A Rapid and Sensitive Method to Detect *Agrobacterium vitis* in Grapevine Cuttings Using the Polymerase Chain Reaction

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ABSTRACT

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Oligonucleotide primers were designed for amplification of the *virA*, *pehA*, and *6a* genes of *Agrobacterium* species and evaluated for their potential use in the identification of *Agrobacterium vitis*. In conjunction with the polymerase chain reaction, the *pehA*-specific primer pair readily identified all strains of *A. vitis* that were tested. Four different extraction/purification methods were tested for the recovery of *A. vitis* DNA from grapevine cuttings. A protocol which relied on exposure to microwaves for cell lysis, followed by purification of DNA by ion exchange chromatography was the most effective in removing, from grapevine samples, inhibitors of the polymerase chain reaction. The reported method was both sensitive and rapid, making it amenable to routine diagnostic testing. It was demonstrated that the lysis in planta of *A. vitis* and analysis of the recovered DNA by polymerase chain reaction provided a more reliable indication of the occurrence of the bacteria in actively growing grapevines than did recovery of viable bacteria and growth on selective media.

Crown gall of grapevine is a serious disease of *Vitis vinifera* L. in most commercial production areas of the world. the disease is caused by infection with *Agrobacterium vitis*, previously referred to as *Agrobacterium tumefaciens* biovar 3 (22). The pathogen is often transmitted through vegetative propagation of grapevine, and the infected plants remain symptomless until frost or physical damage initiates the disease (7,8). Existing programs that screen for potential *A. vitis* infections rely on methods that involve plating samples on selective media (5,28) and identification of bacteria by colony morphology. More recently, methods became available to improve the identification of tumorigenic isolates of *A. vitis* on culture plates by augmenting the screening procedure with serological methods (4) or polymerase chain reaction (PCR) (11). The need to propagate the bacteria in selective plate cultures before testing by these methods seriously retards the rate at which diagnosis can be confirmed.

The objective of this study was to develop a sensitive and cost effective method for rapid detection of *A. vitis* in vine cuttings. This material contains relatively low levels of *A. vitis* that possess a tumor-inducing (Ti) plasmid required for tumorigenicity (9). PCR, a very sensitive method for detecting specific DNA sequences (29), has potential for the detection of tumorigenic bacteria and has previously been demonstrated effective in identifying tumorigenic strains of *A. vitis* grown on selective media (11). However, the presence of contaminating materials in DNA isolated from grapevine samples inhibited the amplification reaction of PCR, rendering the method ineffectual for direct sampling from vines.

A method that provides both a high recovery of bacterial DNA and a low recovery of PCR-inhibiting compounds is desirable. This study examines several extraction methods for the isolation of *A. vitis* DNA and the utilization of the resulting sample in PCR analysis.

MATERIALS AND METHODS

Samples of *Agrobacterium*. Control strains were selected to represent a wide variety of *Agrobacterium* species and Ti plasmid types; 13 strains of *Agrobacterium* spp. (Table 1) were generously supplied by Tom Burr, New York State Agricultural Experiment Station, Geneva. Bacteria were plated on Roy and Sasser (RS) selective medium (28) as modified by Pu and Goodman (24), then cultured at 27°C. Tumorigenicity of *A. vitis* strains was confirmed by inoculation and subsequent gall formation on the stems and/or leaves of Kalanchoë (*Bryophyllum diagremontianum* (Hamet & Perr)) (23).

Grapevine cuttings were collected from four vineyards in the Okanagan Valley of British Columbia; each vineyard had a history of crown gall disease. Xylem flow had commenced when samples were collected in April 1994, but buds had not opened. Cuttings were collected from 1-year-old wood adjacent to the main trunk as close to soil line as possible, typically 0.5 m above the ground. Green vine cuttings were collected from actively growing canes in July 1994. In each case, the samples were immediately placed at 4°C and stored until used.

PCR reactions and analysis. Each PCR (29) reaction contained the following in a final volume of 25 µl: 10 mM KCl, 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.2 mM each dNTP, 1 mM each oligonucleotide primer, and 0.25 U Vent Polymerase (New England Biolabs, Beverly, MA). When added to the PCR reactions, the final concentration of acetylated-bovine serum albumin (BSA) (Bethesda Research Laboratories Inc., Gaithersburg, MD) was 0.1 mg/ml. The amount of target DNA added to the PCR varied. Reactions were overlaid with 30 µl of mineral oil. All manipulations were performed with aerosol-resistant tips (Rainin Instrument, Woburn, MA); each set of reactions included both negative and positive controls.

PCR reactions were performed using a thermocycling block (PHC-2; Techne, Princeton, NJ) programmed with an initial incubation at 96°C for 2 min followed by 40 cycles as follows: 94°C for 1 min, 50°C for 30 sec, 72°C for 30 sec. At the completion of the final cycle, the reaction was held at 72°C for 5 min followed by cooling to 19°C.

From each 25-µl PCR reaction, 10 µl was analyzed by agarose gel electrophoresis and ethidium bromide staining. A 1-kbp DNA ladder (Bethesda Research

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Laboratories) was included on each gel as a DNA molecular weight marker. Samples were run for 1 to 2 h on a 1% agarose (HGT; FMC BioProducts, Rockland, ME) in 0.5 × TBE (1 × TBE = 90 mM Tris-borate, pH 8.0, and 2 mM EDTA) at 60 V, stained for 10 min with ethidium bromide (1.0 µg/ml), and destained for 30 min in distilled water. Bands were visualized with a UV transilluminator and photographed.

Selection of PCR primer sequences. Two sets of primers were designed that would specifically amplify genes borne on the Ti plasmid of either the wide or the limited host range phenotypes of *Agrobacterium* spp. (3). One set of primers specific for the *virA* region was developed based on comparison of sequence information derived from the limited host range Ti plasmid pTiAg162 and the wide host range Ti plasmid pTiA6 (17). The *virA* primers, (TTCAGTCGCGCAAGCAGTT) and (CGGCAATTCGTATCACGGA), delineated a sequence of 480 bp that was specific for the *virA* region of the limited host range Ti plasmids frequently associated with *A. vitis*.

Primers specific for the 6a gene of pTi15955 (2) were produced. The primers (ACATCATCAATCCTGGCGG) and (ACTCGTTGCTGGCCTGAAT) allow amplification of a region of 260 bp characteristically found only in *A. tumefaciens* biovar 1. All octopine/cucumopine strains of *A. vitis* share a deletion in the TA 6a gene (12) that eliminates this target sequence from the Ti plasmid.

Polygalacturonase, a pectic enzyme produced by *A. vitis*, has been shown to be responsible for both root decay and pectolytic activity, and is readily produced in the absence of a Ti plasmid (6,19). Plasmid pCPP2068 (27) containing the pectin enzyme hydrolase (*pehA*) gene of *A. vitis* strain CG49 was kindly supplied by Alan Collmer (Cornell University, Ithaca, NY). The clone was partially sequenced by dideoxy-chain termination (31). From this sequence information, primers (CGATGGCGGCGAGGATTT) and (ATCGGGCGTGAAACAAGT) were designed to allow amplification of 199 bp from the coding region. Genbank and EMBL databases were searched with the primer sequences using the blast algorithms (1,14) to ensure that sequences of other reported bacterial and plant polygalacturonase genes would not be targets for the *pehA* primers.

All primers were synthesized by UCDNA Services, University of Calgary, Alberta, Canada.

Test of primer amplification on laboratory stocks. DNA was isolated from *Agrobacterium* reference strains using a rapid bacterial lysis method (21). A single colony from a streak of *Agrobacterium* on potato-dextrose agar (PDA) plates was touched to the bottom of a 1.5-ml microcentrifuge tube with a sterile wooden toothpick. The bacteria were resuspended in 20 µl of 0.1% (vol/vol) Triton X-100, lysed by the addition of 5 µl of 0.4 N NaOH followed by heating in a boiling water bath for 1 min, then cooled on ice. To neutralize the solution, 5 µl of 1 M Tris-HCl, pH 7.6, was added. One µl of the crude suspension was used as DNA template for each of the subsequent PCR reactions.

Sample preparation from grapevines. Vines were freshly cut to produce an internodal segment of cane approximately 5 cm in length; the segments ranged from 3 to 7 mm in diameter. A short section of tygon tubing was attached to one end of the cane, and the overlap between the cane and the tubing was wrapped with Parafilm to prevent leakage. A 1-ml syringe was snugly fitted into the tygon tubing, and approximately 200 µl of the specified lysis buffer (see below) was slowly injected into each segment until drops of buffer appeared at the opposite end of the cutting. The syringe was detached and the cutting was enclosed in a polypropylene culture tube, capped, and microwaved for 1 min at 180 W followed by cooling on ice for 5 min. This microwaving–cooling procedure was repeated twice more. The Parafilm was replaced and another 250 µl of lysis buffer was forced through the vine cutting; the exudate was collected in a sterile 1.5-ml microcentrifuge tube.

During assessment of the sample preparation methods, the exudates from six samples were pooled prior to DNA extraction to eliminate any potential variation among individual vine cuttings. Trials were repeated at least four times to include grapevines at varying stages of maturity and canes from different cultivars. The internal standard used to evaluate the methods was the Ti plasmid from the laboratory stock of *A. vitis* strain CG49 isolated by alkaline lysis and column purification (Magic Mini-Prep, Promega, Madison, WI) following the manufacturers recommendations. The resulting DNA concentration was determined by measuring the optical density at 260 nm.

DNA isolation methods. *Method 1 (modified Manning method).* Five cuttings were infused with lysis buffer 1 (0.2 M boric acid, 10 mM EDTA; 40 mM Tris, pH 9.6) containing 0.5% sodium dodecyl sulfate (SDS) and 0.28 M 2-mercaptoethanol (18). Cuttings were microwaved and the exudate was collected as described above. The pooled exudate (250 µl) was extracted with phenol–chloroform and the nucleic acids were recovered from the aqueous phase by differential precipitation with 2-butoxyethanol (18). The precipitated nucleic acid pellet was washed with a 1:1 (vol/vol) mixture of 2-butoxyethanol and lysis buffer to remove traces of polyphenolics. The pellet was washed a second time with 70% ethanol, dried under vacuum, and resuspended in 10 µl of sterile distilled water.

Method 2 (column purification). Five cuttings were injected with lysis buffer 2 (40 mM Tris-HCl, pH 7.4; 1 mM EDTA) to which 5% (vol/vol) Triton X-100 had been added. Cuttings were microwaved and the exudate was collected as described above. An aliquot (250 µl) of the pooled exudate was loaded directly onto a QIAGEN tip-20 (Qiagen Inc., Chatsworth, CA). Total nucleic acids were recovered following the manufacturer's directions. To the resulting eluent was added 1/10 vol of 3 M sodium acetate, pH 6, 2.5 µg of polyacrylamide carrier (13), and 3 volumes of 95% ethanol. Samples were incubated for 30 min on dry ice and the precipitate was collected by centrifugation at 13,000 × *g* for 30 min at 4°C. Pellets were washed with 70% ethanol, dried under vacuum, and resuspended in 10 µl of sterile distilled water.

Table 1. The stock cultures of *Agrobacterium* strains used in this study and their responses in the polymerase chain reaction with various primer sets

				Amplification results with PCR primers[a]		
Strain	**Biovar**	**Original host**	**Opine type**	***virA***	***6a***	***pehA***
CG47	3	Grapevine	Nopaline	+	–	+
CG49	3	Grapevine	Nopaline	+	–	+
CG60	3	Grapevine	N/A[b]	+	–	+
CG1005	3	Grapevine	Octopine	+	–	+
NW161	3	Grapevine	Vitopine	+	–	+
NW165	3	Grapevine	Nopaline	+	–	+
NW180	3	Grapevine	Octopine	+	–	+
Sz1	3	Grapevine	Vitopine	–	–	+
R3	2	Rose	N/A	–	–	–
R10	2	Rose	N/A	–	–	–
C58	1	Cherry	Nopaline	–	–	–
R6	1	Rose	N/A	–	+	–
FACH	1	Grapevine	N/A	–	+	–

[a] A crude DNA pellet was prepared by bacterial lysis from colonies growing on a PDA plate. After amplification, the PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. The results were evaluated to determine if the reaction yielded products of the size predicted by available sequence data.

[b] N/A = data not available.

Method 3 (direct precipitation). Five cuttings were filled with lysis buffer 2 (40 mM Tris-HCl, pH 7.4; 1 mM EDTA) containing 5% (wt/vol) SDS. Cuttings were microwaved and the exudate was collected as described above. To 250 µl of pooled exudate, 5 M potassium acetate was added dropwise to a final concentration of 0.3 M. The mixture was centrifuged at room temperature for 15 min to remove precipitated potassium dodecylsulfate. Nucleic acids were precipitated from the supernate by the addition of 1/10 volumes of 3 M sodium acetate, pH 6; 2.5 µg of polyacrylamide carrier, and 3 volumes of 95% ethanol followed by incubation on dry ice for 30 min. The precipitate was collected by centrifugation at 13,000 × *g* for 30 min at 4°C. Pellets were washed with 70% ethanol, dried under vacuum, and resuspended in 10 µl of sterile distilled water.

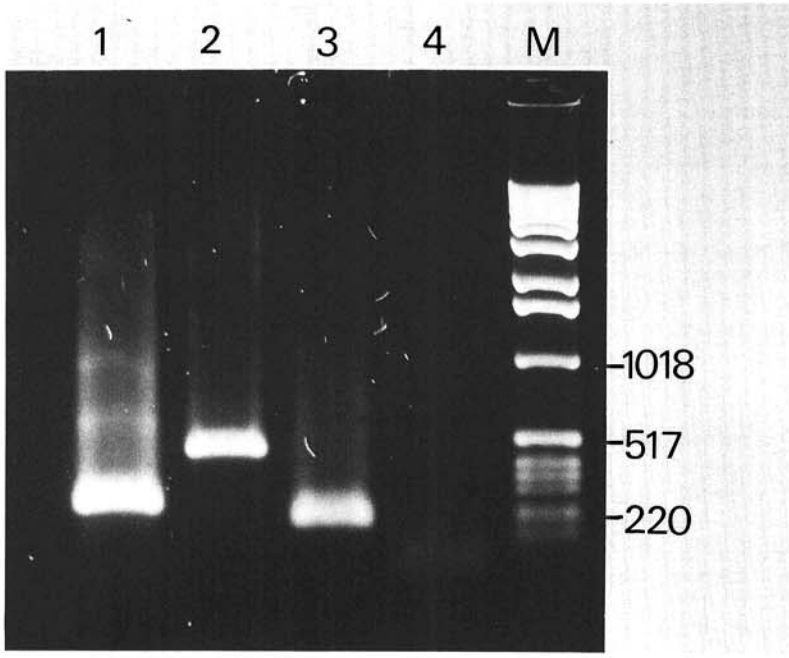

Fig. 1. Reaction of DNA from various *Agrobacterium* strains by polymerase chain reaction (PCR) with various primer pairs. DNA is isolated by bacterial lysis from colonies of *Agrobacterium* growing on potato-dextrose agar plates. Each PCR reaction contains, in a final volume of 25 µl, a different primer pair. Ten µl of the amplification reaction is analyzed by agarose gel electrophoresis. Lane 1: strain FACH amplified with primer pair *6a*; lane 2: isolate CG49 amplified with primer pair *virA*; lane 3: strain CG49 amplified with primer pair *pehA*; lane 4: negative control reaction for *virA* primer reactions (no template); lane M: molecular weight marker (1-kbp ladder).

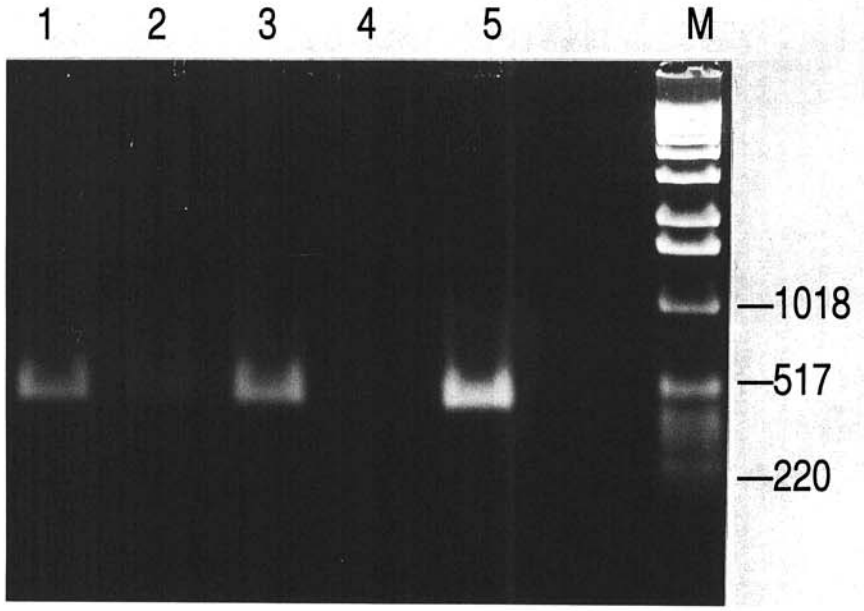

Fig. 2. The effects of the products of various DNA isolation methods on the efficiency of the polymerase chain reaction (PCR) using *virA*-specific primers. To each PCR reaction in a final volume of 25 µl, 0.2 pg Ti plasmid DNA isolated from *Agrobacterium vitis*, strain CG49 is added plus 3 µl of sample, prepared by different methods, from grapevines. Ten µl is analyzed by agarose gel electrophoresis. Lane 1: control DNA (no added grapevine sample); lane 2: control DNA plus DNA from vine exudate prepared by the modified Manning method; lane 3: control DNA plus DNA from vine exudate prepared by column purification; lane 4: control DNA plus DNA from vine exudate prepared by direct precipitation method; lane 5: control DNA plus DNA from overnight cultures prepared by bacterial lysis; lane M: molecular weight marker (1-kbp ladder).

Method 4 (bacterial culture method). A simple cell culture method for the detection of *Agrobacterium* was also tested. A 1-ml syringe was attached by a segment of tygon tubing to one end of the cutting, and the cutting was flushed with water (32) or phosphate-buffered saline. The collected wash, approximately 1 ml, was added to 5 ml of modified RS liquid medium (24) and incubated with shaking at 27°C. Bacteria were pelleted by centrifugation and lysed using the simple bacterial lysis method outlined above for samples from the bacterial stock cultures.

To inoculate cuttings, they were injected with approximately 1×10^2 CFU of *A. vitis* strain CG49 suspended in phosphate buffered saline. The concentration of bacteria was confirmed by dilution plating on agar plates. The vines were incubated overnight at 4°C after inoculation.

RESULTS

Primer evaluation. PCR primer pairs were tested for their ability to facilitate the amplification of specific products from laboratory stocks using the conditions stated above. PCR with the *virA*-specific primers yielded amplification products when the target DNA was prepared from *Agrobacterium* strains CG47, CG49, CG1005, NW161, NW165, and NW180 (Table 1). In all cases, upon electrophoretic analysis, the amplification product appeared as a single, intense band of 480 bp (Fig. 1), the predicted size of the product based on the sequence of pTiAg162 (17). However, PCR with the *virA*-specific primers did not produce an amplification product with *A. vitis* strain Sz1. Amplification with the *virA*-specific primers was exclusive to biovar 3 strains.

The primers specific for the *6a* gene of wide host range Ti plasmids allowed amplification of a single, intense band when DNA from strains R6 or FACH were used as a template. The band was consistent in size with the predicted amplification product of 260 bp. The *6a*-specific primers did not yield a band with strain C58 DNA as template. No amplification products were produced with biovars 2 or 3.

In all instances where DNA from *A. vitis* strains were utilized as template, the use of the *pehA*-specific primers resulted in the amplification of a 199-bp product predicted by the nucleotide sequence derived from clone pCPP2068. No amplification with strains representing other biovars occurred.

To further test the specificity of the *pehA* primers, bacteria were recovered from the vine exudates and cultured on PDA plates. Individual colonies were then transferred to media plates selective for each of the biovars of *Agrobacterium* (5) and to King's B medium (30). Identification of *A. vitis* was determined by growth on these selective media and by colony morphology. Based on these criteria, more than 99% of the bacteria isolated from grapevine vascular fluids were not *A. vitis*. When assayed by PCR with the *pehA*-specific primer pair, no amplification occurred with bacterial colonies that were not identified as *A. vitis*.

PCR sensitivity and sample preparation methods. In preliminary trials, it was found that the addition of acetylated-BSA to the PCR reaction mixture was essential for successful amplification of DNA from grapevine samples; acetylated-BSA was added to all subsequent reactions.

The relative efficiencies of DNA recovery achieved with each of the sample preparation methods were determined. Crude grapevine exudates prepared for the modified Manning, column, or direct precipitation methods were amended with 500 ng of Ti plasmid DNA from *A. vitis* strain CG49 per ml; the isolation procedures were then completed as described. The recovered DNA was analyzed by PCR with *virA*-specific primers. The modified Manning and column purification methods were the most reliable, yielding easily recognizable bands after electrophoresis (data not shown). Amplification of the DNA isolated by the direct precipitation method occurred sporadically, even at the high concentration of target DNA used in these initial trials.

To differentiate between effects of DNA recovery and inhibitor removal by the sample preparation methods, comparisons were performed to establish the maximum amount of purified sample that could be added to a PCR reaction mixture with successful amplification of the target. Samples from grapevine were prepared by either the modified Manning, column purification, or direct precipitation method. To a constant amount of 0.2 pg per reaction of Ti plasmid DNA from *A. vitis* strain CG49, between 0.1 and 3 µl of the purified sample from uninfected vines was added. The column purification method consistently permitted production of an intense amplification product equal in intensity to amplification reactions in the absence of inhibitors (Fig. 2). This level of amplification occurred regardless of the amount of purified exudate added, up to the maximum volume tested. Samples prepared by the modified Manning method also yielded a product, but the intensity of the band was greatly reduced compared to reactions in the absence of inhibitors, even at the lowest sample volumes tested (0.1 µl in a 25-µl reaction). Samples prepared by direct precipitation severely inhibited amplification of the target DNA sequence.

A bacterial culture method was evaluated (method 4). Preliminary trials revealed that incubation periods beyond 10 h had no effect on subsequent DNA isolation or PCR analysis; therefore, a standard overnight incubation of approximately 16 h was used. Bacteria recovered from cuttings that had been inoculated with 1×10^2 CFU were cultured overnight, and the DNA was subjected to PCR analysis. The quantity of the resulting amplification product was typically equal to or greater than that produced by PCR of the control DNA (Fig. 2).

Detection by PCR of *A. vitis* in field-collected samples. The detection of *A. vitis* in cuttings from vineyards was tested by the column purification method with subsequent amplification with the *pehA* primers. A total of 15 vines sampled had visible galls; the PCR analysis of each of these plants yielded a positive reaction (Table 2). In addition, three samples from 14 nonsymptomatic vines also yielded positive results. Overall, positive responses were detected in three out of four varieties, suggesting that varietal differences would not interfere with the PCR reaction. The PCR analysis was repeated with at least three different cane segments, and for each vine sample assayed, all three samples yielded the same results. Amplification of samples from green vines collected in midseason yielded products identical in intensity and frequency to those from samples collected in early spring (data not shown).

To evaluate the effect of in situ lysis on PCR of *A. vitis*, five cuttings were flushed with water (32), and the exudate was used to inoculate modified RS liquid medium. After overnight incubation, DNA was recovered from the cultures by bacterial lysis and used for PCR with *virA* primers; none of the samples yielded amplification products (data not shown). The same pieces of vine were subsequently injected with lysis buffer, microwaved, and the DNA isolated by column purification as described above. In four out of five samples, amplification of the DNA using *virA*-specific primers resulted in the appearance of the bands expected for *A. vitis* (Fig. 3). Amplification levels were similar to those produced with 0.2 pg of Ti plasmid DNA or approximately 10^3 copies per reaction, and the amplification products could be readily identified by agarose gel electrophoresis.

Table 2. Identification by polymerase chain reaction with *pehA*-specific primers of *Agrobacterium vitis* in cuttings from grapevines grown in the Okanagan Valley of British Columbia

	Vines		
Variety	**Sampled**	**Galls**	**Positive**
Bacchus	8	0	3
Heroldrebe	1	1	1
Merlot	6	0	0
Gewurztraminer	14	14	14

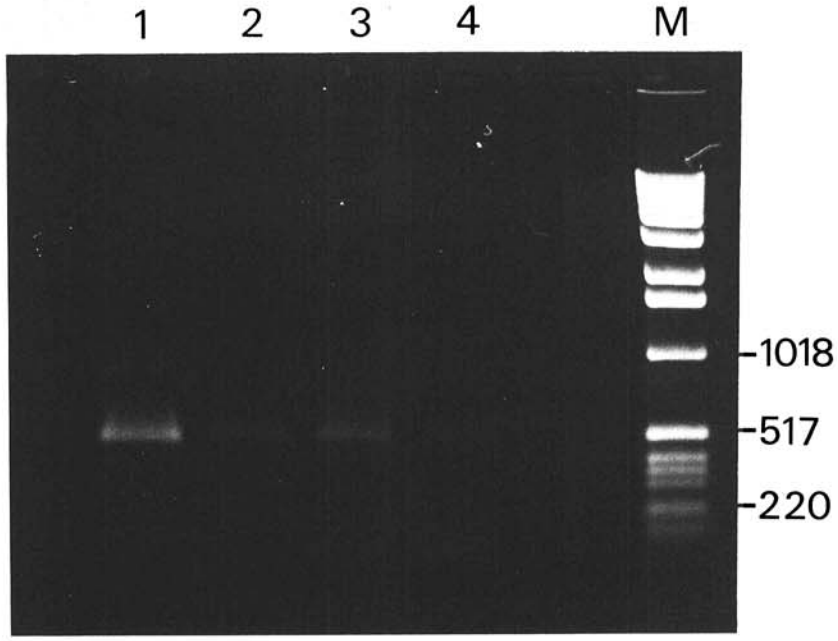

Fig. 3. Gel analysis of amplification products from samples collected from vineyards with a high incidence of crown gall. Vine cuttings were flushed with distilled water, and the DNA remaining in the vascular system was collected by injecting detergent into the cutting, subjecting the cutting to microwaves, flushing the sample again with lysis buffer and purifying the DNA with a QIAGEN tip-20. The resulting DNA was amplified using primers specific for the *virA* region of the Ti plasmid. Lane 1: control reaction containing 0.2 pg Ti plasmid DNA isolated from *Agrobacterium vitis* strain CG49; lanes 2, 3, 4: amplification products from three different field samples; lane M: molecular weight marker (1-kbp ladder).

DISCUSSION

The objective of this study was to develop a rapid and sensitive method for the detection of *A. vitis* in grapevines. PCR provides an extremely sensitive diagnostic tool for detection of specific DNA sequences; however, amplification directly from vine exudate has been problematic due to the presence of polyphenolics and carbohydrate polymers (26) and because vine cuttings can be expected to contain relatively low numbers of *A. vitis* (9,24).

The selective propagation of the *A. vitis* population through overnight culture followed by lysis of the resulting bacterial pellet yielded excellent separation of the target DNA from grapevine inhibitors of PCR. Amplification of the target sequence occurred both through bacterial growth during incubation and through subsequent PCR. However, in our trials, the overnight culture failed to yield amplifiable DNA from the same cane segments that were shown to contain target DNA by lysis in situ and column purification of the released DNA. Favorable attributes of the overnight culture and subsequent bacterial lysis procedure, such as ease of execution and high level of signal amplification, are negated by the inefficient recovery of viable *A. vitis* from grapevines. It has previously been shown that *A. vitis* with Ti plasmids attach to grape cells more readily than those without Ti plasmids (25), and consequently, would defy quantitative elution from vine cuttings with water.

Our results indicate that in planta lysis of *A. vitis*, subsequent column purification of the DNA from grapevine exudates, and specific amplification of pathogen-specific sequences by PCR offer an extremely sensitive method for detection. Lysis of bacteria in situ with detergents and microwaves to release the target DNA avoids the need to isolate intact cells. The subsequent purification through the QIAGEN tip-20 eliminated virtually all inhibitory compounds from grapevine exudates, enabling detection of 0.2 pg of *A. vitis* Ti plasmid DNA. The binding of DNA to the ion exchange media and its subsequent batch elution concentrates the target DNA from larger initial sample volumes with no dilution of the final sample. Therefore, longer vine segments or multiple segments can be used to increase the initial sample size and the subsequent reliability of the overall procedure. Using the column purification method, the entire analysis can be completed in one day, and the minimal number of manipulations reduces the possibilities of cross contamination between samples. Other methods are available for the preparation of DNA from grapevine tissue for the detection of pathogens by PCR (20); however, the column purification method requires fewer transfers and less time; and therefore it is amenable to routine diagnostic testing.

Of the two target regions used for detection of *A. vitis* by PCR analysis, the *pehA*-specific primers offer advantages over the *virA*-specific primers. Since the *pehA* gene is chromosomally located, it is more ubiquitous in the *A. vitis* population than the Ti plasmid, a mobile element. Moreover, it has been reported that the Ti plasmid of *A. vitis* is highly unstable (12), increasing the possibility of further recombination events that may alter the target sequence located on the Ti plasmid. Anomalies in the DNA sequence of Ti plasmid from strain Sz1 have been reported (23) and may suggest an explanation for the failure of the *virA* primer pair to perform as expected. The *pehA* gene of *A. vitis* confers the ability to induce root decay of *V. vinifera* (19), even in the absence of a Ti plasmid. Thus, pathogenicity can be expressed in the absence of tumorigenicity (6), and a detection method that targets the Ti plasmid of *A. vitis* may fail to detect a potentially pathogenic strain. Therefore, the *pehA* region offers a more constant chromosomal target that provides detection of both tumorigenic and pathogenic bacteria. Most plant pathogenic bacteria achieve tissue maceration with pectic lyases, while some, such as *Erwinia carotovora*, employ polygalacturonase activity (10,19). GenBank and EMBL databases were searched for potential primer recognition sites in all reported polygalacturonase genes, including the sequences of *E. carotovora* genes (15,16). No significant matches to the *pehA* primer pair were found. It is interesting to note that strain FACH, a biovar 1 strain originally isolated from grapevine, fails to sustain amplification of a target with the *pehA*-specific primer pair (Table 1). This suggests that the presence or absence of the *pehA* gene is not necessary for successful colonization and gall-induction on Kalanchoë by this isolate. Although aggressive on Kalanchoë, FACH is inefficient relative to biovar 3 strains in initiating and sustaining tumor growth on grape cultivar LN33 (K. C. Eastwell, *unpublished*).

Grapes are an important crop worldwide. The ability to detect important grapevine pathogens such as crown gall is critical in order to develop and maintain stocks free of *A. vitis*. The development of certified grapevine stocks will improve crop yields and ultimately increase the viability of grape production worldwide. The methods described will enhance these efforts to propagate nursery stock free of *A. vitis*.

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