DEVELOPMENT OF A FAST AMPLIFYRP ACCELER8 DIAGNOSTIC ASSAY FOR GRAPEVINE RED BLOTCH VIRUS

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SUMMARY

Polymerase chain reaction (PCR) is routinely used for the detection of grapevine red blotch virus (GRBV), a virus with a circular single-stranded DNA genome, in grapevine tissue. Since the preparation of purified grapevine DNA is time consuming, a user-friendly AmplifyRP Acceler8 assay was developed to quickly, specifically, and sensitively identify GRBV-infected grapevine samples. The sensitivity of AmplifyRP Acceler8 for GRBV detection is approximately 100 times higher than that of PCR. GRBV is consistently detected by AmplifyRP Acceler8 up to a 10^{-8} dilution of infected grapevine leaf crude extracts diluted in healthy grapevine leaf crude extracts and nearly 10 copies of plasmid DNA containing a GRBV genomic fragment in a matrix of healthy grapevine leaf crude extracts. The test has no cross reactivity to grapevine tissue, nor to several grapevine-infecting pathogens, including arabis mosaic virus, grapevine fanleaf virus, grapevine fleck virus, grapevine leafroll-associated virus 1, grapevine leafroll-associated virus 2, grapevine leafroll-associated virus 3, grapevine leafroll-associated virus 4 strain 5, tomato ringspot virus, tobacco ringspot virus, *Xylella fastidiosa*, and *Botrytis cinerea*. Dried GRBV reaction pellets provided in the AmplifyRP Acceler8 kit contain all the necessary reaction components and are stable for at least 5 weeks at –20°C, 4°C, 22°C, and 37°C, providing convenience for transportation and field application. The AmplifyRP Acceler8 assay generated results consistent with PCR and real-time PCR outputs.

Keywords: Grapevine red blotch virus, GRBV, AmplifyRP Acceler8, diagnostic assay

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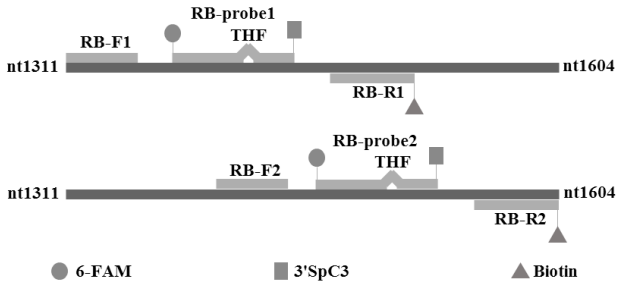
INTRODUCTION

Nearly 70 viruses, viroids, and phytoplasmas are reported from grapevines worldwide (Martelli, 2014). Some of those pathogens cause severe declines and reduce fruit quality and ripening, and thus significantly affect the profitability of vineyards. Grapevine red blotch virus (GRBV) is an emerging virus that is causing unprecedented concern to the grape industry. Disease symptoms similar to those caused by grapevine leafroll viruses were first noticed in 2008 in California (Calvi, 2011). Next generation sequencing revealed GRBV, a virus with a circular single stranded DNA genome of 3,206 nucleotides (nts) in length, in diseased vines (Al Rwahnih *et al.*, 2012, 2013). Concomitantly, GRBV was also identified using rolling circle amplification (RCA) followed by sequencing of material from a vineyard of New York State (Krenz *et al.*, 2012a, 2012b). Subsequently, GRBV was reported from vineyards of Washington State (Poojari *et al.*, 2013), Oregon State (Seguin *et al.*, 2014), Switzerland (Reynard and Gugerli, 2015), and Korea (Lim *et al.*, 2016). GRBV is widespread in the United States (Krenz *et al.*, 2014). GRBV was also found in grapevine tissue collected in 1940 and kept in a herbarium collection in California (Al Rwahnih *et al.*, 2015).

GRBV, formerly known as grapevine red blotch-associated virus (GRBaV), is a member of the genus *Grablovirus* in the family *Geminiviridae* and its genome sequence comprises two distinct GRBV phylogenetic clades (Krenz *et al.*, 2014; Sudarshana *et al.*, 2015; Varsani *et al.*, 2017). The minimum nucleotide sequence identity between the two clades is 91.5%, while it is 94.8% within clade I and 98.8% within clade II. Most of the GRBV isolates belong to clade II with fewer isolates in clade I (Krenz *et al.*, 2014).

GRBV spreads in certain vineyards (Cieniewicz *et al.*, 2017) and the virus was found in free-living *Vitis* spp. (Perry *et al.*, 2016; Bahder *et al.*, 2016b). Recently, the three-cornered alfalfa hopper, *Spissistilus festinus* (Say) (Hemiptera: Membracidae), was suggested as the natural vector of GRBV in the laboratory (Bahder *et al.*, 2016a). Like other grapevine viruses, screening foundation and commercial vineyards for GRBV and eliminating infected



RB-F1	CTGCAAGTGGACATACGTTTAGATTGTATCTA
RB-probe1	FAM/AATAGACCACGCTGTTTATTATGGCGTGTGT/idSp/TGATCAAGTTTCTGAGTTTG/3SpC3
RB-R1	5Biosg/TCGACGTCTCTTCAACGTCTTAACGAATTCAC
RB-F2	ATTATGGCGTGTGTATGATCAAGTTTCTGAGTTTGG
RB-probe2	FAM/TGAATTCGTTAAGACGTTGAAGAGACGTCGAT/idSp/TGAGCGCGGAGAGGTGACA/3SpC3
RB-R2	5Biosg/ATCAGGTTCAGTCAATGCAATTCTTCGCTTGTA

Fig 1. Design of AmplifyRP Acceler8 probes and primers for GRBV detection. Two sets of probes and corresponding primers were designed in the V1-V3 domain of the GRBV genome (nt 1,311 to nt 1,604). The 5' end of each probe was labeled with Fluorescein 6-FAM and the 3' end modified with Spacer C3 to block the extension of DNA synthesis. An abasic Tetrahydrofuran (THF) replaced a nucleotide in a position one-third distance to 3' end. The 5' end of the reverse primer was labeled with biotin.

vines is the only way to prevent the virus from spreading. Since antibodies to GRBV are not available, PCR is the common detection method. PCR requires preparation of purified grapevine DNA for testing, advanced instruments, and skilled technicians, and thus is laborious and time-consuming. A fast and easy-to-implement detection method is urgently needed to determine the presence of GRBV in grapevine tissue.

Isothermal amplification systems such as loop-mediated isothermal amplification (LAMP), cross priming amplification (CPA), RCA, and recombinase polymerase amplification (RPA) (Boonham *et al.*, 2014; James and Macdonald, 2015; Notomi *et al.*, 2000; Piepenburg *et al.*, 2006; Xu *et al.*, 2012) have provided a new approach for the fast detection of target DNA or RNA. In recent years, Agdia Inc. (USA) developed an AmplifyRP platform based on the advanced RPA technology for rapid detection of various plant pathogens (Russell *et al.*, 2011; Doan *et al.*, 2014; Mekuria *et al.*, 2014; Zhang *et al.*, 2014; Hammond and Zhang, 2016; Zhang *et al.*, 2017). The objective of the research reported here was to develop a user-friendly AmplifyRP Acceler8 test to detect GRBV isolates in grapevines.

MATERIALS AND METHODS

Plant materials and pathogens. GRBV isolates used in this study included NY358 (Cornell University) from the phylogenetic clade 2, and NY175 (Cornell University) and CS337 (USDA-ARS at Davis, CA) from the phylogenetic clade 1. Negative controls included healthy grapevine cultivars Cabernet franc and Cabernet Sauvignon. Grapevine material infected with other viral pathogens, including arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV), grapevine fleck virus (GFkV), grapevine leafroll-associated virus 1 (GLRaV-1), grapevine leafroll-associated virus 2 (GLRaV-2), grapevine leafroll-associated virus 3 (GLRaV-3), grapevine leafroll-associated virus 4 strain 5 (GLRaV-4 strain 5), tobacco ringspot virus (TRSV), and tomato ringspot virus (ToRSV), were kindly provided by Foundation Plant Services, University of California at Davis, CA. The DNA samples of *Botrytis cinerea* were provided by Dr. Matthias Hahn (University of Kaiserslautern, Germany) and *Xylella fastidiosa*–infected grapevine leaves were provided by Dr. Hong Lin (USDA-ARS at Parlier, CA).

Grapevine sample preparation. Grapevine leaf DNA was isolated with the DNeasy Plant Mini Kit (QIAGEN, The Netherlands). DNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Grapevine crude extract was prepared by grinding 0.2-0.3 g of the leaf base in sample extraction bags (Agdia, USA) containing GEB2 buffer (1:10 wt/vol) (Agdia, USA) using a pen or a marker to rub the bag on a hard surface. After thorough homogenization, crude extracts were ready for testing. Alternatively, petioles were used and processed as previously described (Krenz *et al.*, 2014).

GRBV probes and primers for AmplifyRP Acceler8. All GRBV full-length genome sequences available in GenBank were aligned using the CLUSTAL 2.1 multiple sequence alignment software. Based on sequence conservation two sets of probes (probe1 and probe2) and their corresponding primers were designed in order to detect all the isolates. The probes and primers were then synthesized by Integrated DNA Technologies (USA) (Fig. 1). The performance of RB-probe1 and RB-probe2 and corresponding primers RB-F1/RB-R1 and RB-F2/RB-R2 for detection of GRBV was tested and compared using AmplifyRP Acceler8 (Fig. 1). The reaction was performed in 10 µl containing 5.9 µl rehydration buffer (TwistDX, UK), 0.5 µl of 280 mM magnesium acetate, 0.48 µl of 10 µM forward

primer, 0.48 µl of 10 µM reverse primer, 0.07 µl of 10 µM probe, 1.57 µl ddH_2O, and 1 µl of template. Leaf crude extracts from GRBV-NY358 infected grapevine cv. Cabernet franc were prepared in GEB2 buffer (1:10 wt/vol) and tested by AmplifyRPAcceler8. GEB2 buffer alone was used as a negative control.

AmplifyRP Acceler8 for GRBV detection. AmplifyRP Acceler8 reaction mix includes numerous components such as two primers, one probe, and the required proteins and enzymes. The reaction mix is lyophilized into a reaction pellet in a PCR tube. The stability of dried reaction pellets was tested after storage at –20°C and 4°C, two optimal temperatures, and at 22°C and 37°C, two suboptimal temperatures. Each GRBV AmplifyRP Acceler8 assay was conducted by dispensing 10 µl of pellet diluent PD1 (Agdia) into a PCR tube containing a reaction pellet, followed by the addition of the sample extract (1 µl) into the rehydrated reaction pellet. The reaction was incubated in a heating block at 39°C for 20 min, immediately followed by transfer of the reaction mixture into the amplicon detection chamber (Ustar Biotechnologies, China). The results were recorded within 20 min after transfer.

Specificity of the AmplifyRP Acceler8 assay. The specificity of AmplifyRP Acceler8 was determined using purified grapevine DNA (1 ng/µl) and crude extracts (1:10 wt/vol in GEB2 buffer) from healthy grapevine leaves or leaves infected by 11 other grapevine pathogens. The sensitivity of AmplifyRP Acceler8 was assessed using purified grapevine DNA (1 ng/µl) and crude extracts (1:10 wt/vol in GEB2 buffer) from grapevine leaves infected with one of three different GRBV isolates.

Detection limit of the AmplifyRP Acceler8 assay. The detection limit of the AmplifyRP Acceler8 assay was tested and compared with PCR and qPCR. DNA purified from GRBV-NY358-infected grapevine leaf tissues was diluted in ten-fold series, from 1,000 pg/µl to 0.001 pg/µl, in water and tested by both AmplifyRP Acceler8 and PCR. At Agdia, PCR was set up using One Step Ex Taq PCR Kit (Takara, Japan) and run in a TC9639 Thermal Cycler (Benchmark, USA). A 20 µl reaction contained 10 µl of 2x master mix, 0.5 µl of Ex Taq mix (5 U/µl), 0.5 µl of forward primer RB-F1 (10 µM), 0.5 µl of reverse primer RB-R2 (10 µM), and 8.5 µl water. The thermal cycling conditions included 3 min at 95°C and then 35 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C, followed by a final 5 min at 72°C. PCR products were analyzed by electrophoresis on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. At Cornell University, a duplex PCR was run according to Krenz *et al.* (2014) and amplification products were analyzed by electrophoresis on 2% agarose gels followed by staining with GelRed™. At Wonderful Plant Health Services (Shafer, CA, USA), PCR was run per Al Rwahnih *et al.* (2013) and amplification products were analyzed by electrophoresis on 1.2% agarose gels, pre-stained with GelRedTM and 1xTAE buffer. Gel-imaging was performed with Gel-Doc XR+ imaging system (www.Bio-Rad.com). The primers and probe sequences for TaqMan qPCR assay of GRBV were provided by Foundation Plant Service, University of California, Davis (M. Al Rwahnih, personal communication). A TaqMan qPCR reaction (12.5 µl) was set up in MicroAmp EnduraPLate Optical 384-well plates (Thermo Fisher Scientific) using AgPath-ID One-Step RT-PCR Reagents (Thermo Fisher Scientific) as follows: 6.25 µl of PCR Master Mix, 2.75 µl of a primer/probe mix (400 nM primers and 120 nM probe), and 3 µl of total RNA template. The reaction plates were sealed with MicroAmp Optical Adhesive Film (Thermo Fisher Scientific) using with Axygen PlateMax Semi-Automatic Plate Sealer (Axygen Scientific, USA) and ran in QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific), programed for 95°C incubation for 10 min followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

RESULTS

AmplifyRP Acceler8 for GRBV detection. In an initial screening of probes and primers, the GRBV-specific RB-probe2 generated a stronger signal line in a lateral flow strip contained inside the amplicon detection chamber than the RB-probe1 (results not shown). Thus the RB-probe2, primer RB-F2, RB-R2, and reaction reagents were subsequently mixed to produce optimal reaction pellets for GRBV detection.

Specificity assays showed that GRBV AmplifyRP Acceler8 reacted only to DNA from plants infected with the three GRBV isolates (Fig. 2). No reaction was obtained with extracts from any of the 11 other grapevine pathogens tested, including ArMV, GFLV, GFkV, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4 strain 5, TRSV, ToRSV, *B. cinerea* and *X. fastidiosa* (data not shown). No GRBV AmplifyRP Acceler8 reactions were observed with healthy grapevine leaf extracts of *Vitis vinifera* cvs Cabernet Sauvignon and Cabernet franc (Fig. 2), water or GEB2 buffer. Other grapevine species, including *V. vulpina*, *V. labrusca*, and several unknown species that were collected locally in Indiana, also tested negative for GRBV.

GRBV AmplifyRP Acceler8 detection limit. AmplifyRP Acceler8 detected GRBV in 0.1 pg of grapevine DNA, whereas PCR detected GRBV in 10 pg of grapevine DNA (Fig. 3), indicating that the detection limit of GRBV by AmplifyRP Acceler8 is approximately 100 times higher than that of PCR. This result was confirmed using four independent batches of pellets and DNA from grapevines infected with GRBV isolates NY175, CS337 or NY358.

The limit of detection of GRBV in a crude extract of GRBV-NY358 infected grapevine leaves was determined in ten-fold serial dilutions (1:10 wt/vol) in GEB2 buffer or

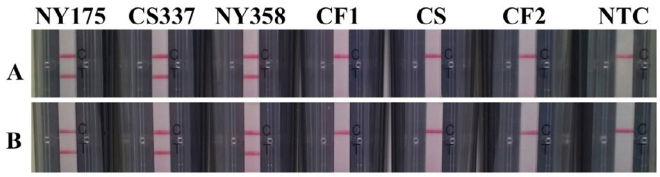


Fig 2. Detection of GRBV in grapevine leaf DNA and grapevine leaf crude extract using AmplifyRP Acceler8. Three GRBV isolates (NY175, CS337, and NY358) and three negative controls (CF1, CF2, and CS) were used in AmplifyRP Acceler8 with (A) DNA (1 ng/µl) and (B) crude extract (1:10 in GEB2 buffer). A non-template control (NTC) was included for each experiment. CF-Cabernet franc, CS-Cabernet Sauvignon.

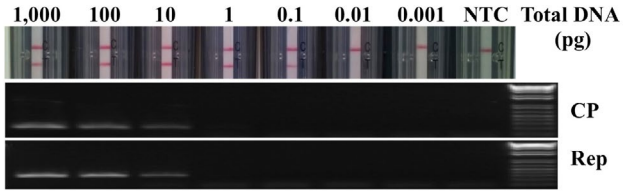


Fig 3. Comparative detection of GRBV in grapevine DNA using AmplifyRP Acceler8 and PCR. One microliter of 10-fold serial dilutions (1,000, 100, 10, 1, 0.1, 0.01, and 0.001 pg/µl) of DNA isolated from GRBV-NY358 infected leaves in water was used in AmplifyRP Acceler8 and PCR. Primer pairs designed in the GRBV coat protein (CP) and replicase-associated protein (Rep) genes (Krenz *et al*., 2014) were used in PCR. NTC: non-template control.

in healthy grapevine crude extracts. Results showed that AmplifyRP Acceler8 could detect GRBV up to a 10^{-8} dilution in GEB2 buffer and healthy crude extracts although the test line signal was weak in the higher dilutions made with healthy crude extracts (data not shown).

A 293-bp PCR amplicon of GRBV was obtained using primers RB-F1 and RB-R2, and cloned into PCR$^{2.1}$ TOPO vector (Invitrogen, USA). Recombinant bacterial colonies were identified and the recombinant plasmid (4201 bp) was purified using ZYPPY plasmid miniprep kit (Zymo Research, USA). One nanogram of this recombinant plasmid DNA contained 2.172×10^8 copies of the plasmid (http://www.modra-stanice.cz/bio/DNAtoCopy.html). AmplifyRP Acceler8 detected 11 and 14 copies of the plasmid prepared in healthy grapevine crude extract across 14 replications in two independent experiments (data not shown).

Stability of AmplifyRP Acceler8 reaction pellets. The stability of AmplifyRP Acceler8 reaction pellets was tested after one to five weeks of storage at –20°C, 4°C, 22°C, and 37°C using a 1:10^6 dilution of a GRBV-NY358 infected leaf crude extract. Tests were replicated six times and each test included a positive control (10 pg of GRBV-NY358 infected grapevine leaf DNA) and a negative control (1:20 dilution of a healthy Cabernet franc crude extract). GRBV was detected in every test, regardless of the temperature and the storage time of reaction pellets (data not shown).

Table 1. Detection of GRBV by AmplifyRP Acceler8 and PCR.

Template	GRBV isolate	GRBV Clade	AmplifyRP Acceler8	PCR
Petiole crude*	G13-4N	I	+	+
	G13-6N		+	+
	G13-18N		+	+
	20-10	II	+	+
	20-11		+	+
	20-12		+	+
	23-6		–	–
	23-4		–	–
	21-16	n/a	–	–
Purified DNA*	G6-2-24N	I	+	+
	G6-2-22N		+	+
	G6-2-10N		+	+
	18-6	II	+	+
	22-3		+	+
	22-5		+	+
	1023 MLH		+	+
	1024 MLH		+	+
	1028 SLH		–	–
	G3-30		–	–
	G3-24		–	–
	G7-1		–	–
	G6-L-2		–	–
	G3-28	n/a	–	–
	G3-2-04	n/a	–	–

* Petiole crude extracts were prepared in a hexadecyltrimethylammonium bromide (CTAB) buffer as previously reported (Krenz *et al*., 2014). Purified DNA was used at 10-50 ng/µl.

n/a: not applicable because these samples correspond to healthy grapevine material.

This result revealed the stability of reaction pellets at suboptimal storage conditions (22°C and 37°C), which can be critical for the integrity of AmplifyRP Acceler8 during shipment and transportation.

Validation of the GRBV AmplifyRP Acceler8 assay. AmplifyRP Acceler8 was tested at Cornell University and Wonderful Plant Health Services for the detection of GRBV. Results of AmplifyRP Acceler8 and PCR were consistent, regardless whether purified grapevine DNA or crude extracts of GRBV-infected grapevines were tested at Cornell University (Table 1). Similarly, no differences between the AmplifyRP Acceler8 assay and qPCR were obtained at Wonderful Plant Health Services for any of the 48 samples from seven grapevine cultivars, demonstrating the reliability of GRBV AmplifyRP Acceler8.

DISCUSSION

In recent years the recombinase polymerase amplification (RPA) technology has emerged as a critical molecular technology for rapid, low-resource diagnostics for human infectious diseases (James and Macdonald, 2015) and plant diseases (Russell *et al*., 2011; Doan *et al*., 2014; Mekuria *et al*., 2014; Zhang *et al*., 2014; Miles *et al*., 2015; Hammond

and Zhang, 2016; Londoño *et al*., 2016; Zhang *et al*., 2017). In this study we report the development of a RPA-based AmplifyRP Acceler8 assay for the detection of GRBV. Compared to PCR, AmplifyRP Acceler8 does not require thermal cycling and DNA purification. The entire process is simple and only requires a portable heating block, a 10 µl mini-pipette, and crude grapevine leaf or petiole extracts. Target is amplified rapidly and detected on a lateral flow strip contained in a contamination-proof amplicon detection chamber. The amplification procedure takes 20 min and detection results are evaluated within 5-20 min. The AmplifyRP Acceler8 assay provides a tool to rapidly determine the presence of GRBV in grapevine samples.

The detection specificity was evaluated based on the use of three GRBV isolates (two from clade I and one from clade II) and 11 other grapevine pathogens including nine viruses, one bacterium and one fungus. No reaction was repeatedly obtained with healthy grapevine samples, confirming the specificity of the GRBV AmplifyRP Acceler8 assay.

AmplifyRP Acceler8 using purified grapevine DNA was 100 times higher sensitive than PCR, and GRBV was detected in crude extract dilutions of $1{:}10^1$ to $1{:}10^8$. Using a cloned 293-bp genomic fragment, 11-14 copies of the GRBV genome were consistently detected using the AmplifyRP Acceler8 assay, indicating that detection is robust. Testing grapevine materials at Cornell University and Wonderful Plant Health Services provided an independent validation for the GRBV AmplifyRP Acceler8 testing results, resulting in GRBV detection in 14 additional isolates representing both subgroups, which were all detected by PCR.

Taken together, the AmplifyRP Acceler8 assay is a detection tool to rapidly test for the presence of GRBV in grapevine samples. It should be invaluable for researchers and grapevine growers. Because of its speed, specificity, sensitivity, reliability, and user-friendliness, it is anticipated that grape growers will use AmplifyRP Acceler8 to determine the presence of GRBV in symptomatic vines. This should facilitate timely and on-site disease management decisions in vineyards.

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