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Chromosomal and plasmid diversity of agrobacterium strains isolated from *Ficus benjamina* tumors

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Abstract

Agrobacteria were previously isolated from tumors developing on branches and aerial and hypogeous roots of weeping fig plants in Italy and in The Netherlands. A representative group of 48 strains was analyzed by PCR–RFLP of 16S and 16S + IGS ribosomal regions, PCR–RFLP of six Ti plasmid (pTi) regions and characterized for plasmid content. Two groups of agrobacteria were separated by cluster analysis of PCR–RFLP profiles of *rrs* gene: seventeen strains were similar to the new species *Agrobacterium larrymoorei*, while the remaining strains were included within the agrobacterium biovar 1 group. Sixteen different plasmid profiles from one to five plasmids were observed. In addition, 21 ribotypes and 20 pTi structures were arranged in many different combinations, showing that fig agrobacteria were characterized by a wide heterogeneity. A general lack of correlation between strain ribotypes and plasmid content was observed.

Introduction

Agrobacteria are soil-borne Gram negative bacteria, responsible for the induction of a neoplastic disease (crown gall) or for abnormal root proliferation (hairy root) on different host plants. On the basis of physiological and biochemical characters most of the members of the genus *Agrobacterium* were distinguished into three biovars (Kersters and De Ley, 1984), two of which were recognized as different species: *A. rhizogenes* (formerly biovar 2) (Sawada et al., 1993) and *A. vitis* (formerly biovar 3) (Ophel and Kerr, 1990). Studies based on DNA–DNA homology (Popoff et al., 1984) indicated that biovar 1 does not correspond to a single species of *Agrobacterium*, but it includes at least nine genomic groups that have not yet received accepted nomenspecies status. Classification and nomenclature of the species belonging to the genus *Agrobacterium* is still under discussion and most recently the inclusion of the *Agrobacterium* species in the genus *Rhizobium* has been proposed (Young et al., 2001).

Most of the pathogenic determinants of agrobacteria are born on a large extra chromosomal replicon termed the Ti plasmid (pTi). During the infection process, part of this plasmid (the T-DNA) is transferred to the wounded plant cells where it is integrated in the nuclear genome (Chilton et al., 1977). The expression of the T-DNA genes in the transformed cells is responsible for their proliferation and leads to the formation of tumors. T-DNA genes also encode the synthesis of low molecular weight compounds termed opines that are specifically metabolized by the bacteria responsible for the induction of the disease. Genetically complex populations of agrobacteria can generate as a consequence of conjugative transfer of pTi to non-pathogenic agrobacteria (Kerr, 1971; Petit et al., 1978), acquisition or loss of sequences (Otten et al., 1992) and nucleotide changes within the pTi (Belanger et al., 1995). The

study of both pTi and chromosomal characteristics of a given population is thus very important to understand the type of agrobacteria involved in a disease outbreak.

The sequence of the 16S genes is highly conserved within strains belonging to the same genomic species (Nazaret et al., 1991) and can be used to differentiate the species, while the intergenic spacer (IGS) between 16S and 23S rDNA genes is sufficiently variable to allow the comparison of closely related strains (Navarro et al., 1992). Oger et al. (1998) showed that PCR–RFLP analysis of 16S and 16S + IGS ribosomal regions is a fast and reliable method to perform the phylogenetic studies of microorganisms. PCR–RFLP can also be employed to study pTi diversity as highly conserved sequences of *vir* and T-DNA regions of pTi can be used to design primers for PCR (Ponsonnet and Nesme, 1994).

Agrobacteria isolated from weeping fig trees in Italy and The Netherlands showed a wide variability for several physiological and biochemical characteristics and were grouped into two main classes. The first included agrobacteria belonging to biovar 1 and to an intermediate biovar and the second included strains which were similar to the proposed new species *Agrobacterium larrymoorei* (Zoina et al., 2001). An analysis of the diversity of agrobacteria provides an understanding of the ecological relationships between strains within the same genus and between agrobacteria and other microorganisms. Moreover, the diversity of agrobacterium populations is considered a critical factor in the management of crown gall disease (Moore and Canfield, 1996). Forty-eight agrobacteria including representative strains from Italy and The Netherlands were analyzed in this study for their 16S and 16S + IGS ribosomal regions, plasmid content and pTi characteristics. The main purpose of this study was to determine the genetic relationship between the two kinds of agrobacteria responsible for the disease outbreaks that were found constantly associated in fig tumors, and to study the phylogenetic similarities of Italian and Dutch isolates with the new species *A. larrymoorei* (Bouzar et al., 1995; Bouzar and Jones, 2001).

Materials and methods

Bacterial strains

The strains of *Agrobacterium* used are listed in Table 1. Biovar attribution was performed by biochemical and physiological tests while the identification of *Agrobacterium* species was done by the BIOLOG ML1™ system (Zoina et al., 2001). Strains isolated from *Ficus benjamina* originated from 30 different plants at one nursery in Salerno (Italy), one plant from a private house in Latina (Italy) and 10 plants from a tropical glasshouse in Arnhem (the Netherlands) and were indicated as Fb, LT and NL, respectively (Zoina et al., 2001). The 48 strains from *F. benjamina* that are considered in this study were chosen on the basis of morphological, physiological and biochemical characteristics as representative of a collection of 241 fig agrobacteria (Zoina et al., 2001). All strains belong to the collection of phytopathogenic bacteria of the Department of Botany, Horticulture and Plant Pathology of the University of Naples 'Federico II' (Italy).

PCR–RFLP analysis of 16S and 16S + IGS ribosomal regions

Amplifications were carried out in a volume of 50 µl containing 100 ng of DNA extracted by the DNeasy System (Qiagen). Primers FGPS6 and FGPS1509′ were used to amplify the ribosomal region of 1479 bp representing 99.5% of the 16S rDNA.

Primers FGPS6 and FGPL132′ were used to obtain the amplification of the ribosomal region of 2500–2700 bp including 16S rDNA and the IGS between the 16S and 23S rDNA (IGS) plus 132 bp of the 23S rDNA (Ponsonnet and Nesme, 1994). Preparation of PCR reaction mixtures and conditions of amplification both for 16S and 16S + IGS were the same as described by Ponsonnet and Nesme (1994).

Ten microliters of the PCR products were digested with 10 U of each restriction enzyme (r.e.). Digestions of 16S rDNA region was performed using *Acc*I (Biolabs), *Alu*I (Biolabs), *Bfa*I (Biolabs), *Hae*III (Gibco BRL) and *Hpa*II (Biolabs). *Cfo*I (GibcoBRL), *Hae*III (GibcoBRL) and *Nde*II (GibcoBRL) were used to digest the 16S + IGS region.

Restriction fragments obtained after 1 h of digestion were separated by horizontal electrophoresis in TBE buffer on a 2.5% (w/v) Nusieve agarose gel containing 1 µg/ml ethidium bromide. 1 Kb and 123 bp ladders (Gibco BRL) were used as molecular weight standards. Gels were run at 2.3 v/cm for 3.5 h and photographed under UV light using Polaroid film type 57. Fragments of less than 100 bp were not considered for the comparison of restriction patterns.

The different ribotypes were indicated with the letters 'L' (for *A. larrymoorei*) and 'T' (for all other fig agrobacteria) followed by a number.

Table 1. Agrobacterium strains used in this study

Strains	Biovar/species[1]	Host plant	Origin
Fb27[2], Fb52[2], Fb80, Fb119[3], Fb122, Fb123[3], Fb126[3], Fb194[3], Fb208[3], Fb251[3], Fb252[3], Fb258[3], Fb262[3], Fb276, Fb283[3], Fb290[2], Fb294[3], Fb313[3] (Strains were isolated from 12 different plants)	1	Ficus benjamina	Italy
Fb215, Fb308[3], Fb310[2], Fb312, Fb321, Fb332[3], Fb342 (Strains were isolated from seven different plants)	Intermediate	*Ficus benjamina*	Italy
Fb23, Fb33, Fb46, Fb55, Fb60, Fb72, Fb86, Fb99, Fb190, Fb233, Fb238, LT3, LT4 (Strains were isolated from nine different plants)	Intermediate (putative *A. larrymoorei*)	*Ficus benjamina*	Italy
NL1[3], NL3.5, NL37[3], NL2,	1	*Ficus benjamina*	the Netherlands
NL32, NL3.7 (Strains were isolated from five different plants)	Intermediate	*Ficus benjamina*	the Netherlands
NL7, NL25, NL29[2], NL360 Strains were isolated from three different plants)	Intermediate (putative *A. larrymoorei*)	*Ficus benjamina*	the Netherlands
B6	1	Undetermined	Colorado (USA)
C58	1	*Prunus cerasus*	New York (USA)
At 20n5	2	*Prunus persica*	Italy
K84	2	Soil	Australia
K305	*A. vitis*	*Vitis vinifera*	Australia
ATCC13335	*A. rubi*	*Rubus ursinus*	USA
AF3.44	*A. larrymoorei*	*Ficus benjamina*	Florida (USA)
AF3.10	*A. larrymoorei*	*Ficus benjamina*	Florida (USA)
AF1.52	1	*Ficus benjamina*	Florida (USA)
AF3.51	1	*Ficus benjamina*	Florida (USA)

[1]Tests for biovar and species identification are described in a previous work (Zoina et al., 2001).
[2]Avirulent strains.
[3]Nopaline catabolizing strains. The remaining strains were unable to utilize mannopine, nopaline or octopine (Zoina et al., 2001).

Plasmid profiles

Plasmid DNA was isolated following the cell lysis and alkaline denaturation procedure of Currier and Nester (1976). DNA was loaded onto a 0.5% ultra pure agarose gel (GibcoBRL) and separated by horizontal electrophoresis at 4 v/cm for 5 h. After staining in a 0.5 μg/ml solution of ethidium bromide, the gels were photographed on a short-wave UV transilluminator with Polaroid film type 57. Strains SW2 and SS104 of *Erwinia stewartii* were used as references for plasmid size determination (Coplin et al., 1981).

The relative mobility (Rm) of each plasmid was estimated and the molecular weight (MW) was determined on the basis of the correlation: log MW = f (log Rm) as described by Meyers et al. (1976). All plasmids were listed in decreasing order according to their size and named in alphabetical order. Different profiles were obtained by the combination of one or more letters.

PCR–RFLP analysis of pTi

Total DNA was extracted by the DNeasy tissue kit (Qiagen). Primers used to amplify pTi regions

were: FGP*tmr*530 and FGP*tmr*701′ for amplifying *tmr* (171 bp), FGP*tms*194′ and FGP*tms*46′ for amplifying *tms* (587 bp), FGP*nos*1236′ and FGP*nos*975 for amplifying *nos* (256 bp), FGP*vir*A2275 and FGP*vir*$B_2$164′ for amplifying *vir* (1673 bp), FGP*vir*B_{11} + 21 and FGP*vir*G15′ for amplifying *vir* (246 bp), ANT*vir*B_{11}887 and FGP*vir*G15′ for amplifying *vir* (418 bp) as described by Nesme et al. (1995) and Pionnat et al. (1999). Preparation of PCR mixtures and conditions of amplification were those described by Pionnat et al. (1999). Analysis of restriction fragments was performed on *tms* (587 bp) with *Cfo*I and *Dde*I, *vir* (370/418 bp) with *Mse*I and *Hpa*II and *vir* (1673 bp) with *Cfo*I.

Data analysis

Similarities between strains were computed from the presence or absence of restriction fragments of amplicons and genetic distances were calculated according to Nei and Li (1979) using the DistAFLP software (http://pbil.univ-lyon1.fr/ADE-4/microb), which delivers a matrix of pairwise comparisons (i.e. rate of nucleotide substitution). A phylogenetic tree was constructed by the neighbor-joining method (Oger et al., 1998).

Results

PCR–RFLP analysis of 16S and 16S + IGS ribosomal regions

The ribosomal region 16S of 1500 bp was amplified in PCR with primers FGPS6 and FGPS1509′ in all the tested strains. Amplified DNA was digested with *Acc*I, *Alu*I, *Bfa*I, *Hae*III and *Hpa*II and the length of DNA fragments of the agrobacterium reference strains was identical to that expected according to the 16S rDNA sequence (Willems and Collins, 1993), thus showing that amplified DNAs corresponded to the 16S gene. Strains of *A. larrymoorei* from fig were distinguished from the biovar 1 reference strains, the other fig agrobacteria and *A. vitis* by RFLP using the enzymes *Alu*I, *Hae*III and *Hpa*II. Distinction between *A. larrymoorei* and *Agrobacterium rubi* was obtained by digesting the 16S amplicons with *Acc*I, *Bfa*I and *Hae*III. Cluster analysis of the restriction profiles of *rrs* gene separated the fig agrobacteria into two groups (Figure 1): Seventeen strains were clustered with *A. larrymoorei* reference strains AF3.10 and AF3.44, the remaining strains, clustered with agrobacterium biovar 1 reference strains. Only Fb194 belonged to the genomic group G6–G8 as C58 reference strain while all other strains belonged to the genomic group G3–G4 as B6 (Figure 1). Since PCR–RFLP analysis of *rrs* gene does not allow the distinction between G3 and G4 and between G6 and G8 genomic groups, results of this analysis show that at least two of the nine hybridization groups included within the biovar 1 were found among fig strains.

Amplification with primers FGPS6 and FGPL132′ gave a product of about 2700 bp as expected for agrobacteria strains (Ponsonnet and Nesme, 1994). The dendrogram obtained by the cluster analysis of PCR–RFLP profiles of 16S + IGS region is reported in Figure 2. The 17 strains shown to be *A. larrymoorei* in this study were grouped into the cluster B2; within this group of strains five different ribotypes were distinguished (L1–L5). Clusters A and B1 included the remaining 31 strains that were distinguished into 21 different ribotypes (T1–T21). All Italian and Dutch strains were distinguishable from Bouzar's reference strain AF3.10.

Plasmid profiles

The analysis of plasmid profiles showed a high variability for this character among fig agrobacteria: eight plasmids ranging from 20 to 360 Kb in size were combined in 16 different profiles (Table 2). Some examples of plasmid profiles are reported in Figure 3. No definite correlation between plasmid profile and agrobacterium species was found. In fact, the different combination of plasmids were found both among the *A. larrymoorei* group and the other fig agrobacteria. The highest variability was found among the Italian strains of ribotype group 'T' that showed nine different profiles while only four profiles were found within the *A. larrymoorei* group (Table 2). Profiles of Italian strains were characterized by the association of a minimum of two to a maximum of five plasmids. Among the plasmid profiles exhibited by the Italian agrobacteria nine of them included a cryptic plasmid of 360 or 340 Kb that was never detected within the Dutch strains. Within these latter strains, those belonging to the 16S + IGS group 'T' harbored 1–4 plasmids combined in five different profiles, while Dutch *A. larrymoorei* strains showed only two types of profiles made of one and two plasmids, respectively. The profile characterized by two plasmids of 240 and 200 bp was the only one that

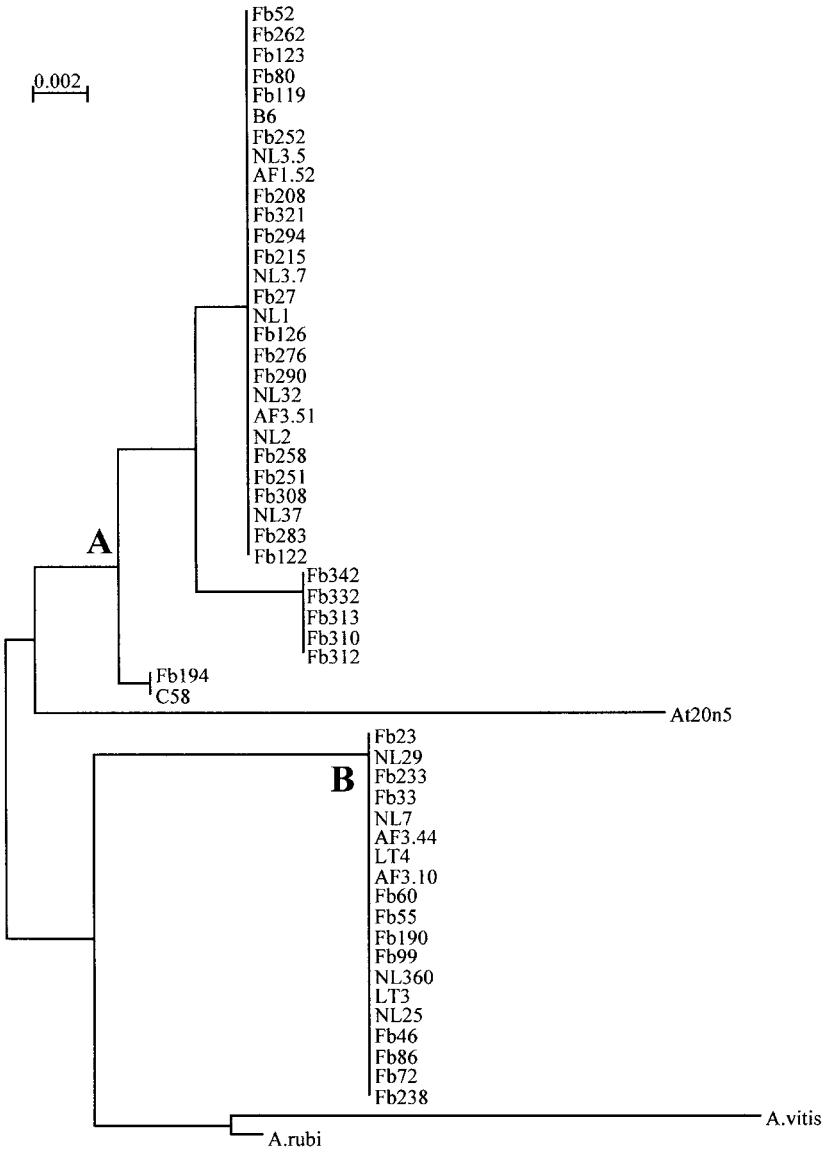

Figure 1. Dendrograms of phylogenetic relatednes between agrobacterial strains isolated from fig tumors based upon RFLP analysis of *rrs* PCR products obtained with *Acc*I, *Alu*I, *Bfa*I, *Hae*III and *Hpa*II. Strains B6, C58 and AF3.10 are respectively members of the genomic species G4, G8 and *A. larrymooreii*. The bar indicates the scale of the genetic distance given in rate of nucleotide substitution.

was found both in Italian and Dutch strains (Table 2). Hybridization with pTi probes was done on 18 strains indicating in all instances that the 200 Kb plasmid was the pTi (data not shown). Combined analysis of plasmid profiles and 16S + IGS restriction patterns of the 48 isolates showed that isolates belonging to a given ribotype could harbor different combinations of plasmids and that genetically different strains could harbor the same plasmid combination (Table 2).

PCR–RFLP analysis of pTi

PCR–RFLP analysis of pTi regions of 43 tumorigenic strains allowed the distinction of 20 different pTi profiles. Amplification products of *tmr*, *tms*, *nos* and three *vir* regions of pTi where obtained from the majority of the Italian strains.

Moreover Fb80 and Fb276 were the only Italian strains that, like the B6 reference strain, produced a

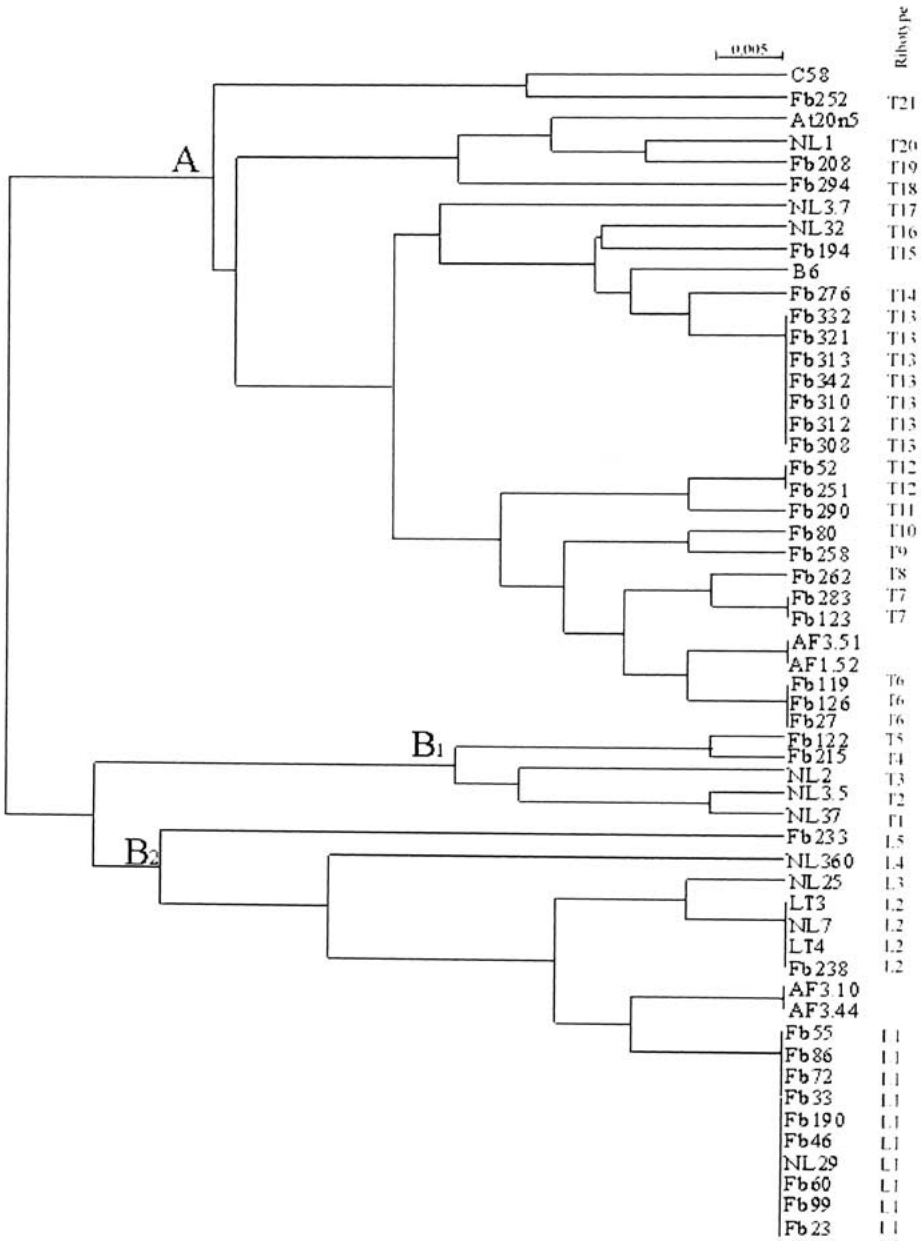

Figure 2. Ribotype relationships among agrobacteria isolated from weeping fig tumors. Cluster analysis was performed using the RFLP patterns obtained by digesting 16S + IGS sequences with *Cfo*I, *Hae*III and *Nde*II. Bar indicates rate of nucleotide substitutions in the 16S + IGS region. A and B indicate the main groups obtained by the 16S analysis. Fb and LT = Italian fig strains; NL = Dutch fig strains; AF = Bouzar reference strains.

fragment of 370 bp when primers ANT *vir*B_{11}887 and FGP*vir*G15′ were used. In the remaining strains this region was 418 bp in size (Table 3). Among Italian strains a certain variability of pTi was seen in the PCR–RFLP analysis of *tms* and two *vir* regions. For these strains the PCR–RFLP analysis of all pTi regions showed that 22 strains formed an homogeneous group, probably harboring the same pTi (M2 H3 C1 D1 C1), while the remaining 12 strains were distributed in ten different groups (Table 3).

Table 2. Relationship between plasmid profiles and strain ribotype

Plasmid profiles	Plasmid sizes (Kb)	Ribotypes
Italian strains		
ACDG	(360, 240, 200, 45)	T21
AD	(360, 200)	T9, T13, T18
ABF	(360, 340, 90)	T11*, T13*
BCD	(340, 240, 200)	T4, T6*, T7, T8, T10, T14, T15
BCDE	(340, 240, 200, 180)	T19
BD	(340, 200)	L1, T7, T13
BDG	(340, 200, 45)	L1
BDEGH	(340, 200, 180, 45, 20)	L2, L5
BF	(340, 90)	T12*
CD	(240, 200)	L2
CDE	(240, 200, 180)	T13
DH	(200, 20)	T12
Dutch strains		
CD	(240, 200)	L3, L4, T1, T16
CDEG	(240, 200, 180, 45)	T20
D	(200)	L2, T3
DE	(200, 180)	T2, L1*
DF	(200, 90)	T17

Plasmids profiles are identified by a combination of capital letters. pTi (200 Kb) is indicated by letter 'D'. 'L' corresponds to agrobacteria belonging to *A. larrymoorei* group and 'T' indicates all other fig agrobacteria. The number indicates a different ribotype according to 16S plus IGS analysis.
*Avirulent strains.

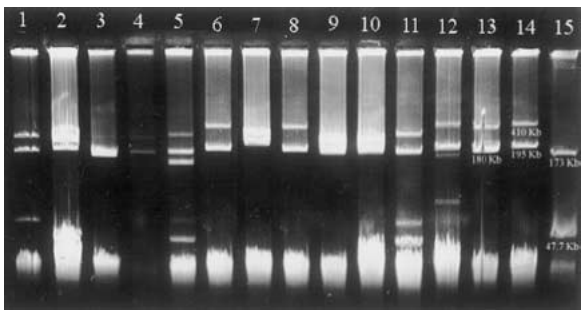

Figure 3. Plasmid profiles of the fig agrobacteria. 1 = Fb23; 2 = NL360; 3 = NL7; 4 = Fb86; 5 = Fb122; 6 = Fb294; 7 = LT3; 8 = Fb313; 9 = Fb99; 10 = Fb46; 11 = Fb208; 12 = Fb252; 13 = B6; 14 = C58; 15 = K84.

A wide variability of pTi structure was also found among the nine virulent Dutch strains, in fact *nos* region was amplified in five strains while *tmr* and *tms* regions were amplified in eight strains. *vir* regions (246 and 1673) were amplified in two strains only, the region amplified by ANT*vir*B_{11}887 and FGP*vir*G15′ primers was of 370 bp in four strains and 418 bp in three others while it was not amplified in two remaining strains (Table 3). Strains NL1 and NL3.7 were positive only with *tmr/tms* and *tmr* primers, respectively showing that their pTis were the closest to the pTiAF3.10 of Bouzar's strain. Results of PCR amplification and PCR–RFLP analysis showed that each pTi of the Dutch strains was characterized by a different combination of regions. No plasmid from the Dutch strains was identical to any pTi of the Italian isolates. Moreover, no correlation between the structure of pTi and the agrobacterium species/genomic groups was observed (Table 3). Cluster analysis of PCR–RFLP of pTi revealed that there were two groups of fig agrobacteria (Figure 4). Group A included five Dutch strains and the two Italian strains (Fb80 and Fb276) that had the *vir* (370) region and AF3.44 and AF3.10 reference strains. Group B included all Italian and Dutch strains that harbored a pTi that was positively amplified by most of the couples of primers and that had the *vir* (418) region and the reference strains B6 and C58. Ribotype groups and results of plasmid contents of fig strains are combined in Figure 4 together with the dendrogram obtained from pTi structure analysis. No clear relationship could be seen among the results of the three kinds of analysis.

Discussion

Unusual European agrobacteria, isolated from tumors developing on branches, aerial and hypogeous roots of weeping fig plants (Zoina et al., 2001), were similar to others isolated from weeping fig in Florida that were proposed as belonging to the new species *A. larrymoorei* (Bouzar et al., 1995; Bouzar and Jones, 2001).

Numerical analysis of PCR–RFLP profiles of the *rrs* gene showed that Italian and Dutch strains were separable into two main clusters. One group of strains (B) was clearly distinguishable from *A. rubi* and *A. vitis* species and also from agrobacteria biovar 1 and 2 and included *A. larrymoorei* AF3.10 and AF3.44 reference strains. All strains belonging to this cluster were thus assumed to be bona fide *A. larrymoorei*. The second group (A) included all other agrobacterium fig strains. Analysis of restriction profiles of 16S region grouped the intermediate strains, which were not included in any of the three recognized biovar by using the classical biovarietal tests (Zoina et al., 2001), within the biovar 1

Table 3. PCR–RFLP analysis of pTi

Italian strains	Species or presumptive genomic group	Amplifications						Restriction profiles				
		nos (256)	*tmr* (171)	*tms* (587)	*vir* (246)	*vir* (370*/ 418)	*vir* (1673)	*tms* (587)		*vir* (370/418)		*vir* (1673)
								*Cfo*I	*Dde*I	*Mse*I	*Hpa*II	*Cfo*I
Fb33, Fb46, Fb55, Fb60	A.L.[1]	+	+	+	+	+	+	C1	D1	M2	H3	C1
Fb72, Fb86, Fb99, Fb190	A.L.	+	+	+	+	+	+	C1	D1	M2	H3	C1
Fb122, Fb123, Fb126, Fb208	G3 or G4[2]	+	+	+	+	+	+	C1	D1	M2	H3	C1
Fb215, Fb258, Fb262, Fb283	G3 or G4	+	+	+	+	+	+	C1	D1	M2	H3	C1
Fb294, Fb308, Fb312, Fb313	G3 or G4	+	+	+	+	+	+	C1	D1	M2	H3	C1
Fb332	G3 or G4	+	+	+	+	+	+	C1	D1	M2	H3	C1
Fb194	G6 or G8[2]	+	+	+	+	+	+	C1	D1	M2	H3	C1
Fb23	A.L.	+	+	+	+	+	+	C2	D1	M2	H2	C1
Fb119	G3 or G4	+	+	+	+	+	+	C1	D1	M2	H3	C2
Fb233	A.L.	+	+	+	+	+	+	C1	D1	M1	H3	C2
Fb251	G3 or G4	+	+	+	+	+	+	C1	D1	M1	H3	C2
Fb80	G3 or G4	+	+	+	+	+*	−	C1	D5	M3	H1	−
Fb238, LT3	A.L.	+	+	+	+	+	+	C1	D1	M2	H3	C4
Fb252	G3 or G4	+	+	+	+	+	+	C1	D1	M2	H3	C3
Fb276	G3 or G4	−	+	+	+	+*	−	C1	D3	M3	H1	−
Fb321	G3 or G4	+	+	+	+	+	+	C1	D4	M1	H3	C2
Fb342	G3 or G4	+	−	+	+	+	−	C1	D1	M2	H3	−
LT4	A.L.	+	+	+	+	+	+	C1	D1	M2	H2	C4
Dutch strains												
NL1	G3 or G4	−	+	+	−	−	−	C1	D1	−	−	−
NL2	G3 or G4	+	+	+	−	+*	−	C1	D1	M3	H1	−
NL3.5	G3 or G4	+	+	+	−	+	−	C3	D7	M2	H4	−
NL3.7	G3 or G4	−	+	−	−	−	−	−	−	−	−	−
NL7	A.L.	+	+	+	+	+	+	C1	D1	M4	H4	C4
NL25	A.L.	+	+	+	+	+	+	C1	D6	M4	H4	C4
NL32	A.L.	+	−	+	−	+*	−	C1	D3	M3	H1	−
NL37	G3 or G4	−	+	+	−	+*	−	C1	D3	M3	H1	−
NL360	A.L.	−	+	+	−	+*	−	C2	D3	M3	H1	−
Reference strains												
AF3.10, AF3.44	A.L.	−	−	−	−	−	−	−	−	−	−	−
B6	G3 or G4	−	+	+	−	+*	−	C1	D2	M3	H1	−
C58	G6 or G8	+	+	+	+	+	+	C1	D1	M2	H3	C3

[1] A.L.: *A. larrymoorei*.
[2] Groups G3, G4, G6 and G8 are defined by the 16S sequences of the genomic groups described by Mougel et al. (2002).

cluster showing that the identification of agrobacteria was more precise and reliable by using the molecular technique.

PCR–RFLP analysis of 16S and the IGS between 16S and 23S, was performed to determine the genetic differences within the two *Agrobacterium* groups. Twenty-six different genotypes were identified using three restriction enzymes showing a wide genetic diversity among fig agrobacteria. High genetic variability was observed among the Dutch strains, since each one showed a unique restriction profile of the 16S + IGS ribosomal region, even though a limited number of strains from The Netherlands was included in the group of agrobacteria analyzed.

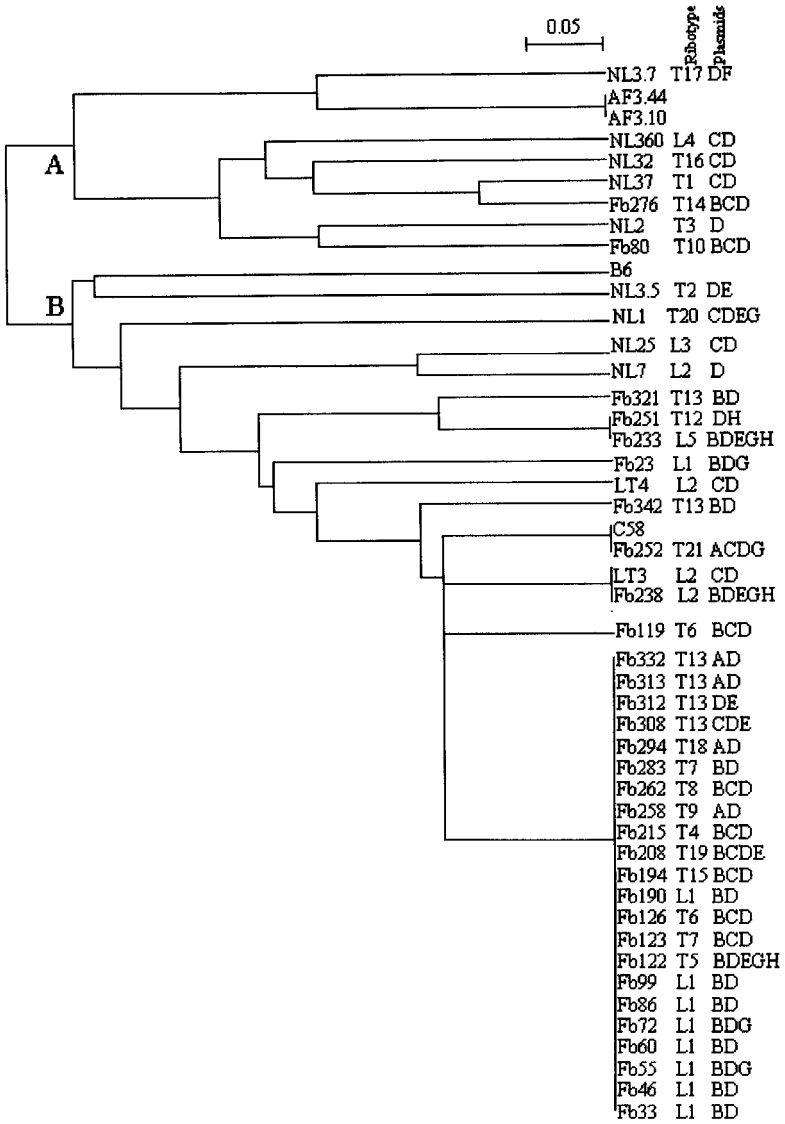

Figure 4. Ti plasmid similarities among agrobacteria isolated from weeping fig tumors and their relationship with ribotype and plasmid profile. Bar indicates pattern similarly based on PCR detection of *nos* (256), *tmr* (171) and *vir* (246), and PCR–RFLP of *tms* (587), *vir* (370/418) and *vir* (1673).

A high level of diversity was also discovered through the study of plasmid content of fig agrobacteria. Up to five plasmids ranging from 20 to 360 Kb, combined in 12 and five different patterns were found in Italian and Dutch strains, respectively. The highest variability observed among Italian strains was most probably due to the large number of strains from Italy included in the group of agrobacteria analyzed. Wide variability of plasmid profiles in agrobacteria had already been reported by Albiach and Lopez (1992) who analyzed a group of agrobacteria isolated from different hosts collected in several Spanish regions. However, the wide variability observed in the group of 48 fig agrobacteria was surprising, since all strains were isolated from aerial tumors of a limited group of host plants of the same species where the expected variability was supposed to be lower than in root tumors, usually colonized by opportunistic agrobacteria. To our knowledge, this is the first report regarding the study of plasmid distribution among a large

number of agrobacteria isolated from one single plant species and from three different locations.

In agreement with other studies (Albiach and Lopez, 1992; Pulawska et al., 1998) no correlation between plasmid profile and geographical origin, virulence, biovar or bacterial ribotypes was found. The casual distribution of plasmids among the fig strains was also revealed by comparing the results of plasmid profile typing with PCR–RFLP analysis of 16S + IGS ribosomal region. In fact, a given plasmid profile was observed in different ribotypes, moreover some strains that were identical at 16S + IGS analysis harbored different plasmid combinations.

Conjugal transfer of plasmids can be the most important source of heterogeneity for plasmid content (Genetello et al., 1997; Stockwell et al., 1990). Probably plasmid exchange occurred between virulent and avirulent fig agrobacteria (Genetello et al., 1997; Hooykaas et al., 1977) or between agrobacteria and other bacteria living as endophytes inside fig plants (Zoina et al., 2002) since transfer of pTi to other bacterial species has been demonstrated among soil microflora (Teyssier-Cuvelle et al., 1999; van Veen et al., 1988).

Fig strains showed some differences when analyzed for some pTi-borne characters such as opine utilization, host range and agrocine 84 sensitivity (Zoina et al., 2001). These observations led us to hypothesize that some differences could exist among the pTi of different strains. PCR–RFLP analyses of *nos*, *tmr*, *tms* and three *vir* regions of pTi demonstrated that most of the strains harbored a pTi nopaline type and that among the strains that were positive at the amplification with *nos* primers 14 different pTi profiles were distinguished. The most frequent of them was shared by a group of 22 Italian strains that included a part of *A. larrymoorei* strains; within these 22 isolates, eight belonged to the same ribotype and could then be considered isolates of the same strain (Tenover et al., 1995). However, nopaline pTis were closely related to one another and probably originated from a common ancestor and were subjected to some minor changes that took place during their respective evolution. Our findings are in agreement with previous studies where a high degree of homogeneity was found within nopaline strains (Knauf et al., 1983; Michel et al., 1990). Homology studies have shown that pTis are composed of homologous and non-homologous sequences that most likely are acquired by horizontal gene transfer since several sequences of octopine and nopaline-type plasmids have been found in other pTi types and even in other bacterial species.

This sequence exchange can explain the evolution of pTi as a mosaic structure (Otten et al., 1992). Moreover, part of pTi variability is due to the insertion elements that are known to produce deletion, rearrangements, amplifications and changes in gene expression (Otten et al., 1992). Tumors are the preferred sites where DNA exchanges take place and it is well known that the opines secreted in tumor tissues induce the conjugative transfer of pTi (Dessaux et al., 1992; Ellis et al., 1982; Moore et al., 1997). This could explain both the association of identical pTis with several different genotypes, even belonging to different *Agrobacterium* species, and the combination of one genotype with different nopaline pTi structures that were found in *F. benjamina* galls.

No pTi of Italian and Dutch strains was identical to the novel plasmid pTiAF3.10 of *A. larrymoorei* reference strain that was negative in all amplifications, confirming its peculiar properties (Bouzar et al., 1995; Vaudequin-Dransart et al., 1995). Heterogeneity of pTi was high within the Dutch group where each strain showed a unique pTi pattern, each one different from those of the Italian strains. Moreover, amplification of *vir* (370/418) showed that the size of 370 bp was more frequent among the Dutch isolates, while only two Italian strains out of the 34 analyzed, had this smaller sized *vir* region.

Results of cluster analysis of PCR–RFLP of pTis showed that Italian and Dutch strains could be separated into two groups that were distinguished mainly by the difference in size of the *vir* (370/418) region. An epidemiological study regarding agrobacteria isolated from rose plants showed that strains harboring this *vir* region of 370 bp in size were able to utilize opines other than nopaline (Pionnat et al., 1999). Similarly, some of the agrobacteria isolated from weeping fig plants could harbor a pTi belonging to an uncommon opine group. This putative uncommon pTi was harbored by different ribotypes belonging to biovar 1 and *A. larrymoorei* strains, thus evidencing that among these fig strains no correlation between pTi structure and ribotype was found. Additional analyses are planned to explore the relatedness of these plasmids with the pTiAF3.10 and the other pTi types in a more exhaustive way. Otten et al. (1996) found a strong correlation between pTi type and ribotype among *A. vitis* strains. In this case, bacterial strains were from different geographic origins and were selected on the basis of their different

opine type plasmids. Nopaline, octopine/cucumopine and vitopine pTi resulted strictly associated to given chromosomal backgrounds. The majority of strains that were analyzed in the present study were isolated from fig galled plants growing in one single nursery that were multiplied by cutting techniques: a homogeneous population of agrobacteria was most likely expected. However overall results showed that the bacterial population was composed at least of two different *Agrobacterium* species, with several ribotypes. Moreover, most of the strains harbored a pTi nopaline type that showed some variability in the oncogenic regions analyzed. This variability appeared unrelated to the chromosomal background of fig strains, thus suggesting that the changes occurred within the pTis were independent from the chromosomal characteristics of strains. The occurrence of agrobacteria belonging to two separate *rrs* genotypes in fig tumors indicates that the disease initiation was likely due to at least two outbreak strains (Tenover et al., 1995). Genetic exchanges that may have taken place between these agrobacteria and other species of bacteria living endophytically in the plants (Zoina et al., 2002), could have produced a certain variability inside the population and consequently a lack of correlation between ribotypes and plasmid content. Endophytic behavior of agrobacteria seems to be a key point in the epidemiology of crown gall on *F. benjamina* (Zoina et al., 2002). Lehoczky (1968) reported that propagation of crown gall disease on grapevines was caused by the use of systemically infected scions and rootstocks. Similarly, it is possible that the cutting technique used to propagate the plants in the Italian nursery may have allowed the dissemination of agrobacteria from the mother plants to the cuttings and consequent disease outbreaks.

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