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# Novel Ti Plasmids in *Agrobacterium* Strains Isolated from Fig Tree and Chrysanthemum Tumors and Their Opinelike Molecules

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**Galls naturally induced on Fig and chrysanthemum plants by strains of *Agrobacterium* contained, in addition to other well-characterized opines such as nopaline, three tumor-specific opinelike molecules. These molecules were identified as deoxy-fructosyl-glutamine (dfg), deoxy-fructosyl-5-oxo-proline (dfop), and chrysopine (Chilton *et al.*, unpublished). Strains isolated from Fig tree and chrysanthemum tumors harbored different and unrelated Ti plasmids as judged by hybridization with various *vir* and T-DNA probes. They also exhibited different opine-catabolic properties. The strains isolated from chrysanthemum plants (Chry strains) and Fig trees degraded chrysopine, but only the Chry strains used dfg and dfop. Remarkably, other strains of *Agrobacterium* catabolized these two molecules: dfg was degraded by most pathogenic and nonpathogenic *Agrobacterium* strains, and dfop by all *Agrobacterium* strains degrading the opine agropinic acid. These results have strong ecological and evolutionary inferences which fit previous speculation on the origin of opine-related functions.**

*Additional keywords:* opine degradation.

*Agrobacterium tumefaciens* is the causal agent of crown gall, a disease that leads to tumor formation on dicotyledonous plants. The disease results from the transfer of a fragment of bacterial DNA to the nuclear genome of host plant cells. This piece of DNA, known as T-DNA (transferred-DNA), is part of the large Ti (tumor-inducing) plasmid of *Agrobacterium*. T-DNA transfer depends on expression of nontransferred genes called *vir* (virulence) genes, located elsewhere on the Ti plasmid. Genes located on the T-DNA are expressed in transformed plant cells. Their products are responsible for the crown gall tumor phenotype which consists of uncontrolled plant cell proliferation and synthesis of tumor-specific, low molecular weight compounds termed opines (for reviews see Citovsky *et al.* 1992; Gelvin 1992; Hooykaas and Schilperoort 1992; Winans 1992). The proliferation of transformed plant cells results mostly from the expression of *iaa* (or *tms*) and *cyt* (or *tmr*) genes responsible for the biosynthesis of auxin and cytokinin, respectively (reviewed in Gaudin *et al.* 1994; Morris 1986). Opine synthesis depends on *ops* genes and proceeds in the tumor cells at the expense of plant metabolites. Remarkably, opines can be specifically degraded by the inducing pathogenic bacterium in which opine degradation functions are Ti plasmid-borne. Furthermore, some opines also induce the conjugal transfer of the pathogenic plasmid between strains of *Agrobacterium*. The presence of opines in the crown gall tumor therefore provides an ecological niche favoring growth of the pathogen and dissemination of the Ti plasmid (reviewed in Dessaux *et al.* 1992; Farrand 1993; Gelvin 1992; Winans 1992).

More than 20 opine molecules have been identified. However, a crown gall tumor synthesizes only a limited number of opine molecules. Indeed, the nature of opines synthesized in plant cells and degraded by the bacteria depends on the type of Ti (or Ri) plasmid found in the inducing bacteria. This feature provides a basis for a tentative classification of Ti and Ri plasmids (reviewed in Dessaux *et al.* 1992). Representative strains of the four main groups of Ti plasmids and some of their opine-catabolic properties are shown in Table 1. Being amino and keto acid derivatives, a limited number of opines occurs in nature as different stereoisomers. This is exemplified by the case of the opine succinamopine (Chilton *et al.* 1984a, 1984b) detected as the L,L isomer in tumors induced by agropine-type *A. tumefaciens* strains (e.g., Bo542) and as the D,L isomer in tumors induced by succinamopine-type *A. tumefaciens* strains (e.g., EU6) (Chilton *et al.* 1985). The degradation of these molecules is correlated with the presence of the relevant Ti plasmid, i.e., agropine-type Ti plasmids encode L,L succinamopine degradation whereas D,L succinamopine degradation is encoded by succinamopine-type Ti plasmids (Chilton *et al.* 1984a, 1984b, 1985). A similar observation can be made for the opines cucumopine and mikimopine and the relevant Ri plasmids (Davioud *et al.* 1988a, 1988b; Isogai *et al.* 1990).

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Besides the above-mentioned opine-related properties, those of a limited number of other plasmids are not clearly known. Thus, occurrence of other Ti plasmids has been reported in strains of *Agrobacterium* isolated from Fig tree (Bouzar *et al.,* 1995) and chrysanthemum (Bush and Pueppke 1991; Kovács and Pueppke 1994). However, only limited data on the nature of opines detected in these tumors or degraded by the bacteria were given. This prompted us to re-examine the pathogenic properties, the opine-related properties and the plasmid content of various *Agrobacterium tumefaciens* strains isolated from Fig tree (Fig strains) and chrysanthemum (Chry strains) tumors. We report here the results of this study which led to the identification of new types of Ti plasmids and to the characterization of three unusual opines.

## RESULTS

### Preliminary investigations on pathogenicity of the new isolates and on opine content of the tumors.

All new strains were first assayed for pathogenicity on various test plants. We retained for further studies only the pathogenic isolates which are listed in Table 2. Among these, strains ANT4, 2788, AF3.10, and AF3.44 were tested on numerous host plants. They exhibited a broad host range since they repeatedly induced tumors on *K. tubiflora, K. daigremontiana, H. annuum, N. tabacum,* and *D. stramonium.* Interestingly, strains AF3.10 and AF3.44 induced tumors exhibiting root formation on *Kalanchoe* whereas strains ANT4 and 2788 induced tumors with teratomalike structures on, respectively, *N. tabacum* and *D. stramonium.*

To determine the opine-type of these new strains, the opine content of the tumors was analyzed by high voltage paper electrophoresis (HVPE) followed by various staining procedures. The phenanthrenequinone staining (not shown) revealed the presence of a compound co-migrating with nopaline at pH 1.9 in extracts obtained from tumors induced by the seven Fig strains and by one of the Chry strains, 2788 (Table 3). The Trevelyan's reagent also revealed the presence of three silver nitrate-positive (SNP) compounds in the extracts obtained from tumors induced by all of the new isolates. These compounds, which migrated at pH 1.9 and 2.8 as the mannityl opines agropine, mannopine, and agropinic acid (Fig. 1), are referred to as SNP compounds I, II, and III, respectively (Table 3). Additionally, in extracts prepared from tumors induced by Chry strains except strain 2788, a silver nitrate-negative spot appeared at pH 2.8 with electrophoretic mobility similar to that of succinamopine. No other opinelike molecules were directly detected by HVPE in the tumors extracts. However, L,L leucinopine was detected in some of the chrysanthemum tumors (Table 3). The presence of this opine

**Table 1.** Reference bacterial strains, plasmids and related opine catabolic properties

| Strains | Opine-type of Ti or Ri plasmid | Observations or opine catabolized |
|---|---|---|
| *Agrobacterium tumefaciens* | | |
| ACH5 15955 | Octopine | Octopine, agropine, mannopine, mannopinic acid, agropinic acid |
| Bo542 | Agropine | Agropine, mannopine, mannopinic acid, agropinic acid, L,L leucinopine, L,L succinamopine, agrocinopines C and D |
| C58 | Nopaline | Nopaline, agrocinopines A and B |
| EU6 | Succinamopine | D,L and L,L succinamopine, D,L succinopine, D,L succinopine lactam D,L and L,L-leucinopine |
| *Agrobacterium rhizogenes* | | |
| 1724 | Mikimopine | Mikimopine |
| 2659 | Cucumopine | Ccucumopine, cucumopine lactam |
| 8196 | Mannopine | Mannopine, mannopinic acid, agropinic acid |
| 15834 | Agropine | Agropine, mannopine, mannopinic acid, agropinic acid |
| Nonpathogenic *A. tumefaciens* | | |
| C58.00 | None | pTi-, pAt-cured derivative of strain C58 resistant to rifampicin and streptomycin |
| SA122 | None | pTi-cured derivative of strain 15955 |
| SA122(pYDH101) | None | Mannopine and agropinic acid |
| SA122(pYDH208) | None | Mannopine and agropine |
| SA122(pYDH301) | None | Mannopinic acid |
| SA122(pYDH303) | None | Mannopinic acid and agropinic acid |
| SA122(pYDH304) | None | Mannopine, mannopinic acid and agropinic acid |
| SA122(pYDH501) | None | Octopine |

**Table 2.** Characteristics of the new isolates

| Strains | Original host plant | Country (year) | Source | Reference | Biotype |
|---|---|---|---|---|---|
| Chry-strains | | | | | |
| ANT4 | *C. x hortorum* | France (1991) | C. Poncet | This work | I |
| Chry-9 | *C. morifolium* | USA (1975) | W.S. Chilton | Miller *et al.* 1975; Bush and Pueppke 1991 | I |
| K224, K289 | Chrysanthemum *sp.* | Colombia (1980) | L. Moore | Unpublished | I |
| 2788 | Chrysanthemum *sp.* | France (1988) | M. Ridé | This work | I |
| Fig-strains | | | | | |
| AF1.72, AF3.9, AF3.10, AF3.43, AF3.44, AF3.53, AF3.81 | *Ficus benjamina* | USA (1991) | H. Bouzar | This work | IV[a] |

[a] These strains belong to a new phenotypic group of *Agrobacterium*, temporarily referred to as biovar IV. This result appears in Bouzar *et al.* 1995.

was assessed by NMR spectroscopy of crystalline molecule isolated from the tumor extracts .

**Analysis of the opine catabolic properties of the strains.**

To clearly identify the opine type of the strains, we investigated their opine catabolic properties using synthetic opines and tumor-purified opines. The catabolic properties of the strains were assessed in liquid medium to minimize the amounts of input opines. In all degradation experiments described in this paper, disappearance of opines from the medium (monitored by HVPE) was strictly correlated with bacterial growth (as judged by visual inspection of the turbidity of the growth medium). None of the new isolates degraded octopine, cucumopine, or mikimopine (Table 4). Strain 2788 and the Fig strains, which induced synthesis of a nopalinelike compound in the tumors, were found to degrade synthetic nopaline. Furthermore, this nopalinelike compound was degraded by the reference nopaline strains (not shown). Strains Chry-9, K224, K289, and ANT4 catabolized the succinamopinelike molecule which was detected in tumors incited by these strains. This molecule was also used by the succinamopine-type strain EU6 (which degrades both L,L and D,L succinamopine) and the agropine-type strain Bo542 (which catabolizes only L,L succinamopine). This molecule therefore appears to be L,L succinamopine. Consistent with this, none of the new isolates nor strain Bo542 completely degraded a mixture of synthetic D,L and L,L succinamopine (not shown). Furthermore, none of the new strains degraded biologically purified D,L succinamopine, but did degrade biologically purified L,L succinamopine (Table 4). Finally, in agreement with the detection of L,L leucinopine in tumors induced by strains Chry-9, K224, and K289 (Table 3), these strains (and strain ANT4) were able to degrade synthetic L,L leucinopine (Table 4).

**Table 3.** Opines produced in tumors induced by the new *Agrobacterium* strains

| | Compounds detected | | | |
|---|---|---|---|---|
| Strains | SNP[a] I, II and III | Nopaline | L,L-Succinamopine | L,L-Leucinopine |
| Chry strains | | | | |
| ANT4 | +[b] | – | + | ND |
| Chry-9 | + | – | + | + |
| K224 | + | – | + | + |
| K289 | + | – | + | + |
| 2788 | + | + | – | – |
| Fig strains | | | | |
| AF1.72 | + | + | – | – |
| AF3.9 | + | + | – | – |
| AF3.10 | + | + | – | – |
| AF3.43 | + | + | – | – |
| AF3.44 | + | + | – | – |
| AF3.53 | + | + | – | – |
| AF3.81 | + | + | – | – |

[a] SNP I, II and III: silver nitrate positive compounds I, II and III.

[b] Symbols are as follows: +, this opine is present in the tumors; –, this opine is not detected in the tumors; and ND, not determined.

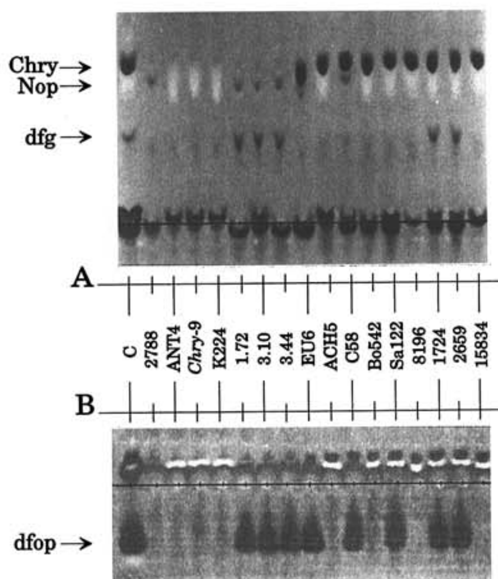


**Fig. 1.** Analysis of the utilization of the three new tumor-specific, silver nitrate-positive compounds. Silver nitrate positive compounds were obtained from tumors induced on *Datura stramonium* by strain 2788. Therefore the extract also contains nopaline (silver nitrate negative spot). The catabolic properties of representative reference Fig and Chry strains were investigated by subjecting 5 µl of the degradation medium to high-voltage paper electrophoresis (HVPE). Strains were as indicated in between the two panels, which were: **A,** HVPE performed at pH 1.9; and **B,** HVPE performed at pH 2.8. For both panels, abbreviations were: Chry, chrysopine; Nop, nopaline; dfg, deoxy-fructosyl-glutamine; and dfop, deoxy-fructosyl-5-oxo-proline. Chrysopine, dfg and nopaline were not resolved at pH 2.8.

**Evidence for the existence of three silver nitrate-positive, opinelike molecules.**

To determine whether SNP compounds I, II, and III were opines, we first investigated the ability of the new strains to degrade them. Interestingly, the Chry strains which could not

**Table 4.** Degradation of synthetic opines by the new *Agrobacterium* strains

| | Opines | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Strains | Oct[a] | Nop | Mop | Agr | AgA | DLSap | LLSap | LLLop |
| Chry strains | | | | | | | | |
| ANT4 | –[b] | – | – | – | – | NT | + | + |
| Chry-9 | – | – | – | – | – | – | + | + |
| K224 | – | – | – | – | – | – | + | + |
| K289 | – | – | – | – | – | – | + | + |
| 2788 | +[c] | + | – | – | – | NT | – | – |
| Fig strains | | | | | | | | |
| 1.72 | +[c] | + | – | – | – | – | – | – |
| 3.9 | +[c] | + | – | – | – | – | – | – |
| 3.10 | +[c] | + | – | – | – | – | – | – |
| 3.43 | +[c] | + | – | – | – | – | – | – |
| 3.44 | +[c] | + | – | – | – | – | – | – |
| 3.53 | +[c] | + | – | – | – | – | – | – |
| 3.81 | +[c] | + | – | – | – | – | – | – |

[a] Abbreviations are as follows: Oct, octopine; Nop, D,L nopaline; Mop, mannopine; Agr, agropine; AgA, agropinic acid; DLSap, D,L succinamopine; LLsap, L,L succinamopine; DLLop, D,L leucinopine; LLLop, L,L leucinopine; Cuc, cucumopine; and; Mik, mikimopine. Note that DLSap was biologically purified from a chemically synthesized mixture of DLSap and LLSap using an agropine-type strain harboring pTiBo542, whereas LLSap was biologically purified from ANT4-induced tumor extracts using strain C58.00. In addition, the assayed strains degraded neither cucumopine, nor mikimopine.

[b] Symbols are as follows: +, opine totally disappeared from the medium in 3–4 days; – opine was still present in the medium after 8–10 days; and NT, not tested.

[c] Octopine disappeared only when nopaline was present in the medium.

catabolize any of the mannityl opines (i.e., agropine, mannopine, and agropinic acid; Table 4) clearly degraded the SNP compounds (obtained from tumors induced by either Chry or Fig strains, Table 5). These observations demonstrated that these compounds were not mannityl opines. Consistent with this, these three molecules were detected on electrophoregrams following oxidation with the tetrazolium reagent, whereas mannityl opines were not (data not shown).

Additional investigations involving analytic chemistry techniques allowed us to determine the structural formulae of these compounds. These results will be published elsewhere (Chilton *et al.*, in preparation). Following structure elucidation, the three tumor-specific molecules were termed chrysopine (d-lactone of N-1-deoxy-D-fructosyl-L-glutamine; SNP I), dfg (N-1-deoxy-D-fructosyl-L-glutamine; SNP II), and dfop (N-1-deoxy-D-fructosyl-5-oxo-L-proline; SNP III).

The data presented above do not suffice to demonstrate that the three silver nitrate positive, tumor-specific molecules are indeed opines. To be regarded as bona fide opines, chrysopine, dfg and dfop must be degraded in a strain-specific manner by pathogenic *Agrobacterium*. To address this question, Chry, Fig, and several wild-type reference strains were inoculated into degradation assay media prepared from tumor extracts. Disappearance of chrysopine and related molecules was then assessed by high-voltage paper electrophoresis (Fig. 1; Table 5). Both the Fig strains and the Chry strains, and only those two groups of strains, degraded chrysopine. In addition, the Chry strains but not the Fig strains degraded dfg and dfop. This was observed whether extracts were prepared from tumors induced by Chry strains or from tumors induced by Fig strains. Interestingly, dfop was also degraded by the octopine-type (ACH5, 15955) and agropine-type (Bo542) *A. tumefaciens* reference strains, and by the mannopine-type (8196) and agropine-type (15834) *A. rhizogenes* reference strains. More surprisingly, dfg which was catabolized by the Chry strains, was also degraded by all assayed *A. tumefaciens* reference strains and by agropine- and mannopine-type *A. rhizogenes* strains. The only bacteria unable to degrade this molecule, in addition to the Fig strains, were the *A. rhizogenes* cucumopine-type strain 2659 and the mikimopine-type strain 1724 (Fig. 1; Table 5).

**Table 5.** Catabolism of the silver nitrate-positive opines contained in tumors induced by the new *Agrobacterium* strains

| Strains | Opines | | |
|---|---|---|---|
| | Chrysopine (SNP I) | dfop[a] (SNP III) | dfg (SNP II) |
| Chry strains | | | |
| ANT4 | +[b] | + | + |
| Chry-9 | + | + | + |
| K224 | + | + | + |
| K289 | + | + | + |
| 2788 | + | + | + |
| Fig strains | | | |
| AF1.72 | + | – | – |
| AF3.9 | + | – | – |
| AF3.10 | + | – | – |
| AF3.43 | + | – | – |
| AF3.44 | + | – | – |
| AF3.53 | + | – | – |
| AF3.81 | + | – | – |
| Reference strains | | | |
| *A. tumefaciens* | | | |
| ACH5 | – | + | + |
| 15955 | – | + | + |
| Bo542 | – | + | + |
| C58 | – | – | + |
| EU6 | – | – | + |
| *A. rhizogenes* | | | |
| 1724 | – | – | – |
| 2659 | – | – | – |
| 8196 | – | + | + |
| 15834 | – | + | + |

[a] Abbreviations are: dfop, deoxy-fructosyl-5-oxo-proline; and dfg, deoxy-fructosyl-glutamine.
[b] Symbols are as in Table 4.

**Cross-catabolism of dfop and agropinic acid.**

Since strains of *Agrobacterium* which catabolized mannopine, mannopinic acid, and agropinic acid also degraded dfop, we investigated whether dfop degradation is related to mannityl opine catabolism. We compared the catabolic properties of strains 15955 and SA122 (a pTi-cured derivative of the

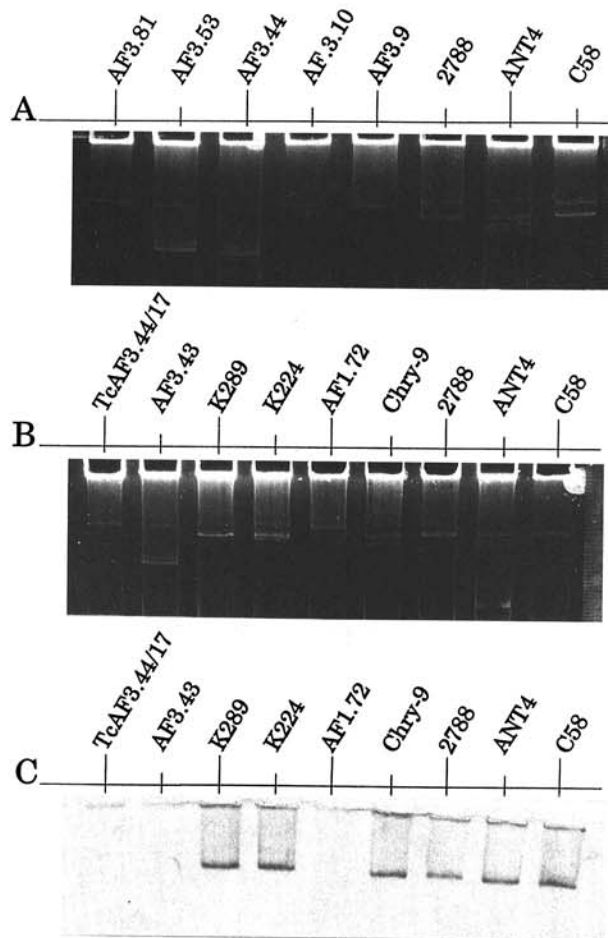


**Fig. 2.** Analysis of the plasmid content of the new *Agrobacterium* strains. **A and B,** Eckhardt gel analysis of the plasmid content of various strains of *Agrobacterium*. Strains are as indicated above the wells. Strain TcAF3.44/17 is a representative pathogenic transconjugant obtained from the cross AF3.44 × C58.00 (see text). **C,** Southern blot transfer and hybridization of the gel shown in panel B with the T-DNA probe obtained from the octopine-type pTi15955. Clear hybridization signals are seen with plasmids pAtK289b, pAtK224b, pAt*Chry*-9b, pAtANT4b and pTiC58 (pAtC58b). Similar results were obtained using the "short" *vir* probe obtained from pTi15955.

former strain). Interestingly, strain SA122 still degraded dfg but did not catabolize dfop (data not shown), confirming that degradation of dfop is encoded by genes located on pTi15955. Clones overlapping the region of pTi15955 involved in mannityl opine degradation (Table 1) were then introduced into SA122 by triparental mating, and the capability of the resulting transconjugants to degrade dfop was investigated. Clones harboring functions involved in agropinic acid catabolism, such as pYDH101, pYDH303, and pYDH304, conferred the ability to degrade dfop upon SA122, whereas clones harboring genes involved in octopine, agropine, mannopine, and mannopinic acid degradation did not confer dfop degradation upon the recipient strain (not shown).

**Identification of the pathogenic plasmids of the Chry strains.**

The biochemical and physiological data presented above do not supply any information on the nature of the pathogenic plasmids of these new *Agrobacterium* strains. To address this question, we first analyzed the plasmid content of Chry and Fig strains using the Eckhardt procedure (Eckhardt 1978). Preliminary experiments yielded only poorly reproducible results. To improve the reliability of the lysis step, which appeared to be the key step in the procedure, the technique had to be modified (see Materials and Methods). Using this protocol, we observed that the number of resident plasmids present in the new strains varied from one (e.g., in strain AF3.10) to four (e.g., strain ANT4; Fig. 2). By comparison to *Agrobacterium* plasmids of known molecular weight (Casse *et al.* 1979), the size of the largest plasmids ranged from approximately 360 kb (pAtANT4a) to approximately 230 kb (pAt3.10).

To determine which of these plasmids may be required for pathogenicity, Eckhardt gels were blotted onto nylon membranes, and the blotted DNA was hybridized with probes specific for pathogenicity. Two probes were first used: the T-DNA region and the "short" *vir* region of pTi15955 (see Materials and Methods). With both probes, hybridization signals were seen (Fig. 2) on lanes containing DNA from all of the Chry strains, indicating that plasmid DNA from these strains harbored sequences homologous to *vir* and T-DNA regions of pTi15955. On this basis, the hybridizing plasmids were called the Ti plasmids.

To evaluate the relatedness of the putative Ti plasmids of the Chry strains, total DNA preparations obtained from these strains were digested with the endonuclease *Bam*HI. The digested fragments were separated by agarose gel electrophoresis, transferred onto a nylon membrane and hybridized with the two probes indicated above, and with two additonal probes which were the pTiC58 *vir* region and the pTiBo542 whole T-DNA region (see Materials and Methods). With all four probes, DNA from strains K224 and K289 exhibited similar *Bam*HI hybridization patterns, as did the DNA from

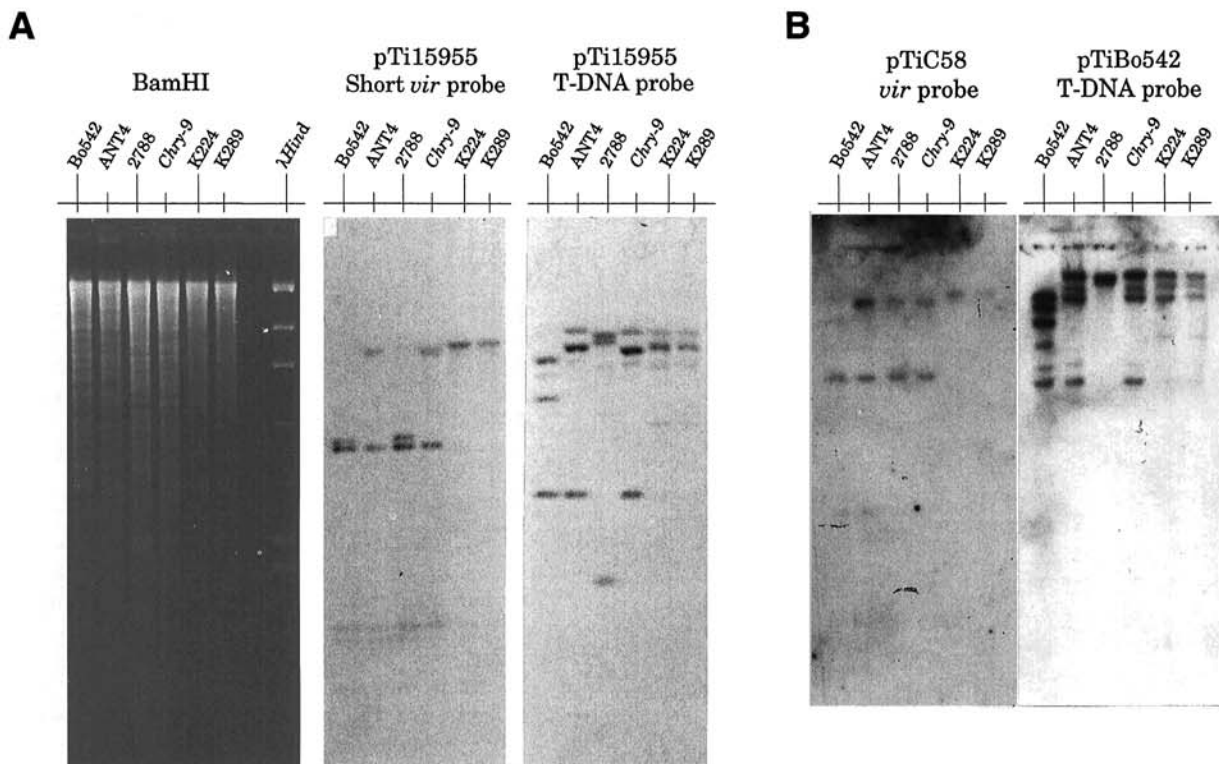


**Fig. 3.** Restriction fragment length polymorphism analysis of the virulence and T-DNA regions of the various Chry strains. Genomic DNA from the Chry strains was digested with the restriction endonuclease *Bam*HI, and the fragments were separated in a 0.8% agarose gel. Lane marked λ*Hind* contains λ phage DNA digested with restriction endonuclease *Hind*III, used as a DNA molecular weight marker (Panel *Bam*HI). Southern blot transfer and hybridization of the gel shown in panel A were performed using various probes and detection techniques. These were: **A,** the X-pho/NBT dye system for hybridizations performed with the T-DNA and the short *vir* probes obtained from the octopine-type pTi15955; and, **B,** the Lumigen-PPD chemiluminescent system for hybridizations performed with the pTiC58 *vir* probe and with the pTiBo542 T-DNA probe.

strains ANT4 and Chry-9 (Fig. 3). Hybridization fragments obtained from strain 2788, however, most often differed from those of the other Chry strains. This was true with both pTi15955-generated probes and with the Bo542-generated probe. Interestingly, the hybridization pattern of strain 2788 DNA, as judged by hybridization with the pTi15955-generated "short" *vir* probe, was identical to that of the agropine-type *A. tumefaciens* strain Bo542.

**Identification of the pathogenic plasmids of the Fig strains.**

To identify the Ti plasmids of the Fig strains, we first attempted to hybridize plasmid DNA of these strains (obtained from nylon-blotted Eckhardt gels) with the T-DNA and the "short" *vir* probes derived from pTi15955. No or very weak signals were seen (Fig. 2) with these two probes on lanes containing Fig strain DNA, even though hybridizations were performed at very moderate stringency (see Materials and Methods). This absence of signal may be due either to the small amount of target plasmid DNA obtained on Eckhardt gels or to the existence of limited homologies only between the Fig strain DNA and the probes. To address these questions, we attempted to hybridize *Bam*HI digestions of DNA obtained from strains AF3.10 and AF3.44 with three other probes which were the *vir* region of pTiC58, the whole T-DNA region of pTiBo542, and the "long" *vir* region of pTi15955. No signals were seen with the pTiC58-generated probe. However, weak signals were detected with the Bo542-generated probe and with the "long" *vir* region from pTi15955 (Fig. 4). Plasmid and chromosome DNA from the Fig strains (Nylon blotted Eckardt gels) were tentatively hybridized with the two later probes. Whatever the probes, no hybridization signal were seen even though hybridization were performed in the same low stringency conditions used thourough this work (not shown).

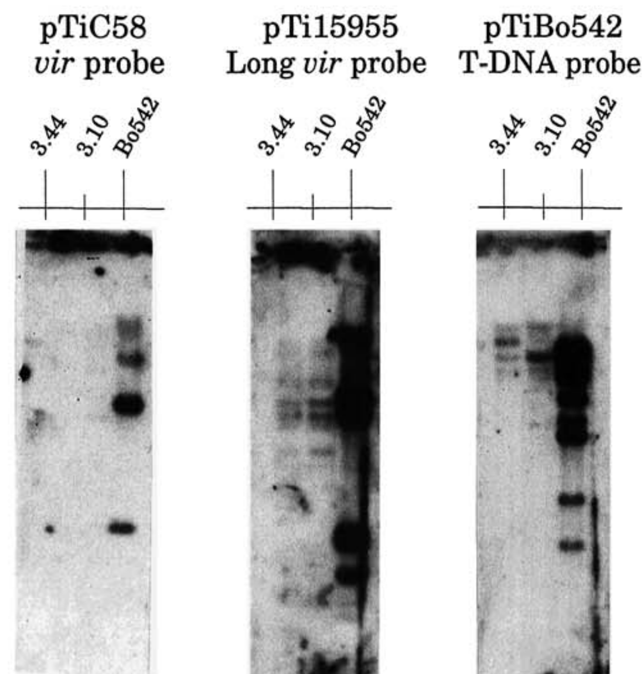


**Fig. 4.** Detection of sequence homologous to *vir* and T-DNA regions of representative Fig strains. Genomic DNA from reference strain Bo542 and from two representative Fig strains was digested with the restriction endonuclease *Bam*HI, and the fragments were separated in a 0.8% agarose gel. Southern blot transfer and hybridization of the gels A were performed using the Lumigen-PPD chemiluminescent system and various *vir* and T-DNA probes. These were: the pTiC58 vir probe; the long pTi15955 *vir* probe; and, the pTiBo542 T-DNA probe. Blots were overexposed to allow visualization of the weak hybridization signals.

To obtain DNA probes that could more efficiently detect homologies to the Ti plasmids of the Fig strains, we attempted to utilize DNA from the Chry strains. Total genomic DNA preparations of strain ANT4 and 2788 were amplified by PCR using various sets of primers. Clear amplification signals of expected molecular weight were seen with DNA obtained from strains ANT4 and 2788 when PCR reactions were performed with primers FGP*vir*A2275 and FGP*vir*B2164′. Signals were also seen when amplifications were performed on ANT4 DNA using *tmr* primers (FGP*tmr*530 and FGP*tmr*701′), and on 2788 DNA using *tms* primers (FGP$tms_2$194′ and FGP$tms_1$884′) (data not shown). Three of the amplification products (ANT4 *tmr*-amplified DNA, 2788 *tms*-amplified DNA, and 2788 *vir*-amplified DNA) were labeled by incorporating a digoxygenin-modified nucleotide, and used as probes in hybridization experiments on blotted Eckhardt gels. Clear hybridization signals were seen with all probes on lanes containing DNA from the positive controls (C58, ANT4, and 2788) but no signals were detected on lanes containing DNA from the Fig strains (results not shown). Since it was possible to obtain PCR-generated DNA from the Chry strains, we also tried to directly amplify DNA from genomic preparations obtained from the Fig strains AF3.10 and AF3.44 using the primers described above. No amplification was seen with any of the primer set under our experimental conditions.

To further attempt to identify the Ti plasmid of the Fig strains, we performed plasmid conjugation experiments using strain C58.00 as the plasmid-free recipient and strain AF3.44 as the donor. The technique, however, was complicated by the unavailibilty of large quantities of chrysopine. We therefore had to purify large amounts of opines by biological depletion of tumor extracts prepared from AF3.44-induced crown galls. The purified extract was then used as the source of nutrients in the selective media which also contained rifampicin and streptomycin. Colonies of potential transconjugants were selected and purified twice on the selective medium. For further studies, we retained only the bacteria which exhibited a C58 chromosomal background (about 95% of putative transconjugant colonies). This was assessed by analyzing phage resistance and genomic DNA *Bam*HI restriction pattern (data not shown). The virulence and the plasmid content of all transconjugants (i.e., bacteria harboring the C58 chromosomal background) were then analyzed by inoculating *D. stramonium* plants and by Eckhardt gel electrophoresis, respectively. Nineteen transconjugants which harbored plasmid pAtAF3.44a (as shown in transconjugant TcAF3.44/17 in Fig. 2) were tumorigenic. All 19 transconjugants degraded chrysopine and nopaline (not shown). From this result, this plasmid was called the putative Ti plasmid.

## DISCUSSION

The work presented above dealt with the identification and characterization of strains of *Agrobacterium* isolated from Fig

tree and chrysanthemum. Our work demonstrates that these strains exhibit unique opine-related properties. In addition, Fig-strains (i) belong to a new phenotypic group (Bouzar *et al.* 1995) and (ii) harbor Ti plasmids that exhibit limited homology to other reference Ti plasmids.

One of the Chry strains (Chry-9) that we analyzed was previously studied by Bush and Pueppke (1991). Additionally, these authors elucidate the physical map of pTiChry-5, the Ti plasmid of another Chry strains (Kovács and Pueppke 1994). Both strains were identified as broad-host range *A. tumefaciens* strains unable to degrade any of the known opines except L,L succinamopine (Kovács and Pueppke 1994). In this work, we confirmed this later result. Furthermore, we report additional data on opines synthesized in tumors and degraded by these strains, on their resident Ti plasmids and we extend our observations to other strains isolated from Fig and chrysanthemum. Thus, the Chry strain 2788 and all of the Fig strains induced synthesis of a compound which, with respect to the observations mentioned above, is indeed nopaline. Similar experiments and reasoning allowed us to identify L,L succinamopine and L,L leucinopine in tumors induced by all Chry strains except strain 2788.

Remarkably, tumors induced by the Chry and Fig strains of *Agrobacterium* synthesized three tumor-specific, silver nitrate-positive compounds, that exhibited electrophoretic mobilities similar to those of the mannityl opines. Degradation studies, however, showed that they were not mannityl opines, but other opinelike compounds. One of the three, chrysopine (which behaved on high voltage paper electrophoregrams as agropine) was specifically degraded by pathogenic Chry and Fig strains of *Agrobacterium* and should be regarded as an opine characteristic of this group of strains. Interestingly, trace amounts of the two other silver nitrate-positive molecules, dfg and dfop, have been previously detected in tumors induced by octopine-type *Agrobacterium* strains (Ellis *et al.* 1984). Thus, dfg was identified as an intermediate in the biosynthetic pathway of the opine mannopine, and dfop as a naturally occurring rearrangement product of dfg. Moroever, dfg and dfop were previously detected in large amounts in tumors induced by the unusual nopaline-type strain IIBV7 (Ellis *et al.* 1984) which, notably, was isolated from chrysanthemum. However, occurrence of these molecules in IIBV7-induced tumors was regarded as a peculiar characteristic of this strain since it was (at that time) the only member of what appears to be a new group of *Agrobacterium* strains.

The degradation of dfg and dfop was not as specific as was the degradation of chrysopine. For instance, dfop was degraded by the Chry strains and by other pathogenic *Agrobacterium* strains. Using cloned genes, we clearly demonstrated that those genes involved in agropinic acid degradation also are involved in dfop utilization. This result is consistent with the close chemical structures of dfop and agropinic acid (Chilton *et al.*, in preparation). It explains why and how dfop is degraded by octopine- and agropine-type *A. tumefaciens* and by mannopine- and agropine-type *A. rhizogenes* strains. The reverse proposal, however, is not true: None of the Chry strains degraded agropinic acid even though they used dfop.

The above data and conclusions support the view that dfop is indeed an opine. The case of dfg is not as simple. This molecule was degraded by most of the *Agrobacterium* strains used in this study. The exceptions were the cucumopine- and mikimopine-type reference strains (respectively 2659 and 1724) and, surprisingly, the pathogenic Fig strains which induced dfg synthesis in the tumors. The reasons accounting for this phenomenon remain unknown. It could be speculated that most of the Ti and Ri plasmids (with the exception of those of the two above strains) do carry genes involved in dfg degradation. However, preliminary experiments performed with the pTi-cured strain SA122 demonstrated that this strain degraded dfg. This partly ruled out the above speculation. In addition, dfg degradation was investigated using 7 randomly selected strains of *Rhizobium meliloti*, *Pseudomonas fluorescens*, *P. syringae* pv. *syringae*, and *P. syringae* pv. *savastanoi*. None of these strains degraded dfg (not shown). This result is of ecological importance. It suggests that dfg-producing tumors may also be an ecological niche for nonpathogenic *Agrobacterium* cells. By favoring growth of both pathogenic and nonpathogenic *Agrobacterium* cells, and not that of other soilborne bacteria, dfg exhibits a broader specificity than usually observed for other opine molecules. Whether this feature allows dfg to be classified as a bona fide opine remains open to discussion.

The identification of dfg as an opinelike molecule fits our model for the evolution of Ti plasmids and opine-related functions (Dessaux *et al.* 1992). To understand this point, the natural occurrence of dfg should first be discussed. To summarize, synthesis of dfg (and other *N*-deoxyfructosyl-amino acids) occurs spontaneously, though at various rates, in all aging biological materials, including plant materials (nonenzymatic browning reactions; Anet 1957; Anet and Reynolds 1957; Heyns and Paulsen 1959). In this respect, and aside from octopine, dfg is the only opine occurring outside grown gall (or hairy root) overgrowths, a feature which may have provided the selective pressure for selection and conservation of dfg catabolic genes in *Agrobacterium*, even before Ti plasmids existed. This speculation is supported by the existence of such genes in numerous pathogenic and nonpathogenic *Agrobacterium* strains (e.g., SA122). As we proposed earlier (Dessaux *et al.* 1992), acquisition of opine biosynthetic genes would have been the second step in the making of the Ti plasmids. How dfg biosynthetic genes have been "captured" by putative ancestral T-DNA remains to be understood. Such an integration, however, would have been clearly favorable to *Agrobacterium* since, in our model, functional dfg catabolic genes were already resident in the bacteria. We therefore suggest that dfg could be an "ancestral opine" from which other opine molecules have evolved. Consistent with our speculation, dfg was found to be an intermediate in mannopine, agropinic acid, and agropine synthesis (Ellis *et al.* 1984), and probably in dfop and chrysopine synthesis (Vaudequin-Dransart *et al.*, unpublished observations).

The search for the pathogenic plasmids of the new *Agrobacterium* isolates revealed that the Ti plasmids of Chry and Fig strains could be distinguished on the basis of their homologies to reference Ti plasmids. Thus, the putative Ti plasmids found in the Fig strains exhibited very little or no homology to these reference plasmids, as judged by Southern blot hybridizations with probes obtained from the three well-known octopine-, nopaline-, and agropine-type Ti plasmids. In agreement with these results, attempts to amplify DNA from strains AF3.10 and AF3.44 by PCR were unsuccessful.

Yet the experiments were performed with primers which were located in putative conserved regions (*vir*, T-DNA *tms*, *tmr*, and *nos* genes) and successfully used to amplify DNA from other Ti plasmids (Ponsonnet and Nesme 1994). Conjugation experiments, however, indicated that pathogenicity is correlated with presence in the transconjugants of pAtAF3.44a, the largest of the three extra chromosomal replicons of strain AF3.44, and with the ability to degrade both chrysopine and nopaline. This experiment strongly suggests that pAtAF3.44a is the Ti plasmid. Interestingly, the Fig strains which induce synthesis of the three new specific molecules in the tumors, degrade only chrysopine, the agropine-related opine. As suggested for *A. rhizogenes* strain HRI (Petit *et al.* 1983; Petit, unpublished results), the Fig strains may have lost one or more replicons that are absolutely necessary for dfg and dfop degradation in the course of the isolation and purification process of the strain.

In contrast to their Fig strain counterparts, the Ti plasmids detected in the Chry strains showed clear homology to the T-DNA and/or the *vir* regions of pTi15955, pTiC58, and pTiBo542. This result is consistent with that reported earlier for strain Chry-5 by Bush and Pueppke (1991) and Kovács and Pueppke (1994) who showed that pTiBo542 and pTi-*Chry*-5 exhibit about 85% overall homology and similar *Bam*HI restriction sites over 12 kb of their *vir* region. Parts of the putative pathogenic DNA molecules of the Chry strains were amplified by PCR using primers chosen among conserved Ti plasmid sequences. This set of results indicates that these putative Ti plasmids should be more closely related to pTi15955 or to pTiBo542 than are the Ti plasmids of the Fig strains. Furthermore, preliminary RFLP experiments showed the existence of polymorphism within the putative T-DNA and *vir* regions of the Chry strains. On the basis of this feature, three subgroups of plasmids were detected. A first subgroup includes the putative pTiK224 and pTiK289 which exhibited identical RFLP patterns whatever the probe used. A second subgroup of plasmids is found in strains Chry-9 and ANT4 and exhibit identical *vir* and T-DNA RFLP patterns. The profiles of *Bam*HI-digested genomic DNA of these two strains, however, were not related, consistent with the fact that they were isolated at different places and times. A third subgroup is limited to the only strain 2788. Interestingly, the RFLP pattern of the *vir* region of strain 2788 and that of strain Bo542 (an agropine-type strain) were identical (Fig. 3) when determined using the pTi15955-generated "short" *vir* probe. The tumor-inducing ability of the Chry strains harboring the putative Ti plasmids remains, however, to be formally demonstrated.

## MATERIALS AND METHODS

**Bacterial strains.**

New isolates of *Agrobacterium tumefaciens* were obtained from tumors of naturally infected chrysanthemum and Fig plants. They are referred to as Chry or Fig strains, depending on their original host plant. Most of the other strains used in this study were previously described wild-type strains. Strain C58.00 is a plasmid-free derivative of strain C58 (Petit, unpublished data). Strain SA122 is a pTi-free derivative of strain 15955 (Dessaux *et al.* 1987). Fragments of pTi15955 cloned in the broad host-range vector pCP13 (Dessaux *et al.* 1987) were conjugated by triparental mating (Ditta *et al.* 1980) into strain SA122 to yield pYDH derivatives of this strain. All strains are listed in Tables 1 and 2.

**Culture media and growth conditions.**

NB, modified LB (containing 5 g $l^{-1}$ NaCl) and modified YEB were used as rich, nonselective media. Modified YEB contained: Bacto-beef extract, 5 g $l^{-1}$; yeast extract, 1 g $l^{-1}$; peptone, 5 g $l^{-1}$; mannitol, 5 g $l^{-1}$; and $MgSO_4.7\ H_20$, 0.5 g $l^{-1}$ (pH 7.5). AT medium (Petit and Tempé 1978), prepared as a 2× solution (AT×2), and dilutions of it were used as minimal media. When necessary, media were supplemented with rifampicin (100 µg $ml^{-1}$) and streptomycin (500 µg $ml^{-1}$) and solidified using 16 g $l^{-1}$ Difco Bacto Agar. Cultures were grown at 28°C. Liquid cultures were incubated with shaking.

**Chemicals.**

All chemicals were from commercial sources except opines which were synthesized in our laboratories using standard procedures (Chilton *et al.* 1984a; Davioud *et al.* 1988a; Dessaux *et al.* 1986; Isogai *et al.* 1990; Petit *et al.* 1983; Tempé *et al.* 1980).

**Pathogenicity assays.**

Pathogenicity was investigated on different test plants (*Datura stramonium*, *Helianthus annuus*, *Kalanchoe daigremontiana*, *K. tubiflora*, *Nicotiana tabacum*) grown in a greenhouse under long day conditions. Plants were wounded on their stem and apex using a scalpel blade and inoculated with bacteria grown overnight on solid rich medium (modified LB or YEB). Results were scored 4 to 6 weeks after inoculation.

**Analysis of the opine content of the tumors.**

Opines were extracted from tumor samples as previously reviewed (Dessaux *et al.* 1992). Opines were separated from other plant metabolites by high-voltage paper electrophoresis (HVPE) in the acetic acid/formic acid (pH 1.9) buffer (Petit *et al.* 1983) or in the formic acid/KOH (pH 2.8) buffer (Chilton *et al.* 1984a, 1984b) and visualized using the appropriate staining reagents. These include the silver nitrate, phenanthrenequinone and tetrazolium reagents (Trevelyan *et al.* 1950; Zweig and Sherma 1972). A negative control (i.e., an extract obtained from the stem or the apex of a healthy plant) was always included.

**Opine utilization assays.**

The opine degradation capabilities of the various *Agrobacterium* strains were first assessed by analyzing disappearance of opine molecules from bacterial culture supernatants. The degradation medium consisted of AT minimal medium supplemented with ammonium sulfate (1 g $liter^{-1}$), yeast extract (0.1 g $liter^{-1}$) and mixtures of the following opines: nopaline, octopine, and L,L-leucinopine (2.5 mM); mannopine, agropine, mannopinic acid, cucumopine, and mikimopine (2 mM). Degradation assays were performed in a final volume of 250 µl. Disappearance of opines was assessed by subjecting 2-µl samples of the degradation medium, taken at various times, to HVPE in the conditions indicated above.

**Degradation of the new opinelike compounds.**

The studies on the degradation of the new tumor-specific compounds required large amounts of these molecules. To

obtain such quantities, tumors (about 80 g, fresh weight) were sliced and the resulting pieces were placed in a beaker. The tumor pieces were submerged in distilled water (about 150 ml) and boiled (1 min in a microwave oven). Preparations were allowed to cool, filtered through glass wool to retain all tumor pieces, and centrifuged (10,000 × *g*, 30 min, 4°C) to eliminate small-size particles. The supernatants were sterilized by filtration through 0.45-µm ultrafiltration membranes. Degradation studies were performed in a 225-µl degradation medium consisting of 75 µl AT×2 stock solution supplemented with 150 µl of tumor extract. The disappearance of opine compounds from culture media was assessed from 2 to 6 days after inoculation by subjecting 4 to 6 µl of this medium to HVPE as indicated above.

**DNA extraction, restriction, and analysis.**

Extraction of genomic (total) DNA was performed on 10 ml of overnight bacterial cultures (NB medium) according to the method of Dhaese *et al.* (1979). Small-scale plasmid preparations were obtained using an alkaline lysis procedure (Maniatis *et al.* 1982). Endonuclease digestion and separation of the restriction products by electrophoresis in horizontal 0.8% agarose gels were achieved according to standard protocols (Sambrook *et al.* 1989).

**Native plasmid analysis.**

Plasmid contents of various *Agrobacterium* strains were analyzed by a modified Eckhardt procedure (1978) performed in 0.8% low EEO agarose (Boehringer, Mannheim, Germany) gels. The modification was as follows. Approximately $2 \times 10^9$ cells from fresh overnight cultures (modified LB medium) were scraped from the plates and suspended in 100 µl of LTE (Tris-HCl, 10 mM, pH 8.0; EDTA, 1 mM, pH 8.0) buffer. The suspension was centrifuged (2 min, 13,000 × *g*, room temperature) and the pellet was vigorously resuspended in 100 µl of 5 M NaCl. Five microliters of 5% Na sarkosyl was added to the cell suspension and gently mixed. The mixture was immediately centrifuged in the above conditions. The pellet of washed cells was then treated as indicated in the Eckhardt protocol.

**Conjugation experiments.**

Conjugation experiments also required purification of large quantities of the tumor-specific compounds. Tumor extracts, obtained as indicated above (see opine utilization assay), were biologically depleted by growing the plasmid-free strain C58.00 for 4 days. The disappearance of sugars and amino acids from the tumor extract was assessed by subjecting 4 to 6 µl of the growth medium to HVPE and by staining the electrophoregrams using silver nitrate (Trevelyan *et al.* 1950) and ninhydrin reagents (Zweig and Sherma 1972). The depleted extract was centrifuged (10,000 × *g*, 30 min, 4°C). The supernatant was filter-sterilized through a 0.45-µm membrane. The filtrate, designated TS solution, was used as the source of tumor-specific nutrients in the conjugation experiments. Donor strain AF3.44 was grown in 2 ml of conjugation medium (CM) which consisted of 80% TS solution (dilution were performed with AT×2, v/v) supplemented with ammonium sulfate (1 g liter$^{-1}$) and yeast extract (100 mg liter$^{-1}$). The recipient strain C58.00 was initially grown on YEB medium supplemented with rifampicin and streptomycin, and transferred to 2 ml of AT medium supplemented with ammonium sulfate (1 g liter$^{-1}$) and mannitol (2 g liter$^{-1}$) prior to conjugation. Conjugation was performed on 0.45-µm ultrafiltration filters left overnight on solid CM. Bacteria were resuspended from the filters in distilled water and spread onto the selective medium which was CM supplemented with rifampicin and streptomycin.

**Amplification of selected *vir* and T-DNA sequences.**

DNA amplifications were performed according to a protocol modified from Mullis and Faloona (1987). The PCR reactions were set up in a 50-µl reaction mix consisting of 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; and 0.01% gelatin. In addition, the media contained the four dNTPs (20 µM each) labeled or not with digoxigenin (Boehringer, Mannheim, Germany), the two primers (0.1 µM each), about 100 ng of genomic DNA (prepared as described above), and 1.25 units of *Taq* DNA polymerase (Gibco BRL, Cergy-Pontoise, France). Cycling parameters were as follows: 35 cycles of denaturation (1 min at 95°C, except for the initial denaturation step, 3 min at 95°C ), annealing (1 min at 55°C) and extension (2 min at 72°C, except for the final extension phase, 3 min at 72°C). Primers were located in putative conserved regions of Ti plasmids (*vir*, T-DNA *tms*, *tmr*, and *nos* genes). They were *vir* primers FGP*vir*A2275 (TCAAA-AGGCAAGCAAGCAGATCTGG) and FGP*vir*$B_2$164′ (TCA-GTGCCGCCACCTGCAGATTG); *tms* primers FGP*tms*$_2$194′ (5′CCTACTCCGGCGTTTCCATG) and FGP*tms*$_1$884′ (5′CGGATCCCCCCCATTTC GGC); *tmr* primers FGP*tmr*530 and FGP*tmr*701′; *nos* primers FGP*nos*975 (5′CATAACGTGCATCATGCATG), FGP*nos*14 (5′GGCA-ATTACCTTATCCGCAA) and FGP*nos*1236′ (5′CACC-ATCTCGTCCTTATTGA) (Ponsonnet and Nesme 1994). Complete gene sequences related to the primers were published elsewhere (Bevan *et al.* 1983; Gielen *et al.* 1984; Rogowsky *et al.* 1990; Yanofsky *et al.* 1985). The results of the PCR reactions, performed in a Dryblock Thermal Cycler PHC3 (Techne Inc., Princeton, NJ), were analyzed by subjecting 5 to 10 µl of the reaction medium to electrophoresis on 0.8 or 2% (according to the expected size of the amplified DNA) horizontal agarose gels.

**Southern hybridization.**

Restriction fragments or native plasmid DNA were transferred onto nylon membrane (Hybond N, Amersham, England) as described previously (Ausubel *et al.* 1989). Three probes generated from the octopine-type pTi15955 were used. They were prepared from a cosmid clone bank of this plasmid (Dessaux *et al.* 1987). The pTi15955 T-DNA probe consisted of the gel-purified (Geneclean II, Bio 101, La Jolla, California.) *Hin*dIII fragment 1 which encompasses TL-DNA genes 1 (*tmr*), 4 (one of the *tms* genes), 6a, 6b, and *ocs* and TR-DNA genes 3′ and 4′. The "short" *vir* probe consisted of the gel-purified *Hin*dIII fragment 3 which overlaps the *vir*G gene and most of the *vir*B operon of pTi15955. The "long" *vir* probe consisted of the following *Hin*dIII fragments: 3, 37, 21c, 19b, 34c, 27b, 39, 21d, and 17, and overlaps *virG*, *virC*, *virD*, most of the *virB* operon, and part of the *virE* region. Other probes used in this study were: (i) a pTiC58 *vir* probe consisting of *Hin*dIII fragments 34, 39, 12, 9, 18, and 14a (which overlaps *tzs*, *virA*, *virB,* and most of the *virG* region;

Hayman, 1989); (ii) a pTiBo542 T-DNA probe consisting of *Bam*HI fragments 5, 10, 13b, 33, 26, 16, 25, 7b (which overlaps the whole TL-DNA and TR-DNA regions, plus sequences in between the two T-DNAs; Hood *et al.* 1984).

Probes were labeled by incorporating a digoxygenin-modified nucleotide using a nonradioactive labeling kit (Boehringer). Hybridizations were performed at 62°C according to standard procedures and to the instructions of the manufacturer of the labeling kit, with the exception of the blocking agent which was used at 2% (w/v) final concentration. Stringency washes were done in the following solution: 2×SSC, 0.1%SDS (2 × 15 min at 62°C). Assuming a 60% G+C content of most of the *Agrobacterium* strains, our conditions provided a hybridization parameter of approximately 35°C below the $T_m$. Detection of heteroduplex DNA molecules was performed directly on the transfer membrane using either the X-pho/NBT dye system or the chimiluminescent Lumigen-PPD substrate of nonradioactive DNA detection kits (Boehringer). During the detection process, the titer of the diluted anti-digoxygenin antibodies was reduced to 1/10,000, and the incubation time was strictly limited to 30 min. Furthermore, the concentration of blocking agent was doubled (1% in place of 0.5%), and the duration of the blocking step was increased to reach at least 1 h.

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