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Molecular Systematics of Biotype III Ti Plasmids of *Agrobacterium tumefaciens*

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Fifty-four oncogenic grapevine isolates of *Agrobacterium tumefaciens* belonging to biotype III and two from biotype I were tested for host range, tumor morphology on *Kalanchoë tubiflora*, opine utilization, and opine production in the tumors. They were subsequently compared at the DNA level by using one T-region probe from a biotype I and two different T-region probes from a biotype III strain. The biotype III probes were selected after cloning and mapping the T-regions of two different types of octopine-cucumopine biotype III strains, Tm4 and AB3. Homology mapping with subfragments of the TL region of the octopine biotype I Ti plasmid Ach5 allowed the localization of a number of putative oncogenes on the Tm4 and AB3 T-regions. By using Ach5, Tm4, and AB3 T-region probes, the various isolates could be classified into four main groups: nopaline strains, octopine-cucumopine strains with a large TA region, octopine-cucumopine strains with a small, partially deleted TA region, and vitopine strains. Octopine-cucumopine Ti plasmids contain characteristic repeats that are closely linked to the T-regions and can also be found, in varying copy numbers, on the chromosome. All vitopine strains contain the same type of repeats, which are plasmid located. A few strains had unique homology patterns and might have special oncogenic properties. The correlations between T-region structure, opine type, and tumor-induction properties are discussed.

Additional keywords: insertion sequence elements, *Agrobacterium* classification.

Agrobacterium tumefaciens is the causative agent of the plant disease crown gall (Smith and Townsend 1907), which affects many dicotyledonous plants, especially fruit trees, ornamental plants, and grapevine (De Cleene and DeLey 1976; Holmes and Robert 1981). *Agrobacterium* is a gram-negative bacterium that has become well known for its capacity to transfer one or two well-defined DNA fragments (T-DNAs) into plant cells (Schell *et al.* 1982; Bevan and Chilton 1982). In the case of two independent T-regions, such regions have been called TA and TB or TL and TR. In the bacterium, the T-regions are parts of a large plasmid, the Ti plasmid, which also contains the functions necessary for transfer of the T-DNA. The transfer is accomplished through the action of the virulence genes (*vir* genes), which are induced by compounds released from wounded plant cells. The transferred DNA or T-DNA is found integrated in the plant genome and, through its expression, is directly responsible for the growth of the tumor (Nester *et al.* 1984; Hooykaas and Schilperoort 1984; Morris 1986). T-DNA expression also leads to the production of large quantities of small molecules, called opines, which the bacterium can use for its growth by specific opine utilization pathways encoded by the Ti plasmid (Guyon *et al.* 1980).

Several *Agrobacterium* strains have been identified. They are commonly classified into three biotypes or biovars, according to chromosomally determined characteristics (Kerr and Panagopoulos 1977). Biotype III strains were first identified on grapevine, and several studies have shown the close association between *Vitis vinifera* and biotype III strains (Loubser 1978; Süle 1978; Perry and Kado 1982; Burr and Katz 1983; Burr *et al.* 1987). In grapevine-growing areas, virulent bacteria have mainly been recovered from grapevine plants and their rhizosphere, whereas the majority of soil isolates was shown to be avirulent (Burr and Katz 1983; Knauf *et al.* 1982). In recent years, propagation of the disease has probably been caused by the planting of infected grapevine plants from nurseries, although infection of apparently healthy nursery material has also been described (Burr *et al.* 1987).

From a phytopathological point of view, the classification of *Agrobacterium* strains in chromosomally determined biotypes is of little use, because the major tumor-inducing determinants are carried by the Ti plasmids that, under laboratory conditions, can be exchanged between strains belonging to different biotypes (Kerr and Brisbane 1983). Another classification is based on the utilization of opines by the bacterium, or on the production of specific opines in the tumors. Both of these properties are plasmid located (Ellis *et al.* 1982; Dahl *et al.* 1983). In the case of biotype III strains, this has allowed a further division into octopine, nopaline, and vitopine strains (Szegedi *et al.* 1988). However, there is no clear relation between opine type and tumor-inducing properties; biotype III octopine strains, for example, show different host range patterns (Thomashow *et al.* 1980, 1981; Knauf *et al.* 1983). Characterization of isolates according to their host range involves inoculation of different plant species under standard conditions, a procedure that is time and space consuming, and the results are not always easy to reproduce in different laboratories. It seems, therefore, that a more reliable and precise way to classify *Agrobacterium* isolates could be based on molecular characterization of their T-regions by comparison with the patterns of thoroughly studied "model" strains. Model strains should be defined after a comparative study of a large number of independent isolates and should represent the most frequently occurring strains.

In an effort to characterize and classify the economically important biotype III strains with regard to their tumor-

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induction properties, we have cloned and mapped the T-regions of two strains of the octopine-cucumopine type with different tumor-inducing properties and used the information obtained to analyze T-region patterns of 54 different biotype III and two biotype I grapevine isolates, which were also characterized with respect to opine production, host range, and tumor morphology on *Kalanchoë tubiflora*. This analysis has revealed the heterogeneity of the T-region structures of the Ti plasmids harbored by the biotype III isolates and enabled us to estimate the frequency of occurrence of various plasmid types. Finally, it has allowed us to propose a molecular classification of biotype III strains found on grapevine.

MATERIALS AND METHODS

Bacterial strains and media. Table 1 lists the strains used in this study. *Agrobacterium* was grown at 28° C in YEB (Leemans *et al.* 1983) and *E. coli* at 37° C in Luria-Bertani.

Plant infections. Four plant species were used: *V. vinifera, Lycopersicon esculentum, Datura stramonium*, and *K. tubiflora*. Plants were kept in the greenhouse at a day temperature of 23° C and a night temperature of 18° C, with a 16-hr light, 8-hr dark period and a relative humidity of 85%.

K. tubiflora was infected at the second, fifth, and sixth internodes (starting from the apex) with 50 μl of a liquid bacterial suspension of about 10^8 bacteria per milliliter. *Lycopersicon* and *Datura* were infected in the same way at the stage of four expanded leaves, at the second and fourth internodes. Results were scored 40 days after infection.

Bacterial opine utilization and detection of opines in tumors. Opine utilization tests and detection of opines in tumors were performed as described (Petit *et al.* 1983; Davioud *et al.* 1988). Briefly, culture media containing synthetic opines (1–2 mg/ml) or partially purified opine-containing tumor extracts were inoculated with strains under study. After growth had ceased, aliquots of the culture media were analyzed for the presence of opines by high-voltage paper electrophoresis (Petit *et al.* 1983; Davioud *et al.* 1988).

Cloning and mapping of T-regions. Ti plasmid DNA was isolated according to Currier and Nester (1976) and purified by isopycnic centrifugation on CsCl gradients. Purified plasmid DNA was partially digested with *Hin*dIII or *Eco*RI, sized on sucrose gradients, and fractions of 15–20 kb were recovered. Fragments were inserted in the dephosphorylated vector pPM1016. This vector is a derivative of pUC18 with the *Hin*dIII-*Sma*I kanamycin-resistance fragment of Tn*5* (Rao and Rogers 1979) filled in and inserted (blunt end) into the *Ssp*I site of pUC18 (Table 1). Clones with inserts were identified on bluogal-IPTG plates and transferred to Whatman 541 paper (Maas 1983).

Clones with homology to the various genes of the pTiAch5 TL region (Leemans *et al.* 1982) were identified by hybridization with pGV201 and pGV153 (De Vos *et al.* 1981), which cover the TL region. Fragments located outside the homology region were identified by plasmid

Table 1. Origin of strains and plasmids used in this study

Agrobacterium strains	Characteristics	Grapevine cultivar	Locale	Year	Reference
Strain					
2179		Danam	Vassal	1982	M. Ride
2608, 2641		Cabernet Franc	Lussac	1985	
2609, 2643, 2673, 2674		Cabernet Sauvignon	Médoc	1985	
2612, 2613, 2614, 2644 2675, 2676		Cabernet Franc	Brissac	1986	
2615, 2616, 2645, 2646 2648		Chenin	Brissac	1986	
2617		Chenin	Savenières	1986	
2618, 2647		Cabernet Sauvignon	Brissac	1986	
2649, 2677		Melon	Le Pallet	1987	
2650		Ugni blanc	La Chade	1986	
2651		Pinot	Le Pallet	1987	
2652, 2678, 2679, 2680		Grenache	Angers	1987	
2653, 2654, 2655, 2656		Cabernet Sauvignon	Médoc	1987	
2657		Cabernet Franc	Chinon	1987	
2681, 2684		Cabernet Sauvignon	Médoc	1987	
2685, 2686		Cabernet Sauvignon	Chinon	1987	
S4, Sz1, Sz2, EK2, AB4, NI1, IS1.1 Tm4, Hm1, AB3, B10/7, Zw2 AT1, AT6, AT66					Szegedi 1985
K305, K308					Brisbane and Kerr 1983 (Australia)
LBA649 (derivative of Ag57:pTiAg57 in biotype I background)					Hoekema *et al.* 1984 and C. Panagopoulos (Greece)
Plasmids					
pPM1016	Kanamycin-resistant pUC18 derivative				This study
pGV201	*Hin*dIII fragment 1 of pTiAch5				De Vos *et al.* 1981
pGV153	*Bam*HI fragment 8 of pTiAch5				De Vos *et al.* 1981
TE-15	Partial *Eco*RI clone of TB *iaa* region of Tm4 in pPM1016				This study

walking.

Characterization of *Agrobacterium* isolates with various probes. Total DNA of *Agrobacterium* isolates was prepared with slight modifications according to Dhaese *et al.* (1979). The DNA of 1.5 ml of an overnight culture was resuspended in 200 μl of distilled water, without ribonuclease treatment, 6 μl was digested with a restriction endonuclease in a total volume of 50 μl, and 3 μl was loaded on a gel to estimate the amount of DNA and to verify whether the digestion was complete. 3–6 μl of the digest was loaded on a 0.8% agarose gel in TEA and run for various amounts of time. The gel was blotted on nitrocellulose, and the dried filters were prehybridized in 50% formamide, 4× saline sodium citrate (SSC), 10× Denhardt's solution (0.2% Ficoll, M_r 400,000, 0.2% polyvinylpyrrolidone, M_r 360,000, 0.2% bovine serum albumin), 0.2% sodium dodecyl sulfate (SDS), and 0.1 mg/ml of sonicated and denatured salmon sperm DNA in a sealed plastic bag at 42° C for 1 hr. The nick-translated and denatured probe was added and hybridized overnight. Filters were washed twice for 15 min with 2× SSC, 0.1% SDS, 15 min with 2× SSC, dried and exposed to Kodak X-O-Mat film with intensifying screens for various amounts of time.

All other molecular biology methods used were standard procedures (Maniatis *et al.* 1982).

RESULTS

Isolation and characterization of biotype III isolates. Thirty-six biotype III and two biotype I isolates were

Table 2. Classification of biotype III isolates[a]

Strain	Infectivity on: *L. esculentum*	*D. stramonium*	*K. tubiflora*	Morphology on *K. tubiflora*	Opine in Tumor	Opine in Utilization	Homology pattern TB *iaa*	TL Ach5	IST-1/2
Octopine-cucumopine strains with small TA region									
2612, 2613, 2617, 2650, 2651, 2653, 2655, 2675, 2677, AB3, AT6, Zw2	+	−	+	re	CO	CO	B	K-1	R-1
2644*									
2656	+	(+)	+	re	CO	CO	B	K-1	R-1
2614, 2676	+	+	+	re	CO	CO	B	K-1	R-1
B10/7	+	+	+	re	CO	CO	B	K-1	R-2
2654	−	−	+	re	CO	CO	B	K-2	R-1
Octopine-cucumopine strains with large TA region									
2649, 2652, 2678	+	(+)	+	sr	CO	CO	A-1	L-1	S-1
2686	+	(+)	+	sr	nt	CON	A-1	L-1	S-1
2680, K305, K308	+	−	+	sr	CO	CO	A-1	L-1	S-1
2657	+	−	+	sr	CO	CON	A-1	L-1	S-1
2679	+	−	+	sr	CO	CON	A-1	L-1	S-1
Tm4	+	−	+	sr	CO	CO	A-1	L-2	S-1
2615	+	+	+	sr	CO	CO	A-1	L-3	S-1
2618	+	+	+	sr	CO	CON	A-1	L-3	S-1
2616	+	−	+	sr	CO	CO	A-1	L-3	S-1
2646, 2648	+	−	+	sr	CO	CON	A-1	L-3	S-1
2645	+	−	+	sr	CO	CON	A-1	L-3	S-1
2647	+	(+)	+	sr	CO	CON	A-1	L-3	S-1
2641	+	(+)	+	s	CO	CON	A-3	L-4	S-1
Hm1	nt	nt	+	sr	CO	CON	A-1	L-5	S-2
2608	+	−	+	sr	CO	CON	A-2	L-6	S-1
LBA649	nt	nt	+	r	nt	nt	C	L-7	R-3
Nopaline strains									
2179, AT66, AB4	+	+	+	srt	N	N	-	M	-
2643, IS1.1	+	+	+	s	N	N	-	M	-
2673, EK2, AT1	+	+	+	sr	N	N	-	M	-
2674	+	(+)	+	sr	N	N	-	M	-
2609	+	(+)	+	srt	N	N	-	M	-
N11	+	+	+	st	N	N	-	M	-
Vitopine strains									
Sz1, Sz2	+	+	+	sr	V	V	-	N-1	T-1
S4	+	+	+	sr	V	V	-	N-2	T-2
2681	+	(+)	+	re	V	V	-	N-3	T-1
Unknown									
2684	-	-	-	-	-	CO	-	O	-
2685	-	-	-	-	-	nt	-	P	-

[a] 2654 and 2655 are biotype I isolates. The isolates have been grouped according to the homology pattern obtained with the TL DNA of Ach5. C, cucumopine; O, octopine; N, nopaline; V, vitopine; s, shoots; r, roots; t, teratomata; e, embryoids (plantlets growing on leaf edges). A–C, K–P, and R–T represent various hybridization patterns, which are defined in the text. nt, not tested; +, tumor formation; (+), weak tumor formation; −, no tumors; *, presence of octopine in tumor uncertain.

obtained from grapevine crown galls in different wine-growing areas in France. Further strains from Hungary (15), Australia (2), and Greece (1), which have been described (Table 1), were also studied. The 56 isolates were tested for opine utilization and for host range. Tumors were analyzed for the presence of opines (see Table 2). On *K. tubiflora* the various isolates induced different tumor morphologies and influenced the growth of the noninfected parts to different degrees. Three different tumor types induced by representative strains of the most frequently occurring strain types (see Discussion) are shown in Figure 1. The data on host range, tumor morphology, and its effect on plant habit on *K. tubiflora*, opine utilization, and opine production in the tumors are summarized in Table 2.

Cloning, mapping, and comparison of T-regions of AB3 and Tm4. Earlier studies in our laboratory had shown that strains AB3 and Tm4 produced different tumor types on *K. daigremontiana*: Tm4 induces shooty tumors, whereas AB3 induces undifferentiated tumors. On *Nicotiana rustica* Tm4 induces nondifferentiating tumors, but AB3 induces rooting tumors. As these different tumor morphologies persisted in sterile tissue culture conditions, we reasoned that AB3 and Tm4 would probably have different T-regions. We thus chose these strains for a more detailed analysis of T-region structure and oncogene content.

The T-regions of strain AB3 and Tm4 were cloned and identified by using the heterologous TL region of pTiAch5 as a probe. Preliminary experiments had shown that pTiAB3 and pTiTm4 lack homology to the pTiAch5 TR region, which contains genes for agropine and mannopine synthesis. A detailed description of these homology studies will be presented elsewhere (Huss *et al.*, unpublished).

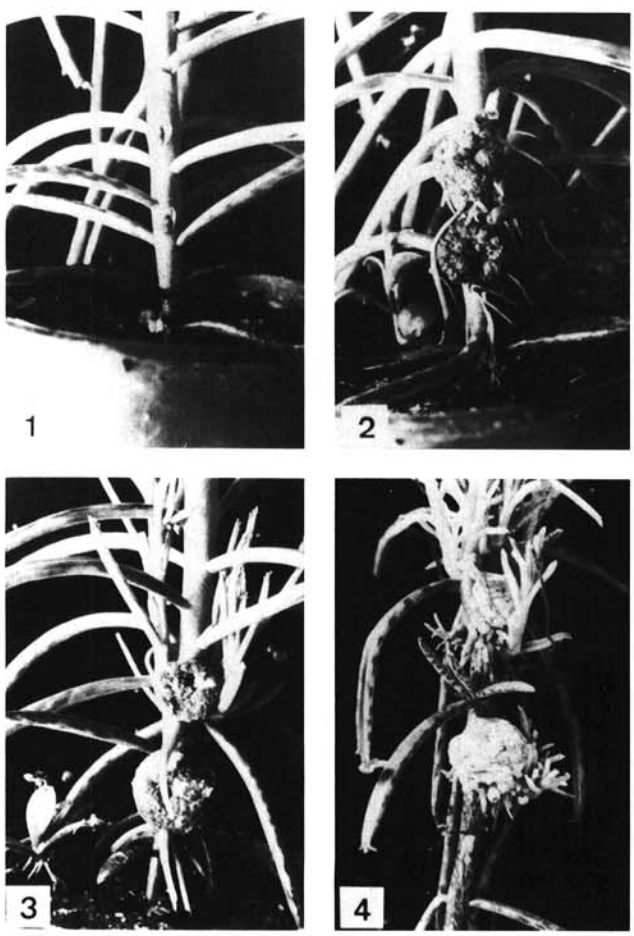

Fig. 1. Tumors induced on *Kalanchoë tubiflora* with representatives of the three major biotype III strains. 1, control infection, no bacteria; 2, 2651 (octopine-cucumopine strain, small TA region); 3, 2615 (octopine-cucumopine strain, large TA region); and 4, AB4 (nopaline strain).

The maps obtained and the homology to the Ach5 TL genes are shown in Figure 2, a and b. Both Ti plasmids contain a region with low homology to the Ach5 *iaa* genes, which is similar to the so-called TB region of the biotype III Ti plasmid pTiAg162 (Buchholz and Thomashow 1984; Knauf *et al.* 1984; Yanofsky *et al.* 1985) (Fig. 2a). In addition to the TB region, pTiAB3 and pTiTm4 contain a region with strong homology to the TL DNA of Ach5, also identified on pTiAg162 and called TA region (Fig. 2b). The TA region of pTiAB3 resembles the TA region of Ag162 and lacks homology to the *iaa* genes and most of the *ipt* gene that is found on pTiAch5 (probably due to a large internal deletion as in pTiAg162; Yanofsky *et al.* 1985). The TA region of pTiTm4, on the contrary, is very similar to the TL region of Ach5, as sequences homologous to all identified Ach5 genes (Leemans *et al.* 1982) are present and in the same relative positions. The TA and TB regions of Tm4 and AB3 are separated by a region of 30 kb, which is highly similar for both plasmids and resembles the equivalent region found on pTiAg162 (Huss *et al.*, unpublished; Paulus, unpublished). Contrary to pTiAB3 and pTiAg162, pTiTm4 contains two *iaa* gene sets, each one on a different T-region. We have started a functional analysis of these two different *iaa* sets (Huss *et al.*, unpublished).

Although the TA region of Tm4 strongly resembles the Ach5 TL region, some interesting differences can be noted. In the Tm4 TA region a deletion has occurred between the *ipt* gene and gene 6b, removing most of the 6a gene, and in gene *iaaH* a 2.7-kb element has been inserted. Sequence data (Bonnard *et al.*, unpublished) show that the insertion in *iaaH* has the characteristics of a bacterial insertion sequence (IS element), which we will call IST-1 (for IS, Tm4). Two similar elements with about 80% homology to the IST-1 element are found on pTiTm4 and pTiAB3: immediately to the right of the octopine synthase gene (IST-2) and in the TB region to the right of the *iaa* genes (IST-3). These two latter elements are indistinguishable for seven out of seven restriction sites and have the same relative orientation. They probably represent variants of IST-1.

Although the maps of pTiAg162 and pTiAB3 are similar for the TA and TB regions (about 90% of the restriction sites is conserved), AB3 induces tumors on tobacco, whereas Ag162 does not (Buchholz and Thomashow 1984). The reasons for this difference are not known, but could be related to differences in virulence genes or oncogenes.

Molecular characterization of various grapevine strains with different probes. To characterize the grapevine isolates, we selected three different probes and hybridized these to blots of total DNA of each strain digested with different restriction enzymes. This enabled us to define the different hybridization patterns. Blots with DNA from strains representative for each pattern were then prepared (Figs. 3, 4, and 5). The fragment sizes of the different patterns are summarized in Table 3; the relation of these patterns with other strain characteristics is shown in Table 2.

TB *iaa* genes. Our studies of the TB region structures of strain AB3 and Tm4, as well as functional studies (Huss

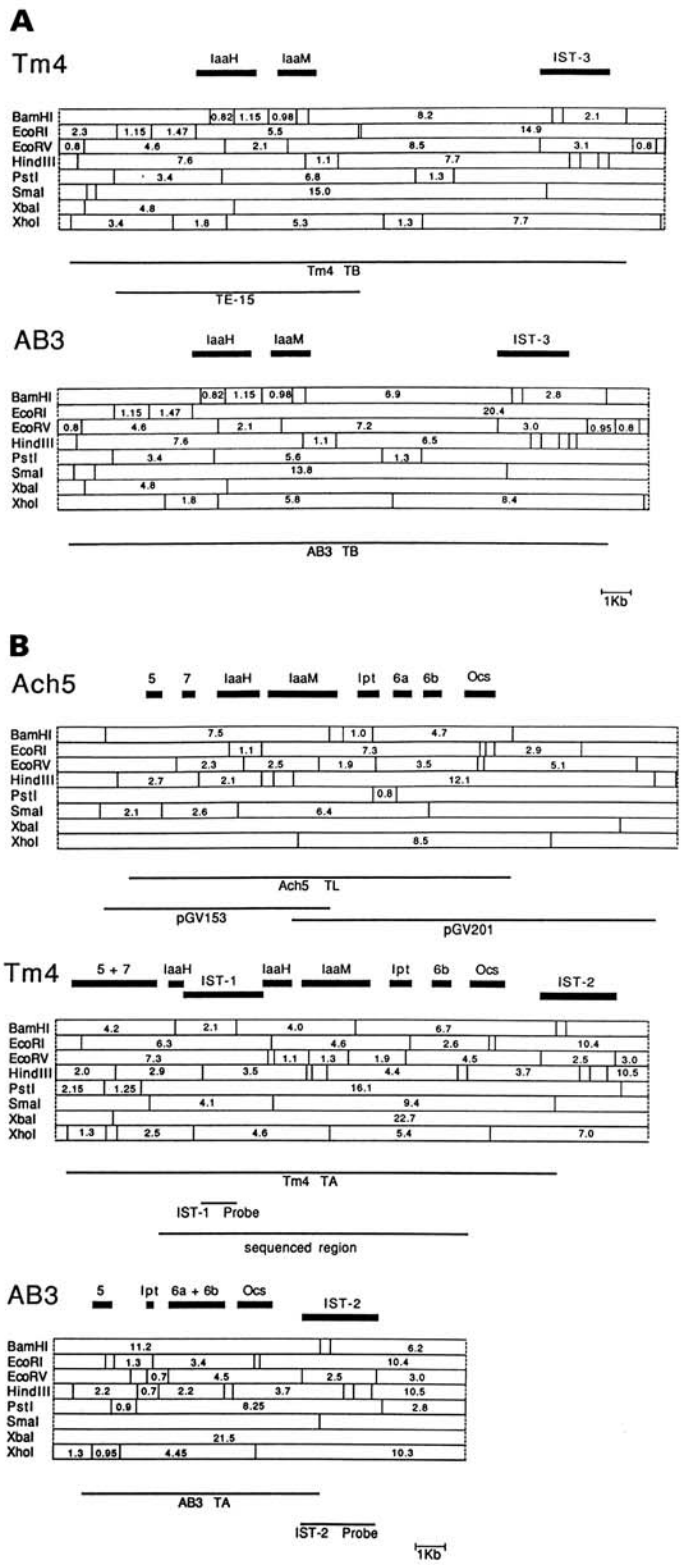

Fig. 2. Maps of the T-regions of Tm4 and AB3. **A**, Physical map of the TB regions of Tm4 and AB3. The *iaaH* and *iaaM* genes were localized by hybridization to Ach5 *iaaH* and *iaaM* probes and by Tn*5* mutagenesis of Tm4 (Huss *et al.*, unpublished). As the restriction sites are the same for Tm4 and AB3 in this part of the TB region, we extended the Tn*5* data for Tm4 to AB3. The TB region of both Ti plasmids corresponds to the TB region as proposed by Yanofsky *et al.* (1985). **B**, Physical maps of the TA regions of Tm4 and AB3, compared with the TL region of Ach5 (Leemans *et al.* 1982). Putative T-region genes were localized by hybridization with Ach5 TL probes and by sequencing (Bonnard *et al.*, unpublished). The sequenced region is indicated.

et al., unpublished), had shown that both strains contained sequences with very low homology to the pTiAch5 auxin genes. Furthermore, studies with the Tm4 TB auxin genes have shown that they have a very weak root-inducing activity on *K. daigremontiana* but are essential for tumor induction on grapevine (Huss *et al.*, unpublished).

It was therefore of interest to determine the distribution of this particular type of auxin genes in other biotype III strains by using the cloned *iaa* region of Tm4 as probe. Clone TE-15, which contains the complete TB auxin gene set TB-*iaaH* and TB-*iaaM* (Fig. 2a), was hybridized to *Hin*dIII-digested total DNA of all isolates. The results are summarized in Tables 2 and 3. Figure 3a shows the hybridization of patterns of representative strains. Three main groups can be defined: strains without homology to TB *iaa*, strains with pattern A, and strains with pattern B.

Strains without homology to TB *iaa*. The first group has no homology to the probe and comprises the nopaline and vitopine strains.

Strains with pattern A. The second group (18 of 39 octopine-cucumopine strains) shows pattern A-1, which is also found in strain Tm4. One octopine-cucumopine strain, 2608, shows an A-type pattern, but contains in addition two bands of 1.0 and 1.25 kb (A-2). Hybridization with subfragments of TE-15 (not shown) showed that these bands have only homology with the TB-*iaaM* region. It is not known whether these bands are part of a T-region. Interestingly, the same two bands are present in the octopine-cucumopine

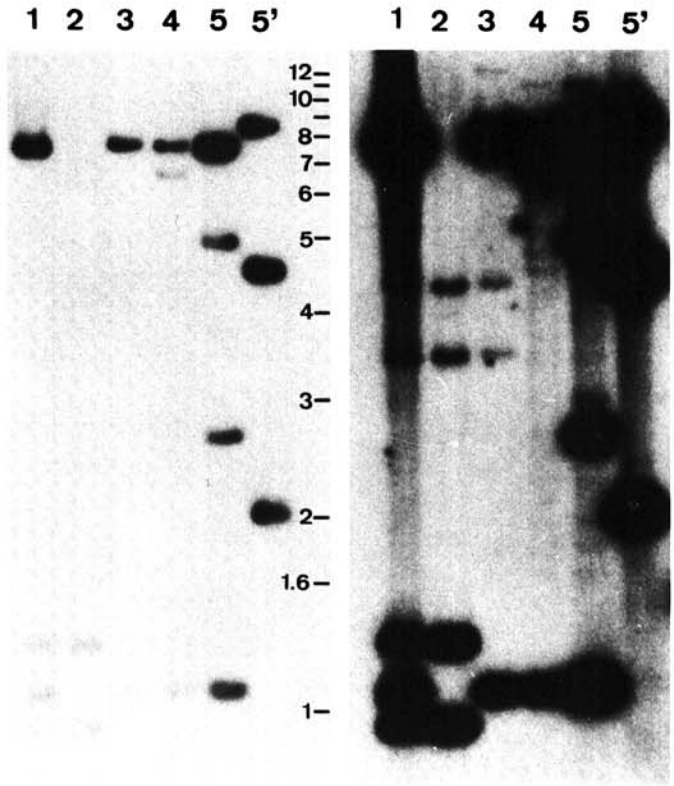

Fig. 3. Hybridization of total *Agrobacterium* DNA, cut with *Hin*dIII (1–5) or *Eco*RV (5'), with the TB *iaa* region as probe. The autoradiogram was exposed for 2 hr (**A**) or 24 hr (**B**). 1, 2608 (A-2); 2, 2641 (A-3); 3, Tm4 (A-1); 4, AB3 (B); 5, LBA649 (C); 5', LBA649, *Eco*RV digested.

strain 2641, which, however, lacks the other TB *iaa* bands. 2641 is probably a natural deletion derivative of an A-type strain because its TA pattern strongly resembles that of Tm4. We therefore call this pattern A-3. Upon overexposure of the autoradiogram (Fig. 3b), the *Hin*dIII fragments of 3.5 and 4.4 kb, which correspond to the *iaa* genes of the TA region, become visible, demonstrating the low homology between the TB- and TA-located *iaa* genes.

Strains with pattern B. A third group (18 of 39 octopine-cucumopine strains, which include the two biotype I strains) shows pattern B (as in AB3). Careful comparison of the Tm4 and AB3 TB maps (Fig. 2a) reveals that the difference between the AB3 and Tm4 *Hin*dIII patterns is due to the lack of a 1.2-kb fragment in AB3 that, in Tm4, is located between the *iaa* genes and the IST-3 repeat (see below).

Strain with pattern C. One exceptional strain, LBA649, shows a unique pattern, C (see Discussion).

From our results it can be concluded that within the biotype III group, the TB *iaa* genes are specific for the octopine-cucumopine strains.

TL-DNA of pTiAch5. As the TA region of Tm4 was shown to be larger than the TA region of AB3, it appeared to be a suitable probe to study the various grapevine isolates. However, it was found in preliminary experiments that the repeated sequences IST-1 and IST-2 associated with the Tm4 TA region were present in many strains in varying copy numbers and thus led to complex hybridization patterns that prevented T-region analysis. Because the TL region of pTiAch5 is strongly homologous to the Tm4 TA region but does not contain the IST-1 and IST-2 repeats, we used this heterologous TL region as probe to reveal the TA-like

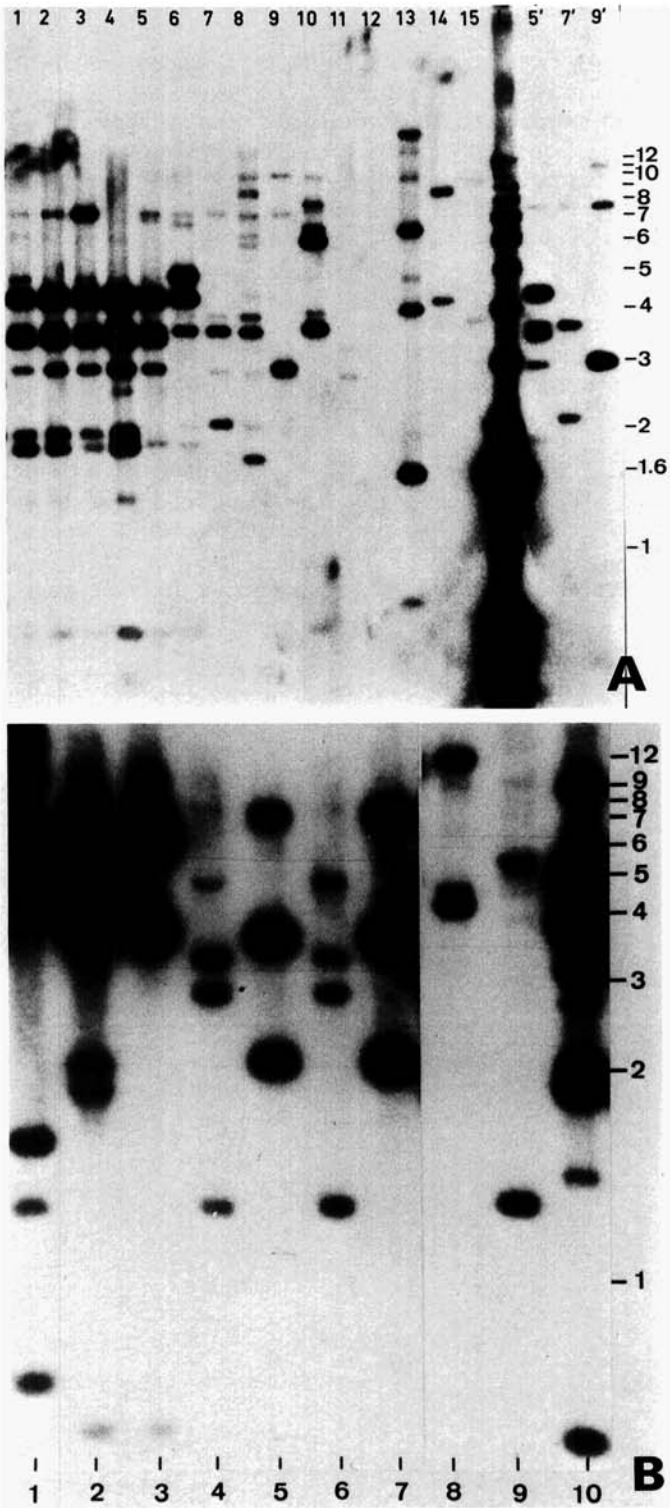

Fig. 4. Hybridization of total *Agrobacterium* DNA, cut with *Hin*dIII, with the TL region of Ach5 as probe. **A**, 1, 2649 (L-1); 2, 2608 (L-6); 3, 2615 (L-3); 4, 2641 (L-4), overloaded; 5, Tm4 (L-2); 6, Hm1 (L-5); 7. 2612 (K-1); 8, 2654 (K-2); 9, LBA649 (L-7); 10, 2179 (M); 11, Sz1 (N-1); 12, 2681 (N-3); 13, S4 (N-2); 14, 2685 (P); 15, 2684 (O). L, kb ladder. 5′, pTiTm4; 7′, pTiAB3; 9′, pTiAg57 (plasmid from LBA649 strain). **B**, Overexposed autoradiogram of selected digests, to show minor bands. 1, S4 (N-2); 2, Hm1 (L-5); 3, AT1 (M); 4, Sz1 (N-1); 5, AT6 (K-1); 6, Sz2 (N-1); 7, AB3 (K-1); 8, 2684 (O); 9, 2681 (N-3); 10, 2680 (L-1).

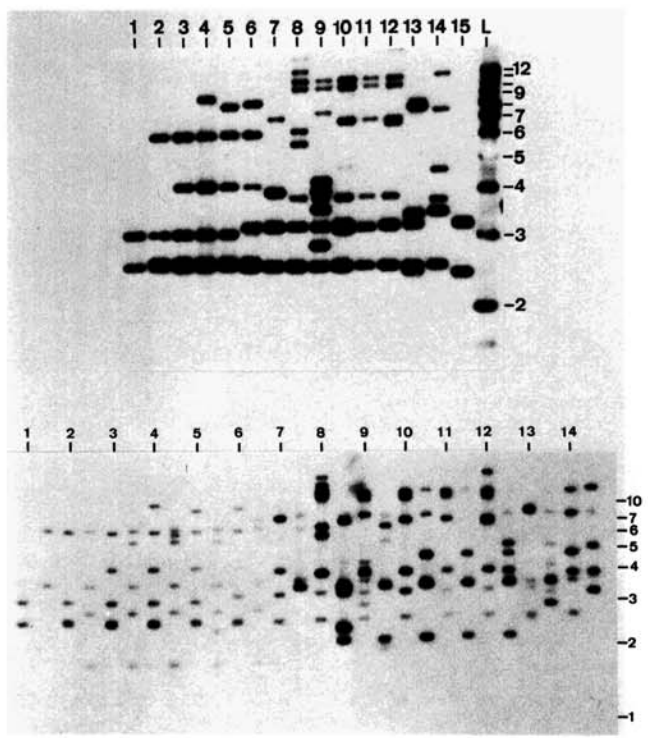

Fig. 5. IST elements in different *Agrobacterium* strains. Upper figure: hybridization of total *Agrobacterium* DNA, cut with *Eco*RV, with an IST-2-specific probe. Lower figure: total DNA, cut with *Eco*RV (every first lane) or with *Hin*dIII (every second lane), hybridized with an IST-1-specific probe. 1, 2654 (R-1); 2, 2675 (R-1); 3, 2656 (R-1), identical to AB3; 4, 2650 (R-1); 5, 2653 (R-1); 6, B10/7 (R-2); 7, Tm4 (S-1); 8, K308 (S-1); 9, 2680 (S-1); 10, 2608 (S-1); 11, 2641 (S-1); 12, 2686 (S-1); 13, 2618 (S-1); 14, Hm1 (S-2); 15, LBA649 (R-3). L, kb ladder.

regions of the various biotype III strains.

The TL region of pTiAch5, represented by pGV201 and pGV153, hybridized to the total DNA of all biotype III strains cut with *Hin*dIII and produced various different patterns: K, L, M, N, O and P (see Tables 2 and 3 and Fig. 4, a and b).

Strains with pattern K. The first pattern (K-1) occurs in 17 of 39 octopine-cucumopine strains and was identified as belonging to the T-regions of the octopine-cucumopine strain AB3 (Fig. 2, a and b). The K-type strains thus have short TA-regions like AB3. Biotype I strain 2654 has a slightly different pattern (K-2).

Strains with pattern L. The second pattern (L-1) is found in nine octopine-cucumopine strains and can be related to the pattern of the octopine-cucumopine strain Tm4. (In Tm4 the 1.4, 1.9, 2.1, and 4.9 *Hin*dIII fragments are absent, and instead, a 2.0-kb fragment is found; the unique Tm4 pattern is called L-2.) Within the L group, some variation occurs: patterns L-2, L-3, L-4, L-5, and L-6. One strain, 2641, which was found to lack homology to the TB *iaa* probe, lacks as expected the 7.6-kb *Hin*dIII fragment on which, according to the Tm4 map, the TB *iaa* genes are located (pattern L-4). The TA-specific bands of 2641 are the same as those of the L-pattern. Strain Hm1 (L-5) lacks the 2.9- and 3.5-kb fragments, which correspond to the TA *iaaH* region (see Fig. 2b), indicating a special TA structure in this strain. Contrary to the K-pattern strains, the L-type strains have a large TA-region like Tm4. LBA649 has a pattern (L-7) that, according to mapping results obtained with LBA649 T-region clones (Paulus, unpublished), might be related to the other L patterns.

Other strains. The third group, containing all the nopaline strains, shows a single pattern, M. The fourth group, strains with pattern N, contains four strains that have been identified as vitopine strains. Sz1 and Sz2 have an identical, very weak pattern, N-1. S4 has pattern N-2. The 1.3 and 4.7 bands seen in S4 are also found in Sz1 and Sz2. 2681 (N-3) has only very weak bands of 1.3 and 5.2 kb. The remaining strains, 2684 and 2685, show patterns O and P, which are unique and cannot be related to the patterns of other groups.

The hybridization results with the TL probe show that all grapevine strains hybridize to this probe, but yield 15 different patterns.

IST-1 and IST-2. As already noted, both AB3 and Tm4 carry repeated sequences on their Ti plasmids, which are closely linked to the T-regions of these strains and absent in the biotype I strain Ach5 (see Fig. 2, a and b). It was therefore of interest to determine whether these sequences

Table 3. Hybridization patterns obtained with three different probes on total DNA of various *Agrobacterium* strains[a]

Pattern	Enzyme	Bands, size (in kb)	Figure/lane
Probe TB *iaa*			
A-1	H	1.1, (3.5), (4.4), 7.6, 7.7	2a, 3a, b/3
A-2	H	1.0, 1.1, 1.25, (3.5), (4.4), 7.6, 7.7	3a, b/1
A-3	H	1.0, 1.25, (3.5), (4.4)	3a, b/2
B	H	1.1, 6.5, 7.6	2a, 3a, b/4
C	H	1.1, 2.8, 5.1, 7.6	3a, b/5
	E	2.1, 4.6, 8.5	3a, b/5′
Probe TL, Ach5			
K-1	H	0.7*, 2.2, 2.2, 3.7, (7.6)	4a/7, 7′, 4b/5, 7
K-2	H	0.7*, 1.7, 3.7, (7.6), various weak bands	4a/8
L-1	H	0.57, (1.4), 1.9, 1.9, 2.1, 2.9, 3.5, 3.7, 4.4, 4.9, (7.6)	4a/1, 4b/10
L-2	H	0.57, (2.0), 2.9, 3.5, 3.7, 4.4, (7.6)	4a/5, 5′
L-3	H	0.57, (1.4), 1.9, 2.1, 2.9, 3.5, 3.7, 4.4, 4.9, 7.6	4a/3
L-4	H	0.57, (1.4), 1.9, 1.9, 2.1, 2.9, 3.5, 3.7, 4.4, 4.9	4a/4
L-5	H	0.57, (1.9), (2.15), 3.7, 4.4, 5.3, (7.0), (7.6)	4a/6, 4b/2
L-6	H	0.57, (1.4), 1.9, 2.1, 2.9, 3.5, 3.7, 4.4, (7.6)	4a/2
L-7	H	2.9, 2.9, (7.6), (11.0)	4a/9, 9′
M	H	0.57, 3.7, (3.9), 6.2, (6.8), (8.0), (11.0)	4a/10, 4b/3
N-1	H	(1.3), 2.85, 3.2, (4.7), (7.7)	4a/11, 4b/4, 6
N-2	H	0.66, (1.3), 1.65, 4.0, (4.7), 6.4, (9.4), (13.0), 15.0	4a/13, 4b/1
N-3	H	(1.3), (5.3)	4a/12, 4b/9
O	H	(3.9), (10.5)	4a/15
P	H	4.2, 8.5	4a/14, 4b/8
Probe IST-1 or IST-2[b]			
R-1	E	2.5 [2], 3.0 [3]	5/1, with additional elements: 5/2, 5/3, 5/4, 5/5
	H	0.35*[2,3], 0.57*[2, 3], 3.7 [2], 6.5[3], 10.5*[2]	
R-2	E	2.5 [2], 2.55, 3.1 [3], 4.0, 6.0, 9.5	5/6
	H	0.35* [2, 3], 0.57*[2, 3], 1.7, 2.65, 3.7 [2], 6.5 [3], 6.6, 7.5, 10.5*[2]	
R-3	E	2.4 [2], 3.1 [3]	5/15
S-1	E	2.5 [2], 3.1 [3], 3.8 (IST-1-like), 7.3 [1]	5/7, with additional elements: 5/8, 5/9, 5/10, 5/11, 5/12, 5/13, 5/14
	H	0.35*[2,3], 0.57*[2,3], (2.9)[1], 3.5 [1], 3.7 [2], 6.6 (IST-1-like), 7.7 [3], 10.5*[2]	
T-1	E	8.2, 12.0	Results not shown
	H	4.0, 4.6	
T-2	E	3.5, 8.2, 12.0	Results not shown
	H	0.95, 1.9, 4.0, 4.6	

[a] H, *Hin*dIII; E, *Eco*Rv. An asterisk indicates a very weak band, not visible on photograph. Numbers in parentheses indicate a weak band. [1], [2], [3] identified as IST-1, IST-2, and IST-3, respectively.

[b] Bands without further indications between brackets hybridized strongly to the IST-2 probe and weakly to the IST-1 probe.

were present in other biotype III strains, whether their presence could be used as a biotype III-specific marker, and whether they could serve in classification schemes. For this analysis, we chose two probes covering IST-1 and IST-2. Sequence data from our laboratory (Bonnard *et al.*, unpublished) have shown the exact location of IST-1. It is inserted in a region homologous to the *iaaH* gene of pTiAch5 and about 80% homologous to IST-2 and IST-3, which are very similar to each other. The incomplete homology between IST-1 on the one hand and IST-2 and IST-3 on the other hand permits discrimination between the two types of sequences. An internal 1.2-kb *Hin*dIII-*Bam*HI fragment from the IST-1 sequence of pTiTm4 (see Fig. 2b) was chosen as an IST-1-specific probe, and an *Eco*RV fragment of 2.5 kb from the IST-2 region of pTiAB3 (see Fig. 2b) was taken as an IST-2-specific probe. Both probes were hybridized to total, *Hin*dIII- or *Eco*RV-digested DNA from the various grapevine strains. The patterns obtained, R, S, and T, are listed in Tables 2 and 3 and shown in Figure 5. Several groups can be distinguished: strains without homology to IST-1 or IST-2, strains with pattern R, strains with pattern S, and strains with pattern T.

Strains without homology to IST-1 or IST-2. The first group, comprising all nopaline strains, has no IST elements.

Strains with pattern R. The second group contains only IST-2-like elements and comprises the octopine-cucumopine strains with a short TA region like AB3. These strains contain varying numbers of hybridizing bands, the simplest pattern being the one showing the two pTi-located repeats, IST-2 and IST-3 (Fig. 2, a and b, and Fig. 5, lane 1). This basic IST-2/IST-3 pattern, found in the two biotype I strains 2654 and 2655, is called R-1. Strain B10/7, which is clearly related to AB3 as shown by its characteristic B and K-1 pattern, has a somewhat larger IST-3 *Eco*RV fragment, pattern R-2. In many strains (see Fig. 5, lanes 2–6) additional IST-2-like elements are found; in the case of AB3 (identical with 2656, lane 3), three additional IST-2-like bands are found, which are not seen with purified plasmid DNA (see Fig. 6, lane 2 and 2′). We therefore assume that these bands represent three additional IST-2-like elements located on the chromosome at different positions. The IST-2 patterns observed are in part additive (Fig. 5) and could have resulted from successive transpositions of the IST elements.

LBA649 has an exceptional pattern (R-3). Cloning of its T-regions (Paulus, unpublished) showed that its two IST-2-like sequences are on the Ti plasmid and correspond in their location to IST-2 and IST-3. A detailed analysis of the location, organization, distribution, and evolution of the IST elements in the various biotype III strains will be given elsewhere.

Strains with pattern S. The third group of strains contains IST-1- and IST-2-like elements, both in varying numbers. This groups comprises all octopine-cucumopine strains with a large TA region and is exemplified by Tm4. The 7.3-kb *Eco*RV IST-1 band of Tm4 is not found in the other strains, but the 2.9- and 3.5-kb *Hin*dIII bands, which also correspond to IST-1, are found in the other strains, with the exception of Hm1. The plasmid-located IST-1, IST-2, IST-3 pattern (see Table 3) is called S-1. As the *Hin*dIII bands corresponding to the IST-1 copy found on the Ti plasmid of Tm4 are seen in all strains of this group (2.9- and 3.5-kb bands; Fig. 5, lanes 7–13), except Hm1 (with pattern S-2, lane 14), we conclude that all these strains carry IST-1 at the same location within *iaaH*. All strains of the S-pattern group contain additional IST elements: Tm4 for example, shows an additional IST-2-like element that is only seen on total DNA, but not on plasmid DNA (see Fig. 6, lane 1 and 1′) and is therefore located on the Tm4 chromosome. Other strains have also additional IST-1- and IST-2-like bands, for which we have not yet determined the location.

Strains with pattern T. The fourth group, which comprises the vitopine strains, has pattern T-1. S4 contains the same bands as Sz1, Sz2, and 2681, and in addition, one more *Eco*RV and two more *Hin*dIII fragments, probably corresponding to an additional IST element (T-2). The IST-2-like elements of Sz1, Sz2, and S4 are plasmid-borne (J. Canaday, unpublished). Our results demonstrate that the IST-1-like elements are specific for octopine-cucumopine strains with a large TA region, whereas the IST-2-like elements are found in all octopine-cucumopine strains as well as in the vitopine strains.

DISCUSSION

The cloning, mapping, and detailed comparison of the two T-regions, TA and TB, of two different octopine-

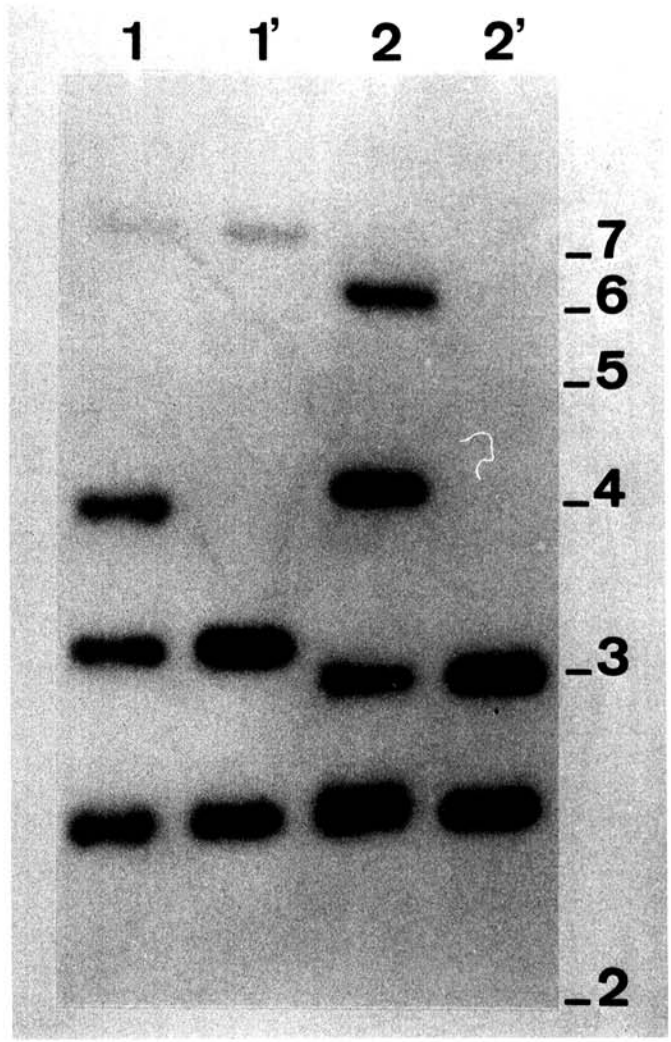

Fig. 6. Localization of IST repeats on the Ti plasmid and on the chromosome. Hybridization of the IST-2 probe with total DNA (1, 2) or purified plasmid DNA (1′, 2′) of Tm4 (1, 1′) or AB3 (2, 2′), cut with *Eco*RV.

cucumopine biotype III strains, Tm4 and AB3, and the already available TL clones of pTiAch5 have allowed us to classify most of 56 oncogenic grapevine isolates (54 of which belong to biotype III) by comparing their hybridization patterns with carefully selected and well-defined probes. These probes were the TL region of Ach5, the *iaa* genes of the TB region of Tm4, which are only weakly homologous to the *iaa* genes of the Ach5 TL-region, and two new, nonidentical T-region-associated IS elements, which we have called IST-1 and IST-2. Our studies have revealed a large amount of heterogeneity within the biotype III group. However, we were able to distinguish four major groups of biotype III strains (Table 3) that could serve as a reference in further molecular studies, both structural and functional: octopine-cumumopine strains with a small TA region, octopine-cucumopine strains with a large TA region, nopaline strains, and vitopine strains.

Octopine-cucumopine strains with a small TA region. The first group (16 strains) is composed of octopine-cucumopine strains like AB3 with a small TA region with a characteristic *Hin*dIII pattern (K) revealed by the TL probe. These strains have also a characteristic *Hin*dIII pattern in the TB *iaa* region (pattern B). They have at least two IST-2-like repeats: one located to the right of the TA region and one in the TB region (pattern R). Additional copies of the IST-2-like elements (the numbers of which vary with the different isolates) occur elsewhere, possibly, as in the case of AB3, in the chromosome. These strains all induce a characteristic response on *K. tubiflora*: tumors with roots and formation of embryoids (small plantlets) on the neighboring leaves (Figs. 1 and 2). Root formation is presumably due to the TB *iaa* genes of these strains. B10/7, which induces no embryoids, constitutes an exception. It might have a different TB structure, as suggested by its unique IST-3 pattern. The strains of this group degrade octopine and cucumopine. The two biotype I grapevine strains 2654 and 2655 belong to this group. Because they only contain Ti-plasmid-located IS elements, they might have acquired their Ti plasmid from a biotype III strain.

Octopine-cucumopine strains with a large TA region. The second group (21 strains) is formed by octopine-cucumopine strains with a large TA region with a characteristic *Hin*dIII hybridization pattern (L) revealed by the TL probe. Strain Tm4, the TA and TB region of which we have cloned and mapped, belongs to this group but seems to have a unique structure in the left half of its TA region (i.e., to the left of the TA *iaa* genes) in the gene 5 region. The strains of the L group, including Tm4, also have a characteristic *Hin*dIII pattern in the TB *iaa* region (pattern A). They have at least two IST-2-like repeats, located on the Ti plasmid at the same position as in the first group. In addition to the IST-2-like repeats, the strains of this group carry at least one IST-1-like repeat situated in the TA-region and interrupting gene *iaaH*. The basic IST-1, IST-2, IST-3 pattern is called S. Additional IST-1 and/or IST-2 copies occur in most of these strains, probably on the chromosome. On *K. tubiflora* they all induce tumors with roots and at some distance from the tumors, shoots (Figs. 1 and 3). Root induction could be due to the TA and TB *iaa* genes, whereas shoot induction could result from the TA-located *ipt* gene, which for Tm4 has been shown to be active (Bonnard *et al.*, unpublished). The strains of this group degrade octopine and cucumopine and, in 11 out of 20 strains, nopaline.

Nopaline strains. The third group contains the nopaline strains (11 strains), which show a characteristic *Hin*dIII hybridization pattern with the Ach5 TL region. This pattern does not resemble the biotype I nopaline T-region *Hin*dIII pattern (Holsters *et al.* 1980). Biotype III nopaline strains lack repeated sequences of the IST-1 or IST-2 type. They also lack the TB *iaa* genes. The T-regions of these strains have not yet been cloned, and a detailed map of their T-region structure is not yet available. In spite of the fact that all nopaline strains have identical TL homology patterns, the response on *K. tubiflora* is variable: the nopaline strains induce shoots that are mostly accompanied by roots and sometimes by teratomata (Figs. 1 and 4).

Vitopine strains. The fourth group (4 strains) contains the vitopine strains (Szegedi *et al.* 1988), which are characterized by a special pattern of IST-2-like elements and by the absence of TB-like *iaa* genes. Remarkably, whereas Sz1, Sz2, and 2681 do not have much homology with the Ach5 TL DNA, the vitopine strain S4 has several bands with strong homology. The vitopine strains induce tumors on *Kalanchoë* with little root and shoot formation. Like the nopaline strains, they require a more detailed study, especially with regard to their (unknown) oncogenes, which hybridize weakly to the biotype I oncogenes. The vitopine strains seem to be rare on grapevine.

Our homology studies clearly define two groups of octopine-cucumopine strains: those with a large TA region and those with a small TA region. We think these differences are phytopathologically relevant because the *iaaM* gene and the *ipt* gene present in the large TA region of Tm4 (and absent in the small TA region of AB3) are active (Huss *et al.*, unpublished; Bonnard *et al.*, unpublished). In addition, the TA *iaaM* gene has been shown to be essential for pathogenesis on grapevine (Tinland, unpublished results). Further experiments will define the role of the various oncogenes. In particular, the presence of two different functional *iaaM* genes on two separate T-regions is of interest.

The presence of an isolated *iaa* set on the TB region is reminiscent of the isolated *iaa* set on the TR region of the *Agrobacterium rhizogenes* Ti plasmids. However, preliminary experiments in our laboratory have shown little homology between the two T-regions.

Our studies demonstrate a considerable T-region variability. Remarkably, the octopine-cucumopine strains seem to vary much more with regard to their T-region structure than do the nopaline strains. With the help of the now-established Tm4 and AB3 maps and the use of specific probes from cloned fragments of these strains, many variant patterns can be studied in more detail and be interpreted in terms of T-region structure and oncogene content. The octopine-cucumopine strain 2608, for example, carries two additional *Hin*dIII fragments hybridizing to the TB *iaaM* region. Such additional fragments might represent additional oncogenes that could confer special pathogenic properties. Another example is strain Hm1, which belongs to the "large TA" strains, but is different in the left part of its TA region, which includes the TA *iaa* genes. Strain 2641, a strain with a large TA region, lacks the TB *iaa* genes, but has the additional *iaaM*-related sequences seen in 2608. Contrary to the strains from the same group, 2641 does not induce root formation on *Kalanchoë*, confirming the root-stimulating role of the TB *iaa* genes on this plant. As 2641

induces the synthesis of cucumopine in the tumors, a trait that is encoded by genes located in the TB region (Tempé, unpublished results), and because the bands for the TB-located IST-3 repeat are still present, part of the TB region is conserved in 2641. It might be interesting to study the structure and function of this remaining fragment.

Other unique strains, like LBA649, are more difficult to classify. The Ti plasmid of LBA649 (pTiAg57) is possibly a derivative of the "large TA" octopine-cucumopine Ti plasmids, as indicated by the detailed map of its T-regions. Also, part of the TB *iaa* gene region of pTiAg57 is repeated to the right of the *iaa* genes (Paulus, unpublished). Two exceptional strains, 2684 and 2685, cannot be related to any group in particular. The absence of IST-like elements or TB *iaa* genes in the two latter strains seems to exclude them from the octopine-cucumopine-vitopine group. Both are avirulent on tomato, *Datura*, and *Kalanchoë*. 2684 is also avirulent on grapevine, whereas 2685 is only weakly virulent on grapevine. 2684 has little homology to the Ach5 TL region, whereas 2685 has a relatively strong homology.

We expect that the IST repeats could help in further classification of the various isolates that contain them. Sequencing results (Bonnard, unpublished) have shown that IST-1 has the characteristics of an IS element and the strong homology between IST-1, IST-2, and IST-3 makes it likely that IST-2 and IST-3 (which are indistinguishable by restriction-site mapping) are also IS elements. As IS elements generally transpose in a replicative way, a bacterium containing them will gradually accumulate more copies.

The various patterns that we have observed may indicate, by careful comparison, how the different strains are derived from each other. Occasional transfer of biotype III plasmids into biotype I strains would have generated strains like 2654 and 2655, which do not contain chromosomal IST elements. In view of the many different IST patterns detected (15 patterns for 21 strains), some isolates might represent local *Agrobacterium* variants. Particular strain types seem to be concentrated at a given location. For example, all seven isolates with the L-3 pattern were found at Brissac. Also, 2608 and 2641, which are the only strains with 1.0- and 1.25-kb *Hin*dIII bands hybridizing with the TB *iaa* probe, have been isolated from the same location. Finally, the French nopaline strains were obtained from Médoc (four strains) and Vassal (one strain), but not from other locations. A detailed study of the IST patterns might allow some insight in the local as well as worldwide distribution of virulent, grapevine-associated *Agrobacterium* strains.

Our detection and localization of the IST elements in the octopine-cucumopine strains constitute strong support for the *Agrobacterium* oncogene origin hypothesis of Yamada *et al.* (1986). These authors proposed, on the basis of homology between IS elements from the gall-inducing bacterium *Pseudomonas savastanoi* (found on plasmids, associated with bacterially expressed *iaa* genes) and sequences immediately to the right of the Ach5 TL region, that the *iaa* genes of *Pseudomonas* were inserted into a primitive *Agrobacterium* Ti plasmid as part of a transposon flanked by two IS elements. The TB *iaa* genes of the octopine-cucumopine strains are flanked by the IS-like elements IST-2 and IST-3, and could thus have been introduced into *Agrobacterium* by a transposition event. Whether the IST-like elements of the vitopine strains are also associated with T-regions of these strains may be shown by the cloning of the vitopine strain T-regions.

Remarkably, IST-1 elements, which can be distinguished from the IST-2 and IST-3 elements because of incomplete homology, are only found in strains that contain the IST-1 element in the TA region. Also, IST-2 and IST-3 elements are only found in strains containing both of these elements on the Ti plasmid. This indicates that the biotype III plasmids are strongly linked to specific chromosomal backgrounds, because otherwise one could expect some nopaline-type or vitopine-type plasmids to be associated with chromosomes containing IST copies or some Ti plasmids with IST-2, IST-3 sequences to be associated with chromosomes containing IST-1 copies. Only in two cases, 2654 and 2655, were IST-containing plasmids found in a biotype I background. It will be of interest to determine whether nononcogenic biotype III strains do contain IST elements.

The type of analysis presented here will eventually allow us to retrace the evolution of the various Ti plasmids and their T-regions. Although at least 15 different TL homology patterns emerged from our studies of 56 strains (K-1 and K-2, L-1 to L-7, M, N-1 to N-3, O, and P), which need to be explained in terms of T-region structure, oncogene content, and phytopathological activity, the maps of only a few model strains might suffice to define the areas where the differences are located. The clones from the model strains will also allow rapid cloning of the T-regions of variant strains. It can be expected that the repeated elements associated with the T-regions will help provide insight into the origin and evolution of the Ti plasmids, as they provide molecular time markers, indicating discrete events (the transpositions) that gave rise to new branches on the evolutionary tree. In combination with functional studies of the individual oncogenes of the T-regions, structural studies of the kind presented here will define which types of oncogene combinations evolved in the biotype III strains of *A. tumefaciens* and enabled them to induce tumorous growth on grapevine. Knowledge of the various ways in which *Agrobacterium* induces grapevine cell division and proliferation might be useful in improving grapevine tissue culture. It might also serve to devise general crown gall protection systems based on transformation of *Agrobacterium* hosts with antisense genes directed against essential oncogenes.

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