Development of a Magnetic Capture Hybridization Real-Time PCR Assay for Detection of Tumorigenic *Agrobacterium vitis* in Grapevines

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ABSTRACT

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Agrobacterium vitis, the causal agent of grape crown gall, can have severe economic effects on grape production. The bacterium survives systemically in vines and, therefore, is disseminated in propagation material. We developed an assay for use in indexing programs that is efficient and sensitive for detecting *A. vitis* in grape tissue. Initially, real-time polymerase chain reaction (PCR) primers specific for diverse tumorigenic strains of *A. vitis* were developed using the *virD*2 gene sequence. To overcome the effects of PCR inhibitors present in plant tissue, DNA extraction methods that included magnetic capture hybridization (MCH), immunomagnetic separation (IMS), and extraction with the Mo Bio Powerfood kit were compared. The assays incorporating MCH or IMS followed by real-time PCR were 10,000-fold more sensitive than direct real-time PCR when tested using boiled bacterial cell suspensions, with detection thresholds of 10^1 CFU/ml compared with 10^5 CFU/ml. DNA extraction with the Powerfood DNA extraction kit was 10-fold more sensitive than direct real-time PCR, with a detection threshold of 10^4 CFU/ml. All three assays were able to detect *A. vitis* in healthy-appearing grapevine cuttings taken from infected vines.

Agrobacterium vitis (26), formerly *A. tumefaciens* biovar 3 (22), is the causal agent of crown gall of grapevines and a limiting factor in grape production worldwide. In addition to causing crown gall, *A. vitis* is capable of causing a root necrosis that is specific to grapevine (10). *A. vitis* infections are induced at injury sites such as those caused by debudding, grafting, and freeze events. Management strategies for grape crown gall include planting resistant rootstock and scion varieties; however, the majority of *Vitis vinifera* varieties that have been tested are susceptible (9). Other management strategies include planting in field sites with no history of crown gall and the use of planting material free of the pathogen. *A. vitis* persists systemically in vines; therefore, whereas nursery stock may seem noninfected, cuttings may carry the pathogen (12). Once present in a field, the bacterium can persist in living and dead grape tissue in the soil for years, acting as a source of inoculum for new plants (12). Therefore, the use of clean planting material in areas with no history of the disease is the best option for control of this disease.

Indexing of grape propagation material for *A. vitis* has been severely limited by the assays that are very time consuming and lacking in sensitivity. One method our laboratory has used involves callusing of dormant cuttings followed by isolation of bacteria from the callus with the aid of semiselective culture media (23). Flushing grapevines with water followed by isolation on selective media and pathogenicity tests have also been attempted (33). Unfortunately, these methods can take 6 weeks or more for completion, and non-target bacteria may mask the presence of *A. vitis*. Polymerase chain reaction (PCR) assays have become increasingly more common for the detection of *A. vitis*, offering improved sensitivity and specificity (14,29). Primers must be able to distinguish between tumorigenic and nontumorigenic strains. The development of specific primers to address this issue targeted pathogenicity genes present on the Ti plasmid (6,7,28). The limitations found with some of these primer sets is the inability to detect a wide range of *A. vitis* strains isolated from different geographic regions. Hence, one of our goals was to develop a primer set specific for detection of a broad range of tumorigenic *A. vitis* strains.

Real-time PCR represents a sensitive and fast approach for pathogen detection, with the advantage of being more sensitive and less time consuming than conventional PCR by not requiring further analysis such as gel electrophoresis. However, real-time PCR is still subjected to the limitations associated with inhibitor compounds in plant tissue and by the requirement for a threshold level of target nucleic acid. To reduce the effects of PCR inhibitors, we initially employed the Powerfood kit (Mo Bio Laboratories Inc., Carlsbad, CA) for DNA isolation from samples followed by conventional PCR for detection of tumorigenic strains of *A. vitis*. This method extracts DNA from all bacteria present in the sample and, therefore, target DNA can be diluted by DNA from non-target bacteria, which may result in false negatives. To address these issues, we tested enrichment of target nucleic acid via magnetic capture hybridization (MCH) and immunomagnetic separation (IMS) followed by real-time PCR. MCH uses a biotinylated oligonucleotide probe, specific for the target sequence, conjugated to streptavidin-coated paramagnetic beads. Incubation of the bead/capture probe complex with a heterogeneous mix of nucleic acids allows target nucleic acid to anneal to the complementary sequences of the capture probe (21). A magnetic force is then applied to concentrate the bead-target DNA complex, and inhibitors and non-target DNA are rinsed away (21). IMS uses antibodies specific for the target bacterial cell that are bound to paramagnetic beads to concentrate target bacteria (35). The bacteria are isolated by magnetic force, and non-target cells and PCR inhibitors can then be washed away. Template DNA is released from bacterial cells for use in real-time PCR. MCH and IMS have been successfully used to enrich target nucleic acid for

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detection of pathogens in many research fields such as food microbiology, plant pathology, and soil microbiology (1,15,17, 19,34). Both methods have the potential to significantly improve the efficiency and sensitivity of grapevine indexing for *A. vitis*. The objective of this study was to compare technologies in the development of an efficient and sensitive method for the detection of *A. vitis* in grapevines and to implement assays on grapevine cuttings naturally infected with crown gall.

MATERIALS AND METHODS

Development of real-time PCR primers for detection of tumorigenic *A. vitis*. It was previously shown that primers derived from the *virD2* gene of the Ti plasmid are able to amplify a product from a high proportion of *A. vitis* strains (6). To improve on published *virD2* primers, the conserved 5′ region of *virD*2 from seven *A. vitis* strains was amplified. Two primers, virD2-F and virD2-R, were designed based on the DNA sequence of the *virD*2 gene from *A. vitis* S4 (avi8212) (Table 1). The PCR reaction mixture contained 1 µl of DNA, 1 µl of forward primer virD2-F (1 pM), 1 µl of reverse primer virD2-R (1 pM),1 µl of dNTP (10 mM each), 2.5 µl of 10× reaction buffer, and 2 units of Taq DNA polymerase (New England Biolabs, Ipswich MA) in a total volume of 25 µl. The PCR reaction was conducted using the following program: initial denaturing at 94°C for 2 min; then, 35 cycles of denaturing at 94°C for 15 s, annealing at 52°C for 20 s, and extension at 72°C for 60 s; and final extension at 72°C for 5 min. The PCR products were purified and ligated to pGEM-T easy vector (Promega Corp., Madison, WI). The ligation mixture was transformed into *Escherichia coli* JM109 and selected on Luria-Bertani agar plates supplemented with ampicillin (100 µg/ml). Plasmid DNA (pGEM harboring PCR product) was purified and sequenced at Cornell University Life Science Core Laboratories Center. The DNA sequences were analyzed with the software DNAStar Lasergene 10.1 (Madison, WI). Four primer pairs were designed within the sequenced fragments using the Integrated DNA Technologies PrimerQuest software (www.idtdna.com). The specificity of the primers in conventional PCR was tested using 27 *A. vitis* strains isolated from different geographic regions in the United States and internationally. Bacterial cells were collected from potato dextrose agar plates and suspended in 100 µl of sterile high-performance liquid chromatography (HPLC) water. Cells were boiled for 10 min to lyse the bacterial cells. Cell debris was pelleted by centrifugation at 13,000 rpm for 2 min. Lysate (2 µl) was subsequently used in PCR. Based on the PCR results, the primer pair that amplified a characteristic product from diverse tumorigenic *A. vitis* was chosen.

Sensitivity and specificity of an *A. vitis* real-time PCR assay. A real-time SYBR green PCR assay was developed for *A. vitis* that would distinguish between tumorigenic and nontumorigenic strains. Primer efficiency of the *A. vitis* real-time PCR assay was determined on 10-fold serial dilutions of *A. vitis* strain S4 DNA extracted using the Ultra Clean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc.). The concentration of nucleic acid used was 100 to 0.0001 ng/µl. The real-time PCR reaction contained 12.5 µl of SYBR Green mix (Bio-Rad, Hercules, CA) and 400 nM virD2.for1 and virD2.rev1 in a total of 25 µl. PCR conditions were as follows: denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 10 s, and extension at 72°C for 60 s. All reactions were conducted in a Bio-Rad CFX1000 real-time PCR machine. The experiment was repeated three times and mean cycle threshold (Ct) values and detection frequencies were recorded. Mean Ct values were plotted against $\log_{10}$ *A. vitis* DNA dilutions and used to generate a standard curve. Linear regression analysis was conducted and the slope of the relationship was used to determine the amplification efficiency of the *A. vitis* real-time PCR assay using the equation efficiency (%) = $10^{[-1/\text{slope}]} - 1 \times 100$ (3,16). To determine the specificity of the assay, the cell suspensions of 27 *A. vitis* strains (inclusive of tumorigenic and nontumorigenic strains), 4 *A. tumefaciens* strains, and 3 *A. rhizogenes* strains isolated from different hosts were tested, as mentioned above. In addition 12 unknown bacteria, isolated from enrichment broth inoculated with grapevine tissue, and *Xylella fastidiosa*, a pathogenic bacterium on grapevines, were also tested.

Extraction and enrichment of *A. vitis* DNA. The efficacy of MCH, IMS, and Powerfood DNA extraction methods followed by real-time PCR was compared with direct real-time PCR for detection of *A. vitis*. The methods used are described below.

Cell suspension preparation. Serial dilutions (10-fold) of *A. vitis* strain S4 cells of ≈1×10^8 to 1×10^1 CFU/ml were prepared in 1× phosphate buffered saline (PBS) solution (137 mM NaCl, 5.6 mM Na_2HPO_4, 2.7 mM KCl, and 1.5 mM KH_2PO_4). The suspensions were used to determine the sensitivity of each assay. For direct real-time PCR, bacterial cells were pelleted by centrifugation at 13,000 rpm for 3 min and suspended in 1 ml of water. Suspended cells were boiled for 10 min and 2 µl was used in direct real-time PCR. To determine the specificity of the MCH and IMS assays, the *A. vitis, A. tumefaciens*, and *A. rhizogenes* strains mentioned previously were used. Suspensions (1×10^8 CFU/ml) of the different strains were made in 1× PBS and suspensions were used in MCH or IMS as mentioned previously.

MCH. To facilitate MCH, a 74-bp capture probe was designed based on nucleotide sequence of the regions flanking the real-time PCR target sequence of *virD2*. The capture probe, VITIS, was modified at the 5′ end with biotin and triethylene glycol (www.idtdna.com) to allow attachment to streptavidin-coated magnetic beads (New England BioLabs). Briefly, beads (20 µl/sample) were washed with binding washing (BW) buffer (10 mM Tris-HCL, 1 mM EDTA, and 100 mM NaCl) to remove the storage solution (21). The beads were then suspended in 1 ml of BW buffer and 2 ng of probe was added per sample. The beads were incubated at 25°C for 1 h with agitation in a hybridization oven. The beads were collected and washed three times with BW buffer before being chemically denatured with denaturation (DN) solution (0.125 M NaOH and 0.1 M NaCl) for 15 min at 25°C. Conjugated beads were again washed three times with 1 ml of BW before being suspended in the original volume of BW buffer. Each 10-fold serial dilution of *A. vitis* strain S4 (1 ml) was pelleted and cells were suspended in 1 ml of Dig Easy Hybridization buffer (Roche, Indianapolis, IN) with 250 µl of 0.1-mm glass beads (BioSpec Products, Bartlesville, OK). Samples were mechanically lysed for 10 min by bead-beating on a vortex adapted with a Mo Bio tube holder. Beads and cell debris were pelleted

TABLE 1. *Agrobacterium vitis*-specific oligonucleotides used for magnetic capture hybridization and real-time polymerase chain reaction

Primer	Sequence (5′→3′)[a]	Function	Reference
virD2-F	CAA CTC ATC ATC CGC ATT GTG CC	*A. vitis* forward sequencing primer	This study
virD2-R	TCGATGAAGTCGTTGCTGAAC	*A. vitis* reverse sequencing primer	This study
virD2.For1	TTG GAA TAT CTG TCC CGG AAG	*A. vitis* forward primer	This study
virD2.Rev1	CTT GTA CCA GCA GGG AAG CTT A	*A. vitis* reverse primer	This study
VITIS capture probe	Bio-TEG-TGT GGA AGG CCG TAA GAT AGT TGT ATT GAC CTC CCC CTT CGC CTG ACC CGA ACG TCT CGG CTG CCC A	*A. vitis* hybridization capture probe	This study

[a] Bio = biotin; TEG = triethylene glycol, 16-atom spacer arm.

and the solution transferred to a new 2-ml tube (Sarstedt, Newton, NC). The DNA was denatured by boiling for 10 min followed by incubation on ice for 5 min. Conjugated beads (20 µl) were then added to each sample and samples were incubated at 37°C for 1 h. After incubation, samples were placed on magnets and beads were washed three times with sterile HPLC water. Beads were suspended in 25 µl of sterile HPLC water and boiled at 100°C for 10 min before being placed on ice for 5 min. The eluted DNA solution (8 µl) was used in real-time PCR.

IMS. Immunomagnetic beads (IMBs) coated with goat anti-mouse antibodies (New England BioLabs) were coated with a monoclonal anti-*A. vitis* antibody that is specific to both tumorigenic and nontumorigenic strains (8). IMBs (10 µl/sample) were washed with cold 1× PBS a total of four times. The beads were suspended in a final volume of 1 ml of 1× PBS and mixed with 2 µl of anti-*A. vitis* antibody per sample before being incubated at 4°C for 1 h with mixing. Following incubation, the antibody-coated IMBs were washed four times with PBS-bovine serum albumen (BSA) and suspended in PBS-BSA. Samples to be tested were prepared for IMS by suspending pelleted cells in 1 ml of PBS-BSA, to which 10 µl of anti-*A. vitis*-coated beads was added, followed by incubation for 1 h at 4°C. After incubation, the beads were rinsed once with PBS-BSA, followed by washing three times with sterile HPLC water. Magnetic beads were resuspended in 25 µl of water. Samples were boiled to release DNA, 8 µl of which was used for real-time PCR, and water was used as a negative control.

TABLE 2. Specificity of primer set 1 in real-time polymerase chain reaction (PCR) using different *Agrobacterium vitis* strains[a]

Bacteria	Opine[b]	Location isolated	Host	Tumorigenic	Direct real-time PCR	Detection frequency	Source
A. vitis							
1860(3)	…	Italy	Grapevine	+	+	3/3	C. Bazzi
AA25	n	Afghanistan	Grapevine	+	+	3/3	Ercolani
AG125	o, n	North Greece	Grapevine	–	–	0/3	Panagopoulos
CG 49	o, n	New York, United States	Grapevine	+	+	3/3	This laboratory
CG 60	n	New York, United States	Grapevine	+	+	3/3	This laboratory
CG 78	v	New York, United States	Grapevine	+	+	3/3	This laboratory
CG 81	v	Michigan, United States	Grapevine	+	+	3/3	This laboratory
CG 102	ol	Virginia, United States	Grapevine	+	+	3/3	This laboratory
CG 106	ol	Mississippi, United States	Grapevine	+	+	3/3	This laboratory
CG127	o, n	Lucas Vineyard, United States	Grapevine	+	+	3/3	This laboratory
CG 135	n	Jim Knapp Farm, United States	Grapevine	+	+	3/3	This laboratory
CG158	…	California, United States	Grapevine	+	+	3/3	This laboratory
CG 213	n	New York, United States	Grapevine	+	+	3/3	This laboratory
CG 435	ol	Virginia, United States	Grapevine	+	+	3/3	This laboratory
CG 442	n	Washington, United States	Grapevine	+	+	3/3	This laboratory
CG 523	…	New York, United States	Grapevine	–	–	0/3	This laboratory
CG 538	…	Black Lake, New York, United States	Grapevine	–	–	0/3	This laboratory
CG 565	…	Grands Isle Park, New York, United States	Grapevine	–	–	0/3	This laboratory
CG 679	…	California, United States	Grapevine	+	+	3/3	This laboratory
CG 1100	…	Taiwan	Grapevine	+	+	3/3	Tze Chung Huang
CG 1115	…	Turkey	Grapevine	+	+	3/3	Nihal Argun
F2/5	…	New York, United States	Grapevine	–	–	0/3	This laboratory
K306	o, n	South Australia	Grapevine	+	+	3/3	K. Ophel
NW11	v	Germany	Grapevine	+	+	3/3	Leon Otten
NW113	v	Germany	Grapevine	+	+	3/3	Leon Otten
NW-180	o	Germany	Grapevine	+	+	3/3	E. Bien
S4	v	Hungary	Grapevine	+	+	3/3	E. Szegedi
A. tumefaciens							
A432/75	…	Oregon, United States	Apple	+	+	3/3	M. L. Canfield, L. W. Moore
CG1028	…	New York, United States	Cherry	+	+	3/3	This laboratory
C58	…	New York	Cherry	+	+	3/3	R. Dickey
CG939	…	Albany New York, United States	Daisy	+	+	3/3	This laboratory
A. rhizogenes							
CG 907	…	New York, United States	Hilton raspberry	+	+	3/3	This laboratory
CG 1063	…	Illinois, United States	Euonymus	+	+	3/3	This laboratory
CG 1109	…	California, United States	Walnut	+	+	3/3	This laboratory
Unknown[c]							
A	…	New York, United States	Grapevine	…	–	0/3	This laboratory
B	…	New York, United States	Grapevine	…	–	0/3	This laboratory
C	…	New York, United States	Grapevine	…	–	0/3	This laboratory
D	…	New York, United States	Grapevine	…	–	0/3	This laboratory
E	…	New York, United States	Grapevine	…	–	0/3	This laboratory
F	…	New York, United States	Grapevine	…	–	0/3	This laboratory
G	…	New York, United States	Grapevine	…	–	0/3	This laboratory
H	…	New York, United States	Grapevine	…	–	0/3	This laboratory
I	…	New York, United States	Grapevine	…	–	0/3	This laboratory
J	…	New York, United States	Grapevine	…	–	0/3	This laboratory
K	…	New York, United States	Grapevine	…	–	0/3	This laboratory
L	…	New York, United States	Grapevine	…	–	0/3	This laboratory
Xylella fastidiosa							
Temecula	…	California, United States	Grapevine	…	–	0/3	Steven Lindow
H_2O	…	…	…	…	–	0/3	…

[a] The experiment was repeated three times.
[b] Abbreviations: n = nopaline, o = octopine, v = vitopine, and ol = octopine/cucumopine plasmid with large TA region (27).
[c] Unknown bacteria isolated from enrichment broth inoculated with vacuum extract collected from grapevine tissue.

Powerfood DNA extraction. Powerfood DNA extraction was conducted according to the manufacturer's instructions. Briefly, 1 ml of each 10-fold dilution of *A. vitis* strain S4 cells was pelleted and the supernatant was completely removed. Cells were suspended in Powerfood DNA extraction buffer 2 and DNA was extracted according to the manufacturer's instructions. DNA was eluted from the column using 80 µl of water, 5 µl of which was used for real-time PCR.

Detection of *A. vitis* in artificially inoculated grapevines. To determine the efficacy of each method for detection of *A. vitis*, grapevine ('Cabernet Franc') tissue was artificially inoculated with dilutions of *A. vitis* strain S4. The vines used were initially propagated from tissue culture and had no visual crown gall infections. Six nodes from each dormant Cabernet Franc grapevine tissue was cut into 0.5-cm pieces and placed in 5 ml of 10^7, 10^5, 10^3, or 10^1 CFU/ml suspensions of *A. vitis* strain S4 and subjected to a vacuum (to help facilitate release of bacteria from xylem vessels) for 2 min for a total of three times. The solution was removed and added to nutrient broth (NB) supplemented with cycloheximide (100 mg/liter). Previously, we determined that NB would support growth of S4 when as few as 1 to 10 cells were added to the medium. Inoculated NB (2 ml) was removed after 1, 2, or 3 days of incubation and the cells were pelleted by centrifugation at 13,000 rpm for 5 min. Nucleic acids were extracted using MCH, IMS, or Powerfood protocols followed by real-time PCR, as described above. As a positive control, crown gall tissue was placed in 1× PBS and exposed to a vacuum, after which the supernatant was used to inoculate media, and samples were collected at 1, 2, and 3 days after inoculation and tested as described above.

Detection of *A. vitis* in naturally infected grapevines. Two dormant canes were collected from 16 crown-gall-infected plants from a 'Pinot Gris' vineyard in New York. Four basal nodes from each cane were cut into 0.5-cm pieces and placed in 5 ml of 1× PBS. The samples were subjected to a vacuum for 2-min intervals three times. The suspension was removed and used to inoculate 18 ml of NB supplemented with cycloheximide (100 mg/liter) followed by incubation at 28°C. Broth (2 ml) was collected at 3 days after inoculation. Samples were centrifuged for 5 min at 13,000 rpm and concentrated cells were subjected to MCH, IMS, and Powerfood DNA extraction protocols followed by real-time PCR. If all samples could not be run on same day, the concentrated bacteria were stored at 4°C.

RESULTS

Development of real-time PCR primers for tumorigenic *A. vitis* detection. A 630-bp fragment of *virD*2 gene was amplified with primer pair virD2-F and virD2-R from all seven of the *A. vitis* strains tested. Sequencing indicated that the cloned PCR products were fragments of the *virD*2 gene. Alignment of DNA sequences from the seven *A. vitis* strains and *A. vitis* strain S4 showed that the identities within the region of the *virD*2 gene were >99% similar. From these sequencing results, four primer pairs were generated. Each was used in conventional PCR with the 27 *A. vitis* strains listed in Table 2, which included the 7 strains mentioned above. Primer sets 2 and 4 were unable to amplify a characteristic product from all the strains; primer set 3 amplified multiple bands from all strains, including the nontumorigenic strains (data not shown). Therefore, primer set 1 was used going forward in development of the detection assay (Table 1).

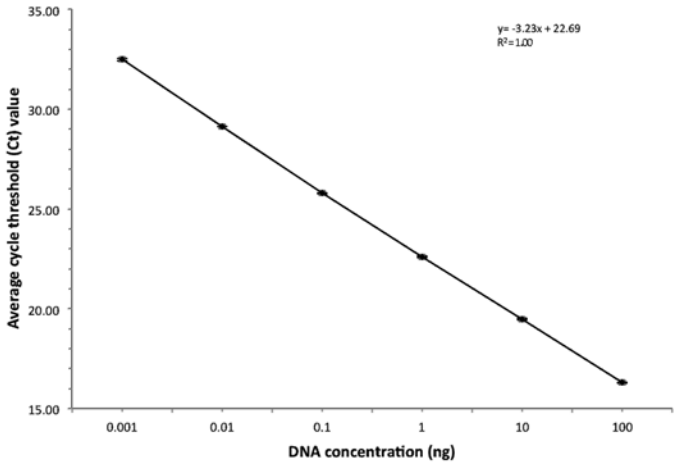

Fig. 1. Linear regression of 10-fold dilutions of *Agrobacterium vitis* strain S4 DNA against corresponding mean cycle threshold values. Data points represent the means of three separate real-time polymerase chain reactions.

Sensitivity and specificity of an *A. vitis* real-time PCR assay. The amplification efficiency of primer set 1, virD2.For1 and virD2.Rev1, was determined using linear regression analysis of the graph generated of $\log_{10}$ 10-fold serial dilutions of *A. vitis* DNA (1.0 to 0.0001 ng/µl) versus Ct value. An R^2 value of 1.00 indicated a very strong correlation between DNA concentration and the Ct values generated, suggesting that primer set 1 was highly efficient in amplification of the *virD*2 target sequence (Fig. 1). The threshold of detection using purified *A. vitis* strain S4 DNA was 0.001 ng/µl, which was detected with 100% frequency, except for 0.0001 ng/µl and water, which remained negative.

Specificity of the virD2.For1 and virD2.Rev1 primer set was determined using 27 *A. vitis* strains, 4 *A. tumefaciens* strains, and 3 *A. rhizogenes* strains. The assay was able to detect all the tumorigenic *A. vitis*, *A. tumefaciens*, and *A. rhizogenes* strains tested (Table 2). Nontumorigenic *A. vitis* strains such as F2/5 and CG538 were not detected. The primers were also tested against 12 unknown bacteria isolated from enrichment broth inoculated with vacuum extract from grape tissue and against *X. fastidiosa*. Bacteria other than tumorigenic strains of *Agrobacterium* did not yield a positive result and were similar to the water sample used as a negative control. It was observed that the melting temperature varied between *A. vitis* strains (likely due to variance in *virD2* sequence), with values of 84.5 to 88°C; the melting temperature of *A. vitis* strain S4 was 85°C.

Development of MCH and IMS real-time PCR assays for *A. vitis* detection. Sensitivity of MCH and IMS real-time PCR assays were compared with direct real-time PCR using 10-fold serial dilutions of *A. vitis* strain S4 cells. The threshold of detection for direct real-time PCR was $\approx 10^5$ CFU/ml, with a frequency of 33% and a mean Ct value of 31.80 (Table 3). Ct values for cell concentrations of 10^8 to 10^6 CFU/ml were 23.89 to 29.39 (Table 3). These dilutions were detected with 100% frequency, except for 10^6 CFU/ml, which had a detection frequency of 66%. Both MCH and IMS had detection thresholds of 10^1 CFU/ml, with detection frequencies (and Ct values) of 33% (30.74) and 66% (28.9), respectively. Powerfood extraction had a detection threshold of 10^4 CFU/ml, with a frequency of 33%. MCH and IMS real-time PCR were 1,000-fold more sensitive than Powerfood DNA extraction and 10,000-fold more sensitive than direct real-time PCR. MCH real-time PCR had <100% detection at 10^3, 10^2, and 10^1 CFU/ml, with 66, 66, and 33% frequency, respectively. IMS real-time PCR also had samples with <100% detection frequency, with 10^3 (66%), 10^2 (33%), and 10^1 (66%) CFU/ml, respectively.

The specificity of the MCH and IMS real-time PCR assay for detection of *A. vitis* was tested using 10^8 CFU/ml of the 27 *A. vitis* strains previously mentioned to determine the ability of these methods to detect diverse bacteria. MCH and IMS real-time PCR assays detected only tumorigenic strains (Table 4), whereas nontumorigenic strains such as CG523 and CG565 remained negative after real-time PCR. Only MCH was used to test strains other than *A. vitis*. All the *A. tumefaciens* and *A. rhizogenes* strains tested positive with MCH. The buffer-only and water-negative

controls did not give positive results with either MCH or IMS real-time PCR.

Detection of *A. vitis* in artificially inoculated grapevines. MCH, IMS and Powerfood DNA extraction techniques were compared using vine tissue artificially inoculate with *A. vitis* strain S4 cells. IMS was found to be the only method able to detect *A. vitis* on grapevines artificially inoculated with bacteria at 10^1 CFU/ml at 1 day postinoculation (dpi) in NB (Table 5). At higher concentrations of 10^2, 10^3, 10^5, and 10^7 CFU/ml, detection frequency was 33 to 100%. The overall frequency of detection of IMS improved with higher inoculum densities and longer incubation times of 2 and 3 days. The MCH real-time PCR assay detected *A. vitis* in samples inoculated with bacteria at 10^1 CFU/ml at 2 and 3 dpi but not at 1 dpi. It is possible that, at 1 dpi, the

TABLE 3. Sensitivity of magnetic capture hybridization (MCH), immunomagnetic separation (IMS), and Powerfood DNA extraction methods compared with direct real-time polymerase chain reaction (PCR) using 10-fold serial dilutions of *Agrobacterium vitis* strain S4 cell suspensions[a]

	Direct real-time PCR		MCH real-time PCR		IMS real-time PCR		Powerfood real-time PCR	
A. vitis (≈CFU/ml)	Ct ± SD	Freq	Ct ± SD	Freq	Ct ± SD	Freq	Ct ± SD	Freq
10^8	21.15 ± 0.32	3/3	18.46 ± 4.11	3/3	21.75 ± 2.42	3/3	19.61 ± 1.72	3/3
10^7	25.59 ± 1.35	3/3	18.53 ± 1.12	3/3	24.79 ± 2.59	3/3	22.42 ± 2.65	3/3
10^6	29.26 ± 0.69	2/3	22.11 ± 1.36	2/3	27.55 ± 1.72	3/3	26.84 ± 1.95	3/3
10^5	32.72 ± 0.59	2/3	25.38 ± 1.01	3/3	29.81 ± 4.44	3/3	29.13 ± 1.2	2/3
10^4	0.00	0/3	29.60 ± 0.38	3/3	32.12 ± 2.63	3/3	31.75	1/3
10^3	0.00	0/3	29.74 ± 1.68	1/3	30.19 ± 3.11	2/3	0.00	0/3
10^2	0.00	0/3	29.74 ± 2.07	3/3	30.97	1/3	34.12	0/3
10^1	0.00	0/3	30.74 ± 2.03	1/3	28.90 ± 3.05	2/3	0.00	0/3
H_2O	0.00	0/3	0.00	0/3	0.00	0/3	0.00	0/3
Buffer	…	…	0.00	0/3	0.00	0/3	…	…

[a] The experiment was repeated three times. Average cycle threshold value (Ct) ± standard deviation (SD) and detection frequency (Freq) are shown.

TABLE 4. Specificity of magnetic capture hybridization (MCH) and immunomagnetic separation (IMS) followed by real-time polymerase chain reaction (PCR) using different *Agrobacterium vitis* strains at 10^8 CFU/ml[a]

	MCH real-time PCR		IMS real-time PCR	
Strain[b]	Ct ± SD	Tm (°C) ± SD	Ct ± SD	Tm (°C) ± SD
A. vitis				
1860(3)	19.02 ± 1.03	86.50 ± 0.00	25.83 ± 2.67	85.00 ± 0.00
AA25	29.74 ± 0.79	86.75 ± 0.35	29.32	85.00
*AG125	0.00	0.00	0.00	0.00
CG 49	28.18 ± 0.91	86.50 ± 0.00	25.5 ± 5.44	86.25 ± 0.18
CG 60	24.44 ± 7.52	86.50 ± 0.00	26.91 ± 5.25	86.00 ± 0.00
CG 78	21.32	86.50 ± 0.00	25.48 ± 1.72	86.00 ± 0.00
CG 81	18.90 ± 0.24	86.50 ± 0.00	23.55 ± 0.59	85.00 ± 0.00
CG 102	28.73 ± 0.31	86.50 ± 0.00	27.77 ± 3.41	86.25 ± 0.18
CG 106	27.89 ± 0.66	87.50 ± 0.00	29.5 ± 1.14	86.50
CG 127	27.56 ± 1.44	86.50 ± 0.00	29.06 ± 2.26	86.25 ± 0.18
CG 135	28.38 ± 1.01	86.50 ± 0.00	26.59 ± 4.31	86.25 ± 0.18
CG 158	28.01 ± 1.24	87.00 ± 0.00	31.5 ± 0.78	86.00 ± 0.00
CG 213	27.61 ± 2.71	86.50 ± 0.00	26.98 ± 5.05	86.00 ± 0.00
CG 435	28.33 ± 1.38	87.50 ± 0.00	27.86 ± 2.35	86.00 ± 0.00
CG 442	28.65 ± 0.51	86.75 ± 0.35	27.22 ± 2.78	86.00 ± 0.00
CG 523	32.84	0.00	0.00	0.00
*CG 538	0.00	0.00	0.00	0.00
*CG 565	0.00	0.00	0.00	0.00
CG 679	28.63 ± 2.07	86.50 ± 0.00	30.76 ± 1.97	86.00 ± 0.00
CG 1100	27.89 ± 0.88	86.50 ± 0.00	29.45 ± 1.34	85.75 ± 0.18
CG 1115	28.33 ± 1.43	87.00 ± 0.00	27.15 ± 1.55	85.5 ± 0.18
*F2/5	0.00	0.00	0.00	0.00
K306	28.46 ± 0.82	86.25 ± 0.35	27.1 ± 5.09	86.00 ± 0.00
NW11	19.67 ± 0.13	86.75 ± 0.35	21.35 ± 0.09	85.00 ± 0.00
NW113	19.54 ± 0.59	86.50 ± 0.00	22.83 ± 1.34	85.00 ± 0.00
NW-180	28.19 ± 1.50	86.75 ± 0.35	28.61 ± 2.02	86.25 ± 0.18
S4	20.26 ± 1.67	86.50 ± 0.00	25.96 ± 0.14	85.00 ± 0.00
A. tumefaciens				
A432/75	19.21 ± 1.45	86.83 ± 0.29	N/A	N/A
CG 1028	19.07 ± 2.56	86.7 ± 0.58	N/A	N/A
C58	22.37 ± 0.03	87.50 ± 0.00	N/A	N/A
CG 939	24.8	86.5	N/A	N/A
A. rhizogenes				
CG 907	18.62 ± 1.66	86.83 ± 0.29	N/A	N/A
CG 1063	19.05 ± 2.44	86.83 ± 0.29	N/A	N/A
CG 1109	17.11 ± 0.89	87.00 ± 0.00	N/A	N/A
Buffer	0.00	0.00	0.00	0.00
Control	18.79 ± 0.49	85.00 ± 0.00	20.06 ± 0.56	85.00 ± 0.00
H_2O	0.00	0.00	0.00	0.00

[a] The experiment was conducted three times. Average cycle threshold value (Ct) ± standard deviation (SD) and melting temperature (Tm) ± SD are shown. N/A= samples were not tested with IMS.

[b] Asterisks (*) indicate nontumorigenic strains and Control = PCR-positive control.

A. vitis populations was below the detection threshold of the assay, and non-target DNA concentration may have been inhibitory. DNA extracted by MCH had 100% detection frequency at the higher initial inoculum concentrations of 10^3, 10^5, and 10^7. At lower inoculum densities of 10^1 and 10^2 CFU/ml, the frequency of detection was 0 to 100%. However, the frequency of detection with MCH is significantly enhanced compared with the Powerfood DNA extraction method, which did not detect *A. vitis* in samples inoculated with 10^1 CFU/ml at any of the days tested or consistently at 10^2 CFU/ml. At higher concentrations and with longer incubation times, Powerfood DNA extraction followed by real-time PCR was able to detect *A. vitis*. All three methods were able to detect *A. vitis* in gall tissue taken from a naturally infected grapevine after incubation at all the days tested. The buffer and water-only controls remained negative.

Detection of *A. vitis* in naturally infected grapevines. MCH, IMS, and Powerfood DNA extraction allowed detection of *A. vitis* in naturally infected grapevines. In all, 18.75% of vines tested with Powerfood DNA extraction or IMS real-time PCR were positive, whereas 68.75% of the samples tested were found to be positive with MCH real-time PCR (Table 6). All the samples found positive by IMS or Powerfood DNA extraction followed by real-time PCR were also found to be positive by MCH. Interestingly, not all samples found positive by IMS real-time PCR were positive using Powerfood followed by real-time PCR and vice versa. One example was vine 9, which was positive using IMS real-time PCR but negative by Powerfood DNA extraction and real-time PCR. The opposite trend was observed with vine 16, which was negative by IMS real-time PCR but found to be positive by Powerfood DNA extraction and real-time PCR. Tissue taken from asymptomatic vines, grown in an experimental vineyard plot, that were initially propagated via shoot tip culture were used as a negative control and assumed to be absent on *A. vitis*. These were found to be negative using the three methods to test for *A. vitis*. MCH allowed a significant increase in sensitivity of detection compared with IMS and Powerfood DNA extraction. Buffer and water served as negative controls and did not produce a PCR product.

TABLE 5. DNA extraction magnetic capture hybridization (MCH), immunomagnetic separation (IMS), and Powerfood followed by real-time polymerase chain reaction for detection of 10-fold serial dilutions of *Agrobacterium vitis* S4 in artificially inoculated grapevine cutting tissues[a]

Tissues[b]	DNA extraction method detection frequency		
	MCH	IMS	Powerfood
10^1, 1 day	0/3	1/3	0/3
10^1, 2 days	2/3	1/3	0/3
10^1, 3 days	1/3	1/3	0/3
10^2, 1 day	3/3	1/3	1/3
10^2, 2 days	3/3	1/3	1/3
10^2, 3 days	1/3	1/2	0/3
10^3, 1 day	3/3	2/3	1/3
10^3, 2 days	3/3	2/3	2/3
10^3, 3 days	3/3	3/3	2/3
10^5, 1 day	3/3	1/3	3/3
10^5, 2 days	3/3	2/3	3/3
10^5, 3 days	3/3	2/3	2/3
10^7, 1 day	3/3	1/3	3/3
10^7, 2 days	3/3	2/3	2/3
10^7, 3 days	3/3	2/3	3/3
R3, 1 day	2/2	1/2	1/2
R3, 2 days	2/2	2/2	1/2
R3, 3 days	2/2	2/2	1/2
Noninfected	0/3	0/3	0/3
Buffer	0/2	0/2	N/A
H_2O	0/3	0/3	0/3

[a] Experiments were done three times. N/A = samples were not tested.
[b] Inoculated grape tissues were incubated in nutrient broth enrichment medium for 1 to 3 days prior to running assays, as described in text. R3 = gall tissue from naturally infected grapevine and Noninfected = noninfected cane.

DISCUSSION

Crown gall of grape is an economically important disease that affects grape production worldwide. Management of disease occurrence and spread of the pathogen can be accomplished by using material free of *A. vitis*. To accomplish this, efficient and accurate indexing methods must be available to test propagation material for the pathogen. Previous protocols included callusing or flushing cuttings with buffer followed by detection of opines or observation of bacterial colonies on semiselective media (4,11). Identification of the isolated bacteria can be accomplished by serology, identification of biochemical markers, or PCR-based protocols (7,8,14,20,30). These methods are time consuming, because callusing alone can take weeks to complete, and may be inaccurate due to lack of sensitivity. Therefore, a more time-efficient method with improved accuracy and sensitivity is required. Real-time PCR is one such method because it allows fast and accurate detection and diagnosis of pathogens such as *X. fastidiosa*, *Grapevine fanleaf virus*, and *Xanthomonas campestris* (5,13,31).

Pathogenic *Agrobacterium* spp. are host to the Ti plasmid, which confers tumorigenesis (36). This makes the genes present on the Ti plasmid ideal targets for PCR detection and allows specific identification of *Agrobacterium* spp. and differentiation of tumorigenic and nontumorigenic strains. The *virD*2 gene, which resides on the Ti plasmid, encodes an endonuclease required for processing and transfer of T-DNA (25). Therefore, the *virD*2 gene is essential for virulence, making it an ideal target for PCR (32). Unfortunately, sequence variability exists between *Agrobacterium* spp. and even between strains, and this may lead to false negatives because of the inability of primers to bind successfully to the target sequence. To overcome this problem, the 5′ region of the *virD*2 gene of seven *A. vitis* isolates was sequenced and the data used to create four real-time primer sets for *A. vitis* detection. The first 684 bases were reported to be most conserved between plasmids expressing different opines; from this, we extrapolated that the 5′ region may be the most conserved between strains and, therefore, would be an ideal target for

TABLE 6. Comparison of magnetic capture hybridization (MCH), immunomagnetic separation (IMS), and Powerfood DNA extraction followed by real-time polymerase chain reaction (PCR) for detection of *Agrobacterium vitis* in dormant cuttings from 'Pinot Gris' grapevines naturally infected with crown gall[a]

Vine	DNA extraction method		
	MCH	IMS	Powerfood
1	+	–	–
2	+	–	–
3	+	–	–
4	–	–	–
5	+	–	–
6	+	+	+
7	+	–	–
8	+	–	–
9	+	+	–
10	+	+	+
11	–	–	–
12	+	–	–
13	+	–	–
14	–	–	–
15	–	–	–
16	+	–	+
Noninfected cane	–	–	–
Buffer	–	–	N/A
PCR control	+	+	+
H_2O	–	–	–

[a] Bacteria were vacuum extracted from dormant grapevine nodes and inoculated into nutrient broth. Bacteria were concentrated after 3 days, and DNA extraction followed by real-time PCR was done. N/A= samples were not tested.

development of real-time PCR primers (32). Sequencing data indicated that the first few bases of the *virD*2 genes sequenced were not conserved, and this may explain the inconsistent PCR results observed when using the virD2A/virD2C primers with *A. vitis* (S. Kaewnum, *personal communication*). Even among the four primer sets designed, variability in specificity among the 27 *A. vitis* strains tested was observed. Of the four primer sets designed, primer set 1, designated virD2.for1/virD2.rev1, was found to amplify all the pathogenic *A. vitis* and *A. tumefaciens* strains tested. virD2.for1/virD2.rev1 had an amplification efficiency of 100%, which is within the acceptable range of 80 to 115% (3). Along with the strong correlation between DNA concentration and Ct value, the results indicated that these primers were suitable for amplification of *A. vitis*. A real-time SYBR green assay was developed for *A. vitis* detection instead of a Taqman assay. Although a Taqman assay may be more specific, this increased specificity may be a disadvantage because the variability in the *virD*2 sequence may lead to a false negative; in addition, the expense of the Taqman probe may be cost prohibitive (2). Unfortunately, the SYBR Green assay has a disadvantage in that the SYBR Green dye binds nonspecifically to double-stranded DNA; therefore, we incorporated the melting temperature analysis to help discriminate nonspecific bands. The production of a Ct value and a melting temperature within the range of 84 to 88°C was used to determine a positive reaction. The variability in Ct value was not unexpected because different nucleic-acid make-up within the target region can lead to a variation in melting temperature (18). Samples were determined to be negative if they lacked both a Ct value and a melt curve within the established *A. vitis* range and in the event that there was a Ct value but no melt curve, as observed with the 10^2 CFU/ml Powerfood extracted sample in Table 3. The presence of a Ct value could be due to the presence of primer dimers or nonspecific amplification of a band that is not the expected size. The specificity of the primer set was tested with unknown bacteria isolated from enrichment media, *A. rhizogenes*, and *A. tumefaciens* from different hosts. The ability of the primers and the assay to detect *A. tumefaciens* from different hosts suggests that this method may be applicable to the detection of *Agrobacterium* spp. from plants other than grape.

MCH and IMS allow for selective concentration of target nucleic acid and whole cells, respectively. MCH is sensitive and specific and relies on a nucleic acid probe to bind to and concentrate target sequences. MCH has been adapted for detection of bacteria, fungi, and viruses from a wide variety of research fields (1,16,24). IMS relies on antibodies for selective targeting of the pathogen for use in real-time PCR. Both methods are simple and rapid and allow concentration of a specific target.

The MCH and IMS real-time PCR assays were both 10,000-fold and 1,000-fold more sensitive than direct real-time PCR and Powerfood DNA extraction followed by real-time PCR, respectively. For both assays, the frequency of detection decreased as concentration of bacterial cells decreased, indicating that sensitivity is affected by the amount of target present. The MCH and IMS real-time PCR assay were both specific to tumorigenic strains of *A. vitis*, and to *A. rhizogenes* and *A. tumefaciens* (in the case of MCH). The antibody used for IMS is specific for *A. vitis*; hence, we did not use IMS for detection of *A. tumefaciens* (8). Nontumorigenic strains were not detected by MCH or IMS followed by real-time PCR. This was expected because the *virD2* real-time primers were not able to detect these strains. Although IMS had a higher frequency of detection compared with MCH when using dilutions of pure cells, reliability of the assay decreased on artificially inoculated grapevine tissue and naturally infected tissue. In all, 75% of naturally infected vines tested with MCH were positive compared with 25% for IMS and Powerfood DNA extraction. In the case of IMS, nontumorigenic *A. vitis* could bind the antibody and nontarget bacteria may also bind to the beads obscuring the target cells. Powerfood DNA extraction may be affected by the amount of background DNA from nontarget bacteria which can dilute *A. vitis* DNA, leading to false negatives; hence the need for an enrichment step such as MCH that specifically concentrates the target nucleic acid. Although MCH real-time PCR was the most sensitive of the methods tested, samples that tested negative may not be indicators that the entire vine is free of the pathogen because the distribution of *A. vitis* in vines is not known .

We have demonstrated the applicability of MCH real-time PCR for detection of *A. vitis* from grapevines and indicated that this protocol may also be adapted for detection of *A. tumefaciens*. This protocol has further potential to be used as a tool to study the distribution of the bacteria throughout grapevines which may help improve efficiency of sampling and testing of apparently healthy propagation tissue. MCH real-time PCR will also be a sensitive assay for reexamining the presence of *A. vitis* in environmental samples such as wild grape species, irrigation water, and soil.

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