since bacteria added to *Zinnia* cells in suspension cultures and tobacco cells in suspension cultures can transform these cells (Douglas, unpublished data; G. An, unpublished data), it is likely that the attachment measured in these types of assays is involved in tumor formation. It has been previously suggested that *Agrobacterium* spp. lack the ability to attach to cells of monocotyledonous plants and that this inability may be the basis for the resistance of monocots to infection by *Agrobacterium* spp. (12). In these previous studies, attachment was measured indirectly by first mixing bacteria with isolated cell walls of the test plant and then assaying the ability of these bacteria to form tumors on pinto bean leaves. Inhibition of tumor formation was interpreted as being due to attachment of the bacteria to the isolated cell walls, thereby rendering them unavailable for attachment to leaf cells. In this type of assay, certain monocot cell walls failed to inhibit tumor formation. In the present study, attachment to plant cells was measured directly, which may account for the difference in results. Alternatively, it is possible that *A. tumefaciens* has the ability to attach to certain monocot cells but not to others. It has been reported that *A. tumefaciens* attaches to corn and oat tissue culture cells in low numbers compared with attachment to carrot cells (13). However, since we showed that *A. tumefaciens* has a specific affinity for bamboo cells which is very similar to that for tobacco cells, the ability to attach to cell surfaces does not seem to control the host range of *Agrobacterium* spp. with regard to all monocots. Our results are consistent with those of Draper et al (6), who found that *Asparagus* cells and possibly other monocot cells were agglutinated by high concentrations of *A. tumefaciens*, a phenomenon which seems to be correlated with attachment. Our results also suggest that the monocot cells we tested must contain any cell surface receptors which are necessary for the attachment of *A. tumefaciens* and that the absence of such receptors is not an absolute characteristic of monocot cell surfaces. Thus, initial attachment to plant cells may not play a major role in determining the host range of *Agrobacterium* spp., in contrast to some *Rhizobium*-legume interactions in which the host range appears to be determined by attachment phenomena (3).

Although we cannot completely exclude the possibility that some qualitative difference between the attachment to the monocot cells and to the dicot cells we tested leads to the resistance of monocot cells to gall formation, our data suggest that this resistance is more likely to be due to a block in subsequent steps in pathogenesis. Such a block might occur in the transfer of Ti-plasmid DNA to the plant cell or in the integration, expression, or function of T-DNA genes within the monocot cell. The identification of the step at which tumor induction is blocked in monocotyledonous plants to which agrobacteria can attach will aid in understanding the mechanism of Ti-plasmid transfer and the regulation of plant cell growth. If the block in tumor formation occurs after Ti-plasmid transfer, agrobacterium-mediated transformation of monocot cells may be possible given the incorporation of the proper selectable markers into the T-DNA of the Ti-plasmid vector used.

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