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Tracking *Agrobacterium* strains by a RAPD system to identify single colonies from plant tumours

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Abstract

A molecular typing system for *Agrobacterium* strains based on the polymerase chain reaction–random amplified polymorphic DNA (PCR–RAPD) procedure was developed. It employs one to four different 10-mer primers and the results are highly reproducible. The band patterns obtained with the four primers for each of the 39 *Agrobacterium* strains analysed were different enough to differentiate the strains from each other. Strains with similar chromosomal background but different plasmid content, e.g. strains C58 and A281, gave the same band pattern with all the primers. Ten host plants were inoculated with eight *Agrobacterium* strains and the isolates obtained from the resulting tumours were analysed using the RAPD system developed here. The procedure allowed rapid identification of isolates recovered from tumours by comparison of their band patterns with band patterns of strains used as inoculum. The procedure also discriminated the various strains analysed. Purified bacterial cell suspensions, used for RAPD analyses, produced the same results as purified DNA, and greatly simplified the procedure. This system can be applied for rapid screening of *Agrobacterium*-like colonies isolated from plant tumours for epidemiological and genetic diversity studies.

Introduction

Agrobacterium tumefaciens, causal agent of crown gall, is a soilborne bacterium that produces tumours in more than 600 plant species (De Cleene and De Ley, 1976). It is considered a quality pathogen in the European Union (Anonymous, 1993) and plants affected by crown gall should not be marketed to avoid the spread of the disease, even when it may only affect, in variable degree, the vegetative growth of the plants. Consequently, it causes severe economic losses to nurseries of fruit and ornamental plants. The taxonomy of the genus has been discussed, but none of the nomenclatural proposals (Sawada et al., 1993; Young et al., 2001) has found universal acceptance. In this work the conventional nomenclature, which considers that tumourigenic strains of biovars 1 and 2 belong to *A. tumefaciens* and those of biovar 3 to *A. vitis* species, will be employed. The identification of *Agrobacterium* strains can be complex (Lelliot and Stead, 1987), and the use of biochemical analyses, such as fatty acid profiles or carbon-source utilisation patterns (Bouzar et al., 1993; Sawada et al., 1992), can differentiate the biovars of *Agrobacterium*, but do not allow distinction between different strains. The use of serological methods for the identification of *Agrobacterium* isolates from tumours is limited because this species is serologically heterogeneous and it is not possible to produce antisera that will recognise all the tumourigenic *Agrobacterium* strains of each biovar (Alarcón et al., 1987; Benjama et al., 1996). There are already several molecular techniques described to study the genetic diversity of *Agrobacterium* strains. Random amplified polymorphic DNAs (RAPDs) were used by Irelan

and Meredith (1996) for biovar (species) identification and classification, and by Momol et al. (1998) for analysing the diversity of *A. vitis.* More complex methods, such as polymerase chain reaction–restriction fragment length polymorphisms (PCR–RFLP) (Oger et al., 1997; Ponsonnet and Nesme, 1994), chromosomal and plasmid typing (Nesme et al., 1992), and rRNA sequencing (Yanagi and Yamasato, 1993) have been used to evaluate strain diversity.

The structure of natural agrobacterial populations is very complex, and different studies have shown the presence of mixed populations containing strains of *Agrobacterium* with different chromosomes and with different types of plasmid genotypes (Anderson and Moore, 1979; Ponsonnet and Nesme, 1994). Moreover, agrobacteria are frequently isolated from soil along with *Rhizobium* and other bacteria (Hofer, 1941; Gaur and Sen, 1976). To advance knowledge of the behaviour of *Agrobacterium* sp. after being introduced in certain ecological niches (e.g., soil, roots, tumours, symptomless plant material), where a complex microflora can be found and specific isolation is not always efficient, techniques that allow rapid but accurate screening of many colonies simultaneously are required. Furthermore, in molecular analysis of bacterial isolates, it is essential to check their identity when there are no markers (e.g. antibiotic resistance) or selective culture media for recovering the selected clones. A RAPD system has been developed which allows the simple identification of individual strains. The method has several advantages: rapidity of analysis, a high degree of polymorphism, distribution of the analysed markers along the whole genome of the bacterium, high reproducibility of the results and the possibility of using bacterial suspensions instead of purified DNA (Mazurier et al., 1992). Furthermore, the data obtained from large collections of strains can be used for phylogeny and diversity studies. To test the RAPD method, colonies of *A. tumefaciens* isolated from tumours induced by eight different strains were analysed. Every strain of *Agrobacterium* was distinguished from each other, and the marked differences amongst the RAPD patterns obtained allowed each isolate to be identified unambiguously.

Materials and methods

Bacterial strains and inoculation

The *Agrobacterium* strains were obtained from the IVIA collection and their origin and host of isolation are shown in Tables 1 and 2. For the inoculation experiments, bacterial strains were introduced to the soil substrate (experiments 1 and 2) or to the stem of plants (experiment 3; Table 2). Inoculation of plants growing in sterilised soil substrate (Vriezenveen potgrond Bv, Holland) was performed by irrigation with a suspension of ca. 10^9 cfu ml^{-1} in water to achieve a final concentration of ca. 10^7 cfu g^{-1}. Inoculation into the stem of plants was done by making a wound in the crown with a scalpel and depositing 10 µl of a suspension ca. 10^8 cfu ml^{-1} of a given strain. Bacterial colonies were recovered from the resulting tumours after 30 days to 9 months, depending on the experiment, using a semi-selective medium for isolating biovar 1 strains (Schroth et al., 1965) and identification by the RAPD–PCR procedure. Strain identity was further confirmed by biochemical tests (urease, aesculin), tomato inoculation (Ridé et al., 2000), PCR with primers specific for the Ti plasmid (Nesme et al., 1989) and, in some cases, analysis of plasmids using a modified Eckhardt method (Fortin et al., 1992).

DNA extraction

After culturing the strains on PYGA medium (Lelliot and Stead, 1987) for 48 h at 26 °C, DNA was extracted from ca. 10^8 cfu ml^{-1} bacterial suspensions (Llop et al., 1999). The DNA was dissolved in water before quantification by spectrophotometry and kept at −20 °C until RAPD analysis. Whole cell suspensions of the same *A. tumefaciens* strains (ca. 10^8 cfu ml^{-1}, OD 0.4 at 600 nm) were also analysed to determine if they would produce comparable RAPD patterns. Duplicates were stored at 4 °C and −20 °C.

Assays performed with the RAPD system

To set up the RAPD system, 39 strains from different origins and hosts were chosen (Table 1). The analyses were performed using purified DNA and bacterial suspensions. Several amounts of DNA were tested and it was found that 70 ng gave good results (data not shown). All the analyses were performed at least twice with each type of sample (purified DNA or cell suspensions). Five sets of random 10-mer oligos (Operon Technologies, Alameda, CA) were screened to determine which would provide a suitable pattern in number and intensity of bands to discriminate between the strains. Among the 50 primers examined, four produced highly polymorphic patterns composed, for most

Table 1. *Agrobacterium* strains used for the RAPD analyses

Collection and strain reference	Origin	Biovar	Opine type	Host
A281*	—	1	Agropine	—
Ach5	USA	1	Octopine	*Prunus* sp.
ATCC 15834	USA	*A. rizhogenes*	ND	Not known
B6	USA	1	ND	Not known
CFBP 42	France	1	ND	Tomato
CFBP 1903 (C58)	USA	1	Nopaline	*Prunus cerasus*
IVIA 014	Zaragoza, Spain	2	Nopaline	Peach
IVIA 66R	Sevilla, Spain	2	ND	Rose
IVIA 251-1	Badajoz, Spain	1	Nopaline	Almond
IVIA 251-21	Badajoz, Spain	2	Nopaline	Cherry
IVIA 251-22	Badajoz, Spain	1	Nopaline	Cherry
IVIA 254-1	Valencia, Spain	ND	Unknown	Peach
IVIA 254-2	Valencia, Spain	2	Nopaline	Peach
IVIA 260-67	Badajoz, Spain	2	Nopaline	Poplar
IVIA 282-64	Tenerife, Spain	2	Nopaline	Rose
IVIA 325-4	Tarragona, Spain	1	Nopaline	Peach × almond
IVIA 325-7	Tarragona, Spain	2	Nopaline	Peach × almond
IVIA 339-26	Ourense, Spain	3	ND	Grapevine
IVIA 347-4	Valencia, Spain	1	Nopaline	Peach
IVIA 354-35	Valencia, Spain	2	Nopaline	Almond
IVIA 360-54	Navarra, Spain	1	ND	Grapevine
IVIA 388-30	Zaragoza, Spain	2	Nopaline	Almond
IVIA 436-46	Zaragoza, Spain	1	Nopaline–mannopine	Peach × almond
IVIA 545-45	Castellón, Spain	2	Nopaline	Quince
IVIA 576-80	Cuenca, Spain	1	Nopaline	Osier
IVIA 678-2	Valencia, Spain	1	ND	Peach × almond
IVIA 796-6	Valencia, Spain	2	ND	Peach × almond
IVIA 1.102	Valencia, Spain	1	Chrysopine	Chrysanthemum
IVIA 1853-2	Zaragoza, Spain	2	ND	Peach
172-17T**	Ourense, Spain	1	Octopine	Grapevine
225-226T**	Ourense, Spain	1	Octopine	Grapevine
194-459V**	Ourense, Spain	3	Octopine	Grapevine
K84	Australia	*A. radiobacter*	Nopaline–octopine	Soil
NCIB 8196	Unknown	*A. rhizogenes*	ND	Not known
NCPPB 1649	South Africa	2	ND	Rose
NCPPB 2437	USA	1	ND	Not known
NCPPB 2659	UK	*A. rhizogenes*	ND	Cucumber
NCPPB 3554	Australia	3	ND	Grapevine

ATCC: American Type Culture Collection, USA; CFBP: Collection Française des Bacteries Phytopatogènes, France; IVIA: Instituto Valenciano de Investigaciones Agrarias, Spain; NCIB: National Collection of Industrial Bacteria, United Kingdom; NCPPB: National Collection of Plant Pathogenic Bacteria, United Kingdom; ND: Not determined.

*Strain C58 with plasmid pTiBo542.

**Strains from PhD Thesis of Lastra B (1998).

of the strains, of three or more reproducible bands, and were consequently selected for further experiments. The oligonucleotides chosen to perform the assays were: OPE-2: (GGTGCGGGAA); OPE-7: (AGAT GCAGCC); OPE-14: (TGCGGCTGAG) and OPE-20: (AACGGTGACC). Since each of the examined strains produced a characteristic pattern with each of the primers, the use of only one of the selected primers was enough to identify a given clone whilst the remaining three were used to confirm the results or to clarify potentially ambiguous results.

The analyses were done using an optimised PCR programme as follows: the PCR mix contained 70 ng of genomic DNA, 1× PCR buffer (20 mM Tris–HCl (pH 8.0), 50 mM KCl); 1.5 mM $MgCl_2$, 60 µM dNTPs, 5 pmols of primer and 1 U of Taq polymerase (Life Technologies) in a total volume of 25 µl. In the case of bacterial suspensions, 3 µl of the suspension

Table 2. Strains employed in this study and the experiments in which they were used

Experiment	Strain	Origin	Biovar	Opine type	Host inoculated
1	NT1pTiC58::289 (C58R)	Von Bodman et al. (1992)	1	Nopaline	GF677*
2	IVIA 325-4	Spain	1	Nopaline	Cherry
2	IVIA 325-4	Spain	1		Pear
2	IVIA 325-4	Spain	1		Adafuel*
2	IVIA 325-4	Spain	1		GF677*
2	IVIA 678-2	Spain	1	ND	Montclar
2	IVIA 678-2	Spain	1		Myrobolan
2	IVIA 678-2	Spain	1		GxN15*
2	IVIA 678-2	Spain	1		GxN22*
2	IVIA 678-2	Spain	1		GF677*
3	Ach5	**	1	Octopine	Tomato
3	IVIA 1.102	Spain	1	Chrysopine	and
3	CFBP 42	France	1	ND	Pepper
3	CFBP 1903 (C58)	USA	1	Nopaline	
3	A281	***	1	Agropine	

CFBP: Collection Française de Bactéries Phytopathogènes, Angers, France; IVIA: Instituto Valenciano de Investigaciones Agrarias; ND: Not determined.
*Peach × almond hybrids.
**Kindly provided by M.D. Chilton, USA.
***Kindly provided by X. Nesme, France.

(ca. 10^8 cfu ml^{-1}) were added to 22 µl of the PCR mix. The amplification cycles were: a denaturation step of 94 °C for 3 min followed by five cycles of 94 °C for 30 s, 36 °C for 1 min and 72 °C for 1 min, with a ramp time of 1 min 48 s and 30 cycles of 94 °C for 30 s; 45 °C for 1 min and 72 °C for 1 min, with a ramp time of 1 min. The thermocycler employed was a Perkin-Elmer 9600. Bands of amplified product were visualised under UV light after electrophoresis on 1.5% agarose gels and staining with ethidium bromide. Dendrograms were obtained by the UPGMA method, with a comparison factor of 1.000 using the index of Jaccard (1908). The analyses were performed at least twice with each of the four primers.

Identification by RAPD analysis of colonies recovered from inoculated plants

The identification procedure was tested in several experiments (Table 2). Experiment 1 was performed with the marked strain C58R (NT1 pTiC58::289 Gen^r; Von Bodman et al., 1992) using as host the hybrid rootstock peach × almond GF677. This strain contains a plasmid, pJB1JI, which confers resistance to gentamicine. This experiment was designed as a control of our system because the isolation medium employed (PYGA) was supplemented with gentamicin (50 mg l^{-1}) and only the colonies that harboured this plasmid were recovered. Ten plants were employed for the analysis. In experiment 2, cherry and pear plants, and hybrids peach × almond GF677 and Adafuel were inoculated with *A. tumefaciens* strain IVIA 325-4. Besides, hybrids GF677, GxN15, GxN22, peach Montclar and plum Myrobolan were inoculated with *A. tumefaciens* strain IVIA 678-2. The inoculation in all these experiments was performed in sterilised soil substrate as described above and five plants of each host were analysed. In experiment 3, *A. tumefaciens* strains Ach5, IVIA 1.102, CFBP42, C58 and A281 were inoculated separately in the stem of tomato and pepper plants (cultivars Roma and Toledo, respectively). The plants were maintained from 30 days to 9 months in a greenhouse at 20–25 °C and 40–60% RH, and a minimum of two plants per strain and host taken for analysis. In the three experiments, 600 colonies obtained by plating 50 µl of tumour tissue macerates on semi-selective medium (Schroth et al., 1965) were analysed by the RAPD system using bacterial suspensions with one of the four RAPD primers selected (primer OPE-7). The PCR procedure was as described previously and all the analyses were repeated at least twice for each colony.

Results

The RAPD analysis yields strain-specific patterns

The RAPD patterns obtained with different strains of *Agrobacterium* using the four selected 10-mer oligos were specific for each strain examined. The pattern of the 39 strains analysed with primer OPE-14 is shown in Figure 1. The dendrogram demonstrates the discriminative power of the technique, since all the strains analysed were readily distinguished (Figure 2) except for three of the strains (C58, A281, C58R), which gave identical patterns. However, these three strains have the same chromosome and only differ in the plasmids they harbour. RAPD analyses performed with isolated DNA and bacterial suspensions gave identical results for all the strains analysed. Hence, for the ease of use, bacterial suspensions of 10^8 cfu ml^{-1} were employed in all subsequent analyses. The *Agrobacterium* suspensions stored at $-20\,^{\circ}$C gave unaltered patterns as did those kept at $4\,^{\circ}$C, even after a period of storage of 4 years at either temperature for the 15 analysed strains.

The RAPD assays are reproducible

This RAPD system was highly reproducible, within and between experiments. In the analysis of the strains from the IVIA collection, some repetitions were included to check the reproducibility of the assay in the same experiment. Duplication of results can be seen in Figure 1 (lanes 16 and 20 or 19 and 25), and were confirmed in the dendrogram analysis (Figure 2). In Figure 3A the banding patterns obtained with several *Agrobacterium* isolates obtained from one tumour are shown. They were all identical to the inoculated parental strain, when analysed with one of the RAPD primers. Different banding patterns were obtained with five *Agrobacterium* strains using the primers OPE-7 and OPE-20 in two sets of experiments performed over 4 months (Figure 3B). Each strain yielded the same banding pattern in the two analyses.

RAPD analysis of the colonies from the inoculation experiments

To test whether the selected oligos would permit the identification of *Agrobacterium* strains recovered from diseased plant samples, isolates from inoculated hosts

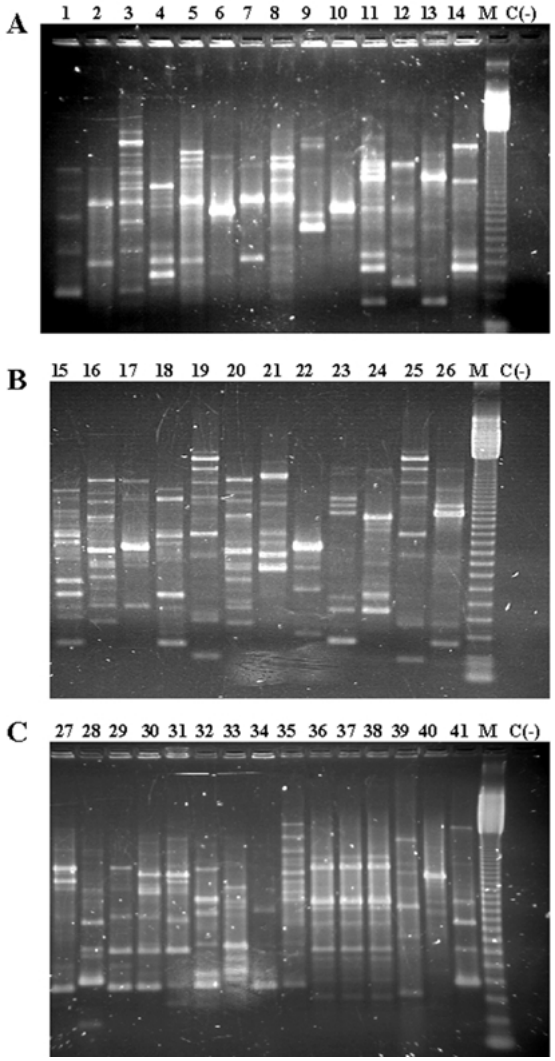

Figure 1. RAPD banding pattern obtained with primer OPE 14 on 39 *Agrobacterium* strains from different origins. (A) Lanes: 1: IVIA 251-22; 2: IVIA 388-30; 3: IVIA 436-46; 4: IVIA 282-64; 5: IVIA 260-67; 6: IVIA 576-80; 7: IVIA 014; 8: IVIA 339-26; 9: 194-459V; 10: IVIA 325-7; 11: IVIA 251-21; 12: IVIA 360-54; 13: IVIA 254-1; 14: IVIA 545-45; M: 123 bp ladder (Life Technologies); C (−): negative control. (B) Lanes: 15: B6; 16: K84; 17: IVIA 347-4; 18: NCPPB 3554; 19: 172-17T; 20: K84; 21: IVIA 251-1; 22: 225-226T; 23: IVIA 325-4; 24: CFBP 42; 25: 172-17T; 26: IVIA 678-2; M: 123 bp ladder (Life Technologies); C (−): negative control. (C) Lanes 27: IVIA 354-35; 28: NCIB 8196; 29: IVIA 1853-2; 30: NCPPB 1649; 31: IVIA 796-6; 32: Ach5; 33: NCPPB 2437; 34: ATCC 15834; 35: IVIA 1.102; 36: C58R; 37: C58; 38: A281; 39: IVIA 254-2; 40: NCPPB 2659; 41: IVIA 66R; M: 123 bp ladder (Life Technologies); C (−): negative control.

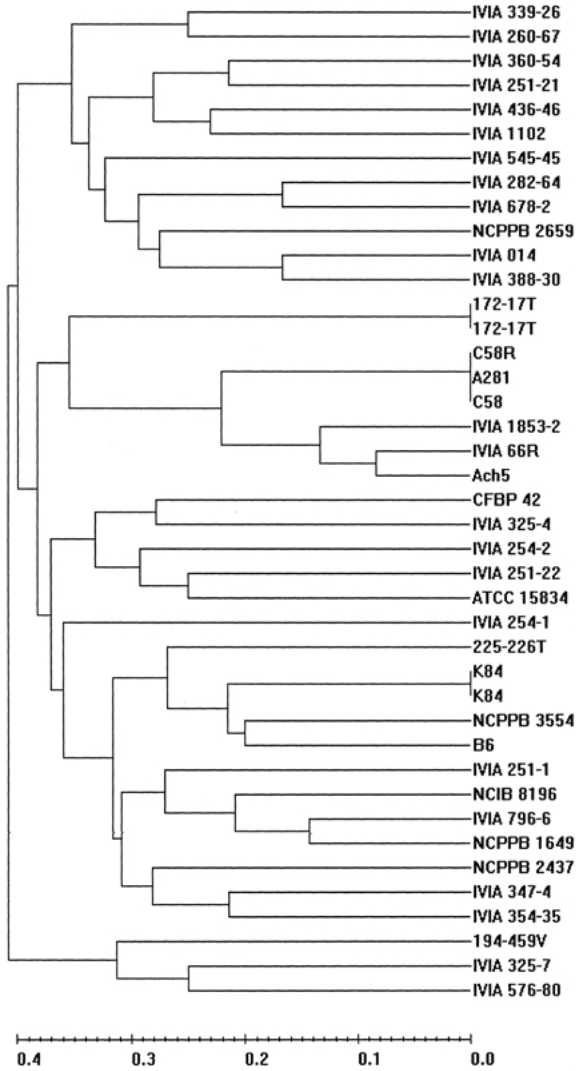

Figure 2. Dendrogram obtained by UPGMA from the analysis of banding patterns of the 39 strains analysed and shown in Figure 1. The index of distance was calculated according to Jaccard (1908). Strains 172-17T and K84 were included twice for reproducibility.

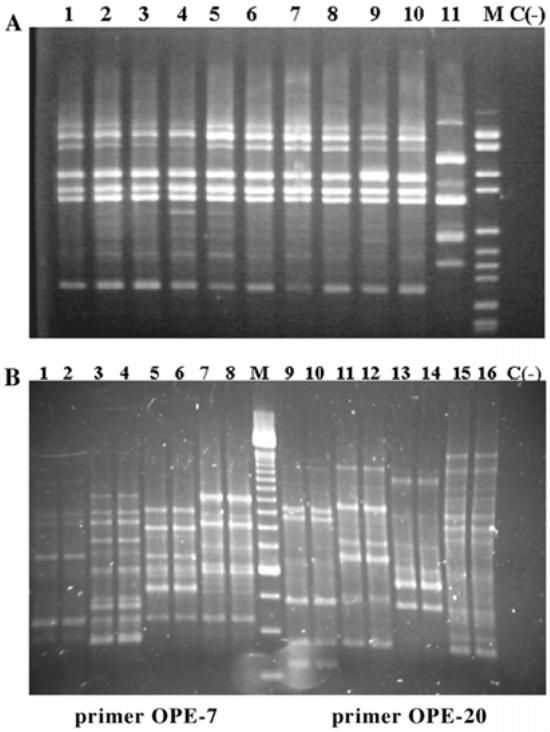

Figure 3. Reproducibility of the RAPD analysis. (A) RAPD banding patterns obtained with primer OPE-7 on colonies recovered on PYGA + Gentamicine (50 mg l^{-1}) from tumours obtained in experiment 1 (GF677 plants growing in soil substrate inoculated with strain NT1 pTiC58::289 (C58R)). Lane 1: strain C58R; lanes 2–10: colonies recovered from tumours; lane 11: strain IVIA 325-4; M: marker VI (Roche-Boehringer Mannheim); Lane C(−): negative control. (B) Comparison of RAPD banding patterns from suspensions of bacterial strains stored for 4 months with initial preparations using primers OPE-7 (left) and OPE-20 (right). Lanes 1–2 and 9–10, strain IVIA 678-2; lanes 3–4 and 11–12, strain Ach5; lanes 5–6 and 13–14, strain 251-1; lanes 7–8 and 15–16, strain IVIA 1.102. M: marker 250 bp (Invitrogen-Life Technologies); Lane C(−): negative control.

were analysed. In experiment 1, all the 50 isolates obtained from tumours were gentamicin resistant, harboured plasmid pJB1JI and produced the same results as the parental strain for the following tests: API 20E, urease, aesculin, PCR analysis with primers from the T-DNA specific for the Ti plasmid (Nesme et al., 1989) and Eckhardt analysis of plasmids (Fortin et al., 1992). Likewise, the parental strain and all the isolates analysed produced identical banding patterns in the RAPD analyses (Figure 3A).

In experiment 2, the banding pattern of 250 *Agrobacterium*-like isolates recovered from the tumours of the nine hosts employed was determined with the RAPD primer OPE-7. An identical pattern to the inoculated parental strain was obtained and the other three primers selected were then employed to confirm the identity of these isolates. When a different profile was obtained with the first primer employed, the isolate was considered different from the one that was inoculated and no further confirmation by the other primers was conducted. Additionally, these isolates were analysed for urease and aesculin activities, as well as for pathogenicity by inoculation on tomato plants.

All the isolates that gave the same banding pattern as the parental strain (146 out of 250) also showed the expected biochemical activities and were pathogenic on tomato, confirming that they were *A. tumefaciens* identical to the parental strain.

In experiment 3, 300 colonies were recovered from tumours in tomato and pepper, from which, 205 were positive in PCR, biochemical and pathogenicity tests, and had the same banding pattern as the respective inoculated strain. The remaining isolates were negative for these analyses and gave a different banding pattern. In two cases, isolates that were positive for all the analyses (biochemical, PCR, urease, aesculin, pathogenicity) produced RAPD profiles different from the inoculated strain. These results could be explained by assuming that they represented endophytic strains of *A. tumefaciens* different from the one used for inoculation (Martí et al., 1999) or, alternatively, that the colonies were mixed cultures containing such endophytic agrobacteria (or other bacteria) and a proportion of the *A. tumefaciens* strain previously inoculated. To test this hypothesis, the isolates were purified by three successive platings of a water suspension of 10^5 cfu ml^{-1} in order to obtain well separated colonies. Ten of the resulting colonies were chosen at random to perform the RAPD analysis. The results of one of these purifications are shown in Figure 4. Six of the colonies produced similar banding pattern as the parental strain, whilst others did not. Only the colonies with the same banding pattern as the parental strain were positive for urease, aesculin, PCR and pathogenicity tests, while the other colonies were negative for these tests, and gave a totally different banding pattern. These results confirmed the efficiency of the RAPD system in discriminating *Agrobacterium*-like isolates from different hosts and origins. The banding pattern of lane 13 in Figure 4, which is the inoculated strain A281, shows slight differences, the fault of one band comparing to lanes 7–12. These are differences that can occur using this technique, but they do not compromise the validity of the analysis. The repetition of the amplification would clarify the doubtful results.

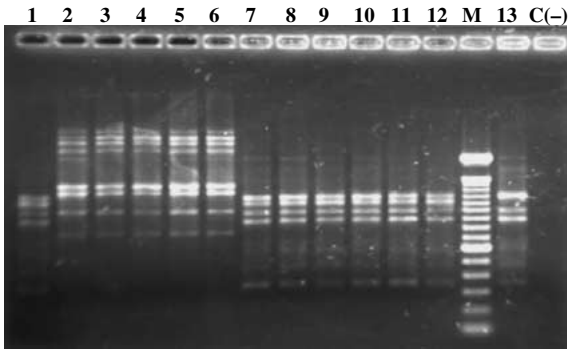

Figure 4. Analysis performed with primer OPE-2 on 10 colonies randomly selected after purifying one colony pathogenic on tomato but providing a RAPD band pattern different than that of the parental strain A281. Lanes 1 and 13: strain A281; Lane 2: colony suspension prior purification; lanes 3–12: colonies purified from the suspension shown in lane 2; M: marker 100 bp Invitrogen-Life Technologies. Only colonies shown in lanes 7–12 were positive for urease and aesculin and pathogenic on tomato.

Discussion

The object of this work was to devise a simple RAPD system to identify *A. tumefaciens* colonies recovered from host plants in epidemiological studies rather than an analysis of phylogenetic relationships as in previous work (Irelan and Meredith, 1996; Momol et al., 1998). The system developed was capable of discriminating between the strains under study. It has been applied both to bacterial suspensions and purified DNA, showing no differences in the results obtained. The banding patterns obtained with the *Agrobacterium* strains from different origins and hosts and from several international collections provided a clear distinction between all of them, and was confirmed by cluster analysis. The possibility of using four different primers allowed the exact discrimination among the strains under study in case there were doubtful results with one of the primers employed. When the analysis of an isolate with one primer gave a different profile from the parental strain it was considered as different, and no further analysis was conducted. By combining the data from the four primers we are able to obtain a unique pattern for each strain tested. Thus, it was possible to develop a database of RAPD profiles for comparison of unknown isolates.

The reproducibility of this technique has been shown (Birch et al., 1997; Maki-Valkama and Karjalainen, 1994; Parent et al., 1996; Wang et al., 1993). However, as pointed out by Penner et al. (1993), the main drawback of the RAPD technique could be the reproducibility between laboratories, which is related to several factors, such as the temperature profile of the thermocyclers and the reagents employed. To avoid these variables that could affect the results in the different PCR amplifications, we performed all the analysis using the same apparatus (Perkin-Elmer 9600) and the same suppliers for reagents and material. With

these variables controlled, good reproducibility was obtained and was confirmed by comparing the results of two assays performed 4 years apart on the same samples, and 4 months apart on different suspensions of the same strains (Figure 3B). The technique must be used only on pure cultures, because with mixed cultures it would not be possible to assign correct banding patterns. In one of the experiments we discriminated between pure and mixed cultures, when an *Agrobacterium* suspension did not show the expected pattern. After purification the bacterial suspension was shown to contain a mixture of two strains with similar colony morphology (Figure 4). This figure also shows some little variations in the banding pattern that can occur with the same samples, but they are too scarce to produce doubtful conclusions. The possibility of working with a mixture of strains, even when proper microbiological practices are followed has been reported (Li et al., 1996). Furthermore, the difficulties in recovering *Agrobacterium* strains from tumours have also been indicated (Cubero et al., 1999; Martí et al., 1999), and the need for extra purification steps for *Agrobacterium* isolates is well known to ensure that after isolation truly axenic cultures are obtained. In experiments 2 and 3, we isolated a high number of colonies from tumours (104 and 95, respectively) that showed *Agrobacterium*-like characteristics but had different banding pattern compared to the inoculated parental strain. Since these isolates were obtained from inoculated plants kept under natural conditions, the origin of these strains is questioned: where did they come from? Are they capable of colonising tumours induced by pathogenic *Agrobacterium* strains and live together in harmony or in competition? Although in our three experiments we used sterilised soil substrates, the plants were obtained from commercial nurseries, and maintained for up to 9 months under greenhouse conditions, irrigated and cultivated as in a nursery. The presence of endophytic *Agrobacterium* in nurseries has already been demonstrated in rose (Martí et al., 1999) and fruit trees (Cubero, 1998), and this could have been the source of the variant strains we isolated. Only in the first experiment, when a marked strain (Gen^r) was employed, were *Agrobacterium* colonies different from the strain inoculated not recovered. This was probably due to the use of the antibiotic in the isolation medium.

The present experiments show that a high proportion of the *Agrobacterium*-like bacteria in a gall (up to 42%) could be unrelated to the strain used as inoculum. Therefore, for certain experiments involving purification of *Agrobacterium* strains from galls, rhizosphere, or soil, the availability of a fast and simple identification technique will be of great help, to discriminate the inoculated strain from endophytic agrobacteria. The RAPD system described here could also be used to monitor the survival of a given strain of *Agrobacterium* in different environments or to follow the origin of a disease outbreak in a nursery.

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