The Cryptic Plasmid of *Agrobacterium tumefaciens* Cointegrates with the Ti Plasmid and Cooperates for Opine Degradation

Valérie Vaudequin-Dransart,¹ Annik Petit,¹ W. Scott Chilton,² and Yves Dessaux¹

¹Institut des Sciences Végétales, CNRS UPR040, Avenue de la Terrasse, F-91198 Gif-sur-Yvette Cedex, France; ²Department of Botany, North Carolina State University, Raleigh 27695, U.S.A.

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We crossed the *Agrobacterium tumefaciens* chrysanthemum strain ANT4, which harbors four plasmids, with the plasmid-free recipient C58.00RS. Transconjugants degrading the Amadori-opines chrysopine and deoxy-fructosyl-oxo-proline (dfop) harbored the Ti plasmid of ANT4, termed pAtANT4b. Upon transfer to the recipient strain C58.00RS, pAtANT4b (pTiANT4) and pANT4a (the largest of the four plasmids of ANT4) could cointegrate. The cointegration of the two plasmids occurs at various places of the pTiANT4, a feature that may affect several functions of the Ti plasmid (e.g., opine degradation). Transconjugants utilizing the opine deoxy-fructosyl-glutamine (dfg) always harbored the large pAtANT4a. Other *Agrobacterium* strains, including nonpathogenic strains such as C58C1, naturally degraded dfg. Remarkably, strain C58C1 carries a large cryptic plasmid termed pAtC58 that also encodes dfg degradation. A screening of physiological traits additionally revealed that this plasmid allows utilization of octopine as sole nitrogen source after mutation. All these results demonstrate that the larger plasmid of *A. tumefaciens* is a catabolic plasmid and that both the “cryptic” plasmid and Ti plasmid cooperate for opine degradation.

Additional keywords: *A. rhizogenes*, conjugation, Ri plasmids.

The genus *Agrobacterium* contains mainly plant-pathogenic bacteria, distinguished by species epithets originally based on pathogenic symptoms. In this paper, we will retain the historical delineation of “species”: *Agrobacterium tumefaciens* is the causative agent of crown gall, and *A. rhizogenes* the bacterium responsible for the hairy root disease. Both diseases affect principally dicotyledonous plants and share numerous common features. Thus, most of the pathogenic determinants of *A. tumefaciens* and *A. rhizogenes* are borne on large extra chromosomal replicons termed Ti (tumor-inducing) or Ri (root-inducing) plasmids, respectively. Part(s) of these plasmids, the T-DNA(s), are transferred during disease induction from the bacteria to the plant cell nucleus, where they integrate into

chromosomal DNA. The transfer is directed by proteins encoded by the virulence (*vir*) genes located on the Ti and Ri plasmids. The expression of the T-DNA genes in the transformed cells is responsible for their proliferation as crown gall tumors or hairy roots, depending on the origin of the T-DNA(s) (for reviews see Gelvin 1992; Hooykaas and Schilperoort 1992; Winans 1992; Zambryski 1992; Gaudin et al. 1994; Ream and Gelvin 1996).

Remarkably, crown gall and hairy root cells produce unusual and specific low molecular weight molecules termed opines. These are synthesized from transformed cell metabolites by enzymes encoded by one or more T-DNA genes. Opines are also specifically degraded by the bacteria responsible for the induction of the disease. The nature of the opines synthesized in the hyperplasias and degraded by the bacteria depends entirely upon the type of Ti or Ri plasmids hosted by the bacteria. Though rudimentary and imperfect, this feature has long been used as a tool to classify the Ti and Ri plasmids, which are named according to the representative opine synthesized by the transformed cells (for reviews see Dessaux et al. 1992; Gelvin 1992; Dessaux et al. 1993).

Among known opines, a class of four molecules called the mannityl opines (agropine, mannopine, mannopinic acid, and agropinic acid) has received considerable attention (Firmin and Fenwick 1978; Tempé et al. 1980; Tate et al. 1982; Ellis et al. 1984), probably because of its broad occurrence in crown gall and hairy root hyperplasias and also because crown gall cells synthesize large amounts of these compounds (up to 7% of their dry weight; Tate et al. 1982). Indeed both the octopine- and agropine-type Ti plasmids, and the agropine- and mannopine-type Ri plasmids encode the capability to degrade one or more of the mannityl opines (for review see Dessaux et al. 1992). The above-mentioned Ti plasmids encode the catabolic functions responsible for the degradation of the four mannityl opines, whereas the mannopine-type Ri plasmid only allows the degradation of mannopine and mannopinic and agropinic acids (Petit et al. 1983). Interestingly, the agropine-type Ri plasmid per se encodes only agropine catabolism, while the catabolic functions for mannopine, mannopinic acid, and agropinic acid are borne on another, smaller replicon that can cointegrate with the agropine-type Ri plasmid (Petit et al. 1983; Jouanin et al. 1986). At least three plasmids are therefore visible in an agropine-type *A. rhizogenes* strain. From the smallest to the largest, they are the opine catabolic

Corresponding author: Yves Dessaux; Telephone: 33 + 1 69 82 36 90; Fax: 33 + 1 69 82 36 95; E-mail: Yves.Dessaux@isv.cnrs-gif.fr

This work is dedicated to the memory of our professor, colleague, and friend, Jean-Paul Aubert, Institut Pasteur, Paris: Thank you so much.

plasmid, the Ri plasmid, and the cointegrate of the former two replicons.

Most *A. tumefaciens* strains harbor a plasmid, pAt, that is larger than the pTi (Rosenberg and Huguet 1984; Albiach and López 1992; V. Vaudequin-Dransart, *unpublished*). Nevertheless, the above-described process of plasmid integration and cooperation for opine degradation has not been reported in strains of *A. tumefaciens* and the pAt has remained a cryptic plasmid. We are studying new types of *A. tumefaciens* strains (isolated from chrysanthemum and named Chry-strains; Bush and Pueppke 1991; Kovács and Pueppke 1994; Vaudequin-Dransart et al. 1995) and their Ti plasmids (Fig. 1), which together induce tumors containing a family of opines known as the Amadori-opines. These are chrysopine, deoxyfructosyl-glutamine (dfg), and deoxy-fructosyl-5-oxoproline (dfop). They are closely related to the mannityl opines agropine, mannopine, and agropinic acid, respectively (Chilton et al. 1995; Vaudequin-Dransart et al. 1995). We report here data showing that the degradation of the Amadori-opines is encoded on the relevant Ti and cryptic plasmids of Chry-strain ANT4, which both therefore appear to be catabolic plasmids. Physiological and molecular data also strongly suggest that these two replicons can cointegrate and dissociate in strain ANT4, as observed in the agropine-type *A. rhizogenes* strains. The catabolic role of the cryptic plasmid of *A. tumefaciens* is further confirmed by the finding that it is also involved in the utilization of the opine octopine as a nitrogen source.

RESULTS

Analysis of the plasmid content and opine-related functions of the *A. tumefaciens* strains isolated from chrysanthemum tumors.

The relevant Ti plasmids of the chrysanthemum *A. tumefaciens* strains were first identified by hybridizing native plas-

mid preparations obtained from modified Eckhardt gels with probes consisting of T-DNA regions or virulence genes of various reference Ti plasmids (Vaudequin-Dransart et al. 1995). To confirm the identity of the pTi of the representative chrysanthemum strain ANT4, we conjugated ANT4 with the plasmid-free recipient C58.00RS, both in vitro on filters and in planta. ANT4 harbors four plasmids: pAtANT4a (approximately 450 kb), pAtANT4b (approximately 180 kb), pAtANT4c (approximately 90 kb), and pAtANT4d (approximately 60 kb). The sizes of these plasmids were estimated by comparison with those of known *Agrobacterium* plasmids. Transconjugants obtained from the filter mating were selected and purified on the selection medium (SM), which essentially consisted of the AT minimal medium supplemented with the appropriate antibiotics and the opines contained in a depleted, ANT4-induced tumor extract. Two techniques were used to ensure that these were indeed transconjugants and not donor or contaminant strains that escaped the antibiotic selection. First, the sensitivity of the putative transconjugants toward phage S2 was assessed. Strain C58.00RS is sensitive to this phage whereas strain ANT4 is not. Only transconjugants sensitive to S2 were therefore retained. Furthermore, genomic DNA fingerprints of these transconjugants were analyzed and only those exhibiting a pattern similar to that of the recipient C58.00RS were kept. Out of 20 colonies isolated on SM medium (Tr1 to 20), 13 were finally identified as bona fide transconjugants (Tr2, 3, 5, 11 to 20) (Table 1).

The pathogenicity and opine catabolic properties of the 13 transconjugants obtained by filter conjugation were evaluated, and their plasmid content was analyzed by the modified Eckhardt procedure. Surprisingly, the transconjugants exhibited quite variable phenotypic traits (Table 1). All transconjugants degraded dfg, but only a subset of these catabolized chrysopine and dfop, and were pathogenic on the test plants. Remarkably, one transconjugant (Tr11) degraded dfg and dfop but not chrysopine, and remained pathogenic. Another one (Tr15) degraded dfg and chrysopine but not dfop and also induced tumors on the test plants. Overall, no clear correlation between opine utilization, pathogenicity, and plasmid content was observed. All analyzed strains harbored an extrachromosomal replicon, the migration of which was generally slightly slower than that of the largest ANT4 plasmid. On the other hand, pAtANT4b, which hybridized to the pathogenicity-related probes used in a previous study, was apparently absent in the transconjugants, even in the pathogenic ones (Tr11, Tr13 to 20), except in Tr12. However, Southern blot hybridizations performed on genomic DNA preparations clearly demonstrated that sequences homologous to the *vir* and agropinic acid catabolism genes and T-DNA region of the octopine-type strain 15955 were present but only in pathogenic transconjugants (Table 1). All these transconjugants were kept frozen (at -80°C as glycerol stock) and maintained on modified LB medium at 4°C for further studies.

A similar analysis was performed on a second series of 17 bona fide transconjugants obtained from in planta crosses (Tr 35 to 55). All transconjugants were able to degrade chrysopine and dfop, but not dfg (Table 1). This metabolic capacity was correlated with the presence of pANT4b. In agreement with the fact that the 17 transconjugants were pathogenic, sequences homologous to the *vir* and T-DNA regions of the octopine-type strain 15955 were detected in all assayed bacteria

Fig. 1. Restriction map of pTiChry5. Coordinates (in kb) are indicated on the inner circle. Clones appear on two external circles with their original numbering. Asterisk indicates region of pTiChry5 that shares no homology with pTiBo542, according to Kovács and Pueppke (1994). Map was redrawn according to that published by Kovács and Pueppke (1994) with kind permissions of S. Pueppke and Springer-Verlag (Berlin), which owns the copyright of the above referenced paper.

(Table 1). Noteworthy, none of the three other plasmids of ANT4 were transferred in planta.

Evidence for plasmid cointegration.

The above results prompted us to reexamine the data obtained with the first series of transconjugants originating from filter matings. We therefore re-analyzed the catabolic properties and plasmid content of the bacteria kept during 3 to 6 months at 4°C. Surprisingly, the results (Table 1) drastically differed from those described above. All pathogenic strains that previously harbored a single, large plasmid eventually harbored two plasmids, the first corresponding to pANT4a and the second one to pANT4b. This latter plasmid was never detected in the nonpathogenic transconjugants (Tr2, Tr3, Tr5). Since it seems possible that pANT4b appeared in the bacteria upon prolonged storage at 4°C, hybridization experiments were performed with the *vir* probe of pTi15955 on blotted Eckhardt gels obtained from these bacteria and from their corresponding frozen stocks. Sequences homologous to the *vir* probe were detected on the unique, large plasmid of the transconjugants kept at -80°C and on both pANT4b and large plasmid (exhibiting an electrophoretic mobility comparable to that of pANT4a) in the transconjugants maintained at 4°C. However, upon very long storage at 4°C (6 to 12 months), the *vir* probe hybridized only to pANT4b, suggesting that (i) the large extrachromosomal replicon seen in the transconjugants kept at -80°C is a cointegrate of both pANT4a and pANT4b, (ii) pANT4b is indeed the Ti plasmid of ANT4, and (iii) long-term, slow growth at 4°C eventually leads to a complete resolution of the cointegrate. Again, neither pANT4c nor pANT4d was transferred under our experimental conditions.

Evidence for multiple cointegration sites.

The catabolic properties of Tr11 and Tr15, which are both pathogenic and which both harbor the pANT4b, suggest that cointegration of pANT4a and pANT4b might occur at different places on the Ti plasmid. This could explain why the first transconjugant has lost the capability to degrade chrysopine, and only chrysopine, whereas the second has lost the capability

to degrade dfop, and only dfop. To evaluate the validity of this hypothesis, we extracted and restricted genomic DNA of transconjugants and control strains, and probed them with cloned regions of the pTiChry5 (Fig. 1), a plasmid that exhibits a restriction map identical to that of pTiANT4 (V. Vaudequin-Dransart, A. Petit, and Y. Dessaux, *unpublished*). Transconjugant Tr11 yielded modified restriction patterns with probes pCH1755 and pCH2546 (Fig. 1; Tables 2 and 3), suggesting that the cointegration site lies within the region where these two clones overlap (*Eco*RI fragments 17a and 22). Further blotting experiments performed with *Eco*RI fragments 17a and 22 and *Bam*HI fragment 18 as probes showed that the integration occurred within the 2.85-kb fragment *Bam*HI 18 (Fig. 2). Similarly, a modified pattern was observed with transconjugant Tr15 in the region shared by clones pCH2546 and pCH1647, but not in that shared by the two former clones and clone pCH549. Examination of the blots (Fig. 2) revealed that integration probably occurred within the 6.7-kb *Bam*HI fragment 8 (not included in pCH549). Numerous cointegrations took place in the region defined by clone pCH2249 (Tr12, Tr13, Tr16 to 19). The cointegrations detected in Tr12 and Tr13 occurred around coordinate 100 on the Ti plasmid (Fig. 1), within *Eco*RI fragments 28b or 28c, as patterns generated with both pCH2249 and pCH1195 appeared modified while probe pCH2711 generated normal patterns. In Tr16, Tr17, and Tr19, hybridization patterns indicated that integrations affected *Bam*HI fragment 12 (Table 2 and data not shown). Finally, in Tr14, the cointegration site remained unidentified, since none of the probes generated abnormal hybridization patterns. Overall, these results provide clear evidence that the cointegration of both Ti and At plasmids may occur at different sites on the Ti plasmid.

The cryptic plasmid of *Agrobacterium* is a catabolic plasmid.

The ability to degrade dfg is correlated with the presence of the cryptic plasmid pANT4a, because the degradation of this molecule is a feature only of the transconjugants harboring pANT4a. Indeed none of the transconjugants harboring only

Table 1. Phenotypic and genetic analysis of the transconjugants (Tr) obtained from a ANT4 × C58.00 cross in planta and in vitro^a

Strains and transconjugants ^b	Opines degraded ^c				Pathogenicity on <i>Datura stramonium</i>	Hybridization ^d			Plasmid content ^e				
	Chry	dfg	dfop	Lop		T-DNA	<i>vir</i> genes	AGA	a	b	Mo ^f	c	d
Parent strains													
C58.00	—	—	—	—	—	—	—	—	—	—	—	—	—
ANT4	+	+	+	+	+	+	+	+	+	+	(0)	+	+
Tr obtained in vitro													
Tr 2, 3, 5	—	+	—	—	—	—	—	—	+	—	—	—	—
Tr 12	+	+	+	+	+	+	+	+	+	+	(0)	—	—
Tr 13, 14, 16 to 20	+	+	+	+	+	+	+	+	+	+	(3 to 6)	—	—
Tr 11	—	+	+	+	+	+	+	+	+	+	(3)	—	—
Tr 15	+	+	—	+	+	+	+	+	+	+	(3)	—	—
Tr obtained in planta													
Tr 35 to 37, 39	+	—	+	+	+	+	+	+	—	+	(0)	—	—
Tr 38, 41 to 47, 51 to 55	+	—	+	+	+	+ ^g	+ ^g	NT	—	+	(0)	—	—

^a Genetic and physiological properties of the transconjugants were assessed as indicated in Materials and Methods.

^b Transconjugants Tr 2 to 20 were obtained from filter matings whereas Tr 35 to 55 came from in planta crosses.

^c Chry, chrysopine; dfg, deoxy-fructosyl-glutamine; dfop, deoxy-fructosyl-oxo-proline; Lop, leucinopine.

^d DNA/DNA hybridizations were performed with probes generated from strain 15955. +, presence of an hybridization signal; — no hybridization signal; NT, not tested.

^e Plasmids were detected by the modified Eckhardt procedure: the delay in the appearance of pANT4b is given between parentheses.

^f Plasmid b visible after (month) at 4°C.

^g Tr 38 was not tested.

the pTiANT4, i.e., pANT4b, can degrade dfg. To extend these results, we investigated dfg degradation by other *A. tumefaciens* strains harboring different At plasmids. Pathogenic *A. tumefaciens* strains such as C58, B6, 15955, and ACH5 and their nonpathogenic, Ti plasmid-free derivatives C58C1, B6m5, Sa122, and ACH5C3, respectively, which harbor only the cryptic At plasmid, degraded dfg. This finding confirms that the Ti plasmid is not necessary for dfg degradation. However, neither the plasmid-free strain C58.00 nor the derived transconjugant C58.00(pTiC58) degraded dfg (Fig. 3), a result that definitely demonstrates that dfg degradation is encoded on the cryptic plasmid of *A. tumefaciens* and not on the Ti plasmid.

Because the pAt of *A. tumefaciens* encodes dfg degradation, we performed an additional series of degradative and enzymatic assays to search for other catabolic functions that might be associated with pAt. The degradation of 150 compounds (API systems 50CH, 50AA, 50AO, Montalieu-Vercieu, France) by strains C58.00 and C58 was investigated, but no

differences were observed. However, the analysis of opine degradation revealed that strain C58 readily mutated toward utilization of octopine as sole nitrogen source in AT minimal medium supplemented with mannitol (1 g liter⁻¹) and this opine (0.5 g liter⁻¹). Under the same conditions, no mutants of C58.00 were obtained. This was confirmed by examination of strains C58C1 and C58.00(pTiC58). C58C1, which harbors pAtC58, frequently mutated toward the utilization of octopine as a nitrogen source, while no mutants of strain C58.00(pTiC58), devoid of pAtC58, were obtained. The capability to mutate toward the utilization of octopine as a nitrogen source therefore appears to be a function encoded on the *Agrobacterium* pAt plasmid.

DISCUSSION

We report two important findings in this paper. First, the pAt of *Agrobacterium* is a catabolic plasmid involved in opine

Table 2. Localization of the cointegration sites of pAtANT4a within pTiANT4^a

Probe	Transconjugants								
	Tr11	Tr12	Tr13	Tr14	Tr15	Tr16	Tr17	Tr18	Tr19
pCH230		ND	ND	ND		ND	ND		ND
pCH77		ND	ND	ND		ND	ND		ND
pCH1755	M	ND	ND	ND		ND	ND		ND
pCH2546	M	ND	ND	ND	M	ND	ND		ND
pCH1647		ND	ND	ND	M	ND	ND		ND
pCH549		ND	ND	ND		ND	ND		ND
pCH2249		M	M			M	M	M	M
pCH1195		M	M						
pCH2711									
pCH5102		ND	ND	ND		ND	ND		ND
pCH3701		ND	ND	ND		ND	ND		ND

^a Detection of the cointegration site of pAtANT4a and pTiANT4 was performed by hybridizing *Bam*HI-digested genomic DNA preparations obtained from the listed transconjugants and from ANT4 with the various probes indicated to the left (see Figure 1). Hybridization patterns obtained with the transconjugants were compared with those generated by ANT4. Identical patterns were observed except where indicated by M (modified pattern) or ND (pattern not determined).

Table 3. Bacterial strains

Strains	Opine-type of Ti plasmid	Observations and opine catabolized	Source
<i>Agrobacterium tumefaciens</i>			
ACH5, 15955, B6s3	Octopine	Octopine, agropine, mannopine, mannopinic acid, agropinic acid, dfop, dfg	Our collection
ACH5C3, Sa122, B6m5	None	pTi-cured derivative of strain ACH5, 15955 and B6, respectively; dfg	Our collection
ANT4	Chrysopine-succinamopine	Chrysopine, dfg, dfop, L, L-succinamopine, L, L-leucinopine	Our collection
C58.00	None	pTi-, pAt-cured derivative of strain C58; resistant to rifampicin and streptomycin	Our collection
C58C1	None	pTi -cured derivative of strain C58; dfg	Our collection
C58.00(pTiC58)	Nopaline	Nopaline, agrocinopines A and B	Our collection
Cloned fragments of pTiChry5 ^a into <i>Escherichia coli</i>			
pCH1755		See Figure 1	Kovács and Pueppke 1994
pCH2546		See Figure 1	Kovács and Pueppke 1994
pCH1647		See Figure 1	Kovács and Pueppke 1994
pCH549		See Figure 1	Kovács and Pueppke 1994
pCH2249		See Figure 1	Kovács and Pueppke 1994
pCH1195		See Figure 1	Kovács and Pueppke 1994
pCH2711		See Figure 1	Kovács and Pueppke 1994
pCH5102		See Figure 1	Kovács and Pueppke 1994
pCH3701		See Figure 1	Kovács and Pueppke 1994
pCH230		See Figure 1	Kovács and Pueppke 1994
pCH77		See Figure 1	Kovács and Pueppke 1994

^a Chry5 is a chrysopine-succinamopine strain as strain ANT4. All clones were obtained in pLAFR1 (Kovács and Pueppke 1994).

degradation. This assertion has been demonstrated for the chrysanthemum strain ANT4 and also for the well-known reference nopaline strain C58. Second, it may cointegrate with the Ti plasmid of pathogenic isolates. The cointegration of both Ti and At plasmids, however, was confirmed only in strain ANT4.

One of the difficulties that will limit the development of further studies on dfg catabolism lies in the limited availability of the opine dfg and its instability. Thus, preliminary attempts to select directly for dfg-utilizing transconjugants on minimal medium supplemented with dfg have failed because of the rapid and spontaneous degradation of this molecule (data not shown).

One of the questions raised by our study deals with the presence of independent transfer systems on both Ti and AtANT4 plasmids. Three transconjugants carry only pANT4a (Tr2, Tr3, Tr5): they were nonpathogenic and Southern blot hybridizations revealed that none of them harbors sequences characteristic of the reference Ti plasmid 15955. These results therefore suggest that pAtANT4a probably has its own *tra* genes encoding plasmid transfer via conjugation. As judged from the isolation of transconjugants harboring the only Ti

plasmid (Tr35 to 55), it is reasonable to assume that the pTi also harbors its own transfer functions, as do most analyzed Ti plasmids (for a review see Farrand 1993). These results therefore suggest that there is one transfer system per plasmid (pAtANT4 and pTiANT4) in strain ANT4. However, more data are obviously necessary to uncover the mechanism accounting for the conjugation of pAtANT4a and pTiANT4, as exemplified by the fact that the opine responsible for the induction of the conjugation remains unknown. Ti and At plasmid cotransfer may be related to an earlier observation, that the Ti plasmid of *A. tumefaciens* strain R10 may promote chromosome mobilization (Dessaux et al. 1989). All these data suggest that the transfer of the Ti plasmid could be a "signal" that triggers major exchange of genetic material, for instance within a crown gall tumor. This point of view, however, should be moderated by our finding that, for unknown reasons, no cotransfer of pTi and pAtANT4a was seen in planta (Table 1).

Most strikingly, the data reported in this paper resemble those described earlier for various *A. rhizogenes* strains (Petit et al. 1983; Jouanin et al. 1986; see the introductory paragraphs above). However, there are several differences between

Fig. 2. Localization of cointegration sites. Genomic DNA preparations obtained from strain ANT4 and the transconjugants were digested with restriction enzyme *Bam*HI and hybridized with various probes. Molecular sizes (in kb) are given in between the two panels. Left panel: probe was pCH1755. Restriction fragment length polymorphism (RFLP) profiles of transconjugants were identical to that of ANT4, except for Tr 11, which lacks *Bam*HI fragment 18 at 2.85 kb. Right panel: probe was pCH1647. RFLP profiles of transconjugants were identical to that of ANT4, except for Tr 15, which lacks *Bam*HI fragment 8 at 6.7 kb.

the two systems. For instance, the Ri plasmid of *A. rhizogenes* strain A4 (also termed pArA4b) encodes only the catabolism of agropine while degradation of agropinic acid and mannopine is encoded on a smaller replicon (Petit et al. 1983). In *A. tumefaciens* strain ANT4, the Ti plasmid encodes the degradation of chrysopine (the agropine "analog") and that of dfop (the agropinic acid analog) while catabolism of dfg (the mannopine analog) is borne on the pAt plasmid, which is larger than the pTi. There are also some interesting similarities. For instance, degradation of agropine in *A. rhizogenes* strain A4 is

thought to proceed via conversion to mannopine by the enzyme mannopine cyclase. The observation that strains harboring only the pRi plasmid are unable to degrade mannopine relates to the absence of an efficient transport system for mannopine (Dessaux et al. 1986; Hong and Farrand 1994). A similar situation is observed in octopine-type *A. tumefaciens* strain 15955 (Hong et al. 1993; Hong and Farrand 1994), where a clone encoding agropine degradation via the mannopine cyclase pathway remains unable to confer efficient growth on mannopine upon its bacterial host, unless the genes

Fig. 3. Assays for degradation of opines. Opine degradation by strain ANT4 and the transconjugants was assessed by monitoring disappearance of opines chrysopine, dfg, and dfop (see text for definitions) from a degradation medium that consisted of ATx2 stock solution supplemented with undepleted ANT4-induced tumor extract. This extract provided opines and nutrients necessary to allow growth of the bacteria. Disappearance of opines was monitored by subjecting 4 μ l of degradation medium to HVPE (pH 1.9; see Materials and Methods). After 5 days at 28°C, chrysopine was metabolized only by the ANT4 strain, while dfop was almost entirely degraded by ANT4 and the octopine-type strains B6s3 and ACH5, as reported previously (Vaudequin-Dransart et al. 1995). The opine dfg was degraded by both pathogenic and nonpathogenic agrobacteria, the exception being the strains devoid of the large cryptic plasmid, e.g., C58.00 and C58.00(pTiC58) termed, on that picture, (pTiC58). On the right, the "T" lane is a migration standard consisting of uninoculated degradation medium incubated and processed as the inoculated media.

encoding the relevant mannopine transport system are introduced into the bacteria (Kim and Farrand 1996). By analogy with these data, the fact that pathogenic transconjugants harboring only the TiANT4 plasmid are unable to degrade dfg may possibly be related to the absence of genes encoding a dfg transport system on this plasmid.

Another interesting feature deals with the observation that the cointegration between pAtANT4a and pTiANT4 occurs at different locations on the Ti plasmid. Since insertion (IS) and transposon (Tn) sequences have been described in *Agrobacterium* spp. and on some Ti plasmids (Fortin et al. 1993; Deng et al. 1995; for a review see Otten et al. 1992) including pTiChry5 (Kovács and Pueppke 1994), it can be proposed that the cointegration event first involves a duplication of IS or Tn sequence(s) and a homologous recombination at the insertion site of the IS or Tn sequence(s). If confirmed, such a cointegration mechanism would differ from that reported for *A. rhizogenes* strain A4 in which pArA4a and pRiA4 (or pArA4b) shared large common regions of homologous DNA where the cointegration takes place (Jouanin et al. 1986).

The cointegration and resolution of the cointegrate of pTiANT4 and pAtANT4a sometimes lead to gene inactivation. Transconjugants Tr11 and Tr15, for example, have lost the ability to degrade chrysopine and dfop, respectively. The data obtained with these two transconjugants allow us to locate the chrysopine and dfop degradative functions around coordinate 50 on pTiANT4, a region that consistently shares little or no homology with the pTiBo542 (Kovács and Pueppke 1994). In agreement with the above, the gene encoding dfg cyclase, an enzymatic activity involved in chrysopine degradation, is located within this region (V. Vaudequin-Dransart, P. Oger, A. Petit, W. S. Chilton, and Y. Dessaux, *in preparation*). The cointegration may also induce a deleterious effect for the bacterium and its Ti plasmid if this event takes place within the region of the pTi involved in plasmid replication and stability. Even pathogenicity might be affected if virulence genes or T-DNA oncogenes are disrupted. In this respect, if the cointegration occurs to the left of the right border of the T-DNA, some pAt sequences might be transferred to the nucleus of the transformed plant cell(s) and integrated into their nuclear genome. If there is a single T-DNA on pTiChry5, the integration of pAt within this T-region will undoubtedly lead to the loss of pathogenicity. However, on the related agropine-type plasmid Bo542 or on the octopine-type pTi15955, there are two T-DNAs: the TR-DNA, which carries opine biosynthetic functions and no oncogenes, and the TL-DNA, which essentially carries the oncogenes (Barker et al. 1983; Ellis et al. 1984; Hood et al. 1984; Salomon et al. 1984; Komari et al. 1986; Hood et al. 1986). If the T-DNA of strain Chry5 is similar to that of Bo542, as suggested by the work of Kovács and Pueppke (1994), the integration of the pAt in a putative TR-DNA may indeed lead to the transfer of pAt sequences into proliferating, transformed crown gall cells. This hypothesis is of importance since it suggests that DNA sequences other than that of the *Agrobacterium* T-DNAs can be naturally transferred into plant cells.

From the results presented and discussed above, degradation of the opine dfg and utilization of octopine as sole nitrogen source after mutation are two functions encoded by pAt of *Agrobacterium*. That pAt encodes dfg catabolism is in agree-

ment with previously published data on mannopine synthesis and degradation, which both involve dfg as an intermediate (Ellis et al. 1984; Kim et al. 1996). As a consequence of our study, the At plasmid of *Agrobacterium* can no longer be regarded as cryptic.

A previous report demonstrated that pAt does not control bacterial virulence (Rosenberg and Huguet 1984). Apart from this result, only speculations on pAt-related functions can be made. In vitro, in spite of a massive screening of catabolic properties, we detected no phenotypic variation related to the presence or absence of pAtC58 in a C58 bacterial background (except the two opine catabolic functions reported in this paper). Even the growth patterns of strains C58.00 and C58C1 did not strikingly differ in rich medium or in minimal AT medium supplemented with mannitol and ammonium sulfate (data not shown). However, no data on the behavior of these two strains in competition in a plant rhizosphere, or in near-field conditions, or in a tumorlike environment are available. Such data would be important to address the question of the biological role of the At plasmid, the size of which represents approximately 8% of that of the whole *A. tumefaciens* genome. We can only suspect that other pAt functions extend beyond the purely metabolic traits presented above, affecting possibly bacterial physiology and ecology.

MATERIALS AND METHODS

Bacterial strains.

Strains and their relevant characteristics and origins are listed in Table 3.

Chemicals.

All chemicals except the opines were commercially available. Chrysopine, dfg, and dfop were extracted from tumor, and partly purified if necessary by biological depletion of the extracts with strain C58.00, all as described by Vaudequin-Dransart et al. (1995). The resulting extracts were filter sterilized (0.45 µm) and kept frozen (-20°C). The depleted extract, designated TS solution, was used as the source of tumor-specific nutrients in conjugation experiments. The opine dfg was also synthesized as described by Chilton et al. (1995). All other opines were synthesized in our laboratories according to previously published procedures.

Growth media and culture conditions.

Bacteria were routinely maintained on modified Luria-Bertani (LB) and modified yeast extract broth (YEB) media. Minimum medium was the AT medium of Petit and Tempé (1978). Liquid cultures were shaken at 28°C. Media were solidified with Difco Bacto-agar (16 g liter⁻¹) for the modified LB and YEB media (Vaudequin-Dransart et al. 1995) and Difco Noble agar (16 g liter⁻¹) for the AT-based media (Difco, Detroit, MI). When required, rifampicin was added at 100 µg ml⁻¹ and streptomycin at 500 µg ml⁻¹.

Bacterial conjugation.

Bacterial conjugations were performed on filters and in planta. Conjugations on filters followed the protocol developed for strains AF3.44 and C58.00RS (Vaudequin-Dransart et al. 1995). Briefly, the donor strain ANT4 was grown in a

diluted TS solution (see Chemicals, above). The recipient C58.00RS was grown in YEB medium supplemented with the antibiotics rifampicin and streptomycin and transferred to an antibiotic-free medium just prior to conjugation. Bacteria were mated overnight at 28°C on conjugation medium (CM), which consisted of 80% TS solution and 20% double strength AT medium, supplemented with ammonium sulfate (1 g liter⁻¹) and Difco yeast extract (0.1 g liter⁻¹). Bacteria were resuspended in distilled water and spread onto the selection medium, which was CM supplemented with the antibiotics rifampicin and streptomycin.

Additional conjugations were performed in planta. Young *Datura stramonium* plants (6 to 8 leaves) were wounded and inoculated at the stem apex and at several sites on their stem with approximately 10⁹ donor (ANT4) and 10⁹ recipient cells (C58.00RS) per wound site. Plants were grown under long day conditions in the greenhouse. Tumors were harvested 6 weeks later. They were roughly surface sterilized for 1 min in a solution of 100 g liter⁻¹ calcium hypochlorite containing 5 drops per liter of commercial liquid detergent. Tumors were washed several times in sterile, distilled water and aseptically cut into approximately 2 × 2 × 2 mm pieces. Several tubes containing a dozen of these fragments and 1 ml of sterile, distilled water were placed on a shaker for approximately 4 h. One hundred microliters of the resulting bacterial suspension was plated onto selection medium. After both types of conjugation, bacteria were left on the selection medium for 3 to 7 days at 28°C. The appearance of putative transconjugants was monitored by daily visual inspection.

Phage typing.

Fifty microliters of a suspension of the assayed strains (approximately 10⁹ cells ml⁻¹) was spread over 55-mm petri plates containing solid, modified YEB medium, and allowed to penetrate fully within the medium. Ten microliters of phage S2 (our collection) suspension (approximately 10¹⁰ infectious units ml⁻¹) was spotted on the dried plate and also allowed to penetrate within the medium. The plates were incubated 24 to 36 h at 28°C and the sensitivity of the assayed strains assessed by the appearance of a clear halo at the place where the phage suspension was spotted.

Pathogenicity assays.

Pathogenicity was assessed by inoculating young *Datura stramonium* plants (6 to 8 leaves) wounded at the stem apex and at various places on the stem. Tumor appearance at the wound site was monitored 6 weeks later. A positive control (ANT4) and a negative control (C58.00RS) were included.

Analysis of the catabolic capabilities of the transconjugants.

Opine degradation by the transconjugants was assessed by monitoring the disappearance of chrysopine, dfg, and dfop from a 225-μl volume of degradation medium, which consisted of 75 μl of double strength AT solution supplemented with 150 μl of undepleted tumor extract, which provided the nutrients necessary to the growth of the bacteria. The disappearance of opines was monitored from 2 to 6 days after inoculation by subjecting 4 to 6 μl of this medium to high voltage paper electrophoresis (HVPE, pH 1.9, silver nitrate staining) as described by Vaudequin-Dransart et al. (1995).

Molecular methods.

Genomic DNA preparations were performed according to the procedure of Dhaese et al. (1979). Small-scale DNA extraction, DNA restriction, and agarose gel separation of restriction fragments were according to standard techniques (Sambrook et al. 1989). Plasmids of *Agrobacterium* strains were resolved on vertical, low EEO agarose (Boehringer-Mannheim, Mannheim, Germany) gels by the Eckhardt procedure (Eckhardt 1978), modified exactly as described in Vaudequin-Dransart et al. (1995). For Southern blot hybridization, restriction fragments or native plasmids visualized from modified Eckhardt gels were depurinated, denatured, and transferred onto a Nylon membrane (Hybond N original formulation, Amersham, Little Chalfont, England) by standard procedures. Southern blot hybridizations were performed with digoxigenin-labeled probes exactly as indicated in Dessaux et al. (1995) and Vaudequin-Dransart et al. (1995). Stringency parameters were as reported in the latter paper, i.e., approximately 35°C below *T_m*. Heteroduplexes were detected with the X-Pho/NBT colorimetric reagent and the CSPD chemiluminescent reagent as reported previously (Vaudequin-Dransart et al. 1995). Probes consisted of various pTi15955 fragments: the *Sma*I fragments 4 and 6 (AGA probe); *Hind*III fragment 1 (T-DNA probe); and *Hind*III fragments 3, 19b, 21c, 26b, 27b, 30c, 31b, 34b, 34c, 37, and 39 (*vir* probe).

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LITERATURE CITED

- Albiach, M. R., and Lopez, M. M. 1992. Plasmid heterogeneity in Spanish isolates of *Agrobacterium tumefaciens* from thirteen different hosts. *Appl. Environ. Microbiol.* 58:2683-2687.
- Barker, R. F., Idler, K. B., Thompson, D. V., and Kemp, J. D. 1983. Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. *Plant Mol. Biol.* 2:335-350.
- Bush, A. L., and Pueppke, S. G. 1991. Characterisation of an unusual new *Agrobacterium tumefaciens* strain from *Chrysanthemum morifolium* Ram. *Appl. Environ. Microbiol.* 57:2468-2472.
- Chilton, W. S., Stomp, A. M., Beringue, V., Bouzar, H., Vaudequin-Dransart, V., Petit, A., and Dessaux, Y. 1995. The chrysopine family of Amadori-type crown-gall opines. *Phytochemistry* 40:619-628.
- Deng, W., Gordon, M. P., and Nester, E. W. 1995. Sequence and distribution of IS1312: Evidence for horizontal transfer from *Rhizobium meliloti* to *Agrobacterium tumefaciens*. *J. Bacteriol.* 177:2554-2559.
- Dessaux, Y., Elasm, M., Glickmann, E., Oger, P., Petit, A., and Vaudequin-Dransart, V. 1995. The use of digoxigenin-labelled probes to detect DNA sequences specific for plant pathogenic bacteria. *Cell. Mol. Biol.* 41:933-943.
- Dessaux, Y., Guyon, P., Farrand, S. K., Petit, A., and Tempé, J. 1986. *Agrobacterium* Ti and Ri plasmids specify enzymic lactonization of mannopine to agropine. *J. Gen. Microbiol.* 132:2549-2559.
- Dessaux, Y., Petit, A., Ellis, J. G., Legrain, C., Demarez, M., Wiame, J. M., Popoff, M., and Tempé, J. 1989. Ti plasmid-controlled chromosome transfer in *Agrobacterium tumefaciens*. *J. Bacteriol.* 171:6363-6366.

- Dessaux, Y., Petit, A., and Tempé, J. 1992. Opines in *Agrobacterium* biology. Pages 109-136 in: Molecular Signals in Plant-Microbe Communications. D. P. S. Verma, ed. CRC Press, Boca Raton, FL.
- Dessaux, Y., Petit, A., and Tempé, J. 1993. Chemistry and biochemistry of opines, chemical mediators of parasitism. *Phytochemistry* 34:31-38.
- Dhaese, P., De Greve, H., Decraemer, H., Schell, J., and Van Montagu, M. 1979. Rapid mapping of transposon insertion and deletion mutations in the large Ti plasmids of *Agrobacterium tumefaciens*. *Nucleic Acid Res.* 7:1837-1845.
- Eckhardt, T. 1978. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. *Plasmid* 1:584-588.
- Ellis, J. G., Ryder, M. H., and Tate, M. E. 1984. *Agrobacterium tumefaciens* TR-DNA encodes a pathway for agropine biosynthesis. *Mol. Gen. Genet.* 195:466-473.
- Farrand, S. K. 1993. Conjugal transfer of *Agrobacterium* plasmids. Pages 225-291 in: *Bacterial Conjugation*. D. B. Clewell, ed. Plenum Press, New York.
- Firmin, J. L., and Fenwick, G. R. 1978. Agropine: A major new plasmid determined metabolite in crown gall tumours. *Nature* 276:842-844.
- Fortin, C., Marquis, C., Nester, E. W., and Dion, P. 1993. Dynamic structure of *Agrobacterium tumefaciens* Ti plasmids. *J. Bacteriol.* 175:4790-4799.
- Gaudin, V., Vrain, T., and Jouanin, L. 1994. Bacterial genes modifying hormonal balances in plants. *Plant Physiol. Biochem.* 32:11-29.
- Gelvin, S. B. 1992. Chemical signaling between *Agrobacterium* and its host plant. Pages 137-167 in: *Molecular Signals in Plant-Microbe Communications*. D. P. S. Verma, ed. CRC Press, Boca Raton, FL.
- Hong, S.-B., Dessaux, Y., Chilton, W. S., and Farrand, S. K. 1993. Organization and regulation of the mannopine cyclase-associated opine catabolism genes in *Agrobacterium tumefaciens* 15955. *J. Bacteriol.* 175:401-410.
- Hong, S.-B., and Farrand, S. K. 1994. Functional role of the Ti plasmid-encoded catabolic mannopine cyclase in mannityl opine catabolism by *Agrobacterium* spp. *J. Bacteriol.* 176:3576-3583.
- Hood, E. E., Jen, G., Kayes, L., Kramer, J., Fraley, R. T., and Chilton, M. D. 1984. Restriction endonuclease map of pTiBo542, a potential Ti plasmid vector for genetic engineering of plants. *Bio/Technology* 2:702-709.
- Hood, E. E., Jen, G., Kayes, L., Kramer, J., Fraley, R. T., and Chilton, M. D. 1986. The hypervirulence of *Agrobacterium tumefaciens* strain A281 is encoded in a region of pTiBo542 outside of T-DNA. *J. Bacteriol.* 168:1291-1301.
- Hooykaas, P. J. J., and Schilperoort, R. A. 1992. *Agrobacterium* and plant genetic engineering. *Plant Mol. Biol.* 19:15-38.
- Jouanin, L., Tourneur, J., Tourneur, C., and Casse-Delbart, F. 1986. Restriction maps and homologies of three plasmids of *Agrobacterium rhizogenes* strain A4. *Plasmid* 16:224-234.
- Kim, K. S., Chilton, W. S., and Farrand, S. K. 1996. A Ti plasmid-encoded enzyme required for degradation of mannopine is functionally homologous to the T-region-encoded enzyme required for synthesis of this opine in crown gall tumors. *J. Bacteriol.* 178:3285-3292.
- Kim, K. S., and Farrand, S. K. 1996. Ti plasmid-encoded genes responsible for catabolism of the crown gall opine mannopine by *Agrobacterium tumefaciens* are homologs of the T-region genes responsible for synthesis of this opine by the plant tumor. *J. Bacteriol.* 178:3275-3284.
- Komari, T., Halperin, W., and Nester, E. W. 1986. Physical and functional map of supervirulent *Agrobacterium tumefaciens* tumor-inducing plasmid pTiBo542. *J. Bacteriol.* 166:88-94.
- Kovács, L. G., and Pueppke, S. G. 1994. Mapping and genetic organization of pTiChry5, a novel Ti plasmid from a highly virulent *Agrobacterium tumefaciens* strain. *Mol. Gen. Genet.* 242:327-336.
- Otten, L., Canaday, J., Gérard, J.-C., Fournier, P., Crouzet, P., and Paulus, F. 1992. Evolution of *agrobacteria* and their Ti plasmids—a review. *Mol. Plant-Microbe Interact.* 5:279-287.
- Petit, A., David, C., Dahl, G. A., Ellis, J. G., Guyon, P., Casse-Delbart, F., and Tempé, J. 1983. Further extension of the opine concept: Plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. *Mol. Gen. Genet.* 190:204-214.
- Petit, A., and Tempé, J. 1978. Isolation of *Agrobacterium* Ti plasmid regulatory mutants. *Mol. Gen. Genet.* 167:147-155.
- Ream, W., and Gelvin, S. B. 1996. Crown Gall: Advances in Understanding Interkingdom Gene Transfer. American Phytopathological Society, St. Paul, MN.
- Rosenberg, S., and Huguet, T. 1984. The pAtC58 plasmid of *Agrobacterium tumefaciens* is not essential for tumor induction. *Mol. Gen. Genet.* 196:533-536.
- Salomon, F., Deblaere, R., Leemans, J., Hernalsteens, J.-P., Van Montagu, M., and Schell, J. 1984. Genetic identification of functions of TR-DNA transcripts in octopine crown galls. *EMBO J.* 3:141-146.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Tate, M. E., Ellis, J. G., Kerr, A., Tempé, J., Murray, K. E., and Shaw, K. J. 1982. Agropine: A revised structure. *Carbohydr. Res.* 104:105-120.
- Tempé, J., Guyon, P., Ellis, J. G., Tate, M. E., and Kerr, A. 1980. Préparation et propriétés de nouveaux substrats cataboliques pour deux types de plasmides oncogènes d'*Agrobacterium tumefaciens*. *C.R. Acad. Sci. Paris (Sér. D)* 290:1173-1176.
- Vaudequin-Dransart, V., Petit, A., Poncet, C., Ponsonnet, C., Nesme, X., Jones, J. B., Bouzar, H., Chilton, W. S., and Dessaux, Y. 1995. Novel Ti plasmids in *Agrobacterium* strains isolated from fig tree and chrysanthemum tumors and their opinelike molecules. *Mol. Plant-Microbe Interact.* 8:311-321.
- Winans, S. C. 1992. Two-way chemical signaling in *Agrobacterium*-plant interactions. *Microbiol. Rev.* 56:12-31.
- Zambryski, P. C. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43:465-490.