APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1995, p. 828–831 Vol. 61, No. 2
0099-2240/95/$04.00+0

PCR Detection of Ti and Ri Plasmids from Phytopathogenic *Agrobacterium* Strains

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Received 5 July 1994/Accepted 9 November 1994

A universal primer set (VCF/VCR) for PCR analysis based on the sequences of the *virC* operon located on Ti and Ri plasmids was designed to detect these plasmids from phytopathogenic *Agrobacterium* strains. With the VCF (sequence, 5′-ATCATTTGTAGCGACT-3′) and VCR (sequence, 5′-AGCTCAAACCTGCTTC-3′) primer set, DNA fragments of 730 bp in length were amplified from cell lysates of 10 rhizogenic and 65 tumorigenic agrobacteria. DNA sequencing and Southern hybridization analysis confirmed that the amplified fragments corresponded to the target region. The PCR method is considered convenient for routine determination of the potential pathogenicity of *Agrobacterium* strains.

Crown gall disease and hairy root disease are caused by *Agrobacterium* strains harboring Ti (tumor-inducing) and Ri (root-inducing) plasmids, respectively. Infection occurs at plant wound sites and involves the transfer of oncogenic DNA (T-DNA), which is located on Ti and Ri plasmids, from plasmid-harboring (phytopathogenic) agrobacteria to the plant cell nucleus. The genes required for T-DNA processing and transfer reside in virulence (*vir*) regions (*virA*, *virB*, *virG*, *virC*, *virD*, *virE*, and so on) that are also located on Ti and Ri plasmids (reviewed in references 10, 22, and 25).

The incidence of crown gall and hairy root diseases affecting a wide range of crops has been a serious problem (3, 6, 9, 12). It is assumed that the frequent occurrence of these diseases may result from transmission by contaminated propagation materials (reviewed in reference 3). One primary control strategy to eliminate these diseases consists of using pathogen-free propagation materials on uninfested land. Thus, it is important to develop a sensitive and rapid technique to detect plasmid-harboring (phytopathogenic) agrobacteria for indexing of propagation materials.

Detection of very low numbers of bacteria by the PCR amplification of a target sequence has already been described (reviewed in reference 19). Dong et al. (5) achieved their objective to amplify part of T-DNA from tumorigenic agrobacteria isolated from muscadine grapes (*Vitis rotundifolia* Michx.). However, because there is much diversity in DNA sequences among Ti plasmids (2, 21, 24) and between Ti and Ri plasmids (20), development of a universal primer set based on a conserved region of these plasmids is necessary for detection of various types of phytopathogenic agrobacteria. In this study, we developed a universal primer set (VCF and VCR) to amplify fragments of the Ti and Ri plasmid-encoded *virC1* and *virC2* regions (4) from cell lysates of pure cultures.

***Agrobacterium* strains and template preparation.** All strains used in this study are listed in Table 1. The origins and pathogenicities of the strains were described earlier (16, 18). In addition to the classical biovar system (11), another nomenclature system is also given in parentheses in Table 1; this system was proposed by Ophel and Kerr (13), Sawada et al. (17), and Bouzar (1). Type strains of closely related *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* species were also used for comparison with agrobacteria.

All strains except *Rhizobium meliloti* and *Azorhizobium caulinodans* were routinely cultured on YEM medium, which contained (per liter of distilled water) 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of NaCl, 0.4 g of yeast extract, 10 g of mannitol, and 15 g of agar (pH 7.2). Strains of *R. meliloti* and *Azorhizobium caulinodans* were cultured on tryptic soy broth (Difco Laboratories) solidified with agar.

Cell lysates of pure cultures were prepared from single colonies formed on a plate medium, using InstaGene DNA purification matrix (Bio-Rad Laboratories) according to the supplier's instructions. Before use, the lysates were centrifuged at 12,000 rpm for 3 min, and the resulting supernatants were subjected to PCR as templates.

Primer design. Of the two separate clusters of pathogenicity genes (T-DNA and *vir* regions) located on Ti and Ri plasmids, T-DNA shows substantial differences in DNA sequences among various types of plasmids (2, 5, 20, 21, 24). In contrast, *vir* regions proved to be highly conserved (7, 8, 15, 23). Thus, a search for published sequence data of *vir* regions in the GenBank database was undertaken to get information for primer design.

On the basis of several aligned sequences, three oligonucleotide sequences derived from the *virG* gene and two sequences derived from the *virC* operon were selected which were highly conserved (Fig. 1). The *virG* gene is a left-hand neighbor of the *virC* operon, which consists of two genes, *virC1* and *virC2* (4, 14). Thus, a forward primer (G1, G2, and G3) was prepared according to each of the three selected sequences derived from the *virG* gene, whereas a reverse primer (C2) and both a forward (C1) and a reverse (C3) primer were synthesized on the basis of sequences derived from the *virC1* and *virC2* genes, respectively (Fig. 1).

PCR amplification. PCR amplification of the target sequence was performed in a total volume of 10 μl of the following reaction mixture: 50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, 2 pmol of each primer, 0.5 μl of template, and 0.5 U of *Taq* DNA polymerase (Toyobo). PCR was performed in a DNA thermal cycler 480 (Perkin-Elmer Cetus), using the following protocol: initial denaturation at

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TABLE 1. Bacterial strains used in this study and PCR results with the universal primer set (VCF/VCR)

Species and strain[a]	Source and location (supplier)[b]	PCR result[c]
Agrobacterium biovar 1 (= *Agrobacterium tumefaciens*)		
Tumorigenic strain harboring Ti plasmid		
NCPPB 2437^T	Unknown; United States (NCPPB)	+
ATCC 33970	Cherry; United States (ATCC)	+
MAFF 03-01001	Cherry; Saitama, Japan (MAFF)	+
MAFF 03-01224	Rose; Osaka, Japan (MAFF)	+
MAFF 03-01222	Marguerite; Shizuoka, Japan (M.AFF)	+
At-M-19, At-M-22	Marguerite; Shizuoka, Japan (M. Togawa)	+
MAFF 03-01276, 03-01278	Chrysanthemum; Shizuoka, Japan (MAFF)	+
CH3, CH5	Chrysanthemum; Shizuoka, Japan (T. Makino)	+
Rhizogenic strain harboring Ri plasmid		
MAFF 03-01724, 03-01725	Melon; Chiba, Japan (MAFF)	+
03-01726, 03-01727	Melon; Chiba, Japan (MAFF)	+
ARM-3, melon-1	Melon; Shizuoka, Japan (Y. Takikawa)	+
MR4, MR40	Melon; Shizuoka, Japan (T. Makino)	+
Nonpathogenic strain		
IAM 12048^T	Unknown; Netherlands (IAM)	−
IAM 1527	Unknown; Unknown (IAM)	−
IAM 1526	Soil; United States (IAM)	−
Ct-Ag-1	Soil; Mie, Japan	−
Agrobacterium biovar 2 (= *Agrobacterium rhizogenes*)		
Tumorigenic strain harboring Ti plasmid		
NCPPB 2303	Almond; Israel (NCPPB)	+
MAFF 03-01546	Rose; Shizuoka, Japan (MAFF)	+
R65, R80	Rose; Shizuoka, Japan (T. Makino)	+
AtR11	Rose; Shizuoka, Japan (K. Ohta)	+
Ro-Ag-10, Ro-Ag-11	Rose; Yamagata, Japan	+
Ro-Ag-12	Rose; Yamagata, Japan	+
Ch-Ag-2, Ch-Ag-3	Cherry; Yamagata, Japan	+
Ch-Ag-6, Ch-Ag-9	Cherry; Okayama, Japan	+
P-Ag-1, P-Ag-2	Pear; Mie, Japan	+
P-Ag-6	Pear; Nagasaki, Japan	+
Peach CG 8331	Peach; Yamagata, Japan (Y. Takikawa)	+
Pch-Ag-2, Pch-Ag-3	Peach; Okayama, Japan	+
Pl-Ag-1, Pl-Ag-2	Plum; Okayama, Japan	+
Rhizogenic strain harboring Ri plasmid		
IFO 13257^T	Apple; United States (IFO)	+
ATCC 15834	Unknown; Unknown (ATCC)	+
Nonpathogenic strain		
Kerr 84	Soil; Australia (T. Makino)	−
Agrobacterium biovar 3 (= *Agrobacterium vitis*)		
Tumorigenic strain harboring Ti plasmid		
NCPPB 2562	Grape; Greece (NCPPB)	±
NCPPB 1771	Grape; Iran (NCPPB)	−
S-4, Sz-1	Grape; Hungary (Szegedi)	+
YGAt 32-3, 33-1, 35-2	Grape; Yamanashi, Japan (Y. Terai)	+
G-Ag-4, G-Ag-9, G-Ag-14	Grape; Shimane, Japan	+
G-Ag-19, G-Ag-21, G-Ag-23	Grape; Nagano, Japan	+
G-Ag-26, G-Ag-27	Grape; Nagano, Japan	+
G-Ag-52, G-Ag-54, G-Ag-56	Grape; Iwate, Japan	+
G-Ag-61	Grape; Aomori, Japan	+
G-Ag-62, G-Ag-63, G-Ag-64	Grape; Yamagata, Japan	+
G-Ag-66, G-Ag-67	Grape; Akita, Japan	+
G-Ag-81	Grape; Toyama, Japan	+
K-Ag-1, K-Ag-2	Kiwifruit; Hiroshima, Japan	+
Agrobacterium rubi		
Tumorigenic strain harboring Ti plasmid		
IFO 13261^T, IFO 13260	*Rubus* sp.; United States (IFO)	+
Unclassified agrobacteria		
Tumorigenic strain harboring Ti plasmid		
NCPPB 1650	*Rosa* sp.; South Africa (NCPPB)	+
K-Ag-3, K-Ag-4	Kiwifruit; Hiroshima, Japan	+
Ch-Ag-4, Ch-Ag-5	Cherry; Okayama, Japan	+
Ch-Ag-7, Ch-Ag-8	Cherry; Okayama, Japan	+
Rhizobium leguminosarum bv. *viceae*		
IAM 12609^T	Unknown; United States (IAM)	−
Rhizobium leguminosarum bv. *phaseoli*		
IFO 14785^T	Unknown; Unknown (IFO)	−
Rhizobium leguminosarum bv. *trifolii*		
IFO 14784^T	Unknown; Unknown (IFO)	−
Rhizobium meliloti		
IAM 12611^T	Unknown; United States (IAM)	−
Rhizobium loti		
IAM 13588^T	*Lotus corniculatus*; New Zealand (IAM)	−
Rhizobium fredii		
IFO 14780^T	Soybean; China (IFO)	−
Rhizobium galegae		
IFO 14965^T	*Galega orientalis*; Finland (IFO)	−
Bradyrhizobium japonicum		
IFO 14783^T	Soybean; United States (IFO)	−
Azorhizobium caulinodans		
IFO 14845^T	*Sesbania rostrata*; Senegal (IFO)	−

[a] For the assignment of strains to taxa and their pathogenicity, see previous reports (16, 18). The taxon of the genus *Agrobacterium* is indicated according to the classical nomenclature (biovar system) (11), followed by another nomenclature system proposed by Ophel and Kerr (13), Sawada et al. (17), and Bouzar (1) given in parentheses. T, type strain. Abbreviations of culture collections: ATCC, American Type Culture Collection, Rockville, Md.; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; MAFF, Ministry of Agriculture, Forestry and Fisheries, Tsukuba Ibaraki, Japan; NCPPB, National Collection of Plant Pathogenic Bacteria, United Kingdom.

[b] Unless a supplier is stated, the strain was isolated by us.

[c] +, 730-bp target band present on agarose gel electrophoresis after PCR using the universal primer set (VCF/VCR) under the conditions described in the text; −, no target band present; ±, faint band present.

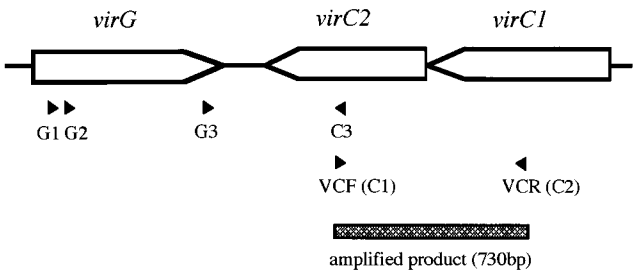

FIG. 1. Positions and directions of the six oligonucleotide primers (G1, G2, G3, C1, C2, and C3 [arrowheads]) used for preliminary PCR analysis on a schematic physical map of the *virC1*, *virC2*, and *virG* genes (open arrows) of Ti plasmid pTiC58 (4, 14). After preliminary analysis, primers C1 and C2 were finally selected as a universal primer set for detection of Ti and Ri plasmids and designated VCF (sequence, 5′-ATCATTTGTAGCGACT-3′) and VCR (sequence, 5′-AGCTCAAACCTGCTTC-3′), respectively (see text).

95°C for 2.5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and an additional extension at 72°C for 7 min. After PCR, the total reaction mixture (10 μl) was separated by electrophoresis in a 2% agarose gel in TAE buffer and the amplified DNA fragment was visualized by ethidium bromide staining and transillumination.

The specificity of each pair of primers was studied preliminarily with templates prepared from several representative strains of each *Agrobacterium* taxon (*Agrobacterium* biovars 1, 2, and 3 and *Agrobacterium rubi*), which were selected from those listed in Table 1 and harbor Ti or Ri plasmids. A total of 37 strains were used in this preliminary experiment.

When a *virG* gene-derived forward primer (G1, G2, or G3) and a *virC* operon-derived reverse primer (C2 or C3) were combined to determine the efficacy of primers for amplification of a target sequence, some strains showed no amplification products and some gave several unexpected bands, which may have been due to nonspecific priming (data not shown). On the

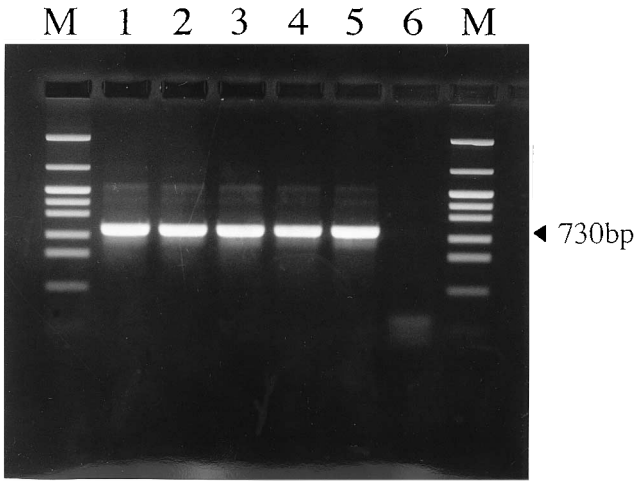

FIG. 2. Agarose gel (2%) showing PCR products of 730 bp in length amplified from various *Agrobacterium* strains harboring Ti plasmids, using the universal primer set (VCF/VCR). Lanes: M, pHY marker (bands from top to bottom: 4,870, 2,016, 1,360, 1,107, 926, 658, 489, and 267 bp) (Takara); 1, *Agrobacterium* biovar 1 strain ATCC 33970; 2, *Agrobacterium* biovar 2 strain NCPPB 2303; 3, *Agrobacterium* biovar 2 strain MAFF 03-01546; 4, *Agrobacterium* biovar 3 (*A. vitis*) strain G-Ag-27; 5, *A. rubi* IFO 13261^T; 6, nonpathogenic *Agrobacterium* biovar 1 strain IAM 12048^T, which does not harbor a Ti or Ri plasmid (negative control).

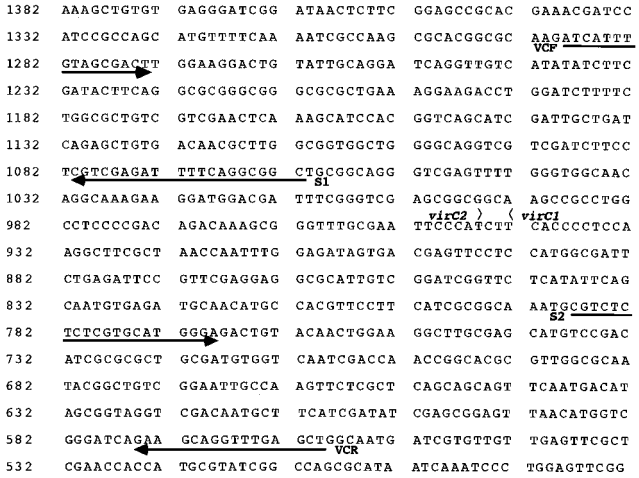

FIG. 3. Partial sequence of the *virC1* and *virC2* genes of pTiC58 (4). The first nucleotide shown corresponds to position 1382 of the *virC* gene of pTiC58 (4). The borders of *virC1* and *virC2* are indicated by the brackets. Arrows indicate the relative priming positions of the primers used in PCR and the sequencing analysis of the amplified products. Primers VCF and VCR are utilized for both amplification and sequencing, and primers S1 and S2 are used for sequencing.

other hand, all of the 37 pathogenic agrobacteria used in this preliminary experiment produced clear bands of about 730 bp in length with C1 and C2 as the forward and reverse primers, respectively (Fig. 2). The primer set (C1/C2) defines a 730-bp region on the *virC* operon (Fig. 1), and the size of the amplified DNAs (Fig. 2) coincided with that of the target region flanked by the primer set. The C1 primer was designated VCF (sequence, 5′-ATCATTTGTAGCGACT-3′) and the C2 primer was designated VCR (sequence, 5′-AGCTCAAACCTGCT TC-3′). The amplified DNAs were analyzed to further confirm the specificity of the primer set (VCF/VCR).

Analysis of the amplified DNA by DNA sequencing and Southern hybridization. The identity of the amplified DNA was confirmed by sequencing analysis. The 730-bp fragment amplified from strain ATCC 33970, harboring pTiC58, whose *virC* genes were sequenced by Close et al. (4), was purified by using Suprec-01 (Takara), a sample recovery tube equipped with a filter cup (Durapore membrane, 0.22-μm pore size), after agarose gel electrophoresis. The purified DNA was sequenced by using a *Taq* DyeDeoxy terminator cycle sequencing kit and a model 373A DNA sequencing system (Applied Biosystems). By using four sequencing primers (VCF, VCR, S1, and S2 [Fig. 3]), the complete sequence of the amplified DNA from strain ATCC 33970 was determined and was found to be identical to that of the 730-bp target region of pTiC58 defined by the primer set (VCF/VCR) (Fig. 3).

Confirmation was further accomplished by Southern hybridization analysis. The amplified DNAs of various strains were electrophoresed and transferred to a nylon membrane (Hybond-N; Amersham International). The entire 730-bp fragment amplified from strain ATCC 33970, harboring pTiC58, was used as a probe. Southern hybridization and detection of the hybridized DNA were carried out with the DIG DNA labeling and detection kit (Boehringer Mannheim) as specified by the manufacturer. In all cases, the 730-bp fragments amplified from different agrobacteria used in the preliminary experiment hybridized with the probe (data not shown). These results suggested that the amplified DNAs corresponded to the target region defined by the primer set (VCF/VCR).

Efficacy of primers for detection of Ti and Ri plasmids. To

evaluate the efficacy of the primer set (VCF/VCR) for detection of various types of Ti and Ri plasmids, a total of 67 tumorigenic and 10 rhizogenic agrobacteria (Table 1), including 37 strains used in the preliminary experiment mentioned above, were utilized. All 10 rhizogenic strains, 8 and 2 of which belong to biovars 1 and 2, respectively, showed amplification bands of the expected 730-bp length. As for tumorigenic strains, 11 of biovar 1, 20 of biovar 2, 2 of *A. rubi*, and 7 of unclassified agrobacteria all produced the clear 730-bp bands. Whereas two strains of biovar 3 (strains NCPPB 1771 and NCPPB 2562) gave no and a faint band, respectively, the other tumorigenic strains of biovar 3 resulted in positive amplification (Table 1).

In contrast, nonpathogenic agrobacteria, four and one strain of which belong to biovars 1 and 2, respectively, showed no amplification of 730-bp bands. Also, negative results were obtained for the type strains of *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* species, which are close relatives to members of the genus *Agrobacterium* but do not have Ti or Ri plasmids.

In conclusion, the data obtained in this study demonstrate that PCR analysis utilizing the primer set (VCF/VCR) can amplify the expected 730-bp products from most (75 of 77) pathogenic agrobacteria harboring Ti or Ri plasmids, suggesting that the amplification of the target band does indicate the potential pathogenicity of agrobacteria. With the universal primer set (VCF/VCR), this method is rapid and easy, can be considered convenient for routine detection of Ti and Ri plasmids from pure cultures, and will extend the capability for studying the molecular epidemiology and etiology of *Agrobacterium* species.

We are grateful to Takahiro Makino and Kouki Ohta (Shizuoka Agricultural Experiment Station, Shizuoka, Japan), Erno Szegedi (University of Horticultural and Food Sciences, Kecskemét, Hungary), Yuichi Takikawa (Shizuoka University, Shizuoka, Japan), Yasuo Terai (Yamanashi Fruit Tree Experiment Station, Yamanashi, Japan), and Masayuki Togawa (Shizuoka Citrus Experiment Station, Shizuoka, Japan), who supplied interesting strains used in this study.

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