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BACTERIAL AND PHYTOPLASMA DISEASES

Short communication

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Detection of tumorigenic *Agrobacterium* strains from infected apple saplings by colony PCR with improved PCR primers

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Abstract With the colony polymerase chain reaction (PCR), the specificity of newly prepared primer sets VCF2/VCR2, VCF3/VCR3, VCF4/VCR4, and VCF5/VCR5 to Ti or Ri plasmids in *Agrobacterium* strains were compared to that of the conventional set VCF/VCR. At first, control strains, which consisted of a nonpathogenic strain and phytopathogenic strains carrying Ti or Ri plasmid, were used. VCF3/VCR3 and VCF5/VCR5 were highly specific to all the phytopathogenic strains, whereas the others were not. These two primer sets were superior to VCF/VCR in their specificity with colony PCR to tumorigenic *Agrobacterium* strains isolated from apple saplings.

Key words *Agrobacterium* spp. · Colony PCR · Ti or Ri plasmid · Crown gall · Apple

Apple growing with dwarf rootstocks is spreading in Japanese apple-growing regions to reduce the labor in orchard management while maintaining the quality of the fruit. Production of apple saplings with dwarf rootstocks is increasing; however, crown gall disease has accompanied the production of such trees and is threatening apple yields (Nekoduka et al. 2001). Kawai et al. (1999) reported that the spread of this disease was likely to be connected with the use of contaminated scions or rootstocks. Because the materials for controlling this disease on apple trees are limited, use of pathogen-free scions or rootstocks is considered a high priority and is expected to be the most effective means for preventing the spread of the disease. Thus, it is important to develop a rapid, sensitive technique to detect tumorigenic *Agrobacterium* strains for diagnosis in apple saplings.

Integration of the T-DNA region of either the Ti or Ri plasmid in *Agrobacterium* strains into the chromosomes of host plants causes crown gall or hairy root disease (Bevan and Chilton 1982). Although T-DNA regions have diverse nucleotide sequences, Sawada et al. (1995) noted that nucleotide sequences of the *virC* gene, which is encoded commonly on Ti and Ri plasmids in various strains of *Agrobacterium* species, are highly conserved. Furthermore, they reported a primer set for the polymerase chain reaction (PCR) to detect Ti and Ri plasmids. Kawai et al. (1998) investigated whether this primer set was useful for diagnosing crown gall disease on apple trees.

Bacterial colony PCR (Guessow and Clackson 1989) is now widely used to detect particular genes in bacterial cells because of its simplicity (Sandhu et al. 1989; Schmitz et al. 1997). However, our preliminary tests have shown that this primer set does not always differentiate phytopathogenic *Agrobacterium* strains isolated from various plant materials, including apple trees, during colony PCR. In this article we report on improved primer sets for Ti and Ri plasmid detection that are more suitable for colony PCR than the conventional one. We also discuss detection of tumorigenic *Agrobacterium* strains isolated from apple saplings by colony PCR using these new primer sets.

Primer design and condition of colony PCR. We prepared four new primer sets – VCF2/VCR2, VCF3/VCR3, VCF4/VCR4, VCF5/VCR5 – for Ti or Ri plasmid detection (Table 1). All the primer sets were designed for universal detection of *virC* genes on Ti or Ri plasmids. Nucleotide sequences of these primer sets were designed based on the published information in nucleotide sequence databases (accession numbers NC003308, AB016260, AF242881, AB027257, AF176227, AP002086, and X12867). Both VCF2/VCR2 and VCF4/VCR4 were derivative degenerate primer sets based on VCF/VCR, and both VCF3/VCR3 (Sawada 2004; Sawada and Tsuchiya 2003) and VCF5/VCR5 were newly

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Table 1. PCR primer sets prepared for Ti or Ri plasmid detection

Primer	Sequence[a] (5′ → 3′)	Length of PCR product (bp)
VCF[b]	ATCATTTGTAGCGACT	730
VCR[b]	AGCTCAAACCTGCTTC	
VCF2	AAGATCATTTGYARMGMYT	736
VCR2	GCTAGCTCAAACMTGCTTY	
VCF3	GGCGGGCGYGCYGAAAGRAARACYT	414
VCR3	AAGAACGYGGNATGTTGCATCTYAC	
VCF4	AAGATCATYTGYARIGMYT	736
VCR4	GCTAGCTCAAACHTRCTYY	
VCF5	GACAAAGCGGGYTTGCGRATNSCCA	93
VCR5	TCAGGATYGCSATGGAGGAASTCGT	

PCR, polymerase chain reaction
[a] H, M, N, R, S, and Y in the primer sequences indicate nucleotide bases as follows. H: A, C, or T; M: A or C; N: A, C, G, or T; R: A or G; S: C or G; Y: C or T. I also indicates inosine
[b] Data are from Sawada et al. (1995)

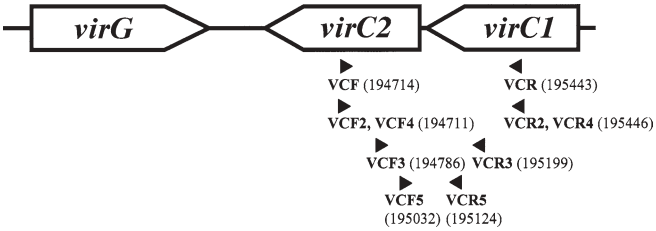

Fig. 1. Positions of conventional and improved polymerase chain reaction (PCR) primers on *virC* genes. Parenthetic numbers represent positions of the heading nucleotides in primer sequences on complete sequence of Ti plasmid in *Agrobacterium tumefacines* strain C58 (accession no. NC003308)

designed to amplify the internal region between VCF and VCR (Fig. 1).

Bacterial strains examined were cultured at room temperature on King's B (20 g peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, 10 g glycerol, and 15 g agar in 1000 ml distilled water, adjusted to pH 7.0) or YP (5 g yeast extract, 5 g peptone, 5 g NaCl, and 15 g agar in 1000 ml distilled water, adjusted to pH 7.0) plate media. A small portion of the growing colony (3–7 days after inoculation) was picked with a sterilized toothpick and suspended in a reaction mixture immediately. The reaction mixture contained the following constituents and was filled with sterilized water up to 20 μl: 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 2 μl of GeneAmp 10x PCR buffer containing 15 mM of $MgCl_2$ (Applied Biosystems), 2 μl of 2 mM dNTPs, 1 μl each of foward and reverse primer (10 μM each). Reactions were performed as follows: initial denaturation (95°C, 10 min), 40 cycles of denaturation (95°C, 1 min), annealing (52°C, 1 min) and extension (72°C, 2 min), and additional extension (72°C, 7 min). The reaction products (5 μl volumes) were separated on 2% agarose gel electrophoresis in TAE buffer (40 mM Tris, 4 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid, pH 8.0) and observed on an ultraviolet (UV) transilluminator after staining in ethidium bromide solution (1 μg/ml distilled water).

Bacterial strains. Eleven strains of *Agrobacterium* species (Table 2) were used to identify strains isolated from apple saplings and as controls for colony PCR. Diseased apple saplings, in which tumors had developed on the graft union or root, were collected from Aomori and Yamagata prefectures. *Agrobacterium* strains were isolated following the method described by Moore et al. (2001) using semiselective media such as 1A, 2E (Brisbane and Kerr 1983), or D1M (Perry and Kado 1982). Isolated bacterial strains were differentiated by the method described by Schaad (2001), and additional physiological properties were examined (Moore et al. 2001). Subsequently, hypocotyls of tomato (cv. Ponderosa) seedlings were inoculated with bacterial strains using scalpels, and tumor induction was examined. Using the physiological properties and pathogenicity on tomato of these bacterial strains, we identified 48 strains of *Agrobacterium* species from apple saplings. Of these 48 strains, 40 were tumorigenic on tomato; 39 strains were identified as *Agrobacterium rhizogenes* (Ti) (=*A. tumefaciens* biovar 2), and the remaining one was *A. tumefaciens* (Ti) (= *A. tumefaciens* biovar 1). The other eight strains were nonpathogenic and were identified as *A. tumefaciens* (nonpathogenic) (= *A. radiobacter* biovar 1) (Table 3).

Specificity of primer sets to Ti or Ri plasmid in control strains. At first, Ti or Ri plasmid detection by colony PCR was performed using the conventional primer set VCF/VCR. This primer set amplified a 730-bp DNA fragment specific to the *virC* genes of Ti and Ri plasmids from only four phytopathogenic strains (MAFF301276, MR4, Ch-Ag-2, Peach CG8331); no DNA fragments were amplified from the rest of the strains (Table 2). On the other hand, when we used the newly prepared primer sets (VCF3/VCR3 and VCF5/VCR5), each pair amplified DNA fragments of the expected size (414 bp or 93 bp in length, respectively) from all strains carrying the Ti or Ri plasmids (Table 3). VCF3/VCR3 amplified no DNA fragments from the nonpathogenic strain, whereas VCF5/VCR5 amplified an unspecific DNA fragment, similar in size to the specific product of phytopathogenic strains, from the nonpathogenic strain.

Table 2. Results of colony PCR in control strains using newly prepared primer sets

Strain	Pathogenic plasmid	Colony PCR results					Results in a previous report (VCF/VCR)[a]
		VCF/VCR	VCF2/VCR2	VCF3/VCR3	VCF4/VCR4	VCF5/VCR5	
Agrobacterium tumefaciens (Ti) (= *A. tumefaciens* biovar 1)							
MAFF 301276	Ti	+	+	+	−	+	+
Agrobacterium tumefaciens (Ri) (= *A.rhizogenes* biovar 1)							
MAFF 301274	Ri	−	−	+	−	+	Not tested
MAFF 301726	Ri	−	−	+	−	+	+
MR4	Ri	+	−	+	−	+	+
Agrobacterium rhizogenes (Ti) (= *A. tumefacines* biovar 2)							
Ch-Ag-2	Ti	+	+	+	−	+	+
Peach CG8331	Ti	+	+	+	−	+	+
Agrobacterium rhizogenes (nonpathogenic) (= *A. radiobacter* biovar 2)							
Kerr 84	None	−	−	−	−	?	−
Agrobacterium vitis (Ti) (= *A. tumefacines* biovar 3)							
NCPPB 2652	Ti	−	+	+	−	+	±
NCPPB 1771	Ti	−	+	+	−	+	−
G-Ag-27	Ti	−	+	+	±	+	+
K-Ag-1	Ti	−	+	+	−	+	+

+, targeted DNA segment amplified; –, targeted DNA segment not amplified; ±, faint fragment was observed; ?, nonspecific DNA segment of size similar to targeted fragment observed

[a] Sawada et al. (1995). Purified DNA samples were used as templates for PCR

Both VCF3/VCR3 and VCF5/VCR5 were highly specific to phytopathogenic strains; VCF2/VCR2 and VCF4/VCR4 were less specific to phytopathogenic strains.

To confirm that the PCR products amplified by VCF3/VCR3 and VCF5/VCR5 originated from the *virC* genes, the PCR products were fixed on a nylon membrane (Hybond-N^+; Amersham Biosciences, Buckinghamshire, UK), and Southern hybridization was performed using the DIG-labeled probe. The 730-bp DNA fragment amplified from the strain Peach CG8331 by use of primer set VCF/VCR was labeled by PCR DIG Labeling Mix (Roche Diagnostics, Mannheim, Germany) and used as a probe. The probe hybridized with the PCR products, indicating that the PCR products derived from the *virC* genes (data not shown).

Specificity of primer sets to Ti plasmid in *Agrobacterium* strains isolated from apple saplings. In the previous experiment, because the two primer sets VCF3/VCR3 and VCF5/VCR5 differentiated phytopathogenic strains efficiently, we compared their specificity with conventional VCF/VCR using *Agrobacterium* strains isolated from apple saplings. The presence of the Ti plasmid in the bacterial strains was investigated by Southern hybridization in advance using the DIG-labeled probe described above. Total DNA was isolated from the bacterial strains by the method described by Louws et al. (1998) with a modification (10% sodium dodecyl sulfate instead of mutanolysin and sarcosyl in the cell lysing step). Isolated DNA (2 μg) was digested with a restriction enzyme *Eco*RI (Takara Bio, Ohtsu, Japan), and transferred to a Hybond-N^+ nylon membrane after 0.7% agarose gel electrophoresis. The result showed that signals of the probe were observed near the 4.4- and 2.3-kb fragments of λ*Hin*dIII DNA ladder marker in all of tumorigenic strains isolated from the diseased apple saplings (Table 3), whereas signals were observed near the 2.3- and 2.0-kb segments for the control strain, Peach CG8331. No signals were observed for nonpathogenic strains. Because the results of Southern hybridization and tumor-inducing ability on the plant materials were in complete agreement, it was confirmed that the tumorigenic strains carried the Ti plasmid. The primer sets VCF/VCR amplified DNA fragments specific to the *virC* genes (730 bp in length) from 35 of the 40 tumorigenic strains at the maximum. VCF3/VCR3 or VCF5/VCR5 amplified specific DNA fragments (414 or 93 bp in length, respectively) from all tumorigenic strains (Table 3, Fig. 2); however, VCF3/VCR3 was superior to VCF5/VCR5 in reproducibility (Table 3). None of the primer sets amplified DNA fragments from nonpathogenic strains. Because the size of the DNA fragment amplified by VCF3/VCR3 was preferable to that by VCF5/VCR5, we concluded that VCF3/VCR3 was more suitable for detecting tumorigenic *Agrobacterium* strains by colony PCR.

In our previous report (Sawada et al. 1995), we found that VCF/VCR could detect Ti or Ri plasmids in 75 of 77 strains of *Agrobacterium* species. In these experiments, VCF/VCR could detect Ti or Ri plasmids in only 4 of 11 of the control strains. We thought that this was due to the

Table 3. Pathogenicity on tomato, Ti plasmid detection by Southern hybridization, and results of colony direct PCR of *Agrobacterium* strains isolated from apple saplings

Strain	Location	Tumorigenesis (tomato seedlings)[a]	*Agrobacterium* classification	Ti plasmid detection[b] (Southern hybridization)	Colony PCR[c]		
					VCF/VCR	VCR3/VCR3	VCF5/VCR5
Graft union (cv. Tsugaru /*Malus prunifolia*)	Aomori						
Tsugaru-1-1		−	*A. tumefaciens* (nonpathogenic (= *A. radiobacter* biovar 1)	−	−/−	−/−	−/−
Tsugaru-1-2		−	*A. tumefaciens* (nonpathogenic) (= *A. radiobacter* biovar 1)	−	−/−	−/−	−/−
Tsugaru-1-3		−	*A. tumefaciens* (nonpathogenic) (= *A. radiobacter* biovar 1)	−	−/−	−/−	−/−
Tsugaru-1-4		−	*A. tumefaciens* (nonpathogenic) (= *A. radiobacter* biovar 1)	−	−/−	−/−	−/−
Tsugaru-2-1		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Tsugaru-2-4		−	*A. tumefaciens* (nonpathogenic) (= *A. radiobacter* biovar 1)	−	−/−	−/−	−/−
Tsugaru-3-3		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Tsugaru-4-2		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	−/−	+/+	+/+
Tsugaru-5-3		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	−/−	+/+	+/+
Tsugaru-6-3		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	−/−	+/+	+/+
Tsugaru-8-2		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	−/−	+/+	+/+
Tsugaru-8-4		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Root (*M. prunifolia*)	Yamagata						
Kourin-1-2		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Kourin-3-2		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Kourin-5-2		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Kourin-5-3		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	−/−	+/−	−/−
Kourin-6-4		−	*A. tumefaciens* (nonpathogenic) (= *A. radiobacter* biovar 1)	−	−/−	−/−	−/−
Kourin-8-2		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	−/+
Kourin-8-3		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	−/±
Root (*M. prunifolia*)	Aomori						
J-1		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
J-2		−	*A. tumefaciens* (nonpathogenic) (= *A. radiobacter* biovar 1)	−	−/−	−/−	−/−
J-3		−	*A. tumefaciens* (nonpathogenic) (= *A. radiobacter* biovar 1)	−	−/−	−/−	−/−
Root (cv. JM7)	Aomori						
J-4		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Graft union (cv. M. 26/*M. prunifolia*)	Aomori						
K-1		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/±
K-2		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Graft union (cv. Kiou/cv. M. 26)	Aomori						
K-3		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+

Graft union (cv. M. 26/*M. prunifolia*)	Aomori						
M-1		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	–/+
M-3		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/−	+/+	–/+
M-5		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	–/+
M-7		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
M-8		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
M-9		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Root (*M. prunifolia*)	Aomori						
2J-1		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
2J-2		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
2J-3		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Root (cv. JM7)	Aomori						
2K-1		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
2K-2		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
2K-3		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
2K-4		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Graft union (cv. M. 26/*M. prunifolia*)	Aomori						
2M-2		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	±/+	+/+	+/+
2M-3		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
2M-6		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
2M-7		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
2M-8		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
2M-10		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Graft union (cv. M. 26/*M. prunifolia*)	Aomori						
3K-1		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Graft union (cv. Kiou/cv. M. 26)	Aomori						
3K-3		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+
Root (*M. prunifolia*)	Aomori						
515④c		+	*A. tumefaciens* (Ti) (= *A. tumefaciens* biovar 1)	+	+/+	+/+	+/+
Root (peach)	Yamagata						
Peach CG8331		+	*A. rhizogenes* (Ti) (= *A. tumefaciens* biovar 2)	+	+/+	+/+	+/+

[a] +, tumor induced by inoculation; −, tumor not induced

[b] +, signals of hybridized probes observed; −, signals not observed. Whereas signals were observed near 2.3- and 2.0-kb fragments in λ*Hin*dIII ladder marker in strain Peach CG8331, they were observed near 4.4- and 2.3-kb fragments in all of the apple strains

[c] Colony PCR was performed twice to confirm reproducibility. +, DNA fragment specific to *virC* gene on Ti plasmid observed on agarose gel electrophoresis; –, no DNA fragment observed; ±, faint DNA fragment observed

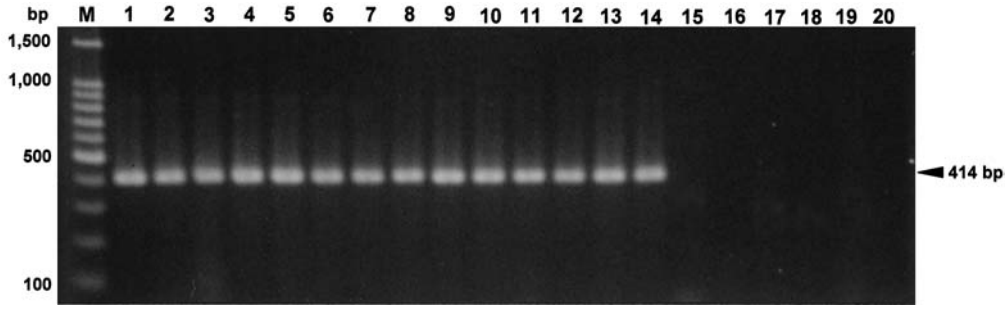

Fig. 2. Detection of Ti plasmids in *Agrobacterium* strains isolated from apple saplings by colony PCR using primer set VCF3/VCR3. Lane numbers represent bacterial strains as follows: *M*, 100-bp DNA ladder marker (Takara Bio); *1*, Peach CG8331 (control); *2*, Tsugaru-2-1; *3*, Tsugaru-3-3; *4*, Tsugaru-4-2; *5*, Tsugaru-5-3; *6*, Tsugaru-6-3; *7*, Tsugaru-8-2; *8*, Tsugaru-8-4; *9*, Kourin-1-2; *10*, Kourin-3-2; *11*, Kourin-5-2; *12*, Kourin-5-3; *13*, Kourin-8-2; *14*, Kourin-8-3; *15*, Tsugaru-1-1; *16*, Tsugaru-1-2; *17*, Tsugaru-1-3; *18*, Tsugaru-1-4; *19*, Tsugaru-2-4; and *20*, Kourin-6-4. Lanes *1* to *14* are tumorigenic strains; lanes *15* to *20* are nonpathogenic strains

following reasons. It is known that constituents of bacterial cells frequently interfere with reproducibility in colony PCR (Tsuchizaki et al. 2000). Although we performed PCR using purified bacterial DNA in our previous report, we added bacterial cells directly to the reaction mixtures in these experiments. Because the sizes of PCR products amplified by VCF3/VCR3 or VCF5/VCR5 were smaller than those amplified by VCF/VCR, the targeted DNA fragments were amplified more efficiently, evading interference by bacterial constituents. As mentioned, we believe that the primer set VCF3/VCR3 is more suitable for colony PCR than conventional VCF/VCR and can be used for large-scale diagnosis of crown gall disease on apple trees. For more rapid diagnosis, we have to put into practice methods for detecting tumorigenic *Agrobacterium* strains directly from apple trees, such as the BIO-PCR method (Schaad et al. 1995). Because we limited the target of diagnosis to tumorigenic *Agrobacterium* strains in this report, by using more rhizogenic strains we have to confirm whether these primer sets are suitable for detecting rhizogenic *Agrobacterium* strains.

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