Crown gall disease was found on cherry trees and raspberry plants in nurseries where K84 had failed to prevent the disease. A total of 109 and 189 Agrobacterium strains were isolated and characterized from seventeen cherry galls and two raspberry galls, respectively. Based on tests for pathogenicity, antibiotic production, agrocin 84 sensitivity, and biovar designations, the cherry and the raspberry strains were characterized into seven and nine distinct phenotypes, respectively. The predominant population in both cherry and raspberry strains was pathogenic and insensitive to agrocin 84. All cherry strains belonged to biovar 2. Raspberry strains were either biovar 1 (32%), biovar 2 (52%), or a group intermediate between biovar 1 and 2, called biovar x (16%). The majority of cherry and raspberry strains utilized nopaline. Only five of the cherry strains were unable to utilize the opines tested; all five were nonpathogenic. One pathogenic and 10 nonpathogenic cherry strains catabolized mannopine as well as nopaline. Of the 189 raspberry strains, 162 utilized
nopaline, 16 utilized octopine, and 11 catabolized both nopaline and octopine, none of them utilized mannopine. Opine utilization by the raspberry strains was distributed among three groups: biovar 1, biovar 2, and biovar x. A high proportion of agrocin 84-insensitive strains was isolated from both plants. Putative transconjugants carrying DNA hybridized to pAgK84 were part of the agrocin 84-insensitive population, as determined by hybridization to the agrocin synthesis gene, SmaIG, antibiotic production, plasmid profiles and fingerprints of pAgK84. Sixty eight percent of the 189 raspberry strains, including both pathogens and nonpathogens, had DNA hybridized to the agrocin synthesis DNA probe, whereas only 15% of the cherry strains showed homology to this probe. Twenty five percent of the cherry strains and 65% of the raspberry strains also produced an antibiotic against agrocin 84-sensitive Agrobacterium strains. However, 10% of the raspberry strains that hybridized to SmaIG produced no antibiotic against these sensitive strains. Most putative transconjugants carried a pAgK84-like plasmid and produced an antibiotic that inhibited the growth of the same bacteria as agrocin 84. Restriction fragment fingerprint of pAgK84-like plasmids from the putative transconjugants and pAgK84 from strain K84 were similar. Reaction of a K84 specific antiserum with putative transconjugants showed that only one nonpathogenic cherry strains and two nonpathogenic raspberry strains were serologically identical to that of strain K84. These result show that pathogenic and nonpathogenic putative transconjugants were recipients of pAgK84, but that K84 was not a recipient of a pTi.
ISOLATION OF PUTATIVE pAgK84 TRANSCONJUGANTS FROM COMMERCIAL CHERRY AND RASPBERRY PLANTS TREATED WITH AGROBACTERIUM RADIOBACTER STRAIN K84

by
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This thesis is dedicated with my love to my dear parents, husband and all my brothers and sisters.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>6</td>
</tr>
<tr>
<td>Sampling strategy</td>
<td>6</td>
</tr>
<tr>
<td>Isolation of <em>Agrobacterium</em> strains</td>
<td>6</td>
</tr>
<tr>
<td>Pathogenicity</td>
<td>7</td>
</tr>
<tr>
<td>Antibiotic production</td>
<td>7</td>
</tr>
<tr>
<td>Opine utilization</td>
<td>9</td>
</tr>
<tr>
<td>Biovar tests</td>
<td>10</td>
</tr>
<tr>
<td>Serological reaction</td>
<td>11</td>
</tr>
<tr>
<td>Plasmid profiles</td>
<td>11</td>
</tr>
<tr>
<td>Purification of plasmid DNA</td>
<td>11</td>
</tr>
<tr>
<td>DNA fingerprints</td>
<td>12</td>
</tr>
<tr>
<td>Probe DNA</td>
<td>12</td>
</tr>
<tr>
<td>Colony hybridization</td>
<td>13</td>
</tr>
<tr>
<td>Southern blots</td>
<td>14</td>
</tr>
<tr>
<td>Characterization of strains harboring pAgK84</td>
<td>14</td>
</tr>
<tr>
<td>RESULTS</td>
<td>16</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>30</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>40</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Growth inhibition of agrocin 84 sensitive and resistant <em>Agrobacterium</em> strains</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Phenotypic distribution of <em>Agrobacterium</em> strains from cherry and raspberry galls</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>Colony hybridization of <em>Agrobacterium</em> strains hybridized with tmsI-tmr or virFAB</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>Pathogenicity and antibiotic production of <em>Agrobacterium</em> strains that hybridized to the SmalG and did not hybridize to the SmalG</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>Opine utilization and biovar distribution for 189 pathogenic and nonpathogenic <em>Agrobacterium</em> strains isolated from raspberry galls</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>Opine utilization and biovar designation of 129 raspberry putative transconjugants</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Immunodiffusion reaction of cherry and raspberry strains against K84 antiserum</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>DNA fingerprints of three serologically identical strains and strain K84</td>
<td>26</td>
</tr>
<tr>
<td>9</td>
<td>Plasmid pattern (A) and Southern hybridization (B) of pathogenic and nonpathogenic <em>Agrobacterium</em> strains from cherry and raspberry galls</td>
<td>27</td>
</tr>
<tr>
<td>10</td>
<td>Restriction fragment fingerprint of pAgK84 of putative transconjugants and strain K84</td>
<td>29</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

1 Distribution of antibiotic production of 129 putative transconjugants *Agrobacterium* strains from raspberry galls

2 Biochemical reactions for biovar designation of the raspberry *Agrobacterium* strains
ISOLATION OF PUTATIVE pAgK84 TRANSCONJUGANTS FROM COMMERCIAL CHERRY AND RASPBERRY PLANTS TREATED WITH AGROBACTERIUM RADIOBACTER STRAIN K84

INTRODUCTION

Crown gall disease has affected a wide variety of dicotyledonous plants and contributed to serious economic losses worldwide (Alconero, 1980; Kennedy et al., 1980; Kerr et al., 1977b; Moore et al., 1979). The disease is caused by the soil-borne bacterium Agrobacterium tumefaciens (Smith and Townsend, 1907) Conn 1942. A. tumefaciens carries a tumor-inducing plasmid (pTi) (van Larebeke et al., 1974; Watson et al., 1975) that is involved in the infection process. As a consequence of infection, oncogenic DNA from the pTi, called T-DNA, transfers from pathogenic Agrobacterium and integrates into the host plant genome (Chilton et al., 1977). After integration of T-DNA, plant tissues become hypertrophic and synthesize low molecular weight carbon and nitrogen compounds called opines (Tempe et al., 1983). The opines have various roles biologically as nutritional sources for A. tumefaciens in the tumor and induction of pTi conjugation (Beck von Bodman et al., 1989; Ellis et al., 1979a; Kerr et al., 1977a; Petit et al., 1978).

The nomenclature of Agrobacterium species is in a state of flux with several propositions for revision having been published (Bouzar
et al., 1993a; Holmes et al., 1981; Kersters et al., 1973; Sawada et al., 1993). Therefore, the nomenclature in this study is based on the Bergey's Manual of Systematic Bacteriology (Kersters et al., 1984).

In this paper, Agrobacterium strains which induce tumors are called A. tumefaciens; strains which cannot induce tumorigenesis are called A. radiobacter (Beijerinck and van Delden 1902) Conn 1942. Subspecies classification is according to biovar designation (Kersters et al., 1984).

Biological control of crown gall disease with a nonpathogen, A. radiobacter strain K84 (New et al., 1972), has been used successfully in plant nurseries in various regions of the world (Moore et al., 1979) to protect plants such as stone fruit trees, nut trees, ornamentals, etc. However, the effectiveness of K84 is limited. K84 has been ineffective on certain hosts (Kerr et al., 1977b; Moore, 1979), and against biovar 3 and agrocin 84-resistant biovar 1 and 2 strains of pathogenic Agrobacterium (Kerr et al., 1977b).

The major mechanism of biological control by K84 is due to the production of a bacteriocin, agrocin 84 (Kerr et al., 1974; Kerr et al., 1984). Agrocin 84 is an analog of an adenine nucleoside (Roberts et al., 1977; Tate et al., 1979), which affects both protein synthesis and DNA replication in sensitive strains (McCardell et al., 1976).

Strains sensitive to agrocin 84 require the presence of certain pTis that encode genes for uptake of agrocin 84 (Engler et al., 1975; Kerr et al., 1976; Murphy et al., 1979). From experimental results, most strains of A. tumefaciens that were sensitive to agrocin 84 in vitro
were controlled by K84 (Kerr et al., 1974; Moore et al., 1979). In addition, crown gall was not controlled by K84 in field experiments when plants were coinoculated with K84 and either native resistant strains (Panagopoulos et al., 1979), strains with an agrocin 84 uptake-mutated pTi (Sule et al., 1980), or sensitive strains harboring a pAgK84 (Ellis et al., 1979a; Ellis et al., 1979b). Examination of Agrobacterium isolates from agricultural fields has shown that not all naturally occurring strains of A. tumefaciens are sensitive to agrocin 84 (Alconero, 1980; Bouzar et al., 1991; Moore, 1979). Therefore, the variability in sensitivity to agrocin 84 of strains complicates the effectiveness of K84.

Both physical and genetic characterization have shown that the genes for agrocin 84 synthesis in strain K84 are found on an agrocin plasmid (pAgK84) (Slota et al., 1982; Farrand et al., 1992). Cooksey et al. (1982) reported that a pAgK84-cured strain of K84 lost the ability to produce agrocin 84 and no longer prevented crown gall disease. Further, pAgK84 encodes genes for self-immunity to agrocin 84 (Ellis et al., 1979b; Ryder et al., 1987) and is conjugally transferrable from one strain of Agrobacterium to another (Farrand et al., 1985). Consequently, transconjugants carrying a pAgK84 produce agrocin 84 and are immune to it.

A report by Panagopoulos et al. (1979) hypothesized that breakdown of biological control by K84 on almond was due to the conjugal transfer of pAgK84 to agrocin 84 sensitive strains of A. tumefaciens which then became resistant to agrocin 84. Further
evidence for this hypothesis was obtained from field experiments after coinoculation of K84 and an \textit{A. tumefaciens} strain resistant to agrocin 84 (Moore et al., 1991; Stockwell et al., 1990; Vicedo et al., 1993). Although these studies demonstrate experimentally that pAgK84 can transfer conjugally to \textit{A. tumefaciens}, there have been no reports of pAgK84 transconjugants found following commercial use of strain K84. To avoid the threat from conjugal transfer of pAgK84 between K84 and pathogenic \textit{Agrobacterium}, a mutant of pAgK84 strain, K1026, was constructed by Jones et al. (1988), which has a deletion in the \textit{tra} region of pAgK84. This mutated pAgK84 is no longer capable of transfer to other agrobacteria. Patent rights and EPA registration are currently being pursued for this genetically engineered strain so that it can be used commercially (Jones et al., 1989; Vicedo et al., 1993).

A breakdown of biological control by K84 also may have occurred in Oregon and California, even though K84 has been long applied successfully to reduce economic losses from crown gall disease in \textit{Prunus} sp. and caneberries in the Western part of the USA (Glenister et al. 1987). Two surprising cases of failure were observed in 1991 and 1992 from nurseries growing cherry trees in Oregon and raspberry plants in California. In both instances, the rootstocks were dipped in a suspension of K84 before planting in the field. To understand why K84 failed to prevent crown gall in these nurseries, we examined \textit{Agrobacterium} isolates from galls on plants from both sites. After reviewing both situations, we hypothesized
that the infections were initiated by agrocin 84-insensitive \textit{A. tumefaciens}, and that the pathogenic strains residing in the galls would be predominantly resistant to agrocin 84. \textit{Agrobacterium} strains isolated from both cherry and raspberry galls were characterized phenotypically and genotypically using physiological and biochemical tests, pathogenicity, production of antibiotics, sensitivity to agrocin 84, plasmid patterns, DNA fingerprints, and non-radioactive DNA hybridization using probes derived from oncogenic pTi genes. In addition, pAgK84 putative transconjugants were distinguished by serological reactions with K84 antiserum, and DNA hybridization using an agrocin synthesis gene as a probe. Presence of pAgK84 in agrocin probe positive strains was confirmed by comparing the fingerprint to that of pAgK84.
MATERIALS AND METHODS

Sampling strategy
Since over 70% of the cherry trees from an Oregon nursery (treated with K84 before transplanting) were galled, we surmised that most of the trees in the field were infected by a K84-resistant A. tumefaciens. Therefore, to obtain an idea of the distribution of strains among the diseased trees, Agrobacterium strains were isolated from 15 galled cherry trees collected randomly across the field. Even though few isolates (approximately five) were characterized per cherry tumor, there was considerable diversity among strains from the 15 galled cherry trees. This raised the question as to whether greater diversity would be observed with larger samples of isolates per tumor. Consequently, only two galled raspberry plants were processed the following year, but many more isolates were characterized. The galled raspberry plants came from a California nursery (with 10-15% gall incidence) that also treated the plants with K84 before transplanting.

Isolation of Agrobacterium strains
Agrobacterium colonies were isolated on a mannitol-glutamate (MG) medium (Moore et al 1988) with 0.2 g L⁻¹ yeast extract (MGY), and selective media 1A (selective for biovar 1 strains) and 2E (selective for biovar 2 strains) (Brisbane et al 1983). A total of 109 cherry strains and 189 raspberry strains were isolated for this
study. Strains were purified, stored at 4°C on potato-dextrose-agar (PDA) slants, and preserved in 30% glycerol in a -80°C freezer.

**Pathogenicity**

Each strain was tested for pathogenicity on each of three 6 week-old tomato seedlings in a greenhouse. Bacteria from a 36 h-old culture on MGY plates was collected with a sterile toothpick and placed onto scalpel-wounded stems. Controls included noninoculated wounded plants and plants inoculated with known pathogenic and nonpathogenic strains. The presence of tumors was recorded four weeks after inoculation. A positive response for pathogenicity was recorded if seedlings were galled.

**Antibiotic production**

Antibiotic production of wild-type *Agrobacterium* strains was determined using methods developed for detection of agrocin 84. We assumed that a zone of growth inhibition produced by the test strains against sensitive strains on agar media was the result of antibiotic production (Stonier, 1960). To expedite testing of multiple strains, a preliminary test was performed by streaking fifty microliters of a cell suspension of strain K84 (10^8 cfu/ml) down the center of a MG agar plate and allowing it to grow for 48 h. Five microliters of each test strain were then streaked perpendicular to the center strip and incubated at 28°C for 36 h. Controls on each plate consisted of *A. tumefaciens* strain C58, which is sensitive to agrocin 84 (positive
control), and *A. tumefaciens* strain B49c/83 which is insensitive to agrocin 84 (negative control). Growth inhibition of the test strains on these plates was measured after 48 h incubation (Fig. 1). The test was repeated twice. All putative producer strains were then tested by the overlay method as described below.

**Fig. 1** Growth inhibition of agrocin 84 sensitive strains, 1. C58 (a positive control), 2. A32/91, 3. A88/91; and lack of inhibition of insensitive strains 4. A50/91, 5. B49c/83 (a negative control), 6. A113/92, 7. A184/92, 8. A55/91. K84 was streaked down the center of a MG agar plate and allowed to grow 48 h before challenging with the wild-type strains.
Confirmation of antibiotic production of strains was performed according to the method of Stonier (1960) as modified by Cooksey et al. (1980). Production of antibiotics by Agrobacterium was determined on MG agar plates. A test strain was toothpicked to the MG agar medium in glass petri dishes and incubated at 28°C for 48 h. The test strain was then killed by a chloroform-soaked filter paper placed on the petri dish lid for 2 h. Agrocin sensitive strains C58 and K24, and agrocin insensitive strains B49c/83 and B6 were suspended respectively in molten phosphate buffer agar (KH₂PO₄, 1.36 g L⁻¹; K₂HPO₄, 1.74 g L⁻¹; agar, 7.2 g L⁻¹; pH 7.0-7.2). The agar was then poured onto plates spotted with test strains to create an overlay. Antibiotic production was indicated by a zone of inhibition around the test strain 24 h after adding the overlay. Each strain was tested at least twice, and a third time for putative pAgK84 transconjugant raspberry strains which induced no zone of inhibition.

Opine utilization

Determination of opine utilization and media preparation were based on Canfield et al.'s (1991) method. Bacterial suspensions (10⁸ cfu/ml) were prepared and placed in wells of a 96-well tissue culture plate and then transferred by a multi-prong replicator to media with 5 mM octopine, nopaline, or mannopine as the only source of carbon and nitrogen. Growth of bacteria on the opine plates was examined 3 to 7 days later. The test was repeated twice for all strains. To confirm opine utilization, one third of the cherry and
raspberry strains were then randomly selected and inoculated to liquid media of the same composition and grown at room temperature on a rotary shaker. An aqueous bacterial suspension of each strain was prepared with an optical density of 0.16 to 0.18 (about $10^8$ cfu/ml) as inoculum. An aliquot of 0.3 ml of bacterial suspension was pipetted into 2.7 ml of the opine liquid medium at 0 h (O.D. = 0.01). Bacterial growth was measured at 16, 20, 24, and 40 h using a Spectronic 20 colorimeter at 600 nm (Bausch & Lomb Inc., Tochester, NY). A strain was rated positive for opine utilization when the optical density of the cell suspension was greater than or equal to 0.2. Some strains reached O.D. 0.2 within 20 h; others reached this value by the 40 h reading.

**Biovar tests**

Biovar characterization of strains was conducted using standard biochemical and physiological tests (Moore et al., 1988). These tests included 3-keto lactose production, cytochrome oxidase reaction, pellicial formation in ferric ammonium citrate, acid or alkali production in litmus milk, acid production from erythritol, and melezitose, alkali production in malonic acid, mucic acid, and L-tartaric acid, and growth in 2% NaCl. Known strains were used as controls: strains C58, and RR9 are biovar 1 strains; K84, and K24 are biovar 2 strains; CG48 and CG64 are biovar 3 strains. The tests were repeated at least two times.
**Serological reaction**

A water phenol extract (WPE) of somatic antigens of each wild-type bacterial strain was prepared for serological analyses by the methods described by Bouzar et al. (1987b). The bacteria were grown overnight on slants of nutrient agar (Difco Lab., Detroit, Mich) and suspended in 0.5 ml of distilled water; 20 ul of 90% liquid phenol was added to the suspension and mixed thoroughly for 45 s. The WPE from each strain was reacted with K84 antiserum in Ouchterlony double-immunodiffusion tests (Chaparas et al., 1983). The experiment was repeated at least two times.

**Plasmid profiles**

Plasmid DNA was isolated following cell lysis and alkaline denaturation procedures of Currier & Nester (1976). The plasmid DNA was loaded onto a 0.5% Seakem agarose gel (FMC BioProd. Co., Rockland, Me.) and separated after electrophoresis at 4 v/cm for 6 h. Gels were then stained for 20 min in 0.5 ug/ml ethidium bromide solution and photographed on a short-wave Ultraviolet Products transilluminator with polaroid type 55 film. The pTi of strain C58 and pAgK84 of strain K84 were used as references.

**Purification of plasmid DNA**

Plasmid DNA from cells was separated from chromosomal DNA in a 0.5% low-melting-point (LMP) agarose (Boehringer Mannheim, Inc., Indianapolis, IN) gel after electrophoresis. Plasmid DNA bands
chosen for further study were cut from the gel and purified using a Gelase™ digestion method (Epicentre Technologies, Inc., Madison, WI). Slices of LMP-agarose gel with plasmids were soaked in GELase buffer (40 mM Bis-Tris, pH 6.0, 40 mM NaCl) for 1 h. Excess GELase buffer was removed and the agarose slices were then melted at 70°C. The DNA was recovered by the addition of GELase™ to digest the long-chain polysaccharides in molten agarose. DNA was then precipitated by addition of 95% ethanol. Precipitated DNA was collected by centrifugation in an eppendorf centrifuge at full speed for 10 min and the pellet was washed once with 70% ethanol and recentrifuged. The pellet was air dried and dissolved in water.

**DNA fingerprints**

DNA isolated from strains or purified from agarose was digested with restriction endonucleases under conditions described by the manufacturer (New England Biolabs, Inc. Beverly, MA). To compare DNA fingerprints of strains A8/91, A117/92, and A118/92, which were serologically identical to K84, restriction endonucleases *BglII* and *SalI* were used. Restriction fragments were separated by electrophoresis in an 0.8% agarose gel according to Maniatis et al. (1982).

**Probe DNA**

Since pathogenic *Agrobacterium* strains carry a tumor inducing (Ti) plasmid with specific, conserved oncogenic DNA sequences, the
DNA probes selected for this study were virFAB from the virulence region (Stachel et al., 1986) and iaaM and iaaH (tmsl-tmr) from the T-DNA region (Garfinkel et al., 1981). An agrocin synthesis gene, SmaIG (SmaI fragment G) (Farrand et al., 1985) was used to detect strains suspected of carrying the agrocin synthesis genes or pAgK84. These DNA probes were labelled by a non-radioactive digoxigenin method (Martin et al., 1990) for DNA hybridization.

**Colony hybridization**

Colony hybridization was performed using a modified protocol of the Genius digoxigenin-nonradioactive-labelling-detection system described by Boehringer Mannheim Co., Indianapolis, IN. *Agrobacterium* colonies were grown on a nylon membrane (Boehringer Mannheim Co.) positioned on a MGY agar plate for 36 h. The membrane was removed, and the bacterial cells on the membrane were lysed with alkali solution. The membrane was then baked in a vacuum oven for 2 h at 80°C. Colony debris was removed by incubating the membrane in a hybridization solution at 68°C for 1.5 h; the membrane surface was then wiped gently with a moistened towel or rinsed with distilled water. Prehybridization and hybridization were both performed at 68°C overnight. The membrane was then washed three times for 10 min at room temperature with a solution containing 2X SSC and 0.1% SDS. The final washes were performed twice in the same solution, but at 55°C and at least 30
min for each wash. Hybridization was determined using an immuno-
colorimetric development method (Martin et al., 1990).

**Southern blots**

Plasmid DNA and restriction endonuclease digested DNA separated by electrophoresis on agarose gels were transferred to a nylon membrane as described by Southern (1975) and the instruction manual of Schleicher & Schuell NCTM (Schleicher & Schuell, Inc., Keene, NH). Lablled DNA probes tmsI-tmr and virFAB were used to determine whether plasmids from the wild-type isolates carried DNA associated with a pTi. To determine whether the suspected pAgK84 isolated from the cherry and raspberry isolates was similar to pAgK84 from K84, SmaIG and SmaI-digested whole pAgK84 were used as probes. The location of pAgK84 was determined in plasmid profiles by Southern hybridization with the SmaIG probe. To identity the similarity of putative pAgK84 and pAgK84, the restriction fragments of them were compared. Purified putative pAgK84 was digested with SmaI and then hybridized to the SmaI-digested pAgK84 probe. Hybridization was conducted using the conditions described previously for colony hybridization.

**Characterization of strains harboring pAgK84**

Colony hybridization with the SmaIG probe was used to screen for strains carrying SmaIG gene. A positive reaction indicated that these strains might harbor pAgK84. The SmaIG probe positive
strains were further characterized by reacting them with K84 specific antiserum to determine whether they were serologically identical to strain K84. Plasmids also were isolated from the putative transconjugants to determine whether they carried a plasmid similar to pAgK84, as determined by plasmid profiles and Southern blots with SmaI G probe. The restriction fragment fingerprint of each putative pAgK84 and that of known pAgK84 were compared to determine their similarity.
RESULTS

Based on tests for pathogenicity, agrocin 84 sensitivity, antibiotic production, and biovar designations, *Agrobacterium* strains isolated from cherry and raspberry tumor tissues were grouped into 7 and 9 distinct phenotypes, respectively (Fig. 2A & 2B). Sixty nine percent of the 109 cherry strains were pathogenic on tomato seedlings, and 85% of these pathogens were resistant to agrocin 84 (Fig. 2A). These agrocin-resistant pathogens were isolated from 15 of the 17 galls sampled, showing that this phenotype was present on trees throughout the planting site. In contrast, all 150 pathogenic strains from raspberry were resistant to strain K84 (Fig. 2B). Each cherry tumor had an average of three phenotypes with a range of 1-4 per tumor. Among raspberry strains, however, six phenotypes were isolated from one gall and seven from the other.

Colony hybridization (Fig. 3) with the two oncogenic labeled DNA probes, tmsl-tmr and virFAB, also allowed us to distinguish pathogens from nonpathogens. There was 100% agreement between colony hybridization data and inoculation of tomato seedlings. Similarly, wild type strains probed with an agrocin synthesis gene (SmalG) allowed identification of strains that had the agrocin synthesis gene. The results from colony hybridization showed that 15 percent of the cherry strains (Fig. 4a) and 68 percent of the raspberry strains (Fig. 4c) had a gene hybridizing with the SmalG probe, including both pathogens and nonpathogens.
(A) Cherry strains

Path⁻ Agr r Ab⁺ (bdfil) 9%
Path⁺ Agr r Ab⁺ (dehijo) 12%
Path⁻ Agr s Ab⁻ (m) 4%
Path⁺ Agr s Ab⁺ (cgp) 5%
Path⁺ Agr s Ab⁻ (adgilmn) 7%
Path⁻ Agr r Ab⁻ (abdfijlpq) 18%

(B) Raspberry strains

Path⁻ BVx Ab⁺ (y) 7%
Path⁻ BVx Ab⁻ (xy) 8%
Path⁺ BV1 Ab⁻ (y) 11%
Path⁻ BV2 Ab⁺ (x) 1%
Path⁺ BV2 Ab⁻ (xy) 13%
Path⁺ BV1 Ab⁺ (xy) 17%
Path⁺ BVx Ab⁺ (x) 1%
Path⁺ BV2 Ab⁺ (xy) 38%
Path⁺ BV2 Ab⁺ (xy) 4%

Fig. 2 Phenotypic distribution of Agrobacterium strains isolated from crown galls on (A) cherry and (B) raspberry plants. Phenotypes were derived from a matrix table that compared phenotypic traits of pathogenicity (Path), antibiotic production (Ab), agrocin 84 sensitivity (Agr), and biovars (BV). The letters in parentheses represent the different tumor sources from which the strains were isolated. The abbreviations of characteristics of each group represent: Path⁺=pathogenic; Path⁻=non-pathogenic; Agr⁺=resistant to agrocin 84; Agr⁻=sensitive to agrocin 84; Ab⁺=antibiotic production; Ab⁻=no antibiotic production.
Fig. 3 Colony hybridization of *Agrobacterium* strains hybridizing with either tmsl-trm or virFAB probes. The dark spots represent a positive reaction with the DNA probe. The white arrow indicates a positive control, *A. tumefaciens* strain B6; the black arrow indicates a negative control, strain K84.

Twenty five percent of the strains from cherry produced a zone of inhibition *in vitro* against both K24 and C58; 60% of these hybridized with SmalG (Fig. 4a and 4b). In contrast, 65% of the strains isolated from raspberry inhibited growth of both K24 and C58 or only K24, and 89% of these strains hybridized to the SmalG probe (Fig. 4c and 4d). Of 129 raspberry SmalG probe positive strains, 106 pathogenic and nonpathogenic strains produced an antibiotic against both C58 and K24; 3 pathogenic strains produced an antibiotic which only K24 was sensitive to. However, 18 pathogenic and 2 nonpathogenic strains did not produce a zone of inhibition against C58 and K24 (Table 1). Ten percent and 7% of pathogenic cherry and raspberry strains, respectively, produced an inhibition zone against K24 and C58 (Fig. 4b & 4d) but did not hybridize with SmalG.
Fig. 4 Pathogenicity and antibiotic production of Agrobacterium strains that hybridized to the SmaIG gene (SmaIG +) or did not hybridize to the gene (SmaIG -). A total of 109 cherry and 189 raspberry strains were analyzed. The abbreviations of each symbol represent: Path- = nonpathogenic; Path+ = pathogenic; Antibio- = no antibiotic production; Antibio+ = antibiotic production.
Table 1 Antibiotic production by 129 SmaIG a) probe positive Agrobacterium strains from raspberry galls

<table>
<thead>
<tr>
<th>Strains</th>
<th>Number of strains producing a zone of inhibition b) against Agrobacterium strain</th>
<th>Both C58 and K24</th>
<th>K24 only</th>
<th>Neither C58 nor K24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogens</td>
<td></td>
<td>88</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Nonpathogens</td>
<td></td>
<td>18</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

a) SmaIG is the SmaI restriction fragment G of agrocin synthesis genes in a pAgK84.
b) A zone of growth inhibition produced by test strains against sensitive strains was determined on a mannitol-glutamate agar medium following a modified Stonier's method.

All the cherry strains belonged to biovar 2. In contrast, the raspberry strains were more diverse: 32.5% were biovar 1, 52% were biovar 2, and 15.5% were intermediate between biovar 1 and biovar 2 (called biovar x in this study). Both pathogenic and nonpathogenic strains were distributed among biovar 1, biovar 2, and biovar x (Fig. 5). Reactions from the biovar tests are shown in Table 2. Among raspberry strains, the SmaIG probe positive strains represented biovar 1, biovar 2 and biovar x, but were more heavily distributed to the biovar 1 (40%) and biovar 2 (47%) (Fig. 6).
Fig. 5 Opine utilization and biovar (BV) distribution for 189 pathogenic and nonpathogenic *Agrobacterium* strains isolated from raspberry galls
Table 2 Biochemical reactions for biovar designation of the raspberry Agrobacterium strains

<table>
<thead>
<tr>
<th>Biochemical tests</th>
<th>BV 1 4)</th>
<th>BV 2</th>
<th>BV 3</th>
<th>Raspberry strains 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C58</td>
<td>RR9</td>
<td>K84</td>
<td>K24</td>
</tr>
<tr>
<td>3-keto-lactose 1)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ferric ammonium citrate 2)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Litmus</td>
<td>B 5)</td>
<td>B</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Oxidase 3)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth in 2% NaCl</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Melezitose</td>
<td>Y</td>
<td>Y</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>Erythritol</td>
<td>NG</td>
<td>NG</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Malonic acid</td>
<td>Y</td>
<td>Y</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>Mucic acid</td>
<td>Y</td>
<td>Y</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>Y</td>
<td>Y</td>
<td>B</td>
<td>B</td>
</tr>
</tbody>
</table>

1) + represents that strains produce a yellow precipitation of CuO₂ from the reaction; - represents no reaction.
2) + represents a pellicle produced in the reaction; - represents no pellicle produced.
3) + represents an oxidase reaction; - represents no reaction.
4) BV represents biovar.
5) The letters represent different colors, B: blue, Y: yellow, NG: no growth, and P: purple.
6) All 189 raspberry strains were tested; 61 strains were biovar 1, 98 strains were biovar 2, and 30 strains were biovar x.
Fig. 6 Opine utilization, and biovar (BV) designation of 129 raspberry *SmaI*G probe positive strains
All cherry strains except five nonpathogens utilized nopaline. These five nonpathogenic strains did not utilize any of the opines tested. None of the cherry strains utilized octopine. However, eleven strains utilized mannopine as well as nopaline (data not shown). Both pathogenic and nonpathogenic raspberry strains utilized either nopaline, octopine, or both. The majority (85.5%) of them (including biovar 1, biovar 2 and biovar x) utilized nopaline. In contrast to cherry strains, none of the raspberry strains utilized mannopine. Sixteen (8%) octopine utilizing raspberry strains were biovar 2 and pathogenic. Six point five percent (11 strains) of the raspberry strains utilized both nopaline and octopine (Fig. 5). In this study, opine utilizations of strains were determined and recorded from the tests on opine solid media, and one third of them also were confirmed in liquid media. Among 129 SmalG probe positive strains from raspberry galls, the majority were pathogenic and utilized nopaline (Fig 6). Five percent of them utilized both nopaline and octopine, and 2% of them utilized octopine only.

The recovery of K84 strains in the galls was surprisingly low. Only one nonpathogenic cherry strain (A8/91) and two nonpathogenic raspberry strains (A117/92 and A118/92) developed a homologous precipitin band to K84 antiserum in the serological reaction (Fig. 7). DNA fingerprints of these three strains were similar to that of strain K84 (Fig. 8). This indicates that these three serologically identical strains were possible strain K84. Since none of the
pathogenic strains hybridizing to SmaIG reacted with K84 antiserum, it is unlikely that K84 was a recipient of a pTi.

Fig. 7 Immunodiffusion reaction of cherry and raspberry strains against K84 antiserum. The center wells of panels A and B contained K84 antiserum. Outer wells of panel A contain water phenol extracts of test strains K84 (well 1 and 4), A8/91 (well 2), A22/91 (well 3), A45/91 (well 5), A29/91 (well 6). Outer wells of panel B contain water phenol extract of test strains K84 (well 1 and 4), A109/92 (well 2), A111/92 (well 3), A118/92 (well 5), A117/92 (well 6). A homologous precipitin band formed between strains A8/91, A117/92, A118/92, and K84 indicating serological identity with strain K84.
Fig. 8 DNA fingerprints of the serologically identical strains A8/91, A117/92, A118/92, and K84. DNA was digested with four restriction endonucleases, (A) BglII and (B) SacII. Putative transconjugants, A82/91 and A11/92, and non-putative transconjugants, A22/91 and A39/92, were used as controls. Lane 1 and 5: K84; lane 2: A8/91; lane 3: A117/92; lane 4: A118/92; lane 6: K24; lane 7: A82/91; lane 8: A11/92; lane 9: A22/91; lane 10: A39/92. The sizes of marker shown in Kb are indicated on both sites of each panel.

Plasmid profiles based on number and size from both the cherry and raspberry strains were diverse (Fig 9A). The number of plasmids detected varied from zero to four. After repeated tests, only 22 raspberry strains yielded no detectable plasmids (data not shown). A large plasmid similar in size to a pTi was found among the nonpathogenic strains, but none of them showed any homology with the oncogenic DNA probes, tmsl-tmr and virFAB (Fig. 9B(1)).
Fig. 9 (A) Plasmid patterns of pathogenic and nonpathogenic cherry and raspberry *Agrobacterium* strains; (B) Southern hybridization with (1) *tmsl-tmr* (or *virFAB*) and (2) *SmaI*G probes. These panels were composited of lanes from 2 or more different results. Lane 3, 5, 8, and 10 are pathogenic strains: A57/91, A39/91, A18/92, and A66/92, respectively. Lane 4, 6, 7, 9, 11, 12 are nonpathogenic strains: A8/91, A56/91, A88/91, A118/92, A150/92, and A189/92, respectively. pTiC58 (lane 1: C58) is indicated by a black arrow; pAgK84 (lane 2: K84) is indicated by a white arrow.
The size of the pTi among the pathogenic strains also was variable (Fig. 9A). Most of the strains that hybridized to the SmaI G probe during colony hybridization also carried a plasmid of similar size to pAgK84 (Fig. 9A). Southern hybridization with the SmaI G probe showed that these plasmids had a gene related to SmaI G of pAgK84 (Fig. 9B(2)).

The restriction fragment fingerprint of putative pAgK84 from the cherry and raspberry strains was compared to that of pAgK84 from strain K84. The putative pAgK84 DNA from both the serologically identical strains (A8/91, A117/92, and A118/92) and non-serologically identical strains (including both cherry and raspberry strains) was similar to that of pAgK84 from strain K84 (Fig. 10).
Fig. 10 Restriction fragment fingerprint of pAgK84. pAgK84 isolated from putative transconjugants of Agrobacterium was digested with Smal restriction enzyme. A Southern blot was performed using Smal-digested whole pAgK84 as a non-radioactive probe. Lane 2-8 are raspberry transconjugants: A117/92, A118/92, A139/92, A126/92, A70/92, A29/92, A1/92 and lane 9-15 are cherry putative transconjugants: A61/91, A60/91, A57/91, A18/91, A50/91, A45/91, A8/91. The size of marker shown in Kb is indicated on the right of strain K84 (lane 1). Banding patterns were compared to Smal-digested pAgK84 from strain K84.
DISCUSSION

This work presents the important finding that many agrocin probe positive strains were readily isolated from galled plants in commercial plantings previously treated with K84. These strains could be pAgK84 transconjugants, or they may have been naturally present in the soil at the planting site or on the rootstocks before transplanting. However, data from our studies (unpublished) and the literature provide circumstantial evidence that these isolates were the result of conjugation between K84 and naturally occurring Agrobacterium strains within the cherry and raspberry tumors. For example, we isolated 200 Agrobacterium strains from the soil where the galled cherry trees from this study were grown, and none of them hybridized to the SmaI G probe. In addition, many Agrobacterium strains have been isolated from tumors and soil in a variety of studies without finding any K84-like strains (Bouzar et al., 1987b; Bouzar et al., 1991; Michel et al., 1990; Nesme et al., 1987), suggesting that this phenotype occurs rarely in nature. An exception to this point is Agrobacterium tumefaciens strain Bo542, an isolate from dahlia gall in Europe, that shows agrocin 84-like antibiotic activity and carries a plasmid very similar to pAgK84 (Slota et al. 1982). Pathogenic pAgK84 transconjugants were isolated from galls on trees that had been treated with K84 and transplanted to soil infested with agrocin-K84 resistant pathogens, but the number recovered was low (nine) (Vicedo et al., 1993). Similiarly, Moore et
al. (1991) isolated pAgK84 transconjugants from cherry tumors, but at a low frequency (about 10^-4). K84-like Agrobacterium strains also were found infrequently among 185 Agrobacterium strains selected from our culture collection; only three Agrobacterium strains reacted with the agrocin synthesis DNA probe and each carried a pAgK84 (Moore et al., 1991). Given these findings, the circumstantial evidence seems substantial in favor of the argument that our strains carrying a plasmid similar to pAgK84 were likely pAgK84 transconjugants. If they were pAgK84 transconjugants, the high proportion (68%) of pAgK84 transconjugants in raspberry strains (Fig. 6) strongly supports the hypothetical threat of transconjugants (Ellis et al., 1979a; Panagopoulos et al., 1979) occurring when strain K84 is used commercially in the field.

In our study, it was unknown whether the conjugal transfer of pAgK84 into pathogenic and nonpathogenic Agrobacterium strains occurred before or after tumor formation. However, given the role of opines in conjugal transfer of pTi between strains of A. tumefaciens (Ellis et al., 1982; Petit et al., 1978), it is probable that conjugation occurred after tumor formation. A report from Hayman et al. (1990) indicated that pAtK84b (a nopaline plasmid) of strain K84 can be conjugally induced by agrocinopine A and B. We don't know if opines have the same effect on the conjugal transfer of pAgK84 as pTis and pAtK84b. If they do, transfer of pAgK84 between K84 and other agrobacteria probably occurs after tumor formation to allow time for opine synthesis in the gall tissues.
If conjugation occurred after tumor initiation, it indicates that K84 had already failed to protect plants before the pAgK84 transconjugants were present. Hence, the transconjugants found in this study (Fig. 4a and 4c) probably do not represent the cause of failure of biological control by K84 to prevent galls on the cherry and raspberry plants, whereas the presence of K84-insensitive strains of A. tumefaciens at the time of planting does.

It also appears that conjugation occurred between these K84-insensitive pathogens and K84 more frequently in raspberry galls than in cherry galls. If the pathogenic putative transconjugants with a pAgK84 survive, future application of K84 to plants to be grown in that same field may be ineffective. Stockwell et al's (1990) work showed that such transconjugants could survive for at least one year in association with cherry seedlings. So that, once pathogenic pAgK84 transconjugant populations are established in fields, the control of crown gall disease by K84 may be reduced if not eliminated. However, this projection does not take into account whether pathogenic transconjugants escape from the galls during the growing season nor the distance which the transconjugant may move in the soil from the point source of infection. Since nursery trees are typically produced for resale, the gall, as a source of transconjugants, would be removed from the field at harvest.

Two major contributions result from transfer of pAgK84 to the transconjugants: agrocin 84 production and immunity to agrocin 84 (Ryder et al., 1987). Indeed, all the putative transconjugants from
cherry galls produced an antibiotic against the same strains as K84. More variability was found, however, in the raspberry strains. Of 129 putative transconjugants from raspberry galls, 106 inhibited the same agrocin 84 sensitive strains of *A. tumefaciens* as strain K84, 3 produced an antibiotic against only K24 but not C58, and 20 did not produce an antibiotic against either K24 or C58 (Table 1). The last two groups of strains may carry a mutated pAgK84 having different specificity to the sensitive strains. Based on Ryder et al's (1987) finding, five regions on pAgK84 were identified and involved in agrocin synthesis by genetic complementation of a series of mutants. The ability of agrocin production among their mutants differed according to the number of encoding regions carried on the mutated pAgK84. Therefore, mutation in any gene of these regions will influence agrocin 84 synthesis. However, it also was not excluded that the antibiotic produced by the strains against only K24 might be different from agrocin 84. In addition to these strains, there also were 11 cherry and 13 raspberry SmaIG negative strains producing an antibiotic against only K24 (data not shown), indicating that other antibiotics rather than agrocin 84 could be produced by *Agrobacterium* strains. Whether spontaneous mutation or deletion/insertion occurred in the pAgK84 of SmaIG positive strains unable to produce agrocin 84, and whether the occurrence is influenced by a specific plant host are intriguing questions.

In this study, only three isolates appeared to be wild type K84; one from cherry (A8/91) and two from raspberry (A117/92 and
A118/92). All three strains were nonpathogenic on tomato seedlings, utilized nopaline, were biovar 2, produced antibiotics against the same strains as K84, were serologically indistinguishable from K84 specific antiserum, carried a pAgK84, and had the same DNA fingerprint as K84, indicating that they were most probably strain K84. Thus, the recovery rate of K84 from crown gall tissues at the end of the growing season was low (1/109 in cherry strains and 2/189 in raspberry strains). This result was similar to Stockwell et al's (1993) study which showed that the population of K84 recovered from cherry galls was ten to one hundred lower than the pathogenic strain. Even so, antibiotic production and biological control by the strain K84 recovered after 2 years' survival in the rhizosphere was indistinguishable from that of the parental strain, showing the stability of these traits. Therefore, the low number of K84 strains detected in tumors shows that it can survive in the tumor, but it appears to be a poor competitor with the natural agrobacteria and other microflora associated in that niche.

In the present study, a diverse population of Agrobacterium was detected in the same plant tumor (especially in the raspberry galls), whereas strains with the same characteristics were found in different tumor tissues (Fig. 2A & 2B), indicating that strain diversity is high within the same field. Seven phenotypes of cherry strains were distributed within 17 cherry galls, in contrast, nine different phenotypes of raspberry strains were isolated from only two tumors. Even though the population of Agrobacterium in both
cherry and raspberry was diverse, the majority of them were pathogenic, as reported in other studies (Bouzar et al., 1991; Michel et al., 1990; Nesme et al., 1987). The main difference between these other studies and ours is the fact that a very high proportion of our pathogenic isolates was also insensitive to agrocin 84. In addition, among our K84-insensitive strains, especially raspberry strains, the majority were putative transconjugants carrying a pAgK84. It is probable that a variety of putative transconjugants arose in the population from conjugation of K84 with the mixed populations of wild-type *Agrobacterium* in that niche.

Another characteristic reported about the population structure of *Agrobacterium* isolates from tumors and soil is that the strains typically belong to a single predominant biovar (Bouzar et al., 1987a; Bouzar et al., 1991; Michel et al., 1990; Nesme et al., 1987). In this study, all cherry strains were biovar 2, and biovar 2 strains predominated in the raspberry galls (Fig. 2A & 2B). Burr et al. (1993) also reported that most of *A. tumefaciens* from raspberry were biovar 2 and insensitive to K84 in vitro.

Different combinations of opine utilization patterns and biovars were found in *Agrobacterium* strains from raspberry but not cherry galls (Fig. 5). This finding suggests that some plant species may support a more diverse population of *Agrobacterium* strains than another. In addition, the presence of different opine utilizers among raspberry strains isolated from the same tumor (Fig. 2B) shows that various opine utilizers co-inhabit the same tumor, indicating that a
particular opine in a tumor is not the sole nutrient source for *Agrobacterium* in tumors.

Among the nonpathogenic cherry (33) and raspberry (39) strains, a high number (all except 5 cherry strains) were capable of utilizing nopaline, octopine or manopine. One explanation for this observation is that opine catabolic genes are not associated with a pTi. Montoya et al. (1978) reported that octopine catabolic genes may locate on the chromosome of some *Agrobacterium* strains. However, the nonpathogenic agrobacteria may carry a disarmed Ti plasmid which has lost the oncogenic genes (e.g. pAtK84b, Clare et al., 1990) but retained the opine catabolic genes, or on a non-Ti plasmid (Wabiko et al., 1990). Clearly, bacteria other than *Agrobacterium* (Bergeron et al., 1990; Canfield et al., 1991; Nautiyal et al., 1990) and fungi (Beauchamp et al., 1990), all lacking a pTi, are capable of utilizing opines as a carbon and nitrogen source.

Because opine synthesis and catabolic genes are typically carried on a pTi, the pTis of *A. tumefaciens* have been designated as specific opine-types according to the ability of the *Agrobacterium* strains to utilize in vitro the opines present in their host tumor cells (Guyon et al., 1980). Accordingly, among the pathogenic raspberry strains, a nopaline-type pTi was scattered broadly in strains with different chromosomal backgrounds, i.e. biovar 1, biovar 2 and biovar x. Conversely, nopaline-, octopine-, and nopaline-octopine-type pTis were found in strains with the same chromosomal traits (biovar 2 strains). This situation was not found, however, in the cherry
strains. All the pathogens from cherry were consistently associated with a nopaline-type pTi and in strains with biovar 2 chromosomal traits. These results are similar to several studies (Bouzar et al., 1993b; Nesme et al., 1992; Schofield et al., 1987), all of which suggest some pTis are stably maintained and highly associated with particular *Agrobacterium* strains. Whether host plants influence the association and distribution of pTis in *Agrobacterium* populations is a very interesting question.

Our plasmid profiles showed that most of the pathogenic and nonpathogenic strains carried a variable number of plasmids (0-4) of different sizes (Fig. 9A). There was no correlation between number of plasmids, pathogenicity, biovar, sensitivity to agrocin 84, opine utilization, and host, as also observed by Albiach et al. (1992). Most of our nonpathogenic strains carried large plasmids of a size similar to the pTi of control strain C58, but there was no homology to the oncogenic DNA probes, *tmsI-tmr* and *virFAB*, indicating that these large plasmids were at least missing these two DNA fragments. However, these large plasmids might represent a disarmed pTi that has lost its oncogenic genes, since the opine catabolic genes are encoded on a pTi, and most of our nonpathogenic strains were capable of utilizing opines. Several studies (Clare et al., 1990; Merlo et al., 1977) have shown that avirulent agrobacteria carry a non-oncogenic large plasmid sharing homology with the pTi (eg. pAtK84b of strain K84, encoding catabolism of nopaline and
belonging to the same incompatibility group as the nopaline-type pTiC58).

In both the cherry and raspberry strains, diverse pathogens and nonpathogens were isolated from the same tumor. It has been reported that opines inside tumors can both promote growth of bacteria that carry a pTi and induce conjugal transfer of the pTi to nonpathogenic Agrobacterium of different chromosomal traits (eg. biovars) (Petit et al., 1978). If true, the process would increase the diversity within pathogenic Agrobacterium. Whether any of the diverse pathogenic strains we isolated were derived from nonpathogenic strains acquiring a pTi through genetic exchange is open to speculation. Even though conjugal transfer of pTi between strains was not proven in this study, our finding of pAgK84 putative transconjugants clearly supports the phenomenon of genetic exchange. From our serological studies, none of the pAgK84 putative transconjugants were serologically identical to K84, indicating that strain K84 was the donor of pAgK84 to pathogenic and nonpathogenic Agrobacterium rather than a recipient of a pTi.

Of the pathogenic and nonpathogenic putative transconjugants that carry a pAgK84, the nonpathogenic may be more important in the long term for preservation of pAgK84 than pathogenic. For example, nonpathogenic strains of Agrobacterium are predominantly isolated from soil (Bouzar et al., 1987a; Kerr, 1969; Schroth et al., 1971), suggesting that nonpathogens survive better in soil than pathogens. Therefore, even though the ratio of nonpathogenic to pathogenic
putative transconjugants from raspberry galls was low (Fig. 4a and 4c), the potential for long survival of nonpathogenic putative transconjugants must be considered. Regardless, once either pathogenic or nonpathogenic putative transconjugants become established in a planting site, new disease management strategies will be needed.
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