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BRIEF REPORT

A rapid, sensitive and inexpensive method for detection of grapevine red blotch virus without tissue extraction using loop-mediated isothermal amplification

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Abstract

Grapevine red blotch virus (GRBV) is an emerging virus of significant viticultural importance throughout North America. Here, we report the development of a simple protocol for point-of-use detection of GRBV. Extraction of nucleic acids is not required; instead, the whole intact plant can simply be pricked with a sterile pipette tip, which is then incubated in sterile distilled water to provide the sample template in a loop-mediated isothermal amplification (LAMP) reaction. This method is 10,000 times more sensitive than conventional PCR, costs under a dollar per sample, and can be completed from sampling to readout in just over half an hour.

Introduction

Grapevine red blotch virus (GRBV) (genus *Grablovirus*; family *Geminiviridae*) is the causal agent of red blotch disease [12], the distribution of which is widespread throughout much of the grape-growing areas of North America [4, 5, 8]. Symptoms vary, but as the name suggests, in the leaves of red *Vitis vinifera* cultivars, such as 'Cabernet Sauvignon', irregular red patches appear on the leaf lamina. In white *V. vinifera* cultivars such as 'Chardonnay', symptoms of chlorosis and marginal necrosis are reported [11, 13]. Infection by GRBV is detrimental to grapevine physiology, affecting both fruit quality and ripening [1, 9]. Management of the disease and limiting its spread in the vineyard are exclusively achieved by regular surveillance and the elimination of infected vines. Symptoms can take time to appear, and horizontal movement of infection as observed in Californian vineyards [2] can only be avoided by accurate and prompt diagnoses. Similarly, the propagation of GRBV-free budwood and the establishment of new vineyards require effective screening methods. At the present time, the molecular diagnostic methods available for GRBV detection are multiplex PCR [5], qPCR [10], and recombinase polymerase amplification (RPA) [6].

In this work, we describe a quick, simple, affordable, and sensitive method for the detection of GRBV in grapevine. To test the utility of loop-mediated amplification (LAMP) [7] for GRBV, we designed a primer set within the predicted coat protein (CP (V1)) open reading frame (ORF) of type strain NY358 (accession no. JQ901105) (Table S1). Our reasoning for this location was twofold, 1) the CP is the most conserved ORF in the genome, and 2) the CP is theoretically one of the most transcribed regions in the genome (this latter aspect is clearly only relevant for those assays that combine the DNA polymerase and a reverse transcriptase). Primers were designed using the online tool PrimerExplorer (https://primerexplorer.jp/e/) with default parameters and ordered (Integrated DNA Technologies) without additional purification. The primer set (see Table S1) was made by mixing together all six primers to give a final working

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concentration for each primer of 1.6 µM for FIP/BIP, 0.4 µM for LoopF/LoopB and 0.2 µM for F3/B3. For all subsequent LAMP reactions (total volume, 12 µl), a mixture of 6.25 µl WarmStart® LAMP Kit (New England Biolabs Cat. No. #E1700S/L), 4.5 µl of sterile distilled water, and 1.25 µl of primer mix was prepared. All LAMP reactions were incubated at 65 °C for 35 min.

The sensitivity of the assay was initially tested against total nucleic acid (TNA) extracted from greenhouse-grown grapevines as described previously [12]. TNA extractions of GRBV-infected grapevine (Cabernet franc GV32) were serially diluted 100-fold (to 1 in 100 million) in TNA extracted from uninfected grapevine (Cabernet franc TJB1-1) while maintaining a constant nucleic acid concentration of 50 ng/

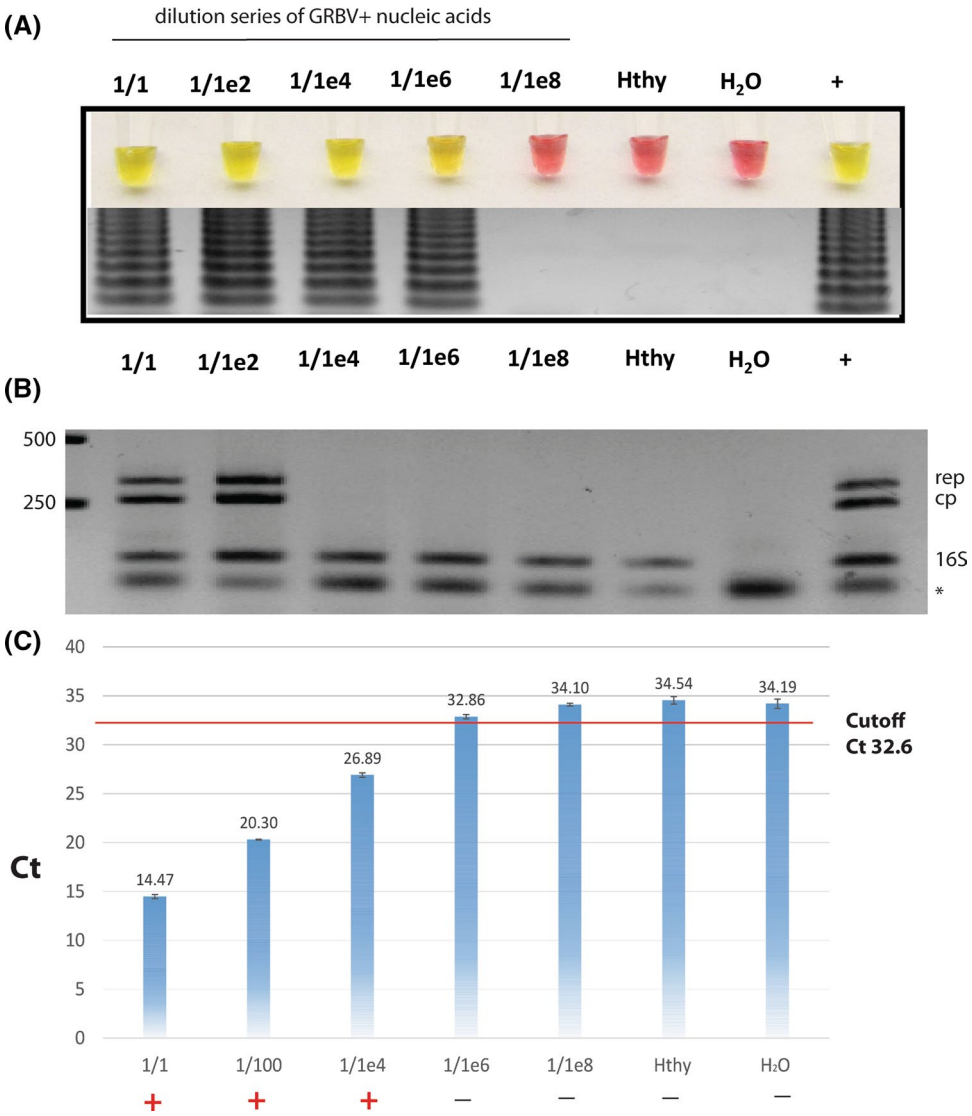

Fig. 1 Comparison of the detection limits of grapevine red blotch virus using LAMP versus multiplex PCR and qPCR using extracted total nucleic acids. A) The upper panel shows colorimetric readout of samples (red, negative; yellow, positive). The lower panel shows 4 µl of LAMP tube reaction products separated by electrophoresis on a 1% agarose gel; the laddering confirms a positive reaction. B) Multiplex PCR results of the same dilution series as tested in panel A, using the assay described by Krenz et al. [5]. PCR products specific for the viral replicase (Rep) and coat protein (CP) regions and the host 16S are visible. Primer dimers are marked with an asterisk. Molecular weight marker sizes (bp) are indicated on the left. C) Quantitative PCR results of the same dilution series as tested in panel A, using the assay described by Setiono et al. [9]. Cycle threshold (Ct) values are graphed with specific values shown over each bar. The calculated Ct cutoff value of 32.6 is indicated by a red line and assigns an infection status (+ or -) to each sample. Total nucleic acid extractions of GRBV-infected grapevine were serially diluted 100-fold (to 1 in 100 million) in TNA extractions of uninfected grapevine (TJB1-1) while maintaining a constant nucleic acid concentration of 50 ng/µl. Hthy, healthy grapevine nucleic acids; H_20, water non-template control; +, positive control of extracted nucleic acids from a GRBV-infected grapevine

µl, as determined spectrophotometrically. These same dilutions were also tested using established multiplex PCR [5] and qPCR [10] methods. The results showed that LAMP outperformed both qPCR and PCR by two to four orders of magnitude, respectively, detecting the virus to a dilution of 1 in 1 million (and at times 1 in 100 million) (Fig. 1). This translates to a detection limit of around 1 fg of target (in the form of plasmid DNA monomer GRBV cloned in pUC19) or 150 molecules (Fig. S1). Next, we examined ways to shorten the time required to complete the protocol at the extraction phase, which, using our standard TNA method, takes about two hours from the initial tissue grinding to TNA pellet resuspension. Given the demonstrated sensitivity of the LAMP assay, simplicity of processing was a major consideration for any isolation method.

The "pin-prick" method developed in this study has only a few steps and can be completed in just over 5 min (Fig. 2). Extraction of nucleic acids from grapevine is notoriously difficult [3], and yet repeatedly stabbing the plant with a sterile 10 µl pipette tip (ART® 10 Reach Barrier Tip, Thermo Scientific) is sufficient to provide enough 'clean' template for the LAMP assay (Fig. S2). Three selected leaves (plus petioles) per plant are removed or left attached to the plant. The plants were in various stages of development, both with and without symptoms that could be attributed to GRBV infection. The youngest vines were agroinoculated as seedlings the previous year with one eight-week period of dormancy. Based on previous observations of GRBV distribution in the plant [10], older leaves proximal to the main stem have generally higher virus titers and should be sampled preferentially. All petioles were carefully pricked three times along their lengths, ensuring that the tip penetrated the epidermis (Fig. S2). Then, the leaves at their base were pricked five times; leaves were stacked onto each other in cases where they had been removed from the plant. To further assess the robustness of the method we tested the specificity and sensitivity of this assay using 43 greenhouse-grown grapevines, the GRBV-infection status of which had been tested by our designated "gold-standard" multiplex PCR [5]. The results (Table S2 and Table S3) show the "pin-prick GRBV LAMP" assay has 100% sensitivity and 96.3% specificity, the latter being due to one plant, the positive infection status (as identified by LAMP) of which was previously not recognized; this plant was also subsequently identified as positive using the RPA-based kit AmplifyRP® Acceler8® (Agdia). These results might suggest that this plant's virus titer was below the detectable threshold of the multiplex PCR. The majority (10) of the 17 plants testing positive were naturally infected or agroinfected with strain NY358, which is a member of the designated clade 2 of the virus species. Other clade 2 (NY147) and clade 1 (NY175) variants also tested positive, thereby in part demonstrating the assay's breadth of detection (see Fig. S3).

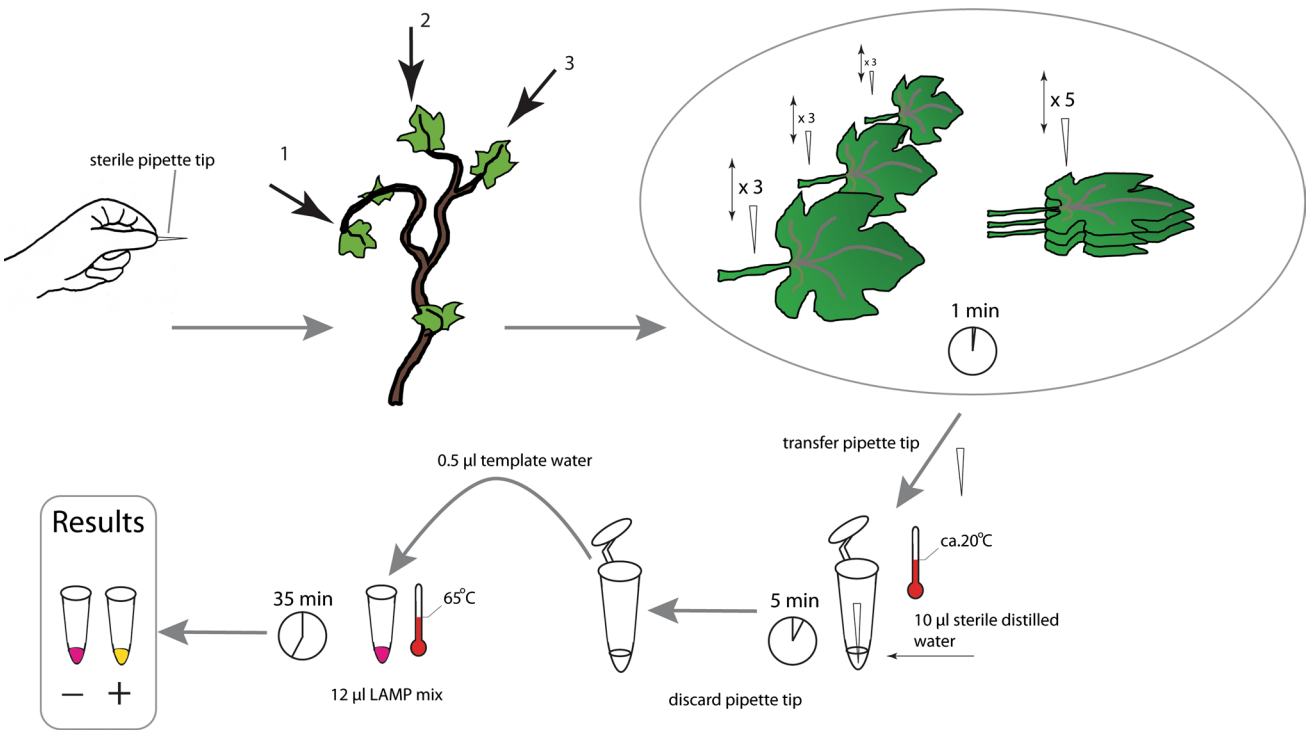

Fig. 2 Flowchart of the steps involved in the "pin-prick GRBV LAMP" assay

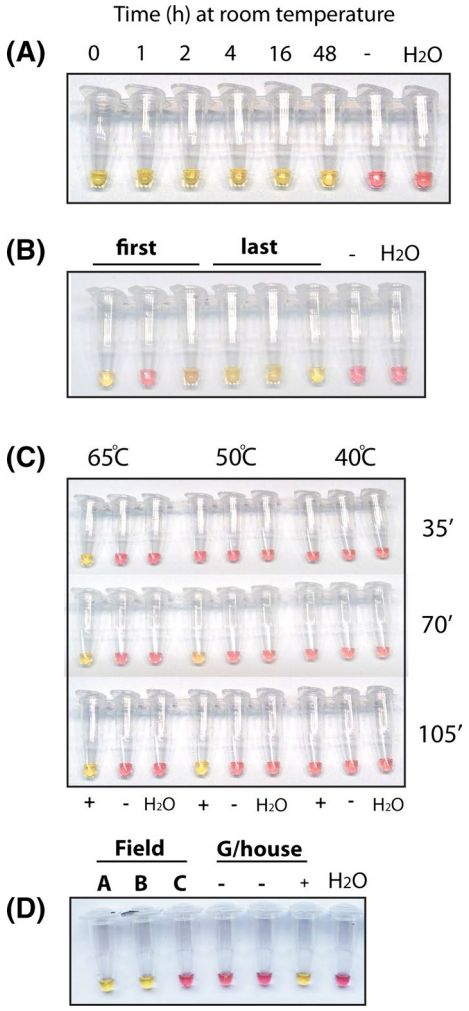

Fig. 3 Testing the versatility of "pin-prick GRBV LAMP". A) Template water from an infected vine was left at room temperature for different times up to 48 hours and tested using LAMP. B) The order of infected sample in the pricking process and the ability to detect grapevine red blotch virus. First (in triplicate): The first stab in each pricking sequence was in the petiole of an infected plant, followed by the standard protocol as shown in Fig. 2, using petioles from an uninfected plant. Last (in triplicate): The last stab in each pricking sequence was in the petiole of an infected plant, preceded by using petioles from an uninfected plant with the standard protocol. C) The effects of different temperatures and times of the LAMP reaction on detection of GRBV. D) Results using dormant cane material. See Fig. S2C and D for source material used in the assay. Lanes A-C refer to individual plants growing in the field. A and B had tested positive previously in multiplex PCR. '-', healthy grapevine nucleic acids; H_20, water non-template control; '+', positive control of extracted nucleic acids from a GRBV-infected grapevine

The robustness and versatility of the pin-prick assay was further tested in a number of ways. First, experiments (n = 3) were carried out to assess the viability of the template water over time. Infected and uninfected tissue was pricked as described, and the template water was left at room temperature (ca. 20 °C), with aliquots taken and frozen at times 0, 1, 2, 4, 16 and 48 hours. The results (Fig. 3a) showed no visible decline in detection, even after two days at room temperature. We also tested how the possible "dilution" effect of pin-pricking uninfected material might reduce the ability to detect GRBV, i.e., whether stabbing of material without virus could remove traces of viral DNA, as might be the case in a new infection or early in the growing season [9]. Infection was detected in more than half (10 of 15) the experiments (n = 5) testing template water derived from tips stabbed first (and once) in a petiole taken from an infected plant followed by the standard pinpricking procedure in uninfected petioles and leaves (Fig. 3b). This reduction was almost definitely due to mechanical cleaning rather than due to differences in virus distribution in the plant, as controls where infected petiole was stabbed last after the complete standard pinpricking procedure in uninfected petioles and leaves produced a positive result in 14 of the 15 experiments. Further modifications to the technique could be tailored according to need. The efficacy of the protocol was also demonstrated for field-collected dormant budwood and at lower temperatures for longer times (50 °C for 70 min) (Fig. 3c and d). In the case of sampling dormant canes, the bark was peeled back, and the pipette tip was used to penetrate the vascular tissue, paralleling the method used on leaves (Fig. S2d)

This simple protocol, which costs approximately 85¢ per sample and takes around 40 min to complete, may prove useful in the detection of multiple plant pathogens but for the time being has the potential to aid in more-rapid identification of GRBV-infected material in the field and thus improve our understanding of GRBV epidemiology.

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Compliance with ethical standards

Conflict of interest All authors declare they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Fig S1

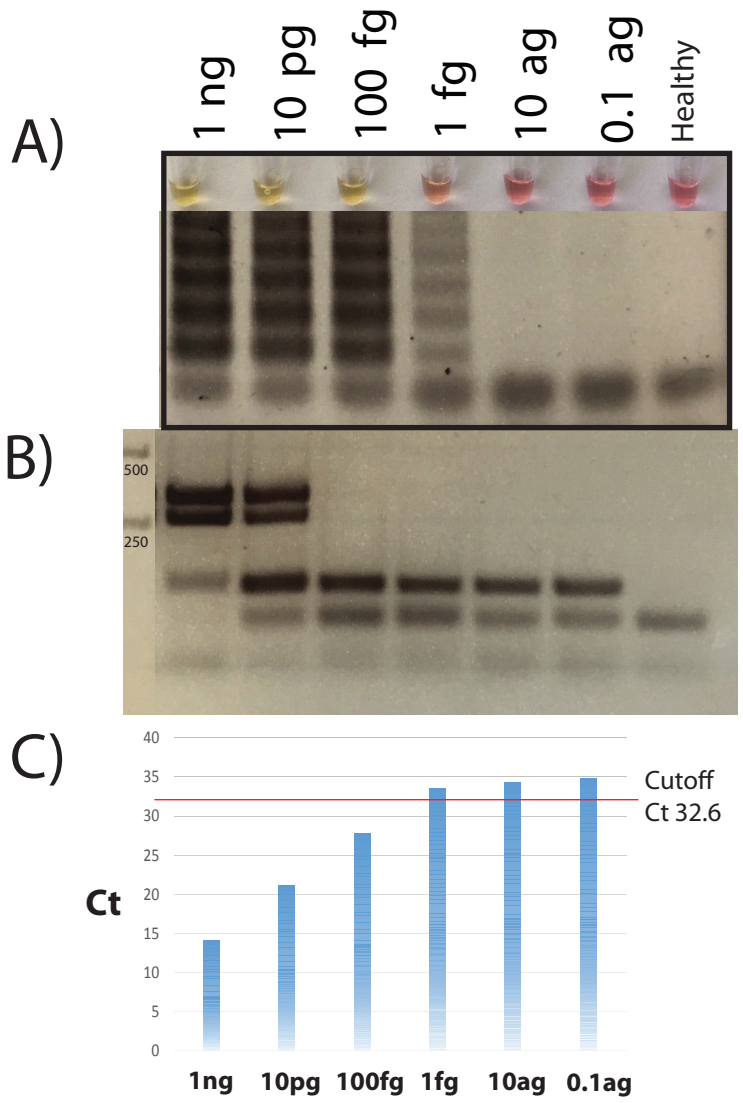

Fig S1. Comparison of the detection limits of grapevine red blotch virus using LAMP versus multiplex PCR and qPCR using plasmidic DNA. A) Upper panel shows colorimetric readout of samples (red – negative; yellow – positive). Lower panel - 4µl of LAMP tube reaction separated by electrophoresis on a 1% agarose gel; the laddering confirms a positive reaction. B) multiplex PCR results of the same dilution series as tested in (A) using assay described in Krenz, et al. [4]. PCR products specific for the viral replicase (Rep) and coat protein (CP) regions and the host 16S are visible. Primer dimers are marked with an asterisk. On the left – molecular weight marker sizes (bp) are indicated. C) quantitative PCR results of the same dilution series as tested in (A) using assay described in Setiono, et al. [7]. Cycle threshold (Ct) values are graphed with specific values shown over each bar. The calculated Ct cut-off value of 32.6 is marked by the red line and assigns an infection status (+ or -) to each sample. Total nucleic acid extractions of GRBV-infected grapevine were serially diluted hundredfold (until 1 in 100 million) in TNA extractions of uninfected grapevine (TJB1-1) while maintaining a constant nucleic acid concentration of 50 ng/µl. Hthy – healthy grapevine nucleic acids, H20 – water non-template control, + - positive control extracted nucleic acids from a GRBV infected grapevine.

Fig S2

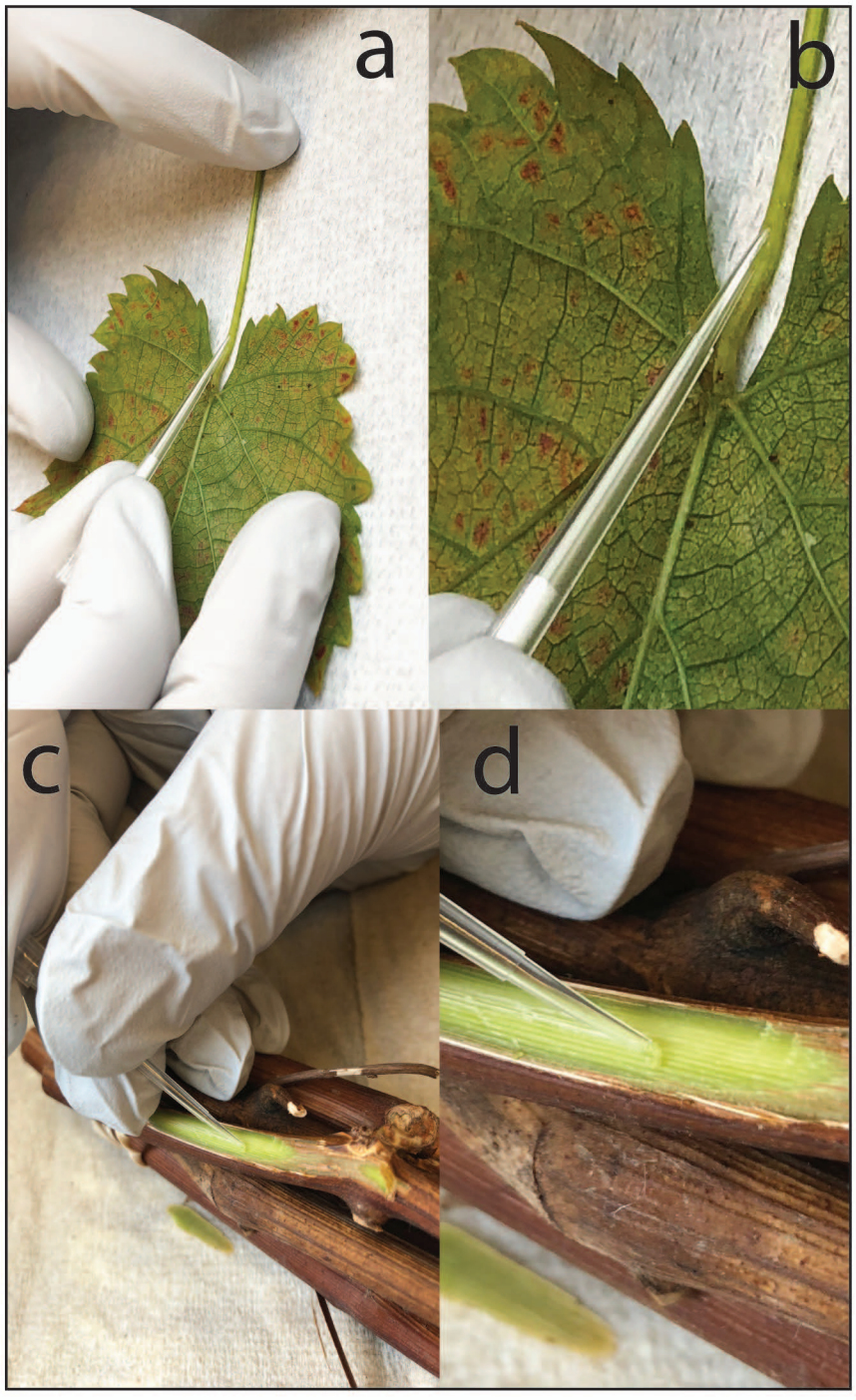

Fig S2. Images depicting the "pin-prick" method used to recover viral nucleic acids for the LAMP assay. A - tip penetration into the petiole of an expanded leaf (b = image magnification). C - tip scraping of exposed greentissue just below the bark (d - image magnification).

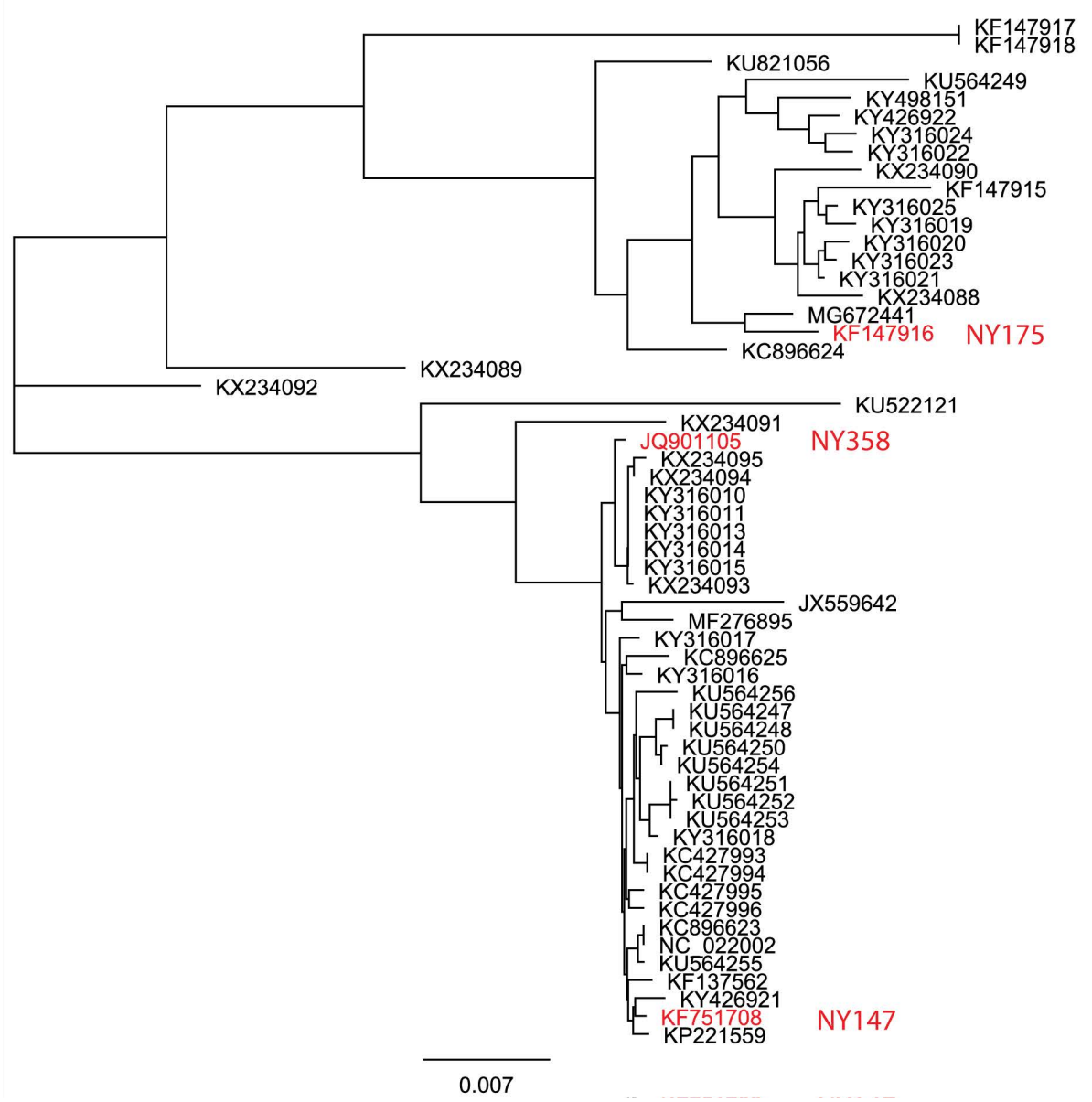

Fig S3. Neighbor-joining phylogenetic tree generated with Geneious 9.1.3 (Biomatters Ltd) software using full-length nucleotide sequences of grapevine red blotch virus. Branch tips show NCBI accession number. In red are those isolates tested using "pin-prick" GRBV LAMP. Bar represents genetic distance.

Table S1. Primers used in LAMP assay for grapevine red blotch virus detection

Primer name	Type[a]	Sequence	Genome position[b]
p1825	F3	GAATCGTTTGAATCGTAAGAGA	1102-1123
p1826	B3	CAGACAAATAAATACGATTCCTTTC	1304-1280
p1827	FIP	AATGACTCCTGCGGCTTCTT*TCGTATTTTGGGTTCGAAGA	1185-1166* 1126-1145
p1828	BIP	TCAAAGACGTCGTCTGGTTGT*CATCATTACGTCCTCCACC	1216-1236* 1277-1259
p1842	LoopB	GCTTTTAAAAACGACGTGT	1238-1256
p1857	LoopF	TTCACGCCAACAACAAGT	1164-1147

a – designated function of primer according to Notomi, et al. [7]

b – reference to original NY358 sequence (Acc. # JQ901105). * denotes intersection between F2/B2 and F1c/B1c sequences and genome positions.

Table S2. Determination of the sensitivity and specificity of the pin-prick LAMP assay for grapevine red blotch virus detection versus multiplex PCR.

		Status determined by multiplex PCR[a]		
		Positive	**Negative**	**Total**
LAMP assay result	Positive	16 (A)	1 (B)*	17
	Negative	0 (C)	26 (D)	26
	Total	16	27	43
	Sensitivity	100%		
	Specificity		96.3%	

[a] Letters in parentheses indicate values used for sensitivity/specificity calculation.

Percentages calculated by formulae: Sensitivity = A/(A+C) x 100, Specificity = D/(D+B) x 100

*The single disparity between the methods (an agroinoculated plant) was further identified as positive using the GRBV AmplifyRP® Acceler8® kit (Agdia).

Table S3. Sample plants or GRBV isolates tested by both multiplex PCR and the pin-prick GRBV LAMP assay.

#	Sample ref[a]	LAMP assay result	PCR Result
1	NY1616	Negative	Negative
2	TJB1-1	Negative	Negative
3	NY629C	Negative	Negative
4	NY1287	Negative	Negative
5	NY662	Negative	Negative
6	NY1414	Negative	Negative
7	NY1287	Negative	Negative
8	NY1353	Negative	Negative
9	NY210	Negative	Negative
10	NY358	Negative	Negative
11	NY358	Negative	Negative
12	NY358	Negative	Negative
13	NY358	Negative	Negative
14	NY358	Negative	Negative
15	NY358	Negative	Negative
16	NY358	Negative	Negative
17	NY358	Negative	Negative
18	NY358	Negative	Negative
19	NY358	Negative	Negative
20	NY358	Negative	Negative
36	NY632	Negative	Negative
37	NY1616A	Negative	Negative
38	NY1616B	Negative	Negative
39	NY1468	Negative	Negative
41	NY1287	Negative	Negative
42	NY1287D	Negative	Negative
21	NY1290	Positive	Positive
22	NY147	Positive	Positive
23	NY358	Positive	Positive
24	NY147A	Positive	Positive
25	NY358	Positive	Positive
26	NY358	Positive	Positive
27	NY358	Positive	Positive
28	NY358	Positive	Positive
29	NY358	Positive	Positive
30	NY358	Positive	Positive
31	NY358	Positive	Positive
32	NY358	Positive	Negative *
33	NY1467E	Positive	Positive
34	NY649D	Positive	Positive
35	NY175B	Positive	Positive
40	GV32	Positive	Positive
43	NY147C	Positive	Positive

a- a sample with the same number is either a different propagated vine from the same mother plant or a vine agroinfected with the same isolate.

*refers to sample that tested positive by AmplifyRP® Acceler8® (Agdia)