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Host Range Encoded by the *Agrobacterium tumefaciens* Tumor-Inducing Plasmid pTiAg63 Can Be Expanded by Modification of Its T-DNA Oncogene Complement

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***Agrobacterium tumefaciens* harboring pTiA6 incite unorganized tumors on *Nicotiana rustica*, sunflowers, carrots, and tomatoes, whereas isogenic strains of agrobacteria harboring pTiAg63 form "rooty" tumors on *N. rustica* and are essentially avirulent on sunflowers, carrots, and tomatoes. In this report we show that the different host range characteristics of these two plasmids were due, in part, to differences in the T-DNA oncogene complements of the plasmids. Specifically, we constructed derivatives of pTiAg63 that contained pTiA6 oncogenes 4, 6a, and 6b inserted into the T_B-DNA region and found that agrobacteria harboring these plasmids could incite unorganized tumors on *N. rustica*, tomatoes, carrots, and the inbred sunflower line HA202R. Undefined host factors, however, also appeared to be involved in determining *A. tumefaciens* host range since three inbred sunflower lines, HA303B, HA89B, and HA290B, were susceptible to tumor formation by agrobacteria harboring pTiA6 but not by strains harboring pTiAg63 or the modified pTiAg63 plasmids.**

Agrobacterium tumefaciens strains harboring the wide-host-range tumor-inducing (Ti) plasmid pTiA6 can incite tumors on a broad range of hosts including common test plants such as tomatoes, sunflowers, *Datura* spp., and *Kalanchöe* spp. (6). In contrast, strains carrying the limited-host-range Ti plasmid pTiAg63 are essentially avirulent on these hosts (17, 18, 21). One can imagine that these host range differences could be accounted for in two general ways. One possibility is that the T-DNA sequences of pTiAg63 can only be stably transferred to relatively few plant species. Alternatively, the pTiAg63 T-DNA sequences might be delivered to a wide range of plants, but the oncogene complement might be effective in converting only a limited number of plant cells to the tumorous phenotype.

In an accompanying report (4), we show that the T-DNA oncogene complements of pTiAg63 and pTiA6 were related but not equivalent; tumor tissues of *Nicotiana rustica* incited by *A. tumefaciens* harboring pTiA6 grew in vitro as loose, friable, unorganized callus, whereas tumor tissues incited by pTiAg63 grew as clumps of rootlike structures. It had been previously shown (7, 8, 16) that agrobacteria harboring derivatives of pTiA6 having mutations in oncogene 4, the cytokinin independence locus (2), incite rooty tumors on some host plants and display an attenuated virulence response on others. We therefore suggested that the oncogene 4 sequences of pTiAg63 might be defective or inactive in some plants and that this might account, in part, for the limited host range that is characteristic of pTiAg63. In this study we tested this hypothesis. We constructed derivatives of pTiAg63 with pTiA6 oncogenes 4, 6a, and 6b inserted into the T_B-DNA region (the functions of genes 6a and 6b are ill defined but seem to limit the size of tumors on some plants [1, 8]) and assayed *A. tumefaciens* strains harboring the modified plasmids for virulence on *N. rustica*, tomatoes, carrots, and sunflowers. The resulting data indicate that this modification does indeed expand the host range specified by pTiAg63. Thus, differences in the oncogene complements of pTiAg63 and pTiA6, which are probably differences in oncogene 4, accounted, in part, for the different host range characteristics of these plasmids. In addition, it appeared that undefined host factors could also influence the host range characteristics of Ti plasmids.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *A. tumefaciens* strains A136(pTiA6) (20) and A136(pTiAg63) (21) and *Escherichia coli* strains C2107, a temperature-sensitive *polA* strain (11), and C2110, a *polA* strain (13), were maintained on modified LB medium (4). Plasmids pRZ102 (10), pNWEco7 (8), pSal9.3 (4), and pRK2501 (12) have been described previously. When antibiotic selection was required for solid media, LB agar was supplemented with 25 μg of tetracycline or 50 μg of kanamycin per ml for *E. coli* and 100 μg of kanamycin per ml for *A. tumefaciens*. The antibiotic concentrations were decreased by half for liquid cultures.

Plant hosts. Seeds for inbred sunflower lines HA89B, HA202R, HA290B, and HA303B were obtained from Stauffer Seeds, Clovis, Calif. Mammoth sunflower and beefsteak tomato seeds were purchased locally. *N. rustica* seeds were obtained from W. J. Kaiser, Western Regional Plant Introduction Station, U.S. Department of Agriculture Agricultural Research Service, Washington State University, Pullman.

DNA isolations. *A. tumefaciens* total DNA was isolated by a modification of the method of Currier and Nester (5). Overnight cultures (2 ml each) were centrifuged, and the pellet was washed once with 50 mM Tris buffer (pH 8.0) containing 50 mM EDTA and 1 M NaCl and then twice with 10 mM Tris buffer (pH 8.0) containing 10 mM EDTA. The cells were suspended in the latter buffer and lysed at 37°C by adding pronase (Calbiochem-Behring, La Jolla, Calif.) and sodium lauryl sulfate at final concentrations of 0.5 mg/ml and 1%, respectively. The lysate was extracted with phenol and then choloroform and was adjusted to 0.3 M sodium acetate, and the nucleic acids were precipitated with 3 volumes of 95% ethanol. After a second round of ethanol precipitation, the pellet was dissolved in 10 mM Tris buffer (pH 8.0)

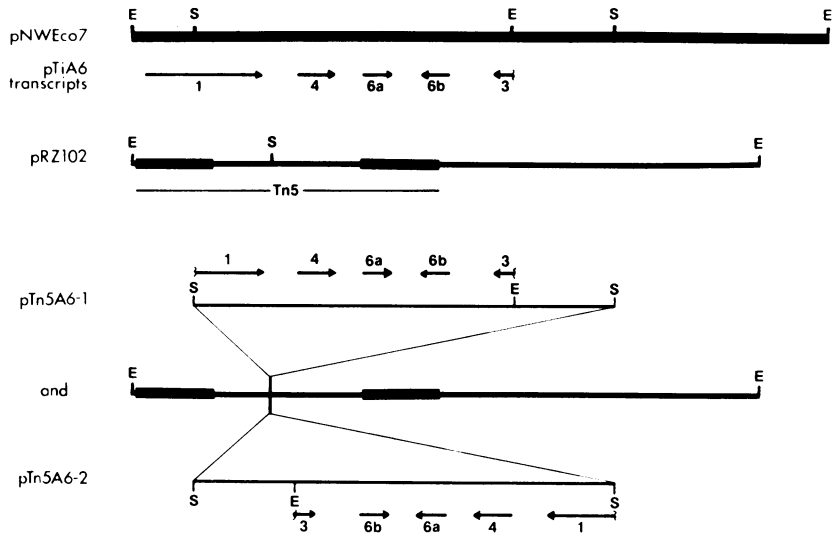

FIG. 1. Construction of pTn5A6-1 and pTn5A6-2. The *Eco*RI (E) and *Sal*I (S) restriction maps of pNWEco7 and pRZ102 are shown as well as the locations and polarities of pTiA6 genes transcribed in tumor tissues (22). pTn5A6-1 and pTn5A6-2 were constructed by inserting the *Sal*I fragment of pNWEco7 that encodes pTiA6 genes 4, 6a, 6b, and portions of 1 and 3 into the unique *Sal*I site of Tn*5* in opposite orientations.

containing 1 mM EDTA. Plasmids were isolated from *E. coli* strains by the method of Birnboim and Doly (3).

Enzymes. Restriction enzymes and T4 ligase were obtained from New England Biolabs, Beverly, Mass., or Bethesda Research Laboratories, Gaithersburg, Md., and were used as recommended by the suppliers.

Bacterial transformations. *E. coli* and *A. tumefaciens* strains were transformed as described by Mandel and Higa (15) and Holsters et al. (9), respectively.

Construction of hybrid Ti plasmids. pNWEco7 is a recombinant molecule composed of pTiA6 *Eco*RI fragment 7 inserted into the unique *Eco*RI site of pBR325 (8; see Fig. 1). Cleavage of this molecule with *Sal*I released a segment of DNA containing pTiA6 oncogenes (8, 14, 22) 4, 6a, and 6b; portions of oncogenes 1 and 3; and a 1.85-kilobase (kb) stretch of pBR325 DNA (see Fig. 1). This fragment was inserted in both orientations into the unique *Sal*I site of transposon Tn*5* (which was present on pRZ102 [10], a derivative of plasmid ColE1) yielding pTn5A6-1 and pTn5A6-2.

The plasmid which served as a target for mutagenesis by pTn5A6-1 and pTn5A6-2 was constructed by inserting the 9.3-kb pTiAg63 T_B-DNA fragment from pSal9.3 (4) into the unique *Xho*I site of the wide-host-range plasmid vector pRK2501 (12). This construction, designated pHIM2 (H. I. Malkawi, M.S. Thesis, Washington State University, Pullman, 1984), was transformed into *E. coli* C2107 (temperature-sensitive *polA*), and then either pTn5A6-1 or pTn5A6-2 was introduced. These strains were grown at 30°C because replication of pTn5A6-1 and pTn5A6-2 is dependent on the

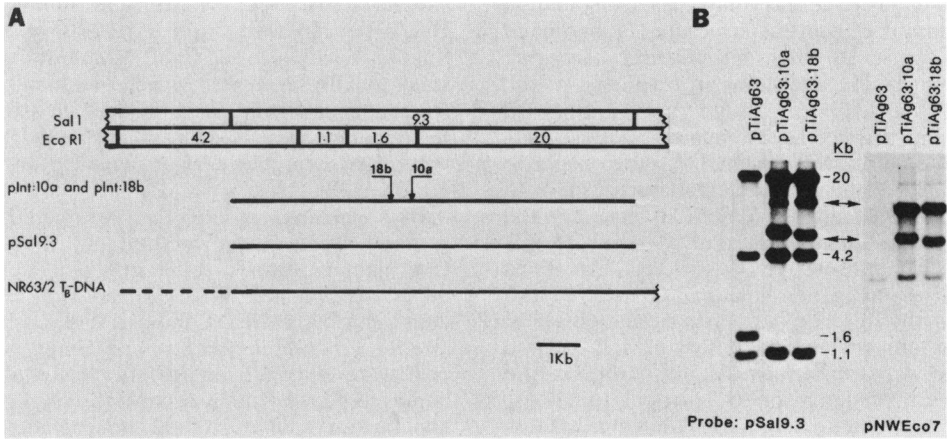

FIG. 2. Hybridization of cloned pTiAg63 or pTiA6 fragments with *Eco*RI digestion products of *A. tumefaciens* total DNA. (A) Restriction map showing the left end of the pTiAg63 T_B-DNA region (4) and the location of the inserts into the 9.3-kb *Sal*I fragment of pInt:10a and pInt:18b. (B) Total DNA isolated from *A. tumefaciens* strains harboring pTiAg63 or putative homologous recombinants, which was digested with *Eco*RI and fractionated by 1% agarose gel electrophoresis. Southern blots of duplicate gels were prepared, hybridized with the indicated ^{32}P-labeled DNA probes, washed under standard stringent conditions (ca. T_m − 17°C), and visualized by autoradiography.

temperature-sensitive *polA* gene product. Isolated colonies of either C2107(pHIM2, pTn5A6-1) or C2107(pHIM2, pTn5A6-2) were subsequently inoculated into LB broth containing kanamycin and tetracycline and incubated at 42°C to select against replication of the ColE1 derivatives and, therefore, for cells in which the Tn*5* derivatives present on pTn5A6-1 or pTn5A6-2 had transposed to either pHIM2 or the chromosome. Plasmid DNA was isolated from these cultures and used to transform *E. coli* C2110 (*polA*) to kanamycin and tetracyline resistance. Restriction and Southern blot analyses of the plasmids contained in the C2110 isolates indicated that they were indeed pHIM2 derivatives carrying the modified Tn*5* inserts.

Southern blot analysis. Gel electrophoresis, preparation of nitrocellulose filters, and hybridization conditions were as described in an accompanying report (4).

RESULTS

Construction of pTiAg63 derivatives containing the oncogene 4 sequences of pTiA6. Insertion of a pTiA6 DNA segment into the T-DNA region of pTiAg63 initially posed a problem since genetic analysis of the pTiAg63 T-DNA had not yet been done and we were unsure as to where the insert could be placed without inactivating genes required for virulence. To circumvent this problem, we first cloned a segment of the pTiA6 T_L-DNA region encoding oncogene 4, the cytokinin independence gene, into the *SalI* site of Tn*5* (Fig. 1). This section of pTiA6 also encodes oncogenes 6a and 6b and portions of genes 1 and 3, auxin independence and octopine synthesis genes, respectively (1, 8, 14, 22). These Tn*5* derivatives present on pTn5A6-1 and pTn5A6-2 were then used to transpose the pTiA6 sequences into random sites on pHIM2, a recombinant molecule containing sequences from the T_B-DNA region of pTiAg63 (see above and Fig. 1 for details of construction). A number of inserts were obtained, and the sites of insertion were determined by standard restriction mapping techniques. The map positions of two inserts relevant to this report, pInt:10a and pInt:18b (resulting from the transposition of the pTn5A6-1 and pTn5A6-2 derivatives, respectively), are shown in Fig. 2A. In these cases the modified Tn*5* transposons were inserted into the 1.6-kb *Eco*RI fragment of the T_B-DNA region.

To recombine the 10a and 18b inserts into pTiAg63, *A. tumefaciens* harboring pTiAg63 were transformed with pInt:10a or pInt:18b, and the marker exchange procedure of Ruvkun and Ausubel (19) was followed. This yielded pTiAg63 derivatives pTiAg63:10a and pTiAg63:18b. Southern blot analysis was used to confirm that the desired recombinations had occurred. When total DNA isolated from *A. tumefaciens* harboring pTiAg63 was digested with *Eco*RI, DNA fragments of 20, 4.2, 1.6, and 1.1 kb were detected by using ^{32}P-labeled pSal9.3 as probe (Fig. 2B). When similar DNA preparations were made from agrobacteria harboring pTiAg63:10a or pTiAg63:18b, the 20-, 4.2-, and 1.1-kb fragments were again detected, but the 1.6-kb fragment was missing. Instead, two fragments, ca. 5 and 9 kb

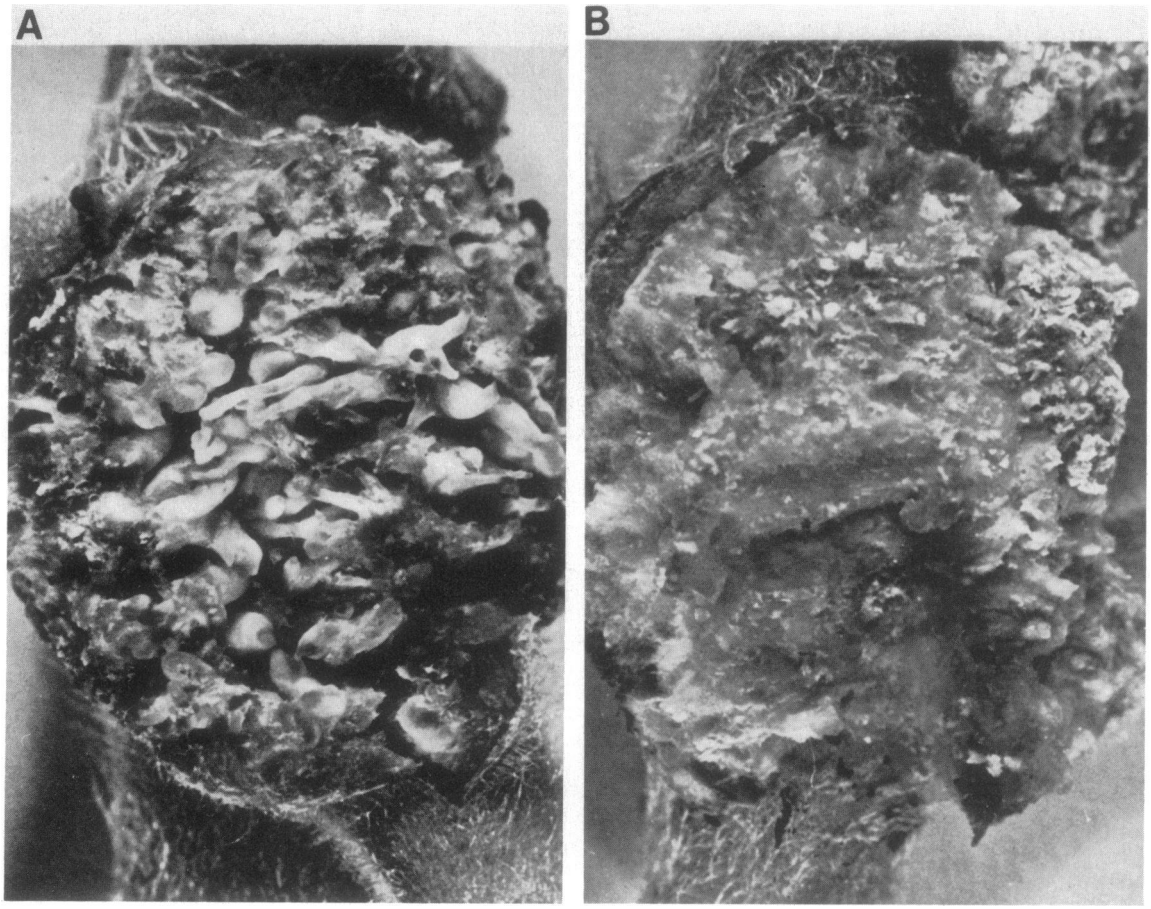

FIG. 3. Morphology of *N. rustica* tumors incited by *A. tumefaciens* harboring pTiAg63 (A) or pTiAg63:10a (B). (See text for details.)

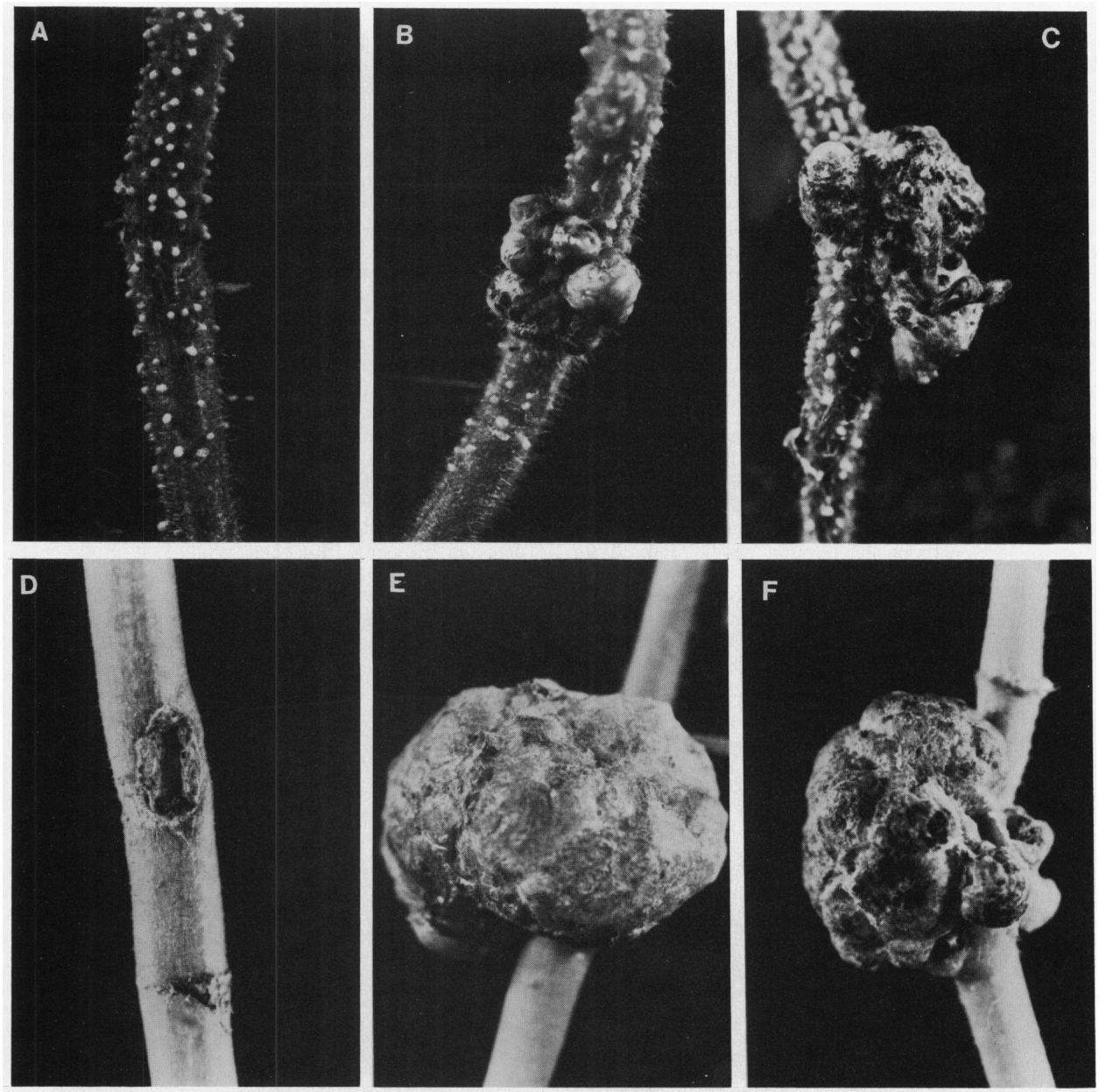

FIG. 4. Response of tomato (A–C) and Mammoth sunflower (D–F) to inoculation with *A. tumefaciens* strains harboring various Ti plasmids. A, pTiAg63; B, pTiAg63:10a; C, pTiAg63:18b; D, pTiAg63; E, pTiAg63:10a; F, pTiAg63:18b. (See text for details.)

in length, were observed. These fragments also hybridized with pNWEco7, the source of the pTiA6 oncogenes (Fig. 2B). These results indicate that the desired recombinations took place. Since both inserts were located in the 1.6-kb *Eco*RI fragment of pTiAg63 and each insert had only one *Eco*RI site, we expected the 1.6-kb *Eco*RI fragment to be replaced by two novel fragments totaling about 14 kb in size (the sum of the Tn*5* construct, ~12.5 kb, plus the 1.6-kb *Eco*RI fragment). Further, because these new fragments should have contained sequences from the pTiA6 insert as well as from the 1.6-kb *Eco*RI fragment of pSal9.3, they should have, and did, hybridize with both pNWEco7 and pSal9.3.

Virulence of the modified pTiAg63 plasmids. Agrobacteria harboring pTiAg63 were virulent on *N. rustica* and incited tumors which were composed primarily of rootlike structures (Fig. 3A). As pointed out in an accompanying paper (4), these data suggested that the pTiAg63 sequences related to pTiA6 oncogene 4 might function poorly in *N. rustica*. If this possibility is true, then the pTiAg63 derivatives containing the pTiA6 oncogene 4 sequences might be expected to produce unorganized tumors on *N. rustica*, as does pTiA6. This was the case. *N. rustica* tumors incited with pTiAg63:10a (Fig. 3B) and pTiAg63:18b (data not shown) were composed entirely of unorganized tissues.

To determine whether the modified pTiAg63 plasmids conferred an expanded host range, we attempted to incite tumors on three plants on which *A. tumefaciens* harboring pTiAg63 are not normally virulent: tomatoes, carrots, and sunflowers. With tomatoes and carrots as hosts, the results

were relatively clear-cut. Agrobacteria harboring pTiAg63 were essentially avirulent on these plants, whereas strains carrying pTiAg63:10a and pTiAg63:18b consistently incited tumors on tomatoes (Fig. 4) and on most carrot slices inoculated (data not shown). With sunflowers, however, the results were not as straightforward.

Agrobacteria harboring pTiAg63 were essentially avirulent on the Mammoth variety of sunflower (Fig. 4). Occasionally, small bumps appeared at the wound site, but these growths did not develop further. In contrast, strains carrying the pTiAg63 derivatives were virulent on this variety of sunflower at a relatively high frequency (Fig. 4); pTiAg63:10a incited large tumors on 13 of 23 plants tested, whereas pTiAg63:18b incited large tumors on 18 of 26 plants. Thus, the host range of the pTiAg63 derivatives seemed to be expanded to include sunflowers, but it was not clear why only about half of the plants developed tumors. One possibility was that the genetic background of the individual plants varied (the Mammoth variety of sunflower is open field pollinated) and that host differences influenced the tumorigenic response. We therefore tested pTiAg63 and its derivatives on four inbred sunflower lines.

Agrobacteria harboring pTiAg63 were essentially avirulent on all of the inbred sunflower lines tested, whereas strains carrying pTiA6 were virulent on all of these host plants. The responses obtained with pTiAg63:10a and pTiAg63:18b varied depending on the particular sunflower line. Agrobacteria harboring these pTiAg63 derivatives were avirulent on inbred lines HA303B, HA89B, and HA290B. However, large tumors consistently developed on the HA202R inbred line (14 out of 14 plants inoculated). Thus, the modified pTiAg63 plasmids had the ability to form tumors on some sunflower lines, but on others, tumor formation was apparently sensitive to unknown host factors, factors which did not seem to affect tumor formation by pTiA6.

DISCUSSION

We have presented data (4) which suggest that the pTiAg63 sequences related to pTiA6 oncogene 4 are defective or function poorly in *N. rustica*, and we have discussed the possibility that this characteristic might be a factor in determining the limited host range that is characteristic of the plasmid. In this study, we tested this hypothesis by constructing derivatives of pTiAg63 having pTiA6 oncogenes 4, 6a, and 6b inserted into the T_B-DNA region and by assaying strains harboring these plasmids for virulence on *N. rustica*, tomatoes, carrots, and sunflowers. The data indicate that, whereas agrobacteria harboring pTiAg63 incite rooty tumors on *N. rustica*, the modified pTiAg63 plasmids form unorganized tumors. These results are consistent with the hypothesis that the pTiAg63 sequences related to pTiA6 oncogene 4 are inactive or are weakly expressed in *N. rustica*. In addition, we show that agrobacteria harboring the pTiAg63 derivatives can incite tumors on tomatoes, carrots, and the HA202R inbred sunflower line, whereas strains carrying wild-type pTiAg63 are essentially avirulent on these hosts. The simplest interpretation of these data is that at least some of the differences in host range encoded by pTiAg63 and pTiA6 are due to differences in the oncogene complements of the plasmids, presumably differences in oncogene 4 sequences.

It is clear from the data, however, that factors other than the oncogene 4 sequences also contribute to the different host range characteristics of pTiA6 and pTiAg63. Agrobacteria harboring pTiA6 not only incited tumors on the HA202R inbred sunflower line, but they also incited tumors on the inbred sunflower lines HA303B, HA89B, and HA290B. Agrobacteria harboring the pTiAg63 derivatives did not form tumors on these latter three sunflower lines. It remains to be determined whether this host range limitation is due to additional differences in the T-DNA oncogene complements of the two plasmids, if the T-DNA sequences of pTiAg63 cannot be delivered to these three inbred sunflower lines, or if other factors, such as an inability of the agrobacteria harboring the pTiAg63 derivatives to grow in the wound sites, may also be involved.

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